



UNIVERSIDADE ESTADUAL DE CAMPINAS  
FACULDADE DE ENGENHARIA DE ALIMENTOS

MANOELA MARTINS

Synergistic Biocatalysis for Sustainable Production of Prebiotic  
Xylooligosaccharides from Agroindustrial Wastes: Chimeric Enzyme  
Synthesis and Techno-Economic Analysis of Integrated Biorefinery

Biocatálise Sinérgica para Produção Sustentável de Xilo-oligossacarídeos Prebióticos  
a partir de Resíduos Agroindustriais: Hidrólise Mediada por Enzima Quimérica e  
Análise Tecno econômica de Biorrefinaria Integrada

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If the only prayer you ever say in your entire life is thank you, it will be enough.

Meister Eckhart

*Life is an unfoldment, and the further we travel the more truth we can comprehend. To understand the things that are at our door is the best preparation for understanding those that lie beyond.*

**Hypatia**

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## Resumo

A presente tese explorou a dinâmica da biotecnologia sustentável, focando na sinergia entre o uso de resíduos agroindustriais, tecnologia enzimática e alimentos funcionais. No centro desta pesquisa estão os xilo-oligosacarídeos (XOS), derivados de biomassa rica em xilana e valorizados por seu potencial prebiótico, promovendo a melhoria da função intestinal, resposta imunológica e outros benefícios à saúde. Esta tese investigou a conversão de três resíduos agroindustriais abundantes—casca de milho, cascas de soja e resíduos de processamento de laranja—em XOS e outros compostos bioativos, enfatizando o aumento da produção de XOS por meio da hidrólise de cascas de soja e milho utilizando uma nova enzima quimérica. Duas enzimas-chave foram fundidas para criar uma quimera bifuncional estável, com o objetivo de reduzir os custos de produção de enzimas e melhorar estabilidade e atividade enzimática. Resultados em destaque incluem a quimera bifuncional de  $\alpha$ -L-arabinofuranosidase GH 62 fundida com endoxilanase GH 10 em *Pichia pastoris*. As investigações abrangeram perfis de hidrólise, condições ótimas, sinergia, especificidade e análises estruturais. GH 62 isoladamente revelou condições ótimas em pH 4,8 e 42 °C, com um efeito sinérgico notável quando associada com GH 10, resultando em um aumento de 2,3 vezes na produção de XOS e coprodução de 0,82 mM de ácido ferúlico e 0,09 mM de ácido p-cumárico a partir de arabinoxilana da casca de milho. O design de uma enzima quimérica bifuncional integrando essas duas atividades demonstrou estabilidade e especificidade aprimoradas, alcançando até 7 g/L de XOS a partir de cascas de milho, e um aumento de 10 °C na temperatura de fusão para o módulo GH 62. Estratégias de evolução dirigida elevaram ainda mais a termostabilidade de GH 62, com o melhor mutante (G205K) apresentando um aumento de 5 °C na temperatura de fusão e melhoria de 41,3% na energia de desnaturação, estabelecendo bases para processos enzimáticos mais robustos. Análises bioinformáticas facilitaram a compreensão e a melhoria da estabilidade enzimática. A quimera apresentou especificidade superior para arabinoxilana, e a ação sinérgica dos dois domínios levou a melhorias nos rendimentos de xilobiose e xilotriose (15% e 33%, respectivamente). Esses avanços na hidrólise de arabinoxilana contribuíram para o avanço de processos enzimáticos sustentáveis, especialmente para a valorização de resíduos agroindustriais. Além disso, a pesquisa se estendeu à biorrefinaria de resíduos de laranja em um contexto distinto, demonstrando a viabilidade técnico-econômica para a produção de XOS, pectina e bioenergia, alcançando um custo de produção de \$3,67/kg com mais de 60% de redução nos requisitos de energia e material. Análises revelaram os efeitos antioxidantes dos produtos, destacando a utilização sustentável de resíduos e o desenvolvimento de alimentos funcionais e nutracêuticos. Esta pesquisa ressaltou a importância de atender às demandas dos consumidores por produtos bioativos naturais, minimizando os impactos ambientais com novas intervenções tecnológicas. No geral, esta tese contribuiu para o avanço de formulações enzimáticas para bioprodutos sustentáveis, reforçando o potencial dos resíduos agrícolas como recursos valiosos em aplicações biotecnológicas.

**Palavras-chave:** Biotecnologia, Hidrólise Enzimática, Prebióticos, Proteínas quiméricas, Resíduos agroindustriais, Xilo-oligosacarídeos.

## Abstract

The present thesis explored the dynamics of sustainable biotechnology, focusing on the synergy between the use of agro-industrial residues, enzymatic technology, and functional foods. At the heart of this research are xylo-oligosaccharides (XOS), derived from xylan-rich biomass and valued for their prebiotic potential, promoting improved intestinal function, immune response, and other health benefits. This thesis investigated the conversion of three abundant agro-industrial residues—corn husks, soybean hulls, and orange processing residues—into XOS and other bioactive compounds, emphasizing increased XOS production through hydrolysis of soybean hulls and corn husks using an innovative chimeric enzyme. Two pivotal enzymes were fused to create a stable bifunctional chimera aimed at reducing enzyme production costs and enhancing enzymatic stability and activity. Highlighted results include the bifunctional chimera of  $\alpha$ -L-arabinofuranosidase GH 62 fused with endoxylanase GH 10 in *Pichia pastoris*. Investigations covered hydrolysis profiles, optimal conditions, synergy, specificity, and structural analyses. GH 62 alone revealed optimal conditions at pH 4.8 and 42 °C, with a notable synergistic effect when paired with GH 10, resulting in a 2.3-fold increase in XOS production and the co-production of 0.82 mM ferulic acid and 0.09 mM p-coumaric acid from corn hull arabinoxylan. The design of a bifunctional chimeric enzyme integrating these two activities demonstrates enhanced stability and specificity, achieving up to 7 g/L of XOS from corn husks, while maintaining superior thermostability with a 10 °C increase in melting temperature for the GH 62 module. Directed evolution strategies further improve the thermostability of GH 62, with the best mutant (G205K) exhibiting a 5°C increase in melting temperature and a 41.3% enhancement in denaturation energy, laying the groundwork for more robust enzymatic processes. Bioinformatics analyses facilitated the systematic understanding and enhancement of enzymatic stability. The designed chimera exhibited superior specificity for arabinoxylan, and the synergistic action of the two domains led to significant improvements in xilobiose and xylotriose yields (15% and 33%, respectively). These advancements in arabinoxylan hydrolysis contributed to the understanding of enzymatic properties and the advancement of sustainable enzymatic processes, especially for the valorization of agro-industrial residues. Additionally, the research extended to the biorefinery of orange waste in a distinct framework, demonstrating the techno-economic feasibility for XOS, pectin, and bioenergy production, achieving a production cost of \$3.67/kg with over 60% reduction in energy and material requirements. Analyses revealed the antioxidant effects of the products, highlighting the sustainable utilization of residues and the development of functional foods and nutraceuticals. This research underscores the importance of meeting consumer demands for natural bioactive products, while minimizing environmental impacts through innovative technological interventions. Overall, this thesis contributed to the advancement of enzymatic formulations for sustainable bioproducts, reinforcing the potential for agricultural residues as valuable resources in biotechnological applications.

**Keywords:** Agroindustrial residues, Biotechnology, Chimeric proteins, Enzymatic hydrolysis, Prebiotics, Xylo-oligosaccharides.

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**CHAPTER 1.**  
**GENERAL INTRODUCTION**  
**AND THESIS STRUCTURE**

## 1. General introduction

The intersection of agroindustrial wastes, enzymes, functional food, and biorefinery marks a dynamic frontier in the landscape of sustainable biotechnology. Xylooligosaccharides (XOS), derived from the hydrolysis of xylan-rich materials, stand out as promising prebiotics, that is, bioactive compounds that nourish beneficial gut bacteria, thereby conferring a spectrum of health benefits upon their human hosts. Xylooligosaccharides are garnering significant attention due to their versatile applications, ranging from their use as prebiotics in the food and feed industries to potential therapeutic benefits, such as promoting gut health.

Concurrently, agroindustrial wastes, comprising residues from agricultural and food processing activities, present both a challenge in waste management and an opportunity for resource valorization (Dong et al., 2023; Pinales-Márquez et al., 2021a). Enzymes, particularly endoxylanases and arabinofuranosidases, emerge as key catalysts in the efficient conversion of complex biomass into valuable XOS. The integration of these elements not only addresses environmental concerns associated with waste disposal but also contributes to the development of functional foods with enhanced nutritional profiles. This approach, rooted in the principles of biorefinery, seeks to optimize the utilization of agroindustrial wastes through enzymatic processes, thereby generating a spectrum of bio-based products for the food industry, in alignment with the growing demand for health-promoting consumables (McDonagh, 2021).

Furthermore, the narrative of agroindustrial waste utilization is significantly enriched by enzymatic engineering. Chimeric enzymes, designed from the fusion of distinct functional domains from different parent enzymes, offer a unique blend of catalytic activities that can be precisely tailored for optimal performance in agroindustrial waste hydrolysis (Saadat, 2017). Through recombinant techniques, the genetic blueprints of these chimeric enzymes are constructed, introducing hybridized sequences into microbial hosts. The chimeric design allows us to leverage the synergistic effects of multiple catalytic functionalities, enhancing substrate specificity and overall efficiency in breaking down complex polysaccharides into valuable products such as XOS. The advantages of recombinant chimeric enzyme expression extend beyond the laboratory to the industrial scale, where cost-effective and sustainable production becomes increasingly feasible.

This study focused on exploring the potential of three agroindustrial wastes—corn husks, soy hulls, and orange waste processing—to produce XOS and other bioactive compounds. These residues were chosen based on their significant annual production and

commercialization. After characterizing the biomass, the enzymatic hydrolysis approach utilized the most suitable hemicellulases cocktail for efficient XOS release.

For arabinoxylan-rich residues like soy hulls and corn husks, a fusion enzyme comprising GH 62  $\alpha$ -L-arabinofuranosidase and GH 10 endoxylanase was employed to enhance XOS production. The primary aim was to generate a stable chimeric protein with the ability to act on diverse biomass sources. Conversely, waste from orange processing underwent a distinct biorefinery approach utilizing a commercial cocktail rich in endoxylanase activity to assess economic viability (don't worry, all this will be explained in the following chapters). This analysis aimed not only to characterize the bioactive compounds derived from the enzymatic hydrolysis but also to discern their potential applications in promoting health and nutrition, thereby underscoring the versatility and value of the developed chimeric enzyme strategy and of the proposed biorefinery for orange waste processing.

## 2. Objectives

### 2.1. General objective

The objective of this thesis was to develop a streamlined process for producing xylo-oligosaccharides using three abundant agroindustrial wastes: soy hulls, corn husks and orange processing wastes. This entails employing a novel chimeric enzyme to yield functional ingredients, while also proposing integrated biorefinery solutions customized for the food industry. Through this approach, the thesis aimed to optimize resource utilization, enhance sustainability, and generate value within the food sector by implementing innovative and efficient methodologies.

### 2.2. Specific objectives

The first specific objective of this study was the characterization of chemical composition of three major agroindustrial wastes produced in Brazil – soy hulls, corn husks, and orange processing waste. Both corn husks and soy hulls are rich in arabinoxylan, a polysaccharide that we targeted in our research. On the other hand, orange processing waste had to proceed with a different approach.

Therefore, for soy hulls and corn husks, the aim of producing XOS involved the development of a bi-functional chimeric enzyme to enhance arabinoxylan depolymerization into XOS. For this end, the specific objectives concluded were:

- Expression of GH 62  $\alpha$ -L-arabinofuranosidase from *Aspergillus fumigatus* in *Pichia pastoris*.
- Optimizations of activity and hydrolysis profile of recombinant GH 62  $\alpha$ -L-arabinofuranosidase and synergistic analysis with endoxylanase for XOS production.
- Design, characterization, and expression of a novel bi-functional chimeric protein resulting from the tandem fusion of GH 10 endoxylanase from *Papilio-trema. flavescens* and GH 62  $\alpha$ -L-arabinofuranosidase from *Aspergillus fumigatus*.
- Thermostability analysis and enhancement of GH 62 arabinofuranosidase.

- Optimization of hydrolysis conditions of the chimeric enzyme to produce xylooligosaccharides from soy hulls and corn husks.

The final objectives focused on utilizing the orange processing waste. Despite its low hemicellulose content, its potential for industrial applications prompted a different research approach. The specific goal achieved was:

- Pectin and xylan extractions optimization;
- Technoeconomic analysis of sequential extractions and enzymatic hydrolysis of recovered xylan for XOS production
- Development of an integrated biorefinery approach to extract pectin and produce and purify xylooligosaccharides from orange waste using a commercial endoxylanase cocktail.



### 3. Thesis structure

The stages performed in this thesis are organized in 15 chapters (**Figure 1-1**). The agroindustrial wastes selected for the elaboration of this thesis include corn husks, soy hulls, and orange processing waste. While the first two are lignocellulosic materials rich in xylan, the third primarily comprises pectin and sugar. The selection of these specific agroindustrial wastes is influenced by the substantial quantities generated annually in Brazil. Our research group systematically investigates the chemical composition of these agroindustrial wastes, with a keen focus on identifying the biochemicals that can be derived through their depolymerization. Notably, our attention centers on prebiotic fibers, particularly xylooligosaccharides (XOS). Therefore, **Chapter 1** introduced the xylooligosaccharides and our objectives, and presented the **doctoral period memorial**. These objectives unfold in a comprehensive manner across **Chapter 2** and four dedicated review studies, meticulously detailed in: **Appendix I** and **Appendix II** serve as a comprehensive introduction to the chemical conversion of lignocellulosic materials within the broader context of biorefinery practices. **Appendix III** specifically accentuates the production and application of nutraceutical oligosaccharides, especially XOS. **Chapter 3** offers a systematic and bibliometric analysis as these compounds constitute the primary products derived from this research. This review lays the groundwork for the exploration of enzymatic hydrolysis of lignocellulosic biomass, with a special emphasis on chimeric enzymes, discussed in detail in **Chapter 4** and **Chapter 5**.

The insights from these review studies inform our experimental design for xylooligosaccharides, ferulic acid, and p-coumaric acid, which leverages the synergistic effects of GH 62 arabinofuranosidase and GH 10 endoxylanase, expounded upon in **Chapter 6**. The identified functionality of GH 62 arabinofuranosidase and the yields enhancement lay the foundation for the subsequent study on the novel bi-functional chimeric enzyme design, elucidated in **Chapter 7**, while **Chapter 8** delved into the examination of point mutations for enhanced thermostability and prediction tools. The distinct composition of orange processing waste led to a separate experimental study for optimization and technical economic analysis of an integrated biorefinery for XOS, pectin and bioenergy, presented in **Chapter 9**. Finally, **Chapter 10** presents the general discussion and **Chapter 11** the general conclusion and suggestions for future studies. In these, we discussed the main results and conclusions observed in each review and experimental article. **Chapter 12** presents the references used in the general introduction and discussion, the supplementary material of the experimental research articles, and the book chapters, the patent file, publishers authorization, and the original pdf files.

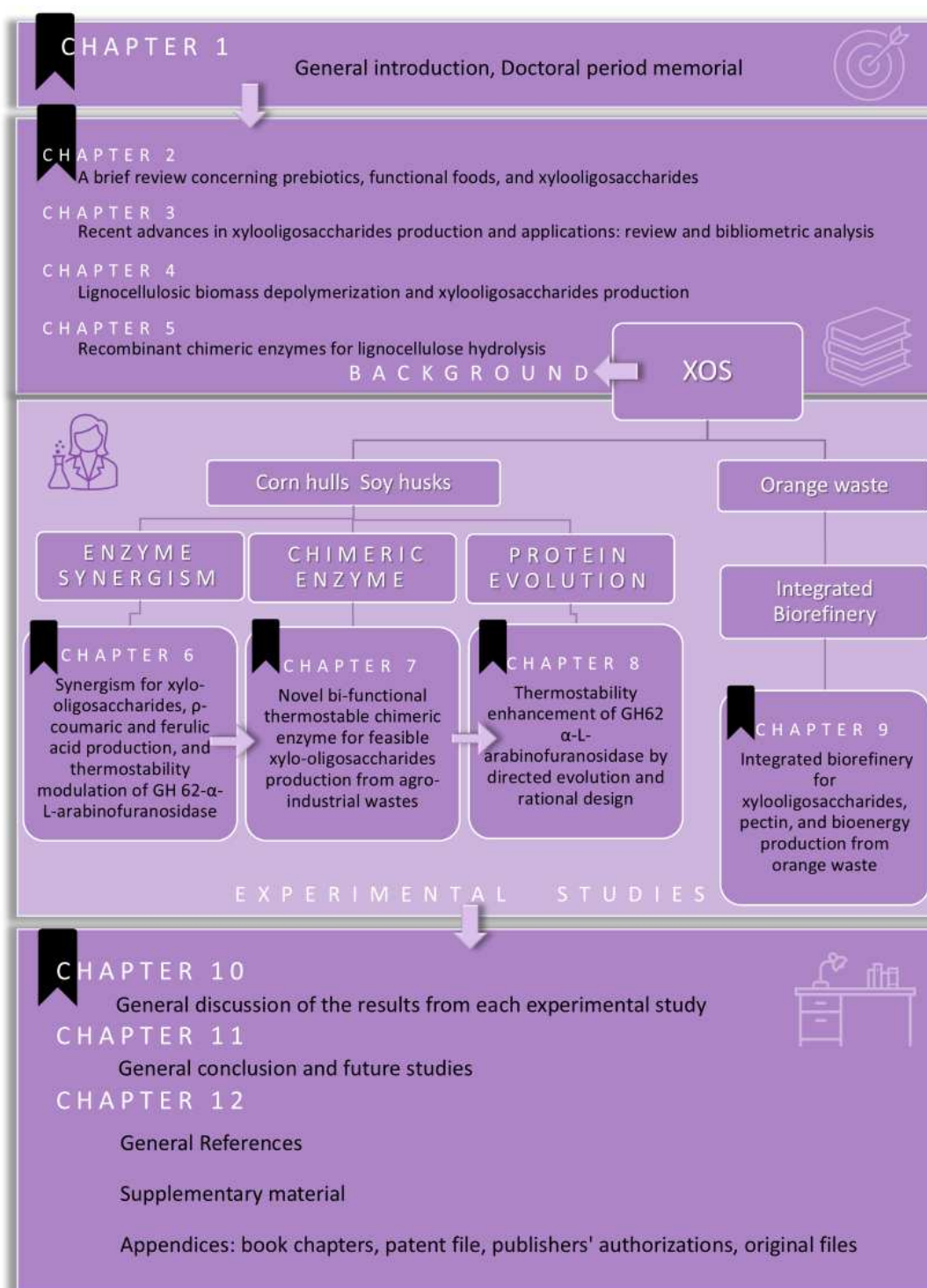


Figure 1-1: Thesis structure

#### 4. Doctoral Period Memorial

Ph.D. candidate Manoela Martins enrolled in the doctoral program at Unicamp in 2020 through the selection process of the Food Engineering Postgraduate Program. Her studies are financially supported by **FAPESP (2020/02871-2)**. Having completed the three mandatory subjects (Fenômenos de Transporte I (TP322), Termodinâmica (TP320), Fenômenos de Transporte II (TP323 -A)) during her master's program, in the initial year of her Ph.D. (2020), Manoela attended one mandatory subject, Seminários (TP199), and three elective subjects offered by the Food Engineering department: Tópicos Especiais em Engenharia de Alimentos (TP159, 2 credits), Processos de Transformação de Biomassa em Biocombustíveis (BI002), Produção Biotecnológica de Ingredientes para Indústria de Alimentos (TP359), totaling 8 credits. Additionally, she took one elective subject offered by the School of Philosophy, Sciences and Letters of Ribeirão Preto at the University of São Paulo FFCLRP, Estrutura e Função de Proteínas, with 4 credits. Furthermore, she started a voluntary teaching training internship PED C (CD003), on the subject of Unit Operations accumulating 2 credits, totaling 16 credits. The candidate achieved the **highest grade, A**, in all subjects.

In the second semester, the candidate performed the bioinformatics analysis of the proteins and started the design of the chimera. Besides that, she assisted in the covid research in **FIOCRUZ**. That was a time when significant efforts were being directed towards research for the development of an effective vaccine and rapid testing for COVID-19. Considering the candidate's expertise and the need for researchers in COVID-19-related projects, she sought an opportunity as a research assistant to collaborate on research at **Biomanguinhos** at **Fiocruz** in Rio de Janeiro, in the project "Development of improvements and new tests for the detection of SARS-CoV-2 and other respiratory viruses - MELKITCOVID." It is relevant to highlight that the research at Fiocruz contributed to a valuable gain of experience that facilitated the development of the candidate's doctoral work. The activities included nucleotide sequencing, the expression of recombinant chimeric peptides, as well as learning new methods that accumulated new knowledge and experience, such as the Phage-display technique.

In the first semester of 2021, the candidate initiated the experimental characterization of GH 62 arabinofuranosidase. The research related to her doctoral project was conducted in collaboration with prof Dr. Taisa Magnani Dinamarco laboratory. This period covered the review papers writing and the first experimental paper. In the second semester of 2021, she focused on the characterization of the agroindustrial wastes and the expression of the chimeric enzyme, followed by an in-depth analysis of the produced products. This period yielded another

published paper and underscored the imperative for additional investigations into the thermo-stability of GH 62.

Moving into the first semester of 2022, the candidate diversified the scope of her study to encompass the comprehensive characterization of orange processing waste and the conceptualization of a biorefinery designed to optimize its potential. She also guided and contributed to the experimental activities of her mentored scientific initiation student of the Lepeb research group. This period rendered a scientific paper reporting the technical economic feasibility of the proposed biorefinery and a report for **patent** application “*Processo de produção de pectina e xilo-oligossacarídeos a partir de resíduo industrial de suco de laranja*” (BR 10 2022 021023), filed in the national institute of intellectual property on October 17, 2022. Recognizing her accomplishments, Manoela was also honored with the **Inventor Award** in 2023, in the category deposited patent. Her biorefinery work attracted attention and was featured in notable publications in [Veja](#), [Forbes](#), and [FAPESP](#) magazines.

In the second semester of 2022, she undertook a **sandwich period** at **Johns Hopkins University (USA)** financially supported by **FAPESP (2022/04557-9)** and advised by Professor Dr. Marc Ostermeier, from the department of Chemical and Biomolecular Engineering. There, she had the opportunity to learn about fitness landscape and protein evolution by directed mutagenesis, expanding the horizon towards enzyme engineering, thereby expanding her research horizons through international collaboration with Professor Marc and Professor Dr. Jeffrey J. Gray and exposure to diverse academic perspectives.

Upon returning to Brazil at the end of the first semester of 2023, she engaged in further bioinformatic analysis of the results obtained during her exchange period, collaborating with Professor Dr. Munir Skaf from the Chemistry Department at UNICAMP, resulting in two scientific papers. Also, she was **awarded the second-best poster** presented in the 15<sup>th</sup> Latin American Symposium of Food Science and Nutrition (SLACAN).

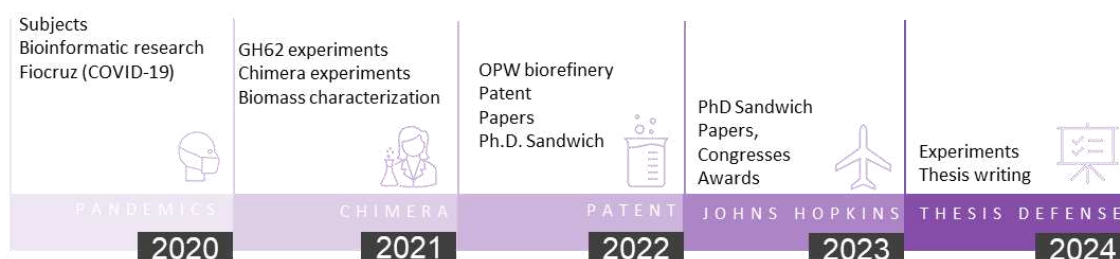


**Figure 1-2 Main activities performed during the Ph.D. period.**

The doctoral project's development resulted in 3 book chapters, 2 review papers, 4 experimental articles (so far), and 1 patent. Furthermore, together with her research group and collaborators, she produced 7 scientific papers, 1 book chapter, and 10 abstracts published in conference proceedings (see **Table 1-1** for detailed list).

The chronological progression outlined a multifaceted approach to her doctoral project, integrating laboratory experimentation, collaborative efforts, publication outcomes, and international exposure.

**Table 1-1 Quantitative index of doctoral period**

|    |                                               |
|----|-----------------------------------------------|
| 6  | First authored published articles             |
| 7  | Publications performed in partnership         |
| 4  | First authored book chapters                  |
| 2  | Awards                                        |
| 1  | Patent application                            |
| 1  | International internship                      |
| 5  | Conferences attended                          |
| 10 | Abstracts published in conference proceedings |

*Patent (see Appendix IV)*

**Manoela Martins**, Rosana Goldbeck.; PROCESSO DE PRODUÇÃO DE PECTINA E XILO-OLIGOSSACARÍDEOS A PARTIR DE RESÍDUO INDUSTRIAL DE SUCO DE LARANJA E XILOOLIGOSSACARÍDEOS (2022). INPI - Instituto Nacional da Propriedade Industrial. Country: Brazil. Patent of Invention. Register number: BR 10 2022 021023  
3. Deposit date: 10/17/2022. Depositor/Holder: University of Campinas.

***First authored articles published in peer-reviewed journals (see appendix VI)***

4. **Martins, M.**, dos Santos, A. M., Costa, C., Canner, S. W., Chungyoun, M., Gray, J. J., Skaf, M. S., Ostermeier, M., & Goldbeck, R. (2024). Thermostability enhancement of GH 62  $\alpha$ -L-arabinofuranosidase by directed evolution and rational design. *Journal of Agricultural and Food Chemistry*.
5. **Martins, M.**, Sganzerla, W. G., Forster-Carneiro, T., & Goldbeck, R. (2023). Recent advances in xylo-oligosaccharides production and applications: A comprehensive review and bibliometric analysis. *Biocatalysis and Agricultural Biotechnology*, 102608.
6. **Martins, M.**, Silva, M. F., Dinamarco, T. M., & Goldbeck, R. (2022). Novel bi-functional thermostable chimeric enzyme for feasible xylo-oligosaccharides production from agro-industrial wastes. *Process Biochemistry*, 122, 331-340.
7. **Martins, M.**, Tramontina, R., Squina, F. M., Dinamarco, T. M., & Goldbeck, R. (2022). Synergism for xylo-oligosaccharides,  $p$ -coumaric and ferulic acid production, and thermostability modulation of GH 62  $\alpha$ -l-arabinofuranosidase. *Biocatalysis and Agricultural Biotechnology*, 44, 102469.
8. **Martins, M.**, Dinamarco, T. M., & Goldbeck, R. (2020). Recombinant chimeric enzymes for lignocellulosic biomass hydrolysis. *Enzyme and microbial technology*, 140, 109647.
9. **Martins, M.**, & Goldbeck, R. (2023). Integrated biorefinery for xylooligosaccharides, pectin, and bioenergy production from orange waste. *Biofuels, Bioproducts and Biorefining*.

*Abstracts published in conference proceedings*

1. CELLO-OLIGOSACCHARIDES PRODUCTION FROM MULTI-STAGE ENZYMATIC HYDROLYSIS BY RICH CELLULOSE AGROINDUSTRIAL WASTES. Patrícia Ávila, **Manoela Martins**, Fatima Aparecida de Almeida Costa, Rosana Goldbeck. In: XXIII SINAFERM & XIV SHEB & XIV ENZITEC, 2022.
2. BI-FUNCTIONAL THERMOSTABLE XYLANOLYTIC CHIMERA FOR PREBIOTIC XYLO-OLIGOSACCHARIDES PRODUCTION. **Manoela Martins**, Taísa Magnani Dinamarco, Rosana Goldbeck. In: XXIII SINAFERM & XIV SHEB & XIV ENZITEC, 2022.
3. EVALUATION OF THE PREBIOTIC AND ANTIOXIDANT CAPACITIES OF SHORTCHAIN XYLO-OLIGOSACCHARIDES PRODUCED FROM AGRO-INDUSTRIAL RESIDUES. João Rafael Fernandes Baptista, Marcos Fellipe da Silva, **Manoela Martins**, Rosana Goldbeck. In: 2nd International Congress on Bioactive Compounds and 3rd International Workshop on Bioactive Compounds: Personal Nutrition, Ageing and Food Science, 2022.
4. TECHNO-ECONOMIC ANALYSIS OF INTEGRATED BIOREFINERY OF INDUSTRIAL ORANGE PROCESSING WASTES. **Manoela Martins** and Rosana Goldbeck. In: WCCE11 - Biotechnology, Bioeconomy, Bioindustry, 2023.
5. INTEGRATED BIOREFINERY OF ORANGE WASTES FOR XYLOOLIGOSACCHARIDES, PECTIN, AND BIOENERGY PRODUCTION. **Manoela Martins**, Rosana Goldbeck. In: 15th Latin American Symposium of Food Science and Nutrition (SLACAN), 2023. (*awarded the second-best poster presented*)
6. BUTTER ADDED WITH ENCAPSULATED XYLOOLIGOSACCHARIDE: THE IMPACT ON THE PHYSICAL PARAMETERS. Mariana Rosa, Elson Rogério Tavares Filho, **Manoela Martins**, Rosana Goldbeck, Jamile Maureen



de Sousa Oliveira, Erick Almeida Esmerino, Tatiana Colombo Pimentel, Marcia Cristina, Adriano Gomes da Cruz. In: 15th Latin American Symposium of Food Science and Nutrition (SLACAN), 2023.

7. FREE COMMENT ATTACK-EVOLUTION-FINISH (FC-AEF) AS AN APPROACH TO ESTABLISH A TEMPORAL SENSORY PROFILING OF BUTTER ADDED WITH ENCAPSULATED XYLOOLIGOSACCHARIDE. Mariana Rosa, Benjamin Mahieu, Elson Rogério Tavares Filho, **Manoela Martins**, Rosana Goldbeck, Erick Almeida Esmerino, Tatiana Colombo Pimentel, Marcia Cristina, Adriano Gomes da Cruz. In: 15th Latin American Symposium of Food Science and Nutrition (SLACAN), 2023.
8. COMPUTATIONAL INSIGHTS INTO THERMOSTABILITY ENHANCEMENT OF GH 62 ARABINOFURANOSIDASE FROM *ASPERGILLUS FUMIGATUS* authored by Alberto Monteiro, Clauber Henrique Souza da Costa, **Manoela Martins**, Rosana Goldbeck, Munir Skaf. In: 47th Annual Meeting of the Brazilian Biophysical Society, 2023.
9. DYNAMIC PROPERTIES OF CHIMERIC GLUCOSYL HYDROLASES PROTEIN IN THE PRESENCE OF CALCIUM IONS. Clauber Henrique Souza da Costa, Alberto Monteiro, **Manoela Martins**, Rosana Goldbeck, Munir Skaf. In: 47th Annual Meeting of the Brazilian Biophysical Society, 2023.
10. DEVELOPMENT OF SINGLE-CHAIN VARIABLE FRAGMENT (SCFV) ANTIBODY AGAINST COVID-19 BY PHAGE DISPLAY AS A POSSIBLE TOOL TO DIAGNOSTIC AND TREATMENT. Alexandre Bezerra Conde Figueiredo, Thiago dos Santos Chaves, Fernando de Paiva Conte, Rodrigo Nunes Rodrigues da Silva, Milena Mouta Verdan França Carvalho, **Manoela Martins**, Adriana de Souza Azevedo Soares, Sheila Maria Barbosa de Lima, Patrícia Cristina da Costa Neves. In: V Internacional Symposium of Immunobiologicals, 2021.

11.

***Publications performed in partnership during the Ph.D. period***

1. Rosa, M. C., Mahieu, B., Tavares Filho, E. R., Cavalcanti, R. N., **Martins, M.**, Sobral, L. A., ... & Cruz, A. G. (2023). Impact of adding xylooligosaccharides encapsulated in butter: Microstructural, optical, rheological and sensory aspects. *Food Research International*, 170, 113003. <https://doi.org/10.1016/j.foodres.2023.113003>
2. Wagle, A., Angove, M. J., Mahara, A., Wagle, A., Mainali, B., **Martins, M.**, ... & Paudel, S. R. (2022). Multi-stage pre-treatment of lignocellulosic biomass for multi-product biorefinery: A review. *Sustainable Energy Technologies and Assessments*, 49, 101702. <https://doi.org/10.1016/j.seta.2021.101702>
3. Lopes, A. M. M., **Martins, M.**, & Goldbeck, R. (2021). Heterologous expression of lignocellulose-modifying enzymes in microorganisms: current status. *Molecular Biotechnology*, 63, 184-199. <https://doi.org/10.1007/s12033-020-00288-2>
4. Ávila, P. F., Silva, M. F., **Martins, M.**, & Goldbeck, R. (2021). Cello-oligosaccharides production from lignocellulosic biomass and their emerging prebiotic applications. *World Journal of Microbiology and Biotechnology*, 37, 1-11. <https://doi.org/10.1007/s11274-021-03041-2>
5. Avila, P. F., **Martins, M.**, & Goldbeck, R. (2021). Enzymatic production of xylooligosaccharides from alkali-solubilized arabinoxylan from sugarcane straw and coffee husk. *BioEnergy Research*, 14, 739-751. <https://doi.org/10.1007/s12155-020-10188-7>
6. Dos Santos, A., Costa, C., **Martins, M.**, Goldbeck, R., Skaf, M. (2024). Exploring the Structural and Dynamic Properties of a Chimeric Glycoside Hydrolase in the presence of calcium ions. *Journal of Agriculture and Food Chemistry*.
7. Tramontina, R., Scopel, E., Cardoso, V. G. K., **Martins, M.**, da Silva, M. F., Flaibam, B., Salvador, M. J., Goldbeck, R., Damasio, A., Squina, F. (2024). Establishing agroindustrial lignocellulosic sources for lignin valorization strategies and application through production of flavor compounds and antioxidant supplements. *ACS Sustainable Chemistry & Engineering*.

*Book Chapters (see appendices)*

1. **Martins, M.,** Ávila, P. F., da Silva, M. F., de Melo, A. H. F., Lopes, A. M. M., & Goldbeck, R. (2022). Lignocellulose Bioconversion through Chemical Methods, Platform Chemicals, and New Chemicals. Lignocellulose Bioconversion Through White Biotechnology, 221-247. <https://doi.org/10.1002/9781119735984.ch9>
2. **Martins, M.,** Jiménez Castro, M. P., Soares Forte, M. B., Goldbeck, R. (2022). Brettanomyces: Diversity and Potential Applications in Industrial Fermentation. Júnior, S. L. A., Treichel, H., Basso, T. O., & Stambuk, B. U. (Eds.), Bentham Science Publishers. In: Yeasts: From Nature to Bioprocesses, volume 2. ISBN: 978 981 5051 07 0
3. **Martins, M.,** Ávila, P. F., Poletto, P., & Goldbeck, R. (2023). Polysaccharide degradation for oligosaccharide production with nutraceutical potential for the food industry. In Polysaccharide Degrading Biocatalysts (pp. 335-363). Academic Press. <https://doi.org/10.1016/B978-0-323-99986-1.00004-1>
4. **Martins, M.,** Ávila, P. F., da Silva, M. F., de Melo, A. H. F., E.X. Ramalho, & Goldbeck, R. (2022). Enzymatic Bioconversion Trends for Lignocellulosic Biorefineries. Handbook of Biorefinery Research and Technology, 1-20. Springer. [https://doi.org/10.1007/978-94-007-6724-9\\_29-1](https://doi.org/10.1007/978-94-007-6724-9_29-1)

## Awards

### I. Inventors Award 2023, deposited patent category, INOVA



### II. Second best presented poster in 15<sup>th</sup> SLACAN



**CHAPTER 2.**  
**A BRIEF REVIEW CONCERNING PREBIOTICS, FUNCTIONAL**  
**FOODS, AND XYLOOLIGOSACCHARIDES**

## Functional Foods and Prebiotics

To offer a new health tool for disease prevention and increased life expectancy, functional ingredients of natural origin have been increasingly sought after, showing significant effects on the quality of life of regular consumers. The term "functional foods" refers to a group of foods that contain bioactive molecules capable of benefiting various bodily functions, making their consumption relevant both for improving health status and reducing the risk of disease development in individuals (Ozen et al., 2012).

They are not pills or capsules but part of a food formulation. Thus, a functional food is a natural product or a product that has been modified to exhibit functional influence on the consumer's health. The concept of functional food relates to the improvement of the circulatory system, immunoregulation, disease prevention, and slowing down of aging. They include bioactive substances naturally present in foods (e.g., dietary fibers); foods supplemented with bioactive substances (such as probiotics and antioxidants); ingredients derived from other sources introduced into food formulations, such as **prebiotics** (Al-Sheraji et al., 2013).

Any ingredient that resists the action of digestive enzymes in the gastrointestinal tract and stimulates the growth of beneficial bacteria present in the intestine can be considered a potential **prebiotic**. A deep explanation about this topic is presented in the **published (first-authored) book chapter** presented in **Appendix III: Polysaccharide degradation for oligosaccharide production with nutraceutical potential for the food industry**, devoted to describing the properties of a wide range of prebiotic fibers for functional food applications. The use of prebiotics in foods presents multiple advantages, as they also act on sensory characteristics and promote a more balanced nutritional composition. Prebiotics increase the fiber content of foods and prolong the shelf life of cereals by preserving their crispness for longer. Their water solubility also allows them to be added to beverages, such as yogurts and salad dressings. Due to their gel-forming property, prebiotics improve the texture of low-fat foods without affecting the taste. Moreover, they are frequently added to low-calorie foods, such as diet chocolates and fruit yogurts, given their sweet taste (Yan et al., 2022).

Oligosaccharide-based prebiotics have recently attracted considerable interest due to the discoveries of their multidimensional benefits and the possibility of obtaining them from low-cost agricultural residues, which are abundant and renewable in nature. These agricultural residues consist mainly of cellulose and hemicellulose, a cheap for new functional ingredients to be added to food formulations through (chemical or enzymatic) hydrolysis of these

polysaccharides into low-degree oligomers, which include xylooligosaccharides (XOS) with remarkable prebiotic and antioxidant activity (Faryar et al., 2015).

### **What are Xylooligosaccharides and why study them?**

Xylo-oligosaccharides (XOS) are oligomers consisting of 2 to 10 xylose monomers linked by  $\beta$  1-4 bonds named according to the number of monomers, naturally found in vegetables, fruits, milk, honey, and bamboo. Their industrial-scale production comes from the hydrolysis of lignocellulosic materials. XOS are used in food formulations due to their prebiotic properties that benefit consumer health. For food applications, degrees of polymerization from 2 to 4 are desirable. Although xylotriose (degree of polymerization equal to 2) is not considered an oligosaccharide, for food applications, it is considered a xylo-oligosaccharide due to its characteristics and health effects (V. Kumar et al., 2021; Poletto et al., 2020; Santibáñez et al., 2021).

In addition to the prebiotic effect, XOS has been explored in agriculture as growth stimulants, and in the pharmaceutical industry as medication related to obesity control and treatment of gastrointestinal infections. Another aspect is their use, along with other prebiotics, in animal feed, replacing antibiotics either totally or partially, as excessive use of these drugs can lead to bacteria resistance, including pathogenic ones, and there is a possibility of finding antibiotic residues in their meats. Regarding industrial production, XOS have favorable properties for incorporation into foods, as they demonstrate acceptable taste and odor, are non-cariogenic, are present greater stability at acidic pH and pasteurization temperatures. Their effect of delaying carbohydrate absorption, combined with their low energy value and sweet taste (about 30% of the sweetness of sucrose), allows for efficient application in products for diets with sugar restrictions for diabetics (H. H. Chen et al., 2012; Freitas et al., 2019a).

As the name suggests, the precursor of xylo-oligosaccharides is xylan, which accounts for 25% to 35% of the dry biomass of lignocellulosic materials. Exploring the best substrates rich in xylan to increase the efficiency of XOS production enhances the economic viability of the process. Starting from a xylan-rich source, glycosidic and heterocyclic ether-type bonds of the xylan chain are hydrolyzed, releasing compounds with low degrees of polymerization (XOS). Due to the increasing production of agro-industrial residues generated each year, the application of these materials in the development of high-value-added products has been the subject of intense research. An important fraction of these residues is hemicellulose (xylan), which can be converted into XOS, increasing the energy efficiency of lignocellulosic material

conversion plants, aiming to achieve significant economic and environmental impact in the sectors where this material originates (Freitas et al., 2019a; Santibáñez et al., 2021).

As demonstrated by the literature, XOS exhibit their prebiotic effects at a lower concentration compared to other classes of prebiotics, making their commercial production competitive. This has led to a focus on research for developing alternatives to improve the efficiency of the production process, thus reducing costs. Recent advancements have been made in environmentally friendly processes, integrating strategies for producing XOS from agricultural residues and using genetic engineering to enhance hydrolytic enzymes and host microorganisms. Additionally, the increasing *in vivo* information about the effects of XOS on the body further increases their commercial value (Amorim et al., 2019).

According to a study by "Global Info Research," The global Xylooligosaccharides Polymer market size is expected to reach \$ 34 million by 2029, rising at a market growth of 1.4% CAGR during the forecast period (2023-2029). Fructo-oligosaccharides (FOS) are currently the main functional oligosaccharides consumed in developed countries; however, their efficacy is not as good as that of XOS. Besides, the growing public's health consciousness in developed countries hints that products with health care function will become more popular (Report, 2018). Despite their benefits, XOS are still relatively expensive, with prices ranging from \$22 to \$50 per kilogram, depending on the level of purity (from 30 to 95% in powder form). Thus, their production from abundant sources rich in xylan from agro-industrial residues can significantly contribute to cost reduction, enabling better integration of XOS into the global market and application in food and pharmaceutical formulations (Otieno & Ahring, 2012).

The next **CHAPTER 3** is a bibliometric analysis that elucidates the state of art of xylooligosaccharides production and applications. Through bibliometric analysis, emerging trends, research gaps, and future opportunities were scrutinized, encompassing XOS production via both chemical and enzymatic routes, purification techniques, health benefits, and advancements in new product development. Recent studies have predominantly focused on innovating technologies for XOS production, aligning with the ethos of biorefinery models, particularly emphasizing the valorization of agricultural and industrial byproducts within a circular bioeconomy framework.

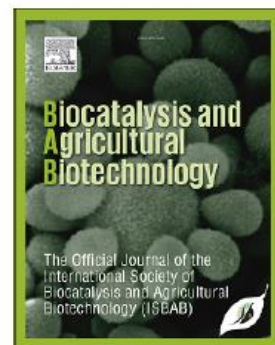


**CHAPTER 3.**  
**RECENT ADVANCES IN XYLO-OLIGOSACCHARIDES**  
**PRODUCTION AND APPLICATIONS: A COMPREHENSIVE**  
**REVIEW AND BIBLIOMETRIC ANALYSIS**

## Recent advances in xylo-oligosaccharides production and applications: A comprehensive review and bibliometric analysis

Manoela Martins, William Gustavo Sganzerla, Rosana Goldbeck  
Research article published in Biocatalysis and Agricultural Biotechnology  
Volume 47, January 2023, 102608.

DOI: [10.1016/j.bcab.2023.102608](https://doi.org/10.1016/j.bcab.2023.102608)



### First things first

This bibliometric analysis was conducted to examine the current landscape of xylooligosaccharides (XOS) production and applications. This analysis scrutinized emerging trends, identified research gaps, and highlighted future opportunities. The key areas explored include XOS production methods utilizing both chemical and enzymatic routes, purification techniques, health benefits, and advancements in new product development. Recent studies have primarily concentrated on technological innovations in XOS production, reflecting the principles of biorefinery models. This focus emphasizes the valorization of agricultural and industrial byproducts within a circular bioeconomy framework, which is the whole purpose of this present thesis. Therefore, following this bibliometric analysis is the introduction about the enzymatic conversion of lignocellulosic materials as well as the presentation of the three agroindustrial residues chosen for this thesis research in **CHAPTER 4**.

### **Abstract**

This comprehensive review elucidates the state of the art of xylo-oligosaccharides (XOS) production and applications. Emerging trends, gaps, and opportunities for future research were evaluated by bibliometric analysis, covering XOS production by chemical and enzymatic methods, purification, health benefits, and new products development. In total, 1256 publications (1217 articles and 39 reviews) were recorded from 2000 to 2021, the selected timespan for the bibliometric analysis. The network analysis of keywords revealed that XOS research had been divided into five main knowledge areas: prebiotics, autohydrolysis, enzymatic hydrolysis, xylan pretreatment, and xylanase. The prebiotic activity of XOS is a deeply investigated field, and this hot topic still demands future research. Autohydrolysis was shown to be a base niche, while enzymatic hydrolysis is gaining more interest. Most recent research is focused on the development of new technologies for XOS production toward a biorefinery concept, especially with the valorization of agro-industrial wastes in a circular bioeconomy. In conclusion, XOS is a value-added biological macromolecule with scientific and industrial interest that can be obtained from lignocellulose biorefineries and applied to develop functional foods.

**Keywords:** Bioeconomy; Biorefinery; Lignocellulosic biomass; Prebiotic; Xylan

### 3.1. Introduction

The high occurrence of public health problems, especially obesity, hypertension, and diabetes, raises people's awareness and increases the demand for natural foods with reduced fat, salt, and sugar, as well as functional foods containing prebiotics and probiotics, as they are considered potential therapeutics with health benefits (Ozen et al., 2012). Non-digestible oligosaccharides (NDOs) are prebiotic carbohydrates made up of low molecular weight compounds that stimulate the growth of beneficial intestinal microbiota. Among the different classes of NDOs, xylo-oligosaccharides (XOS) are an emergent food ingredient produced from inexpensive and renewable sources such as agro-industrial by-products. The multidimensional influence of XOS on human health makes it a potential agent against several gastrointestinal disorders, insulin resistance, and even cancer (Vázquez et al., 2000). There is a growing awareness of the role of human gut microflora in maintaining host health. Several studies have revealed the beneficial effects of XOS for its bifidogenic activity, reduction in blood cholesterol and glucose levels, and increase in mineral absorption, immunomodulation, antioxidant, and anti-inflammatory activities (Ávila, Martins, Costa, et al., 2020; T. Li et al., 2022; Valls et al., 2018; X. Yu et al., 2015).

At present, more investigation is necessary to fully understand the molecular mechanisms underlying the beneficial effects of XOS on health. However, the scientific community and industries have made efforts to prove that it is possible to produce XOS from several types of agro-industrial wastes composed of cellulose, hemicellulose, and lignin (Ávila, Martins, Costa, et al., 2020; Gullón et al., 2011), including forest and pulping waste from hardwood and softwood. Xylan, the hemicellulosic main chain, is highly important for XOS production via autohydrolysis or enzymatic hydrolysis. Many treatments have been investigated to optimize the recovery of xylan from these wastes and its conversion to short-chain xylose oligomers (i.e., XOS). Additionally, from the lignocellulosic material, several bioproducts can be produced and applied in the food, energy, and chemical industries in a biorefinery concept advocating circular bioeconomy (S. F. Ferreira et al., 2021; Sganzerla et al., 2021). The production of bio compounds and biofuels from lignocellulosic biomass is increasing due to the new policies of replacement of fossil-based carbon, sustainability, waste management, and land competition for food production (Álvarez et al., 2017; Gullón et al., 2011; B. Pereira et al., 2021; Pinales-Márquez et al., 2021b; Praveen et al., 2017).

Recent reviews presented the production of XOS from lignocellulosic biomass, purification technologies, applications, and health benefits (Anarado et al., 2022; Pinales-Márquez

et al., 2021b; Santibáñez et al., 2021). However, a bibliometric analysis of XOS was not reported in the literature. The use of bibliometric tools can be a promising approach to perform a complete analysis of scientific publications related to all research areas, addressing gaps, identifying trends, and supporting the establishment of future research and perspectives (Aria & Cuccurullo, 2017; van Eck & Waltman, 2010). Due to the increasing interest in XOS production from sustainable sources and their application in functional food, this study aimed to address the status of XOS research regarding the technological routes of production, the health benefits investigation, and advances toward the development of an integrated biorefinery for the valorization of agro-industrial wastes as well as the application as a functional food ingredient.

### **3.2. Methodology for literature search and bibliometric analysis**

The bibliometric study used scientific output data from the core collection of Science Citation Index Expanded (SCI-E) – Clarivate Analytics' ISI – Web of Science®. The type of search applied was “advanced search” applying the following logic operation: TS= ("xylo-oligosaccharides" OR "xylooligosaccharides" OR "xyloligosaccharides" OR "xylo-oligosaccharide" OR "xylooligosaccharide" OR "xyloligosaccharide") NOT KP= ("xylo-oligosaccharides" OR "xylooligosaccharides" OR "xyloligosaccharides" OR "xylo-oligosaccharide" OR "xylooligosaccharide" OR "xyloligosaccharide").

This search query was used to obtain reliable and accurate details based on selected words included in the title, abstract, author's keywords and not in the keywords-plus of each document on the topic. The types of documents were filtered to “article” and “review”. The systematic search was conducted from 2000 to 2021, aiming to observe the publication's evolution over the years and the most frequent research areas. This timespan was selected based on guidelines established in previous studies (Gabriel da Rosa et al., 2022; Sganzerla & da Silva, 2022; Sillero et al., 2022).

In the selected timespan, 1256 publications were recorded (1217 articles and 39 reviews). The data from the documents obtained were exported and analyzed using bibliometric software. The “Bibliometrix” package (R language) was used to obtain a three-field plot and the top authors' production over the timing diagram (Aria & Cuccurullo, 2017). After that, the dataset was investigated on VOSviewer© software to construct the authors, sources and country's networks, and the authors' keyword clusters (van Eck & Waltman, 2010). All the data used in the bibliometric analysis were obtained from the Web of Science©. The most cited documents were evaluated to understand the importance and repercussions of different aspects of XOS.

### 3.2.1. Bibliometric analysis of xylo-oligosaccharides

The bibliometric analysis of XOS production and application is presented in the following sections, providing insightful data on research progress, updates, and trends.

### 3.2.2. Publication evolution and research areas

**Figure 3-1** reveals a total of 1256 publications (1217 articles and 39 reviews) related to XOS over the selected timespan (2000 – 2021), with significant increases from 2020 and 2021, jumping from 105 to 174 publications. The constant increase in the number of publications represents the active interest of researchers in the subject and market trend. Biotechnology Applied Microbiology is the predominant area (22.7%), followed by Food Science Technology (16.2%), which can be associated with the increased demand for functional foods and is considered a hot topic in the field. Notwithstanding, the market of XOS presents an annual growth rate of approximately 4.1% and is projected to reach US\$ 130 million by 2025 (L. B. Brenelli et al., 2022). The application of XOS in food mainly includes the development of symbiotic foods (containing both prebiotic and probiotic microorganisms) such as drinks composed of probiotic bacteria, XOS, tea extract, and minerals (Amorim et al., 2019).

### 3.2.3. Study of the main keywords

Co-occurrence keyword analysis has been widely used to identify hot research topics and gaps (**Table 3-1, Table 3-2**). From this search over the previous 21 years, there are 2717 keywords. The 20 most used keywords are displayed in Table 3-1. In addition, the bibliometric coupling of the most used keywords is presented in **Figure 3-2**, where the 75 most used keywords were divided into 5 clusters. Each cluster was associated with a specific research field, and the keywords were considered given a minimum of 7 occurrences. The keyword “xylo-oligosaccharides” was the keyword with the highest link strength and occurrence. As previously elucidated, there are different keyword combinations to express the XOS, but with less occurrence. The most frequent keywords used in the ranked documents according to the strongest link were “xylan” and “xylanase”, which reveals the importance of the enzymatic hydrolysis technology of xylan bioconversion into XOS. Interestingly, xylanase appears in a different cluster than enzymatic hydrolysis, suggesting the greater importance of xylanase in this field for XOS production compared to other xylanolytic enzymes. In fact, the hemicellulose fraction of the lignocellulosic biomass utilized for XOS production is mainly composed of xylose units, followed by branched chains of other types of sugars, such as arabinose and glucose. Moreover, it was observed that the keyword “prebiotic” was presented in a separate cluster, which indicates a distinct research field with a strong connection between the other clusters.

The network analysis of keywords can be plotted over the publication years (**Figure 3-2**). The colors ranging from blue to yellow illustrate the trend of XOS over the years, considering the average terms used. The research fields related to “prebiotic”, “microbiota”, and “enzymatic hydrolysis” were most expressive between 2016 and 2018, while the fields related to “autohydrolysis” were expressive in approximately 2012 but not in most recent years. Hence, there is a trend in studying XOS production by enzymatic methods rather than autohydrolysis, as well as the bioactivities of XOS as prebiotic and their effects on microbiota.

**Figure 3-3** shows a thematic map grouping the keywords according to the relevance and development degree of the research field. This map is an intuitive research theme plot divided into four quadrants: i) niche (very specialized) themes; ii) motor themes; iii) emerging or declining themes, and iv) basic themes (de Oliveira et al., 2022) In this bibliometric study, the keywords “xylooligosaccharides”, “xylan”, and “xylanase” are considered basic themes, which means that they are important for the research field but are not fully developed, that is, these keywords present low density and high centrality and more research and analysis on this matter is required, and when the research around these keywords will be further substantiated, they may become motor themes. The results suggest that the technology around xylan pretreatment and xylanase production still requires improvements to increase XOS yield and feasibility (Pinales-Márquez et al., 2021b). Further studies could be conducted to elucidate biomass pretreatment technologies, the choice of xylan sources, and enzyme engineering to develop thermostable and highly specific xylanases.

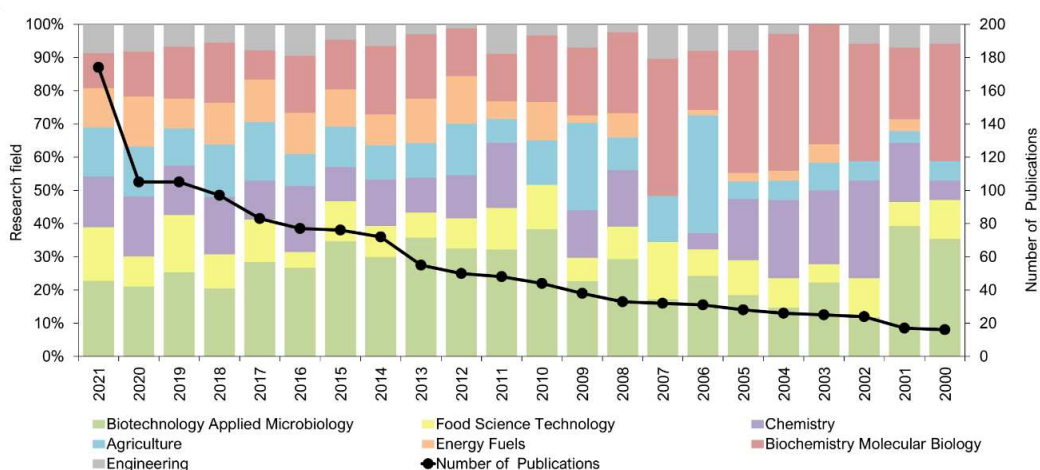


**Figure 3-1** Evolution of the number of publications over the years (2000-2021) in the field of xylo-oligosaccharides

**Table 3-1 Clusters of the main keywords obtained on VOSviewer software**

| Clusters | # of items | Keywords on the VOSviewer network                                                                                                                                                                                                                                                                                                                                                                                              |
|----------|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1        | 21         | Alpha-l-arabinofuranosidase, <i>Aspergillus niger</i> , $\beta$ -d-xylosidase, $\beta$ -xylosidade, carbohydrate-binding, characterization, crystal structure, endo-xylanase, glycoside hydrolase, heterologous expression, hydrolysis, immobilization, <i>Pichia pastoris</i> , purification, steam Explosion, thermostability, thermostable xylanase, trans-glycosylation, xylan hydrolysis, xylanase, xylo-oligosaccharide. |
| 2        | 15         | Arabinoxylan, <i>Bacillus subtilis</i> , bifidobacteria, fermentation, growth performance, gut microbiota, microbiota, oligosaccharides, prebiotic, prebiotics, probiotic, probiotics, synbiotic, XOs, xylooligosaccharide.                                                                                                                                                                                                    |
| 3        | 12         | Antioxidant activity, arabinofuranosidase, corncob, glucuronoxylan, glycoside hydrolase Family, NMR, substrate specificity, wheat straw, wheat bran, xylo-oligosaccharides, xylobiose, xylooligosaccharides (XOs).                                                                                                                                                                                                             |
| 4        | 12         | Bioethanol, biomass, cellulase, cellulose, enzymatic hydrolysis, hemicellulose, hydrothermal pretreatment, lignin, lignocellulose, poplar, <i>Trichoderma reesei</i> , xylooligosaccharides.                                                                                                                                                                                                                                   |
| 5        | 8          | Autohydrolysis, biorefinery, furfural, hemicelluloses, oligosaccharide, response surface methodology, sugarcane bagasse, xylose.                                                                                                                                                                                                                                                                                               |
| 6        | 7          | Corn stover, endoxylanase, lignocellulosic biomass, pretreatment, xylan, xylanases, xylosidade.                                                                                                                                                                                                                                                                                                                                |

**Table 3-2 Clusters of the main keywords obtained on Bibliometrix software**

| Clusters | Main keyword          | # of items | Keywords on Bibliometrix network                                                                                                                             |
|----------|-----------------------|------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1        | B-xylosidase          | 10         | B-xylosidase, xylose, xylo-oligosaccharide, glycoside hydrolase, substrate specificity, xylobiose, crystal structure, wheat bran, cellulase, oligosaccharide |
| 2        | Enzymatic hydrolysis  | 10         | Enzymatic hydrolysis, autohydrolysis, biorefinery, bioethanol, corncob, lignin, hemicellulose, hydrothermal pretreatment, biomass, pretreatment              |
| 3        | Xylooligosaccharides  | 10         | Xylooligosaccharides, xylanase, xylan, xylooligosaccharide, hemicellulose, purification, sugarcane bagasse, xylanases, hydrolysis, characterization          |
| 4        | Xylo-oligosaccharides | 10         | Xylo-oligosaccharides, oligosaccharides, prebiotic, XOs, fermentation, gut microbiota, arabinoxylan, synbiotic, growth performance, probiotic                |
| 5        | Prebiotic             | 4          | Prebiotic, probiotics, synbiotics, Bifidobacterium                                                                                                           |



**Table 3-3 The top 20 keywords in the field (rank based on the occurrences).**

| <b>Ranking</b> | <b>Keyword</b>        | <b>Occurrences</b> | <b>Total link strength</b> |
|----------------|-----------------------|--------------------|----------------------------|
| 1              | Xylooligosaccharides  | 243                | 429                        |
| 2              | Xylanase              | 172                | 324                        |
| 3              | Xylan                 | 103                | 233                        |
| 4              | Xylooligosaccharide   | 75                 | 124                        |
| 5              | Enzymatic hydrolysis  | 72                 | 144                        |
| 6              | Xylo-oligosaccharides | 72                 | 110                        |
| 7              | Prebiotics            | 61                 | 97                         |
| 8              | B-xylosidase          | 54                 | 109                        |
| 9              | Oligosaccharides      | 47                 | 62                         |
| 10             | Prebiotic             | 44                 | 82                         |
| 11             | Autohydrolysis        | 43                 | 93                         |
| 12             | Hemicellulose         | 40                 | 90                         |
| 13             | Xylose                | 38                 | 92                         |
| 14             | Purification          | 29                 | 66                         |
| 15             | Biorefinery           | 26                 | 59                         |
| 16             | Xylo-oligosaccharide  | 26                 | 27                         |
| 17             | Sugarcane bagasse     | 24                 | 45                         |
| 18             | Glycoside hydrolase   | 23                 | 57                         |
| 19             | Xylanases             | 22                 | 37                         |
| 20             | Bioethanol            | 20                 | 54                         |

Otherwise, “xylose” and “ $\beta$ -xylosidase” are classified as motor themes. However, these keywords are not related to XOS production since  $\beta$ -xylosidase is responsible for the cleavage of xylobiose and xylose release. The enzymatic cocktail for XOS production must be poor in  $\beta$ -xylosidase activity to improve yields and avoid an excessive production of xylose, which is considered an impurity of the production and prejudice the downstream purification process (Carvalho et al., 2013). “B-xylosidases” are often studied to improve the efficiency of saccharification and fermentation of XOS. Bioethanol production from xylose (product of XOS depolymerization) is essential for reducing enzymatic inhibition and taking full advantage of lignocellulose biomass (Niu et al., 2019).

At the center of relevance and occupying the niche and motor themes, “enzymatic hydrolysis”, “autohydrolysis”, and “biorefinery” are important keywords found in every research paper related to XOS production. There is constant investigation toward the development of an integrated biorefinery of XOS using sustainable biomass from lignocellulosic wastes

(straws, bagasse, peels, and husks from crops) by autohydrolysis or enzymatic hydrolysis. Both are efficient technologies, but each presents specific bottlenecks that must be assessed (V. Kumar et al., 2021).

The keywords “prebiotic”, “probiotic”, and “synbiotic” are located at the center of development degree (research density) between niche themes and emerging or declining themes. Two conclusions may be obtained from this chart: i) the prebiotic potential of XOS was the main reason for the growing interest in XOS production; and ii) the development of functional food with XOS could be of industrial interest, especially if obtained from sustainable sources, to replace artificial sweeteners, emulsifiers, and fat while providing several health benefits, such as antioxidant and prebiotic activities. Therefore, the bioactivities of XOS should be deeply investigated to consolidate XOS as an emerging prebiotic to raise industrial interest. At the same time, new investigations are being made to explore further the potential application of XOS in the field of gut microbiota. Therefore, the research field of XOS bioactivities is very specialized and represents a recent research trend.

#### 3.2.4. The most cited publications

A good indicator to identify the most advanced and influential topics in a research field is the most cited publications. **Table 3-4** presents the ten most cited research articles and review papers in the XOS field over the last 20 years. The first four papers and the 7<sup>th</sup> are related to the prebiotic potential, mainly *in vitro* and one in mice. These papers did not exclusively report XOS effects, but XOS presented the most promising results compared to the other evaluated prebiotics. The attention given to these articles shows the strong interest in establishing XOS as a functional ingredient in the food industry. The 3<sup>rd</sup> paper was the most recent, from 2014, while the 1<sup>st</sup> is the oldest, published in 2001. The following most cited articles reported the production of XOS by autohydrolysis of agro-industrial by-products. Only the 9<sup>th</sup> paper of this list reported enzymatic hydrolysis using xylanase.

In contrast, the most cited review presented a comprehensive overview of manufacturing and application of XOS. The 1<sup>st</sup> and 2<sup>nd</sup> papers are quite similar and present the production from lignocellulosic biomasses, application in food, and market perspectives. The reviews occupying the 3<sup>rd</sup>, 7<sup>th</sup>, 9<sup>th</sup>, and 10<sup>th</sup> positions compile the health-related effects, but they are not focused solely on the XOS, discussing other classes of prebiotic and cereal-derived oligosaccharides. The 4<sup>th</sup> paper discusses the utilization of xylan to obtain bioproducts, including XOS. Although not focused on XOS itself, this review establishes xylan from agro-industrial wastes as the most important source for XOS production. The 5<sup>th</sup> paper was wrongly submitted as a

**Figure 3-2 Clustering of the 75 most frequent authors' keywords. (a) Term map based on different clusters; (b) Term average year map; (c) Density visualization map.**

Based on the most cited documents, the prebiotic potential is well established and still under constant investigation; autohydrolysis is commonly associated with its production; however, enzymatic hydrolysis from agro-industrial wastes and the development of new enzymes seems to be a trend in the XOS research field, following **Figure 3-3**.

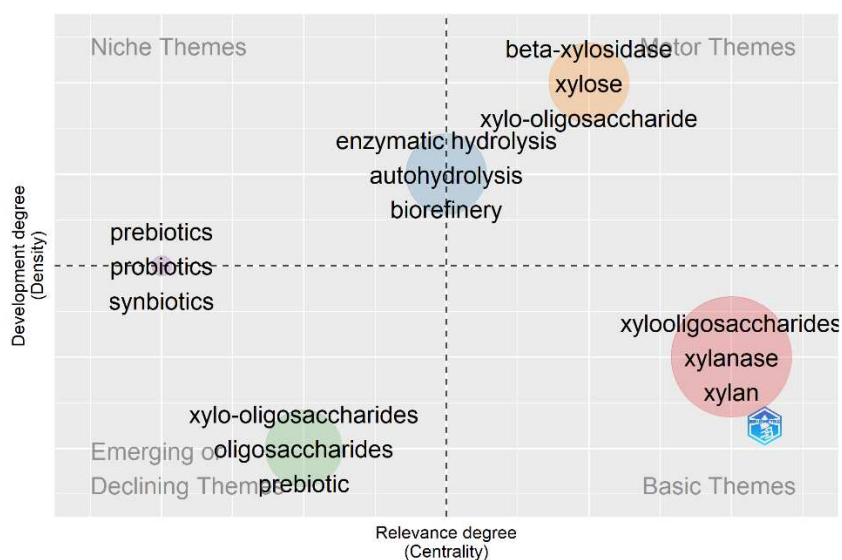


**Figure 3-3** Thematic map of the clusters from the 100 most frequent authors' keywords.

Table 3-4 The top 10 most cited articles and reviews.

| Ranking          | Title                                                                                                                                | Issue                                                                                                                                                                                                                                                                                                               | Journal                                        | Citations | Year | Reference                    |
|------------------|--------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------|-----------|------|------------------------------|
| <b>Articles</b>  |                                                                                                                                      |                                                                                                                                                                                                                                                                                                                     |                                                |           |      |                              |
| 1 <sup>st</sup>  | A comparative <i>in vitro</i> evaluation of the fermentation properties of prebiotic oligosaccharides                                | Comparison of <i>in vitro</i> fermentation properties of commercial prebiotic oligosaccharides.                                                                                                                                                                                                                     | Journal of Applied Microbiology                | 467       | 2001 | (Rycroft et al., 2001)       |
| 2 <sup>nd</sup>  | The Impact of a Consortium of Fermented Milk Strains on the Gut Microbiome of Gnotobiotic Mice and Monozygotic Twins                 | Characterization of the fecal microbiomes and metatranscriptomes of mice after consumption of fermented milk containing probiotics. <i>B. lactis</i> was the dominant member of the consortium due to the catabolism of xylooligosaccharides.                                                                       | Science Translational Medicine                 | 384       | 2011 | (McNulty et al., 2011)       |
| 3 <sup>rd</sup>  | Prebiotic stimulation of human colonic butyrate-producing bacteria and <i>bifidobacteria</i> , <i>in vitro</i>                       | Reports carbohydrate utilization patterns for representative butyrate-producing anaerobes on different substrates. XOS was the most selective growth substrate.                                                                                                                                                     | Fems Microbiology Ecology                      | 235       | 2014 | (Scott et al., 2014)         |
| 4 <sup>th</sup>  | <i>In vitro</i> fermentation of cereal dietary fiber carbohydrates by probiotic and intestinal bacteria                              | A range of probiotics and other intestinal bacteria were examined for their ability to ferment the dietary fiber. XOS was efficiently fermented by <i>Bacteroides</i> isolates, <i>Bifidobacterium</i> , and <i>Lactobacillus</i> but not by <i>E. coli</i> , enterococci, and <i>Clostridium</i> species examined. | Journal of the Science of Food and Agriculture | 225       | 2002 | (Crittenden et al., 2002)    |
| 5 <sup>th</sup>  | Production of oligosaccharides by autohydrolysis of brewery's spent grain                                                            | Various temperatures and reaction times were investigated for the hemicellulose treatment of brewery's spent grain. The maximal XOS yield (61% of the feedstock xylan) was obtained at 190 °C after 5 min of reaction.                                                                                              | Bioresource Technology                         | 214       | 2004 | (Carvalho, 2004)             |
| 6 <sup>th</sup>  | Autohydrolysis of corn cob: the study of nonisothermal operation for xylooligosaccharide production                                  | Up to 94% of xylan was removed, producing XOS and other byproducts (including xylose and furfural). Series reactions modeled the autohydrolysis.                                                                                                                                                                    | Journal of Food Engineering                    | 208       | 2002 | (Garrote et al., 2002)       |
| 7 <sup>th</sup>  | Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats | This study reports that XOS is superior to FOS in microbiota growth, decreasing the cecal pH and serum triglyceride concentration and increasing the total cecal weight and <i>bifidobacteria</i> population.                                                                                                       | Journal of Nutrition                           | 200       | 2004 | (C.-K. Hsu et al., 2004)     |
| 8 <sup>th</sup>  | Autohydrolysis of agricultural byproducts for the production of xylo-oligosaccharides                                                | Six agricultural residues were tested for XOS production. The yield of XOs depended on the content of xylan and its accessibility and was proportional to the acetyl content of the raw materials.                                                                                                                  | Carbohydrate Polymers                          | 185       | 2007 | (Nabarlatz et al., 2007)     |
| 9 <sup>th</sup>  | Production of xylooligosaccharides from corn cob xylan by fungal xylanase and their utilization by probiotics                        | Corn cob xylan was extracted using the alkaline treatment and hydrolyzed by fungal xylanase. XOS purification was done using activated charcoal. Prebiotic effects were also evaluated <i>in vitro</i> .                                                                                                            | Bioresource Technology                         | 176       | 2012 | (Chapla et al., 2012)        |
| 10 <sup>th</sup> | Hydrothermally treated xylan-rich byproducts yield different classes of xylo-oligosaccharides                                        | Branched XOS was obtained by autohydrolysis of 4 xylan-rich agricultural residues                                                                                                                                                                                                                                   | Carbohydrate Polymers                          | 176       | 2002 | (Kabel, 2002)                |
| <b>Review</b>    |                                                                                                                                      |                                                                                                                                                                                                                                                                                                                     |                                                |           |      |                              |
| 1 <sup>st</sup>  | Xylooligosaccharides: manufacture and applications                                                                                   | A comprehensive review on xylooligosaccharides production from lignocellulosic materials by chemical or enzymatic methods, purification, and application as food ingredients                                                                                                                                        | Trends In Food Science & Technology            | 459       | 2000 | (Vázquez et al., 2000)       |
| 2 <sup>nd</sup>  | Advances in the manufacture, purification, and applications of xylo-oligosaccharides as food additives and nutraceuticals            | A comprehensive review on XOS production from lignocellulosic materials by chemical or enzymatic methods, utilization in synbiotic preparations, technological properties, and market perspectives.                                                                                                                 | Process Biochemistry                           | 383       | 2006 | (Moure et al., 2006)         |
| 3 <sup>rd</sup>  | Prebiotic and Other Health-Related Effects of Cereal-Derived Arabinoxylans, Arabinoxylan-Oligosaccharides, and Xylooligosaccharides  | This review focuses on the AXOS and XOS prebiotic effects in the colon of humans and animals. <i>In vitro</i> experiments and <i>in vivo</i> intervention studies on animals or humans are discussed that have investigated potential health-related effects resulting from the dietary intake of AX, AX            | Critical Reviews in Food Science and Nutrition | 349       | 2011 | (Broekaert et al., 2011)     |
| 4 <sup>th</sup>  | From plant biomass to biobased chemicals: Latest developments in xylan research                                                      | This review is focused on xylan and xylan-derived products (ethanol, xylitol, xylo-oligosaccharides). It discusses extraction and purification methodologies, chemical modification, and analytical methods.                                                                                                        | Biotechnology Advances                         | 187       | 2012 | (Deutschmann & Dekker, 2012) |
| 5 <sup>th</sup>  | Production of xylooligosaccharides by autohydrolysis of lignocellulosic materials *research article classified as a review paper     | This study determined the composition of xylan-containing raw materials and produced nonlinear XOS by autohydrolysis under optimal conditions deduced from reported kinetic models.                                                                                                                                 | Trends In Food Science & Technology            | 166       | 2004 | (Parajó et al., 2004)        |
| 6 <sup>th</sup>  | Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families                      | This study analyzes the 3D structures of xylanases to provide insight into the mechanisms and strategies by which xylanases bind and hydrolyze structurally different heteroxylans and XOS.                                                                                                                         | Critical Reviews In Biotechnology              | 164       | 2010 | (Pollet et al., 2010)        |
| 7 <sup>th</sup>  | Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine                                   | This review focused on the prebiotic potential of AXOS and the intestinal microbiota involved in their transformation processes                                                                                                                                                                                     | Trends In Food Science & Technology            | 164       | 2007 | (Grootaert et al., 2007)     |
| 8 <sup>th</sup>  | Toward enzymatic breakdown of complex plant xylan structures: State of the art                                                       | This review highlights the possibility of the discovery of novel xylanolytic enzyme construction of multifunctional chimeric enzymes and xylanosomes in parallel with increasing knowledge of XOS structure.                                                                                                        | Biotechnology Advances                         | 145       | 2016 | (Biely et al., 2016)         |
| 9 <sup>th</sup>  | Prebiotics and gut microbiota in chickens                                                                                            | This review presents the current knowledge of the chicken gastrointestinal microbiota and the most recent publications related to prebiotics in the modulation of the gut microbiota and immune functions.                                                                                                          | Fems Microbiology Letters                      | 143       | 2015 | (Pourabedin & Zhao, 2015)    |
| 10 <sup>th</sup> | The functionality of prebiotics as immunostimulant: Evidence from trials on terrestrial and aquatic animals                          | This review covers the scientific outcomes of the direct and indirect mechanisms through which prebiotics can affect the intestinal immunity of animals.                                                                                                                                                            | Fish & Shellfish Immunology                    | 108       | 2018 | (Nawaz et al., 2018)         |

\* The number of citations was recorded until 2020.

### 3.2.5. Bibliometric study of authors, journals, institutions, and countries

The most productive researchers in the field can be identified after the evaluation of the most cited papers. **Figure 3-4** presents the analysis of the top 20 authors' productivity over the last 20 years. The size of the circles is defined by the number of articles, and the marker color represents the citations per year, where the more transparent the marker is, the lower the number of total citations. Additionally, the authors shown in **Figure 3-4** are not necessarily the first authors of the paper. Of the listed authors, only Parajo JC, Garrote G., and Biely P. are among the most cited authors. Biely P was the most productive researcher in the field, accounting for 24 publications. In 2016, the number of citations registered for this author reached a maximum number of 20 citations. Xu was the author with the most publications (10) and citations (20) in 2021.

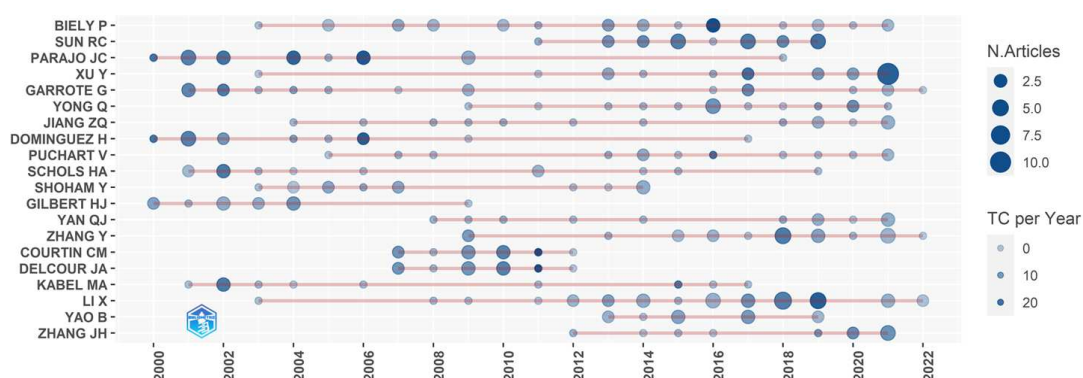


**Figure 3-4** Bibliometric analysis of the top 20 authors over time.

The main journal dedicated to publishing XOS research was *Bioresource Technology*, with 92 publications and representing 7.3% of total publications, followed by *Applied Biochemistry and Biotechnology* and *Industrial Crops and Products*, both with 34 publications (2.7%). The scope of the listed journals is similar and related to biomass, waste management and feedstock utilization, biotransformation, bioprocesses, and bioproducts. The 5<sup>th</sup> and 9<sup>th</sup> most cited research articles were published in *Bioresource Technology*. In addition, *Carbohydrate Polymers* is the 5<sup>th</sup> journal listed, with 33 publications, including the 8<sup>th</sup> and 10<sup>th</sup> most cited research articles (**Table 3-5**).

The countries and institutions related to XOS research were ranked according to the number of publications (**Table 3-5**). Globally, 68 countries are engaged in research in this field. **Figure 3-5** shows the distribution of publications among the countries. China is the most productive country, accounting for 345 publications (27.46%), followed by the USA (139

publications, 11.06%), Japan (125 publications, 9.95%), Brazil (95 publications, 7.56%), and Spain (90 publications, 7.16%). The massive contribution of China to this field is notable. In addition to China, this topic is well spread throughout the globe, with assistance from all continents.

Regarding the most prestigious institutions, *Nanjing Forestry University* and the *Chinese Academy of Science*, both from China, stand out, followed by the *United States Department of Agriculture* (USA) and the *University of Virgo* (Spain). The University of Campinas (Brazil) is the 6<sup>th</sup> most prestigious institution in XOS research. **Figure 3-5b** illustrates the collaboration network between the countries. The number of citations per country defines the size of the circles, and the lines among the authors represent the number of co-authorships, with the distance between them determined by the strength of the relation. In general, they are assembled into 4 clusters, and the shorter the distance between the circles is, the more similar the research fields are.

**Figure 3-6** presents a three-field plot relating the top 15 authors, keywords, and journals to illustrate how they are connected through a Sankey diagram. This plot is widely used to depict a flow from one item to another, where the heavier the links are, the stronger the connection between the items. Xu and Yong presented the heaviest link to enzymatic hydrolysis; Garrote and Parajo, to autohydrolysis. These two keywords share the weighty link to *Bioresource Technology Journal*, suggesting that this journal covered most publications with these keywords. Garrote, Parajo, Xu, and Dominguez presented the heaviest link to the keyword “xylo-oligosaccharides”, which also showed the strongest link to *Bioresource Technology Journal*.

### 3.3. Advances and trends of xylo-oligosaccharides

#### 3.3.1. General properties and production methods

The linear XOS known for their prebiotic potential and health benefits are xylobiose (degree of polymerization (DP) = 2), xylotriose (DP = 3), xylotetraose (DP = 4), xylopentose (DP = 5) and xylohexose (DP = 6) (**Figure 3-7**). They are low-calorie sweetener (1.5 kcal/g), solid, and colorless, although this is dependent on the xylan source and purification process. The recommended daily intake is 1.4-2.8 g/day for healthy adults (Finegold et al., 2014); it is suitable for diabetic patients and has antioxidant and cytoprotective activity. XOs are generally recognized as safe (GRAS); however, excess intake could result in distension of the intestine, nausea, flatulence, and diarrhea (Samanta et al., 2015).

In the depolymerization process (chemical or enzymatic), xylan is converted into high molecular weight xylooligomers, branched or linear, or is degraded to furfural, depending on the severity of the treatment and biomass nature. Xylan is a large hemicellulosic fraction found in different cheap raw materials, especially lignocellulosic biomass of agricultural wastes. From the xylan fraction in the biorefinery context, XOS and several high value-added products (ethanol, xylitol, lactic acid, furfural, xylose, arabinose) can be obtained for the food, bioenergy, and pharmaceutical industries. XOS are prebiotic compounds with 2 to 6 xylopyranosyl residues linked by  $\beta$ -1,4 xylosidic bonds. It is important to mention that these compounds stand out for their overall thermal and pH stability since XOS are stable at temperatures above 100 °C and are capable of withstanding pH variations between 2.5 and 8 (Gullón et al., 2011). The fundamentals of XOS production start with drying and grinding biomass to reduce the crystallinity and enhance the contact surface. The next stage generally involves a treatment to remove lignin and reduce biomass recalcitrance. These treatments can be physical (ultrasound and microwave), hydrothermal (steam explosion or liquid hot water) (Ruiz et al., 2023), biological (using microorganisms), or chemical (acid, alkali, ionic, or oxidative). These treatments solubilize xylan and can produce a wide range of oligosaccharides and several degradation products (Poletto et al., 2020).

Enzymatic treatment for XOS production has gained relevance as it is a controlled process that prevents the formation of undesirable compounds such as furfural and phenolic acids. The hemicellulosic enzymes used in these processes are mainly composed of  $\beta$ -1,4-endoxylanases (E.C.3.2.1.8), which divide the  $\beta$ -1,4-xylose bonds inside the heteroxyylan chain. The accessory enzymes (mainly esterases and arabinofuranosidases) act on the side chains of xylan. To improve the enzymatic production of XOS, the activity of  $\beta$ -xylosidase must be low to release the lower content of monomers possible. The ability of endoxylanases to release short XOS and thermostability are some of the crucial selection parameters (A. M. M. A. M. M. Lopes et al., 2021; Pinales-Márquez et al., 2021b; M. L. T. M. Polizeli et al., 2005).

XOS can be produced from xylan depolymerization by chemical or enzymatic methods; however, both processing routes have advantages and disadvantages compared to each other. Chemicals are cheaper than enzymes; nevertheless, improper treatments can lead to the degradation of monosaccharides and the production of toxic compounds (**Figure 3-8**). On the other hand, the use of enzymes means much more specific, environmentally friendly, and controlled processes carried out under mild temperature and pH conditions (Poletto et al., 2020). The combination of chemical and enzymatic methods can be established to modulate the proportion of various DP. The hydrothermal treatment and subsequent endo-xylanase hydrolysis



resulted in a XOS with conversion yield of 44.6% containing 78.7% xylobiose and xylotriose starting from the initial xylan in raw poplar (Su et al., 2021). In another study, the acidic condition of subcritical carbon dioxide and inorganic salts in seawater significantly promoted the degradation of xylan and increased the contents of low-DP XOS, enhancing XOS yields (51.44%) and increasing the pore area and porosity of the pretreated biomass, furtherly enhancing enzymatic hydrolysis (W. Zhang et al., 2022). Also, chemical methods may present synergistic effects and can be combined for a better exploitation of biomass, achieving the coproduction of XOS, lignin fractionation, and cellulose-rich substrates, for example hydrothermal and deep eutectic solvent pretreatments (Shen et al., 2021).

**Table 3-5 Ranking 10 top publication areas, affiliations, countries, journals, and authors.**

| <b>Ranking</b>   | <b>Research areas</b>                                                      | <b>Number</b> | <b>%<sup>1</sup></b> |
|------------------|----------------------------------------------------------------------------|---------------|----------------------|
| 1 <sup>st</sup>  | Biotechnology Applied Microbiology                                         | 448           | 35.67                |
| 2 <sup>nd</sup>  | Biochemistry Molecular Biology                                             | 310           | 24.68                |
| 3 <sup>rd</sup>  | Chemistry                                                                  | 265           | 21.09                |
| 4 <sup>th</sup>  | Agriculture                                                                | 195           | 15.52                |
| 5 <sup>th</sup>  | Food Science Technology                                                    | 190           | 15.12                |
| 6 <sup>th</sup>  | Energy Fuels                                                               | 163           | 12.97                |
| 7 <sup>th</sup>  | Microbiology                                                               | 124           | 9.87                 |
| 8 <sup>th</sup>  | Engineering                                                                | 109           | 8.67                 |
| 9 <sup>th</sup>  | Polymer Science                                                            | 70            | 5.57                 |
| 10 <sup>th</sup> | Science Technology Other Topics                                            | 58            | 4.61                 |
| <b>Ranking</b>   | <b>Affiliations (Country)</b>                                              | <b>Number</b> | <b>%<sup>1</sup></b> |
| 1 <sup>st</sup>  | Nanjing Forestry University (China)                                        | 46            | 3.66                 |
| 2 <sup>nd</sup>  | Chinese Academy of Sciences (China)                                        | 39            | 3.10                 |
| 3 <sup>rd</sup>  | United States Department of Agriculture (USA)                              | 35            | 2.78                 |
| 4 <sup>th</sup>  | University of Vigo (Spain)                                                 | 32            | 2.54                 |
| 5 <sup>th</sup>  | National Centre for Scientific Research (France)                           | 31            | 2.46                 |
| 6 <sup>th</sup>  | University of Campinas (Brazil)                                            | 30            | 2.38                 |
| 7 <sup>th</sup>  | South China University of Technology (China)                               | 30            | 2.38                 |
| 8 <sup>th</sup>  | Slovak Academy of Sciences (Slovakia)                                      | 29            | 2.30                 |
| 9 <sup>th</sup>  | Beijing Forestry University (China)                                        | 28            | 2.22                 |
| 10 <sup>th</sup> | National Research Institute for Agriculture, Food and Environment (France) | 28            | 2.22                 |
| <b>Ranking</b>   | <b>Countries</b>                                                           | <b>Number</b> | <b>%<sup>1</sup></b> |
| 1 <sup>st</sup>  | China                                                                      | 345           | 27.46                |
| 2 <sup>nd</sup>  | United States of America                                                   | 139           | 11.06                |
| 3 <sup>rd</sup>  | Japan                                                                      | 125           | 9.95                 |

| 4 <sup>th</sup>  | Brazil                                             | 95     | 7.56           |
|------------------|----------------------------------------------------|--------|----------------|
| 5 <sup>th</sup>  | Spain                                              | 90     | 7.16           |
| 6 <sup>th</sup>  | India                                              | 87     | 6.92           |
| 7 <sup>th</sup>  | England                                            | 51     | 4.06           |
| 8 <sup>th</sup>  | Portugal                                           | 51     | 4.06           |
| 9 <sup>th</sup>  | France                                             | 47     | 3.74           |
| 10 <sup>th</sup> | Thailand                                           | 41     | 3.26           |
| Ranking          | Journals                                           | Number | % <sup>1</sup> |
| 1 <sup>st</sup>  | Bioresource Technology                             | 92     | 7.32           |
| 2 <sup>nd</sup>  | Applied Biochemistry and Biotechnology             | 34     | 2.70           |
| 3 <sup>rd</sup>  | Industrial Crops and Products                      | 34     | 2.70           |
| 4 <sup>th</sup>  | Applied Microbiology and Biotechnology             | 33     | 2.62           |
| 5 <sup>th</sup>  | Carbohydrate Polymers                              | 33     | 2.62           |
| 6 <sup>th</sup>  | Biotechnology for Biofuels                         | 29     | 2.30           |
| 7 <sup>th</sup>  | Applied and Environmental Microbiology             | 28     | 2.22           |
| 8 <sup>th</sup>  | Carbohydrate Research                              | 28     | 2.22           |
| 9 <sup>th</sup>  | Process Biochemistry                               | 26     | 2.07           |
| 10 <sup>th</sup> | International Journal of Biological Macromolecules | 24     | 1.91           |
| Ranking          | Authors                                            | Number | % <sup>1</sup> |
| 1 <sup>st</sup>  | Biely P                                            | 24     | 1.91           |
| 2 <sup>nd</sup>  | Sun RC                                             | 20     | 1.59           |
| 3 <sup>rd</sup>  | Parajo JC                                          | 19     | 1.51           |
| 4 <sup>th</sup>  | Xu Y                                               | 19     | 1.51           |
| 5 <sup>th</sup>  | Garrote G                                          | 18     | 1.43           |
| 6 <sup>th</sup>  | Yong Q                                             | 15     | 1.19           |
| 7 <sup>th</sup>  | Jiang ZQ                                           | 14     | 1.11           |
| 8 <sup>th</sup>  | Dominguez H                                        | 13     | 1.03           |
| 9 <sup>th</sup>  | Puchart V                                          | 13     | 1.03           |
| 10 <sup>th</sup> | Schols HA                                          | 13     | 1.03           |

<sup>1</sup> Percentage of 1,256 documents (automatically calculated in WoS).

### 3.3.2. Development of biorefineries for XOS production

Most recent publications in the field of XOS production focus on the development of integrated processes in a biorefinery concept. A biorefinery is an industrial complex using organic raw materials, especially wastes, as feedstock to produce biomaterials and bioenergy (Pinales-Márquez et al., 2021b). Processing wastes through the biorefinery to obtain multiple value-added products leads to cost reduction and creates a competitive advantage, given the increased interest in the food and cosmetics industries due to their biological and physicochemical properties. Different processing stages can be designed to couple byproducts from

cellulose, hemicellulose, and lignin, promoting better use of the feedstock (Tsouko et al., 2020). Agro-industrial wastes represent an advantageous raw material to produce XOS and other bioproducts due to their quantity, availability, and elevated xylan content, ranging from 20 to 50% depending on the biomass (**Table 3-6**) (Maity, 2015). Important environmental benefits emerge with this strategy, as it helps manage millions of tons of waste generated annually (Amorim et al., 2019).

The industrial application of XOS has been greatly limited due to product quality and impurities. The expensive purification methods have become the key to successful industrial application. Additionally, the autohydrolysis method has high energy consumption and production cost, and the corrosive acid used in the process generates many undesirable compounds (Yan et al., 2022). Therefore, XOS separation and purification steps still need further study to improve the purity and lower the production cost. The bibliometric analysis showed that bioconversion of xylan by enzymatic hydrolysis had gained relevance within the scientific community as it is a controlled process and prevents the formation of undesirable compounds (such as furfural, 5-hydroxymethylfurfural, and formic acid), reducing costs related to downstream processing. However, the costs of enzyme production and long reaction times raise the value of the process (Pinales-Márquez et al., 2021b).

There are several technological opportunities for increasing the performance of pretreatments and enzymes toward greener polymerization (Pinales-Márquez et al., 2021b). Physical or chemical pretreatment is still required for enzymatic hydrolysis because of the biomass recalcitrance to dissolve the hemicellulose. Among the methods, the steam explosion was shown to be an effective green pretreatment with low energy consumption and short reaction time, reducing the chemicals usage (Yan et al., 2022). For example, Shandong Longlive, one of the most important XOS producers, patented a preparation method for pure XOS production by which the first step is a continuously steam explosion pretreatment with diluted acid (0.1 – 1.5 %) (SHANDONG LONGLIVE BIO-TECHNOLOGY Co Ltd, 2014).

The search for sustainable energy and food resources accrues from the population increase, raising the need to develop technologies and policies to regulate the use of soils to avoid the conflict between energy and food production. Additionally, the 1.3 billion tons of lignocellulosic biomass generated annually does not compete with the land for food cultivation. It takes advantage of the development of second-generation biorefineries, becoming a sustainable economic model (Nanda et al., 2015). The global demand for bioproducts in the biorefinery concept and circular bioeconomy sets the investment at 2.6 to 5.8 trillion dollars from 2025 to 2030. In 2023, XOS will have an estimated value of 130 million dollars in the prebiotic market,

which shows an excellent opportunity for the economic development of compounds produced from xylan (Poletto et al., 2020). According to the global prebiotic ingredient market, the annual growth rate for 2019-2024 is forecasted to be 12.2% and valued at 3.85 billion dollars (Pinales-Márquez et al., 2021b).

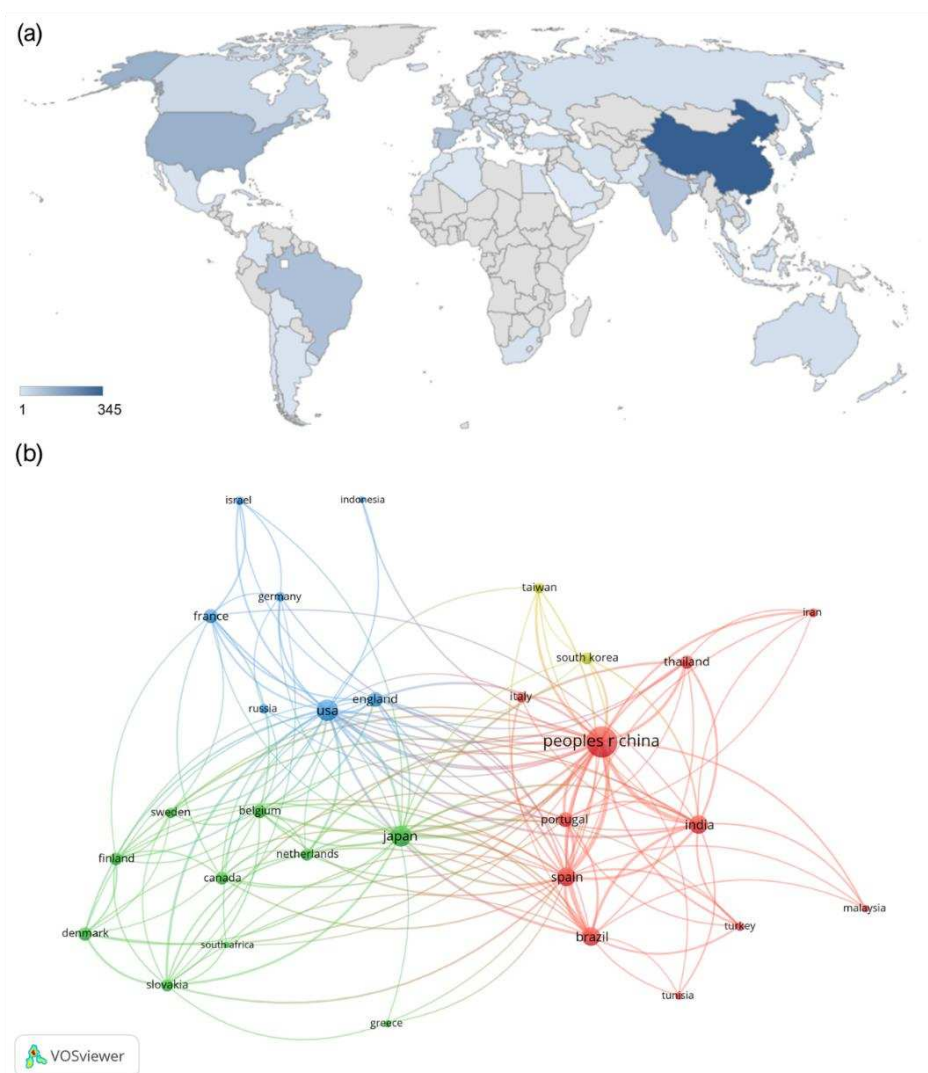


**Figure 3-5** The most expressive countries. (a) Map representation of the number of publications per country. (b) Collaboration network strength between the countries according to co-authorship.

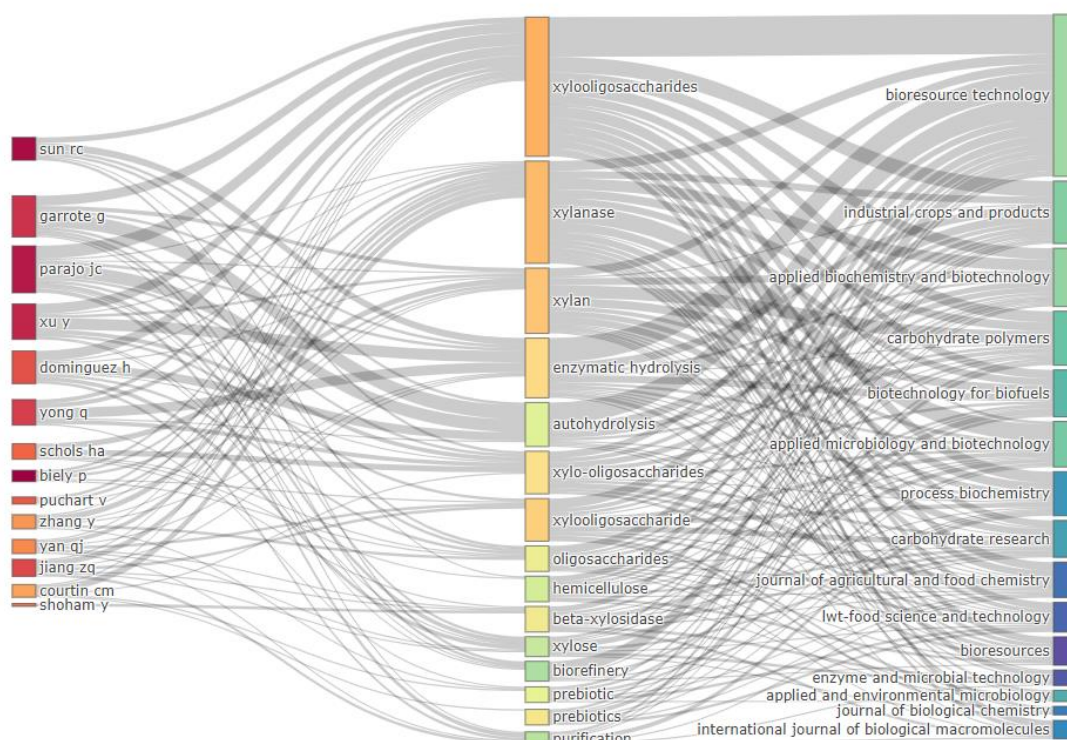


Figure 3-6 The three fields plot correlating the top 15 authors, keywords, and journals

### 3.3.3. XOS as a functional food ingredient

Longlive (Shandong, China), YuanLong Chem (Tianjin, China), Henan Shengtai Biotechnology Co. (Zhengzhou, China), Henan Kangwei (Xinjiang, China), HFsugar (Xinjiang, China), and Yibin Yatai (Sichuan, China) are the most important XOS industries worldwide. XOS can be purchased in bulk at US\$ 20 to 52, depending on the powder or syrup purity. Several researchers have made efforts the investigation the use of XOS as a novel food ingredient for its bioactive properties, flavor, and physicochemical properties. Although XOS is a potential sugar and fat replacement and emulsifier in beverages, dairy products, chocolates, and bakery products, the studies are directed toward the development of cost-effective production routes rather than their application fields. Here, the most recent studies regarding the application of XOS in food are described.

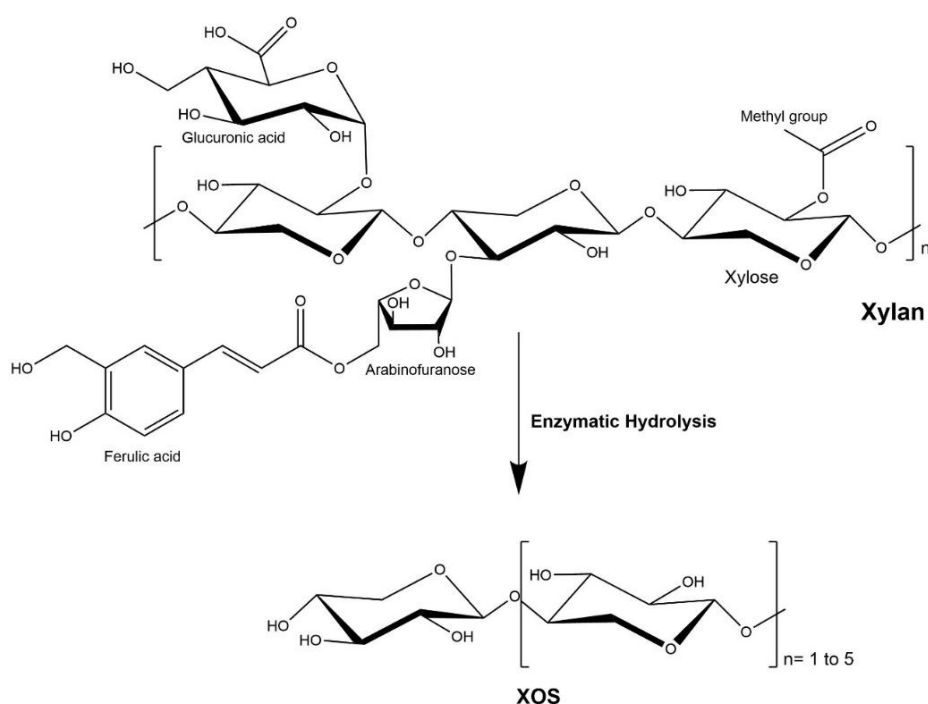


**Figure 3-7 General structure of arabinoxylan and obtainment of XOS by enzymatic hydrolysis**

A pioneering study elucidated the cryoprotectant effect of a mixture of xylobiose and xylotriose on porcine myofibrillar proteins during frozen storage (Chou & Lin, 2010). The gel strength increased with increasing XOS concentration up to 6%, and protein solubility improved with increasing XOS concentration up to 14%. XOS provided the lowest syneresis compared to other oligosaccharides.

In another study, different combinations of XOS and sucrose were incorporated into a meatball recipe. The 4% XOS treatment provided the lowest cooking loss and a similar value of sucrose, and no difference in overall acceptability was found. There was no significant difference in the water holding capacity or pH after cooking or frozen storage. Incorporating XOS into meatballs resulted in a product comparable to the traditional sucrose-containing recipe in terms of sensory and physicochemical properties (Wu & Lin, 2011).

XOS was also utilized as a cryoprotectant to increase the emulsion stability of the meat batters compared to sucrose, trehalose, and sorbitol. Non-significant difference was observed in the water holding capacities and freeze-thaw stabilities during the frozen storage of any samples. However, these values increased with increasing cryoprotectant concentration. The water-soluble protein remained higher for the XOS samples during frozen storage. The relative surface hydrophobicity indicated that the XOS containing batters exhibited less change in protein structure during frozen storage (Wu & Lin, 2014).

The functionality of plant proteins may also be improved by conjugation with XOS. The application of plant proteins is limited due to their low solubility. The combination of XOS and pea protein increased thermal stability, antioxidant activity, and solubility from pH 3 to 10. These results can be the foundation for the development of novel plant-based foods (S. Zhao et al., 2022). Additionally, whey beverages enriched with XOS presented better functional characteristics, such as greater antioxidant activity, angiotensin-converting enzyme (ACE) inhibitory activity,  $\alpha$ -amylase, and  $\alpha$ -glucosidase inhibition. The inhibition of ACE activity leads to a decrease in the angiotensin II concentration and an increase in the bradykinin level, and consequently in the reduction of hypertension, one of the major causes of chronic diseases and a high-risk factor of cardiovascular diseases worldwide (Paiva et al., 2017), while the  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition contributes to blood sugar modulation as it decreases the digestion of carbohydrates into glucose, promoting anti-diabetic effect (Jeong et al., 2012). The beverages also presented higher viscosity, volatile profile, and sensory acceptance, in addition to providing adequate concentrations of the prebiotic (1.25 g/mL) (F. P. Souza et al., 2019).

XOS may also substitute sugar in food. For instance, snack hawthorn leathers were formulated with 25% XOS and 75% fructo-oligosaccharides (FOS) and exhibited comparable morphology and sensorial properties to the traditional one containing sucrose, showed anti-obesity activity with suppressed body weight gain, blood glucose, and oxidative stress, improved the histology of liver and subcutaneous fat tissues, and decreased inflammatory responses in obese mice (T. Li et al., 2022); cookies enriched with 5% XOS were found to be the most acceptable and were stable for 21 days at room temperature ( $25 \pm 2$  °C) (Ayyappan et al., 2016).

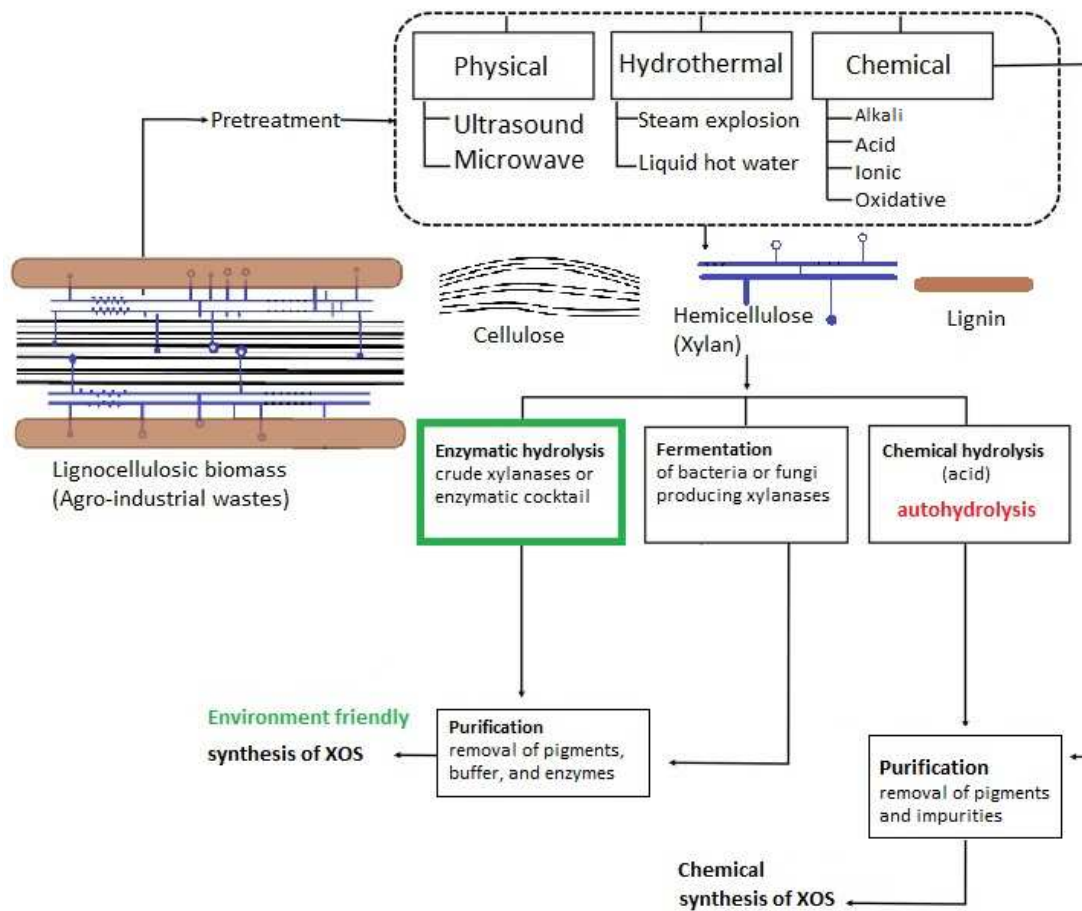


Figure 3-8 Steps for XOS production from lignocellulosic biomass

The incorporation of XOS in vegan products can already be found commercially, such as the sparkling fermented tea and kombucha from Wonder Drink, and many others are being developed. For example, berry juice containing XOS improved skin brightness, moisture, elasticity, spots, UV spots, and brown spots of subjects, indicating that prebiotic juice may provide an alternative route to daily skincare. However, the underlying mechanism of XOS for skin improvement is still unclear (Chang et al., 2020). In another study, the addition of XOS to rice cakes effectively reduced cooking loss and promoted the regeneration of starch, decreasing the digestibility rate (C. Yang et al., 2021).



**Table 3-6 Agro-industrial wastes as raw materials to produce XOS with different pretreatments.**

| Feedstock            | Method                                            | XOS yield<br>(xylan conversion) | Other value-added bi-products                            | Ref.                      |
|----------------------|---------------------------------------------------|---------------------------------|----------------------------------------------------------|---------------------------|
| Brewers spent grains | Steam explosion and amyl-glucosidase treatment    | 74.2%                           | Xylitol                                                  | (Swart et al., 2021)      |
| Miscanthus           | Extrusion and auto-hydrolysis                     | 32.5%                           | Cellulose microfibrils and biogas                        | (Lan et al., 2021a)       |
| Paper mulberry       | Acetic acid pretreatment and enzymatic hydrolysis | 40.72%                          | Amino-acids and monosaccharides                          | (X. Zhao et al., 2022)    |
| Rice and corn bran   | Enzymatic hydrolysis                              | 32 and 33.9%                    | Polyphenols                                              | (Mehta et al., 2022)      |
| Sugarcane bagasse    | Steam explosion                                   | 29.62%                          | ethanol, cellulose nanofibrils, and lignin nanoparticles | (B. Pereira et al., 2021) |
| Wheat straw          | Steam explosion                                   | 91%                             | -                                                        | (Álvarez et al., 2017)    |

XOS is also incorporated into hydrogel structures to improve gel strength and better conserve compounds such as vitamins or probiotics. Microparticles of alginate containing 3% XOS increased the survival rate of encapsulated *Lactobacillus acidophilus* during refrigerated storage and simulated digestion by increasing the gel strength and reducing the pore size of the capsule (Martins, Silva, et al., 2020). In another study, XOS influenced the rheological properties, contributing to forming a gel network with high viscosity to protect the encapsulated vitamin complex (A + D + E) based on pectin and lentil protein (Mekala et al., 2022).

### 3.3.4. Perspectives and opportunities

At present, most XOS production methods still face drawbacks. Although enzymatic hydrolysis provides greener and more efficient production, it requires biomass pretreatment and downstream decolorization. Additionally, XOS-related products are not widely present in the market, so future research should focus on the following aspects: i) optimization of the enzymatic hydrolysis and purification processes in a biorefinery concept of valorization of lignocellulosic biomass; ii) development of food, medicines, feed, and health products containing XOS to increase its market presence and further investigate its bioactivities.

Agro-industrial wastes represent an ideal source for obtaining XOS due to their low cost and high availability. The recalcitrance of the material remains a challenge, so biomass fractionation technologies should be focused on improving the sustainability of processes, preferably those that are easy to operate and under continuous mode in the context of a circular bioeconomy. More research should be conducted at a pilot-scale and industrial level to improve the feasibility of the production processes.

### 3.4. Conclusion

The bibliometric analysis of XOS research showed that it is a growing and global research field with many well-established concepts and emerging technologies that direct toward new potential industrial solutions and applications. Globally, 68 countries are engaged in XOS research, being China the most engaged country, followed by the USA, Japan, and Brazil. The field of gut microbiota showed to be both a consolidate and a trend topic. Based on the most cited documents, enzymatic hydrolysis of agro-industrial wastes is a current focus of XOS research, as well as XOS separation and purification, which still need further study to improve the purity and lower the cost. These studies may have a strong impact and lead to cost reduction for XOS production from agro-industrial wastes, pretreatment technologies, and enzyme engineering in a biorefinery concept, as well as a better understanding of the health effects of XOS regarding the microbiota and immunomodulation.

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**CHAPTER 4.**  
**LIGNOCELLULOSIC BIOMASS DEPOLYMERIZATION AND**  
**XYLOOLIGOSACCHARIDES PRODUCTION**

## The role of lignocellulosic biomass in functional food production

Various vegetables are cultivated in large quantities in agro-industries, generating significant amounts of agro-industrial waste. The variability in hemicellulose composition offers numerous opportunities for application in the development of new food products, as well as raw materials for the chemical and pharmaceutical industries. The increasing knowledge and willingness to develop new biopolymers, coupled with the abundant source of hemicellulosic material derived from agro-industrial waste, lead to the growing commercial application of xylan derivatives (Freitas et al., 2019a).

Brazil, as one of the world's leading agricultural producers, generates vast quantities of agroindustrial residues, among which **corn husk**, **soy hulls**, and **orange processing waste** stand out prominently. These residues, abundant by-products of corn, soybean, and orange cultivation and processing, respectively, represent valuable resources with considerable potential for various applications across multiple industries given their diverse chemical compositions and properties, making them suitable for applications in animal feed, bioenergy production, biorefining, and the extraction of high-value compounds. Understanding and harnessing the potential of these agroindustrial residues and their lignocellulosic fractions are crucial not only for waste management and environmental sustainability but also for enhancing the economic viability and competitiveness of Brazil's agroindustrial sector.

The particular interest in arabinoxylan polymers, as discussed earlier, arises from their abundance in the agro-industrial residues and potential for obtaining oligosaccharides with prebiotic potential, namely xylo-oligosaccharides. The challenge lies in the treatment and development of enzymatic cocktails that economically enable the deconstruction and bioconversion of this complex into high-value-added xylo-oligosaccharides (Ávila, Cairo, Damasio, et al., 2020a; Ávila, Martins, & Goldbeck, 2020; Ávila, Martins, Costa, et al., 2020; Goldbeck et al., 2014, 2016; Gonçalves et al., 2012; Martins, Ávila, et al., 2020).

The structural diversity of hemicellulose allows for the generation of specific high-value-added products, and its abundance in nature reduces industrial process costs. Furthermore, optimizing the application of this material from agro-industrial waste reduces the environmental impact generated by the production of these residues. Many products can be derived from hemicellulose using methods such as fermentation or chemical processes, to form films, hydrogels, or biocomposites of interest. The structure of xylan makes it ideal for enzymatic modifications, a promising pathway for generating environmentally friendly products from renewable sources, yielding, for example, ethanol, lactic acid, and xylitol, commercially



important products, as well as, particularly for the present study, xylo-oligosaccharides (Naidu et al., 2018).

XOS can be obtained from lignocellulosic materials by three methods: enzymatic treatment, chemical fractionation (alkaline treatment to isolate or solubilize xylan) followed by enzymatic hydrolysis, hydrothermal degradation (physical process), or acid hydrolysis (chemical process). However, the physical and chemical processes (or self-hydrolysis) present the inconvenience of producing undesirable components that inhibit the growth of probiotics and complicate the purification of XOS. The production of a wide range of undesirable by-products, such as xylose, hydroxymethylfurfural, and furfural, requires complex purification steps (Samantha et al., 2015).

On the other hand, the enzymatic process is more advantageous and desirable because it is a selective method operated under mild conditions, avoiding the use of solvents, resulting in lower production of inhibitory byproducts. Enzymatic preparations with high endoxylanase activity maximize the production of these oligomers, at the expense of xylose production. Additionally, XOS obtained from self-hydrolysis typically exhibit a higher degree of polymerization, unlike XOS produced through enzymatic processes, which, being shorter-chain oligosaccharides, have a greater prebiotic potential. This is since endoxylanases specifically act on the glycosidic bonds of the xylan chain, while chemical treatments randomly hydrolyze cross-links between arabinoxylans, lignin, and cellulose (hydrogen and covalent bonds) (Samantha et al., 2015; Y. Zhang et al., 2018; Z. Zhang et al., 2014).

During enzymatic hydrolysis, as the substituents are removed, xylan becomes less soluble, forming aggregates that further hinder and delay its degradation. Simultaneous removal of these substituents and cleavage of the glycosidic bonds in the main xylan chain increase the degradation rate. Thus, mixtures of synergistic enzymes are desirable and the subject of intense investigation to understand their mode of action and optimize concentrations. The details of the enzymes involved in xylan depolymerization are discussed in **two published book chapters (both first-authored)**, presented in **Appendix I: Lignocellulose Bioconversion through Chemical Methods, Platform Chemicals, and New Chemicals**, and **Appendix II: Enzymatic bioconversion trends for lignocellulosic biorefineries**.

Exploring the potential of agroindustrial residues such as corn husk, soy hulls, and orange peels offers Brazil a dual opportunity: to address environmental concerns and to pave the way for economic expansion and technological progress. Now, let's examine the distinctive features and versatile applications of these three primary agroindustrial residues.

### Soy hulls



Brazil is one of the leading soybean producers globally. In the 2022/23 crop season, Brazil produced 154.566.3 million metric tons of soybeans, with a planted area of 44.062.6 million hectares and an average yield of 3,508 kg/ha. This positions Brazil as the largest soybean producer worldwide. The United States ranks second, with an annual production of 120,707,230 tons, while Argentina is the third-largest producer, with 46,217,911 tons, according to Embrapa and Abiove. It's worth noting that Brazil also leads in soybean exports, accounting for 50% of the global trade in this commodity.

For every ton of soybeans, approximately 180 kg of oil (18%), 710 kg of soybean bran with 48% crude protein (71%), and about 50 kg of hulls (5%) are obtained. The soybean hull is separated during the dehulling process of the grain, in the oil production process, as well as in the production of milk and other derivatives such as tofu and tempeh. The average fraction of hemicellulose present in the hull represents around 18% of the dry biomass. The grains harvested in the fields are cleaned and dehulled in dehullers, consisting of beaters or rotating knives, which separate the hull without crushing the pulp, collected by vibrating screens and air blowing (Mandarino et al., 2001).

In a soybean oil industry, for example, to obtain a ton of crude oil, 5 tons of grains are required, generating around 250 kg of hulls. After its removal, it is usually ground or pelleted to reduce transportation costs. It is considered an agroindustrial residue of little use for both human consumption and industrial application. However, due to its fiber-rich composition, it is commonly used for animal feed (Ipharraguerre & Clark, 2003). Physical, chemical, and economic factors combine to make soybean hulls more than just an alternative feed for livestock. Its considerable hemicellulose content makes it an attractive raw material to produce high-value compounds, namely xyloligosaccharides.

### Corn husks



Brazil is also one of the leading producers of corn in the world. According to data from the Brazilian Institute of Geography and Statistics (IBGE), in the 2021/22 crop season, 113.1 million tons of corn were harvested in Brazilian soil. In the last completed season, farmers produced 131.8 million tons of this cereal. Furthermore, Brazil surpassed the United States and became the world's largest exporter of corn, accounting for 32% of global exports in the 2022/2023 crop season. The United States, which has led the corn market for over a century, accounted for about 23% of global exports. This achievement is made possible by the strength of the Brazilian agribusiness, which generates jobs, income, and contributes to feeding one out of every eight people in the world (Instituto Brasileiro de Geografia e Estatística, 2023).

Corn kernels are typically processed to obtain starch (and flour) and ethanol, with corn oil considered a by-product, which can also be used for biodiesel production. Regardless of the final product of interest from corn kernels, the first step of the process usually involves dry or wet milling to separate the pericarp (the husk), which represents around 5.5 to 6% of the dry biomass of the kernel (Veljković et al., 2018). In corn milling, especially through wet milling, a yield of 68% starch is obtained, extracted from the endosperm, relative to the dry weight of the kernel, and only a 5% yield of oil, extracted from the germ. The husk, or pericarp, is a non-commercial residue rich in arabinoxylan, and its utilization to produce xyloligosaccharides has been reported in the literature. Corn husk with a hemicellulose content of 37.39% was hydrolyzed by a commercial endoxylanase, primarily producing xylobiose, with a concentration reaching 1.91 mg/mL, a result that demonstrates its potential application (Samanta et al., 2016).

### Orange processing waste



According to the Global Agricultural Information Network, during the marketing year 2022/2023, Brazil was the leading global orange producer, with a production volume of about 16.75 million metric tons. This significant output contributes to Brazil's prominence in the citrus industry. Oranges are one of the most produced fruits worldwide, and Brazil's efficient cultivation practices and favorable transport conditions have contributed to its success in orange production. The country's orange production is primarily used for juice processing, with approximately 70 percent of the crop serving this purpose.

The citrus fruit peels represent one of the most underutilized residues on the planet. After juice extraction, peel residue can account for over 50% of the fruit's wet weight (**Figure 4-1**). Orange peels consist of 20% dry matter (sugar, cellulose, hemicellulose, pectin, and D-limonene) and 80% water. The reuse of this residue has been primarily focused on the extraction of D-limonene and pectin. Traditionally, these peels are dried, and the compounds of interest are extracted with acids, with a small portion of dried peel utilized in animal feed. However, most of this material is inadequately disposed of in soil due to drying costs and acid usage, leading to serious organic pollution due to the high demand for biological oxygen for its decomposition. An alternative to address the issue is the utilization of its lignocellulosic fraction. The lower lignocellulosic content present in orange peels can be offset by the high volume of waste generated annually in the juice industries (Balu et al., 2012a; Rezzadori et al., 2012).

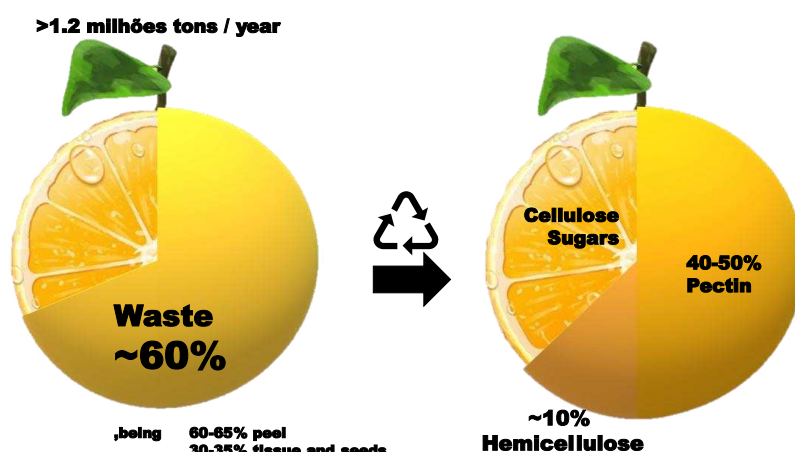


Figure 4-1: Annual processing of orange for juice production.

## Xylanolytic enzymes for arabinoxylan degradation

The structure of xylan found in plant cell walls exhibits considerable variety depending on the plant type and tissue. However, they always contain the main chain consisting of D-xylose monomers linked by  $\beta$ -1,4 glycosidic bonds. Due to its heterogeneous structure, complete depolymerization relies on a complex of xylanases, mainly produced by organic matter-decomposing microorganisms. Endo-1,4- $\beta$ -xylanases (EC 3.2.1.8) cleave the glycosidic bonds between the xyloses in the main xylan chain, releasing lower degree polymerization xylose oligomers and xylo-oligosaccharides. However, for the production of xylo-oligosaccharides, other enzymes acting on xylan side chains are required: acetyl xylan esterase (EC 3.1.1.6) removes O-acetyl groups from positions 2 and/or 3 on  $\beta$ -D-xylopyranosyl residues;  $\alpha$ -glucuronidase (EC 3.2.1.131) hydrolyzes  $\alpha$ -1,2 linkages between glucuronic acid and  $\beta$ -D-xylopyranosyl residues found in glucuronoxylans; arabinase removes L-arabinose residues from positions 2 and/or 3 on  $\beta$ -D-xylopyranosyl residues; ferulic acid esterase (EC 3.1.1.-) and p-coumaric acid esterase (EC 3.1.1.-) cleave ester bonds of xylan side chains.  $\beta$ -1,4-D-xylosidases (EC 3.2.1.37) hydrolyze small xylo-oligosaccharides and xilobiose, releasing xylose (M. L. T. M. Polizeli et al., 2005). Information about the characteristics and classifications of xylanolytic enzymes can be found in the Carbohydrate-Active Enzyme (CAZy) database (<http://www.cazy.org>).

### Endoxylanases

These glycoside hydrolases (GH) catalyze the hydrolysis of the  $\beta$ -1,4 linkages between xyloses in the main xylan chain, leading to the formation of a hemiacetal sugar and its corresponding aglycone. Initially, the primary hydrolysis products are  $\beta$ -D-xylopyranosyl oligomers, followed by the production of mono-, di-, and trisaccharides of  $\beta$ -D-xylopyranosyl in subsequent steps (Motta et al., 2013). Attempts have been made to classify these enzymes based on molecular weight and isoelectric point, crystalline structure, kinetic properties, or substrate specificity and products formed. However, due to many exceptions to suggested classifications, a more comprehensive system based on primary structure and comparison of catalytic domains was introduced, analyzing both structure and mechanism of action (Collins et al., 2005).

The hydrolysis of xylan can result in either retention (GH 5, 7, **10**, and 11) or inversion (GH 8 and 43) of the anomeric center of the substrate's reducing sugar monomer (**Figure 4-2**). Enzymes that hydrolyze with retention of the anomeric configuration possess two glutamic acid residues responsible for the catalysis, by double displacement, in which a covalent intermediate is formed with the enzyme and the glycosyl group, which is then hydrolyzed,

and two carboxylic acid residues present in the active site involved in the formation of this intermediate, with one being the acid catalyst for protonation of the substrate and the other performing the nucleophilic attack, resulting in the release of the leaving group and formation of the  $\alpha$ -glycosyl intermediate ( $\beta$  to  $\alpha$  inversion). In the second step, the first carboxylic group acts as a base, abstracting a proton from the water molecule of the nucleophile, which attacks the anomeric carbon. This leads to a second substitution, again resulting in the inversion of the anomeric carbon from  $\alpha$  to  $\beta$  (Collins et al., 2005).

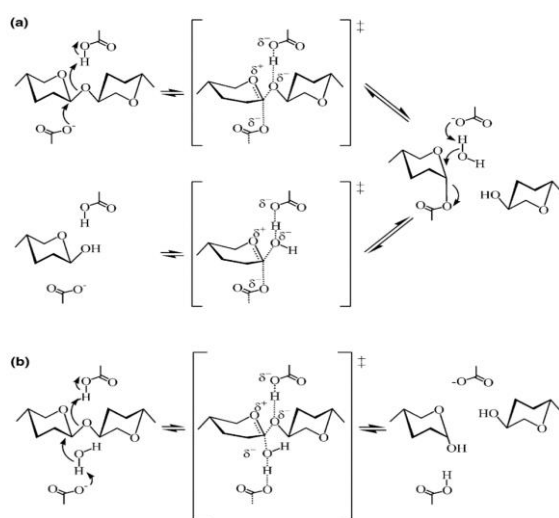


**Figure 4-2: General mechanism of hydrolases that act by (a) retention or (b) inversion of the anomeric centre. Source: COLLINS; GERDAY; FELLER (2005).**

Endoxylanases from GH families 10 and 11 are the most studied. In fact, these two families are primarily constituted by endoxylanases, unlike the others. Catalytic domains of endoxylanases belonging to other families still have limited information available, and thermophilic endoxylanases belonging to other families, apart from GH 10 and 11, have not been reported. Most GH 10 enzymes are endo-1,4- $\beta$ -xylanases, followed by endo-1,3- $\beta$ -xylanases, and cellobiohydrolases. The specificity of endoxylanases in this family is not restricted to xylan but can also show activity on low molecular weight cellulose. Typically, they have high molecular weight and low isoelectric points, with peak activity between 40°C and 80°C and pH between 4 and 6.5. Analyses of crystal structure and kinetics on xylan oligosaccharides of various sizes and final hydrolysis products indicate that GH 10 endoxylanases typically have 4 or 5 substrate-binding sites. Moreover, they have demonstrated a better ability to cleave glycosidic bonds near other substituents, such as acetic acid and methylglucuronic acid, being less affected by the presence of branches in the xylan chain (Collins et al., 2005; Motta et al., 2013).

Unlike all other families, GH 11 is exclusively composed of endoxylanases, showing activity only on D-xylose polymers, in addition to having low molecular weight and high

isoelectric points. However, they exhibit less catalytic versatility than GH 10. In fact, their final products can still undergo hydrolysis by GH 10 endoxylanases. They also act on aryl  $\beta$ -D-glycosides; however, substituents of  $\beta$ -1,3 represent a significant obstacle to their catalytic activity, resulting in higher degrees of polymerization compared to GH 10. GH 11 enzymes are more active on larger chain xylan and have broader substrate interaction sites, with at least 7 subsites, while GH 10, as mentioned earlier, has 4 or 5 subsites (**Figure 4-3**). Regarding their structural shape, GH 10 typically exhibits a barrel shape ( $\beta/\alpha$ )<sub>8</sub>, consisting of eight concatenated "strand-loop-helix-turn" units. The active sites are in the  $\beta \rightarrow \alpha$  loops (Silverman et al., 2001).

Side chains hinder enzymatic activity; this difference in specificity has important implications in xylan deconstruction to obtain desired products. GH 10 endoxylanases require two unsubstituted xylopyranosyl residues between branches, whereas GH 11 requires three consecutive unsubstituted residues. The greater catalytic versatility of GH 10 compared to GH 11 can be attributed to their tertiary structure. GH 11 molecules are smaller and more compact, with the catalytic site located in a groove accommodating 5 to 7 xylopyranosyl residues, in contrast to the GH 10 site, which is not located in a groove and accommodates 2 or 3 residues. Additionally, larger molecules exhibit greater flexibility, which may contribute to the lower specificity for a single substrate for GH 10. Given the characteristics of the two main families of hydrolases to which endoxylanases belong, the hydrolysis profile and final products guide the selection of the most suitable enzyme for a specific process. However, as mentioned earlier, the cooperation of synergistic enzymes is essential for efficiently converting xylan. Complex substrates, such as corn and soybean husks, contain large amounts of arabinoxylans, which are not easily degraded without prior treatment with  **$\alpha$ -L-arabinofuranosidase** (Motta et al., 2013; M. L. T. M. Polizeli et al., 2005).

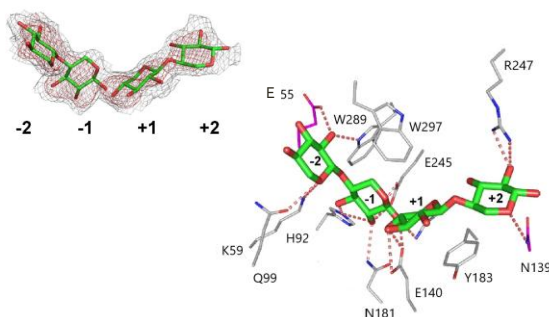
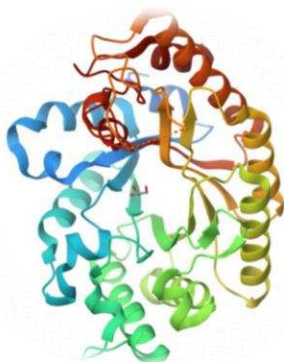


**Figure 4-3:** GH 10 substrate binding site (xylotetraose represented).



### ***GH 10 Endoxylanase from *Papiliotrema flavescens****



Xylanases are primarily produced by the genera *Aspergillus* and *Trichoderma*. However, a high level of expression should not be the only factor considered. Economically, one seeks an enzyme that exhibits characteristics to fit a specific process, such as inhibitor tolerance and high temperature or certain hydrolysis profiles. Therefore, enzymes produced by other microorganisms have been explored, such as those produced by yeasts (F. Lopes et al., 2011). Xylanases from *Papiliotrema flavescens* (strain LEB-AY10 (CCT 7725)) were first described by Lopes et al. (2011). The gene sequence responsible for the expression of this enzyme was isolated and sequenced, available in GenBank under the accession code KF3181871.

Xyn10cf GH 10 showed optimal activity at 77.5°C (pH 4.8) and greater stability at pH values close to 5.3, with an isoelectric point of 4.6. The half-lives of this enzyme at pH 4.8 were 1.11 min at 77.5°C, 47.5 min at 70°C, and 4.84 h at 67°C. At pH 5.3, the half-lives were 9.2 minutes at 77.5°C, 50.25 min at 72°C, 1.93 h at 70°C, and 33.74 h at 67°C. The kinetic parameters  $K_M$  and  $v_{max}$  for the substrate of birch xylan were 4.13 g/L and 2.32 U/mL, respectively, with the main hydrolysis products being xylobiose and xylotriose, demonstrating its potential for XOS production. The purified xylanase showed activity on 4-nitrophenyl- $\beta$ -D-xylopyranoside (0.02 U/mg) and exhibited some cellulase activity on carboxymethyl cellulose (0.99 U/mg) (C. Andrade et al., 2015; C. C. P. de Andrade, 2014).

The nucleotide sequence and conserved residues suggest that it belongs to family GH 10. A single gene was identified, encoding this enzyme formed by 1265 bp, and the cDNA by 1035 bp, due to the removal of 6 introns. The predicted signal peptide is composed of 13 amino acids and the mature protein of 331 amino acids, with an estimated molecular weight of 37.1 kDa. It exhibits about 11 kDa of glycosylation, since the purified protein showed an average molecular mass of 48 kDa. The enzyme was expressed in *Pichia pastoris* constitutively, using the pGAPZ $\alpha$ A vector, produced in a bench reactor, and applied in the hydrolysis of xylan extracted from sugarcane straw (Martins, Ávila, et al., 2020). Thermostability and up to the mark XOS yield were the motivating factors for further studies with this enzyme, as presented in **CHAPTER 7**.



### Arabinofuranosidases

After being largely neglected for a long time, this group of enzymes (EC 3.2.1.55) has currently gained considerable attention due to the high frequency of L-arabinose residues found linked to arabinoxylan and arabinogalactan chains, which can be associated with pectic substances, such as citrus fruit. The L-arabinofuranosyl residues are distributed along the main xylose chain and impair the hydrolysis of xylan and pectins.  $\alpha$ -L-arabinofuranosidases are broad-specificity accessory exoenzymes that cleave  $\alpha(1\rightarrow2)$ ,  $\alpha(1\rightarrow3)$ , or  $\alpha(1\rightarrow5)$  linkages of L-arabinofuranosyl residues of non-reducing hemicellulose polysaccharides and oligosaccharides, as well as pectins and different glycoconjugates. Based on similarities in amino acid sequences and analysis of hydrophobic groups, they are classified as belonging to the glycoside hydrolase families GH 3, 10, 43, 51, 54, and 62 (Biely et al., 2016).

Like other glycoside hydrolases,  $\alpha$ -L-arabinofuranosidases mediate the cleavage of glycosidic linkages via acid/base-assisted catalysis with retention or inversion of the anomeric configuration (**Figure 4-2**). In both mechanisms, hydrolysis requires two carboxylic groups, which are conserved within each hydrolase family. In the retention group of anomeric configurations are members of GH 3, 51, and 54, which cleave glycosidic linkages through a double displacement mechanism. The group with inversion of the configuration is represented by members of GH 43 and 62, which use a single displacement mechanism, in which a carboxylate acts as a basic catalyst, deprotonating the nucleophilic water molecule, which attacks the glycosidic linkage, while the other carboxylate acts as an acidic catalyst, protonating the corresponding aglycone (Numan & Bhosle, 2006; Wilkens et al., 2017).

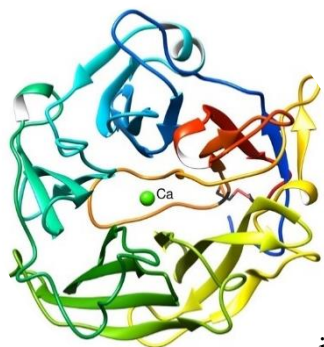
GH 62 is the only family of hydrolases that contains only arabinofuranosidases, despite being the smallest and least studied. Phylogenetic analysis indicates the existence of four subfamilies, but it is still unclear if this division represents distinct enzymatic properties among the members of these two subfamilies. Members of this family are found in bacteria and primarily in fungi. Only in 2014 did the first published works on the structural determination of some of the GH 62 arabinofuranosidases enzymatically characterized begin to emerge. GH 62 preferentially hydrolyzes L-arabinose residues from arabinoxylan side chains, in contrast to most characterized GH 51 enzymes, which show specificity mainly for arabino-oligosaccharides (Phuengmaung et al., 2018). Due to this characteristic, GH 62 are also termed arabinoxylan arabinofuranohydrolases. In other words, GH 62 remove especially  $\alpha$  1-2 and  $\alpha$  1-3 linkages, although they are also active against arabinans and arabinogalactans, in which  $\alpha$  1-5 linkages predominate (Kaur et al., 2015).

The structures share a  $\beta$ -propeller catalytic domain with 5 arranged blades. Each blade has 4 antiparallel  $\beta$ -sheets radially oriented around a central axis. The N-terminal and C-terminal together form the so-called "molecular velcro," typical of proteins with this conformation. This circular proximity is important for protein stability, and some  $\beta$ -propeller forms additionally have disulfide bridges connecting the N- and C-terminal of the "velcro". Differences in the sizes of loops between the  $\beta$ -sheets may be the reason for the variation in substrate specificity (Numan & Bhosle, 2006; Wilkens et al., 2017).

The nature of GH 62 produced by fungi appears to be associated with mechanisms of plant invasion. The synergistic action of GH 62 with other xylanolytic enzymes, such as endoxylanases, xylosidases, and esterases, illustrates its powerful role in the efficient degradation of plant cell walls and its potential industrial applications, which include bioethanol production, oligosaccharide synthesis, juice clarification, pharmaceuticals, and the improvement of the quality of wines, bread, and animal feed. Although no study has yet been published applying these enzymes in the food industry to enhance the processing of the hemicellulosic fraction to obtain biomolecules of interest, several studies already point to their synergistic effect in association with endoxylanases and potential application to produce xylo-oligosaccharides (Ávila, Martins, Costa, et al., 2020; Gonçalves et al., 2012; H. Li et al., 2015).

Bacteria, fungi, and actinomycetes play a fundamental role in the decomposition of organic matter and have been reported as producers of various arabinofuranosidases. Many fungi, such as *Penicillium* sp. and *Aspergillus* sp., have had their arabinofuranosidases identified and characterized using different carbon sources containing L-arabinose as induction (Poria et al., 2020). Thermostable enzymes produced by various organisms are of particular interest, here are some examples: GH 51 from *Thermobacillus xylanilyticus* (Debeche et al., 2000), stable at temperatures up to 90°C and pH 4.0 to 12.0; GH 51 from *Bacillus pumilus* (Degrassi et al., 2003), maintaining 80% of its activity for 2 hours at 65°C; GH 62 from *Streptomyces thermoviolaceus* (W. Wang et al., 2014), with a half-life of 60 minutes at 60°C, and GH 62 from *Streptomyces* sp. SWU10, with optimal activity at 50°C and pH 5.0 (Phuengmaung et al., 2018). Generally, enzymes produced by thermophilic organisms are more stable than those produced by mesophilic organisms. Identifying these enzymes has great potential for industrial processes to accelerate the catalytic process and reduce possible contaminations (Shi et al., 2014). As mentioned earlier, GH 62, although still underexplored, are already known for their superior ability to hydrolyze arabinoxylans, in addition to the reported synergistic activity with endoxylanases (Y. Xue et al., 2020).

### *GH 62 Arabinofuranosidase from Aspergillus fumigatus*



Filamentous fungi play an important role in secreting CAZymes (carbohydrate active enzymes), which act synergistically in enzymatic cocktails. The genus *Aspergillus* includes about 250 species, many of which secrete hydrolytic enzymes of interest. *Aspergillus fumigatus* is an opportunistic pathogenic ascomycete fungus that can cause various allergic reactions. However, it is an important producer of CAZymes. This fungus is capable of secreting thermostable glycosyl hydrolases, such as  $\beta$ -glucosidases, endoglucanases, and endoxylanases (de Gouvêa et al., 2018; Sharma et al., 2016). Proteomic analysis and transcriptome studies of *Aspergillus fumigatus* have revealed the induction of expression of several CAZymes necessary for the hydrolysis of lignocellulosic biomass, such as GH 62 and GH 10. Most of the enzymes produced by the fungus are endoxylanases, cellobiohydrolases, endoglucanases, and arabinofuranosidases. Studies have identified high expression of hemicellulases, including  $\alpha$ -L-arabinofuranosidases GH 43 and GH 62. The latter is less studied and has the potential to act on recalcitrant biomass. Despite its pathogenicity, the potential of *A. fumigatus* can be harnessed to produce a variety of enzymes, which can be expressed in non-pathogenic microorganisms, contributing to the optimization of enzymatic cocktails, and enabling various bioprocesses (de Gouvêa et al., 2018; Pérez & Eyzaguirre, 2016). For evident reasons, this enzyme was of particular interest for this research, whose results are presented in **CHAPTER 6**, **CHAPTER 7**, and **CHAPTER 8**.

But first let's conclude this: enzymatic hydrolysis is crucial for converting lignocellulosic biomass, offering controlled, selective processes with lower energy requirements. However, enzyme costs are significant, prompting efforts for more efficient cocktails. Recombinant enzymes are sought for their improved properties, and yeasts like *Saccharomyces cerevisiae* and *Pichia pastoris* are the preferred hosts due to their post-translational modification, high-density growth, and efficient secretion, besides being toxin-free and safe. Yeast secretion pathways facilitate favorable disulfide bridge formation and glycosylation, enhancing enzyme functionality. The upcoming published bibliographic review presents a comprehensive explanation of recombinant enzymes, heterologous expression systems, and chimeric enzymes. This bibliographic exploration provides insights into the advancements, methodologies, and applications in the field. Furthermore, we will explore the potential of **chimeric enzymes**, which combine elements from different (but synergic) enzymes to enhance catalytic efficiency, substrate specificity, and stability.

**CHAPTER 5.**  
**RECOMBINANT CHIMERIC ENZYMES FOR**  
**LIGNOCELLULOSE HYDROLYSIS**

## Recombinant chimeric enzymes for lignocellulosic biomass hydrolysis

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### A brief introduction before we start

Continuing the explanation about lignocellulosic biomass and the importance of enzymes and recombinant expression, this published review article introduces the chimeric enzymes concept. Chimeric enzymes offer tremendous potential in various applications by combining the advantageous properties of multiple proteins within a single peptide chain. This synergistic approach not only enhances hydrolysis efficiency but also reduces enzyme production costs, making it highly attractive for industries dealing with lignocellulosic biomass hydrolysis, plant-based products, food additives, waste treatment, and biofuels. This review provides a concise overview of the construction of recombinant enzymes, with a focus on methods commonly employed in the *Pichia pastoris* host system and design strategies for chimeras, emphasizing the desired properties necessary for specific applications, such as the production of xylooligosaccharides from arabinoxylan. Furthermore, highlighted studies in the field of lignocellulosic biomass hydrolysis showcase the effectiveness of chimeric enzymes in breaking down complex substrates like arabinoxylan into valuable products. Additionally, advancements in structure prediction tools that facilitate the rational design of chimeric proteins, enabling the identification of optimal fusion partners and linkers to achieve enhanced performance and functionality. This comprehensive examination elucidates the potential and challenges associated with the utilization of chimeric enzymes in various biotechnological applications.

**Abstract**

Chimeras with improved properties can be designed by synergistically incorporating multiple proteins into one peptide chain. Such design enhances hydrolysis efficiency and lowers enzyme production costs for numerous applications, particularly in the fields of lignocellulosic biomass hydrolysis, plant-based industries, food additives, waste treatment, and bio-fuels. In this minireview, we summarize a brief, common approach to construct recombinant enzymes, especially in the *Pichia pastoris* host. We present the design strategies and the desired properties of chimeras and provide examples of recent studies in the area of lignocellulosic biomass hydrolysis. Subsequently, we describe the progress in structure prediction tools that facilitate the design of chimeric proteins toward identifying the best combinations of fusion partners and linkers.

**Keywords:** Protein engineering; chimeric enzymes; lignocellulosic biomass.

## 5.1. Introduction

The application of enzymes in various industrial processes is well established. Enzyme-catalyzed reactions occur at moderate temperatures, are stereospecific, and eliminate the use of toxic reagents, which is particularly beneficial for the synthesis of biomolecules for food or pharmaceutical purposes (Minshull & Stemmer, 1999).

The advent of purification techniques and genetic engineering improved enzymatic processes applied in the manufacture of biofuels, resins, processed meats, cheeses, juices, oils, and alcoholic beverages, among other products (A. M. Lopes et al., 2018). Special attention has been given to the hydrolysis of lignocellulosic materials: these structures are varied and complex, so their complete degradation requires a coordinated action of an enzymatic complex (Ebringerová et al., 2005).

Several vegetables are grown in large quantities in the agroindustry and generate massive amounts of waste. The diverse constitution of these wastes opens up numerous application opportunities for the development of new products in the food, chemical and pharmaceutical industries. Increasing knowledge about biopolymers, willingness to develop new bioproducts, and the fact that agro-industrial residues constitute an abundant source of lignocellulosic biomass have intensified efforts to develop enzyme cocktails that can deconstruct and convert these materials in an economically viable way (Ebringerová & Heinze, 2000; Freitas et al., 2019b; Gonçalves et al., 2012).

## 5.2. Lignocellulosic Enzymes

Lignocellulosic materials are the major constituents of plant biomass and consist mainly of three macromolecules (Naidu et al., 2018): cellulose (40–50%), hemicellulose (heteropolysaccharide derived from monosaccharides, such as arabinose, xylose, mannose, and rhamnose) (20–30%), and lignin (4-hydroxyphenylpropanoids aromatic blocks from hydroxycinnamyl, coniferyl and sinapyl alcohols) (10–15%) (Vanholme et al., 2010), all of which can be hydrolyzed to products of interest. A smaller portion of the biomass comprises pectin,

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### Aminoacids abbreviations

|               |        |
|---------------|--------|
| Alanine       | Ala, A |
| Glutamic acid | Glu, E |
| Glycine       | Gly, G |
| Lysine        | Lys, K |
| Proline       | Pro, P |
| Serine        | Ser, S |

proteins, waxes, and extracts. The amount of each component varies with the biomass source, part, and age (Naidu et al., 2018).

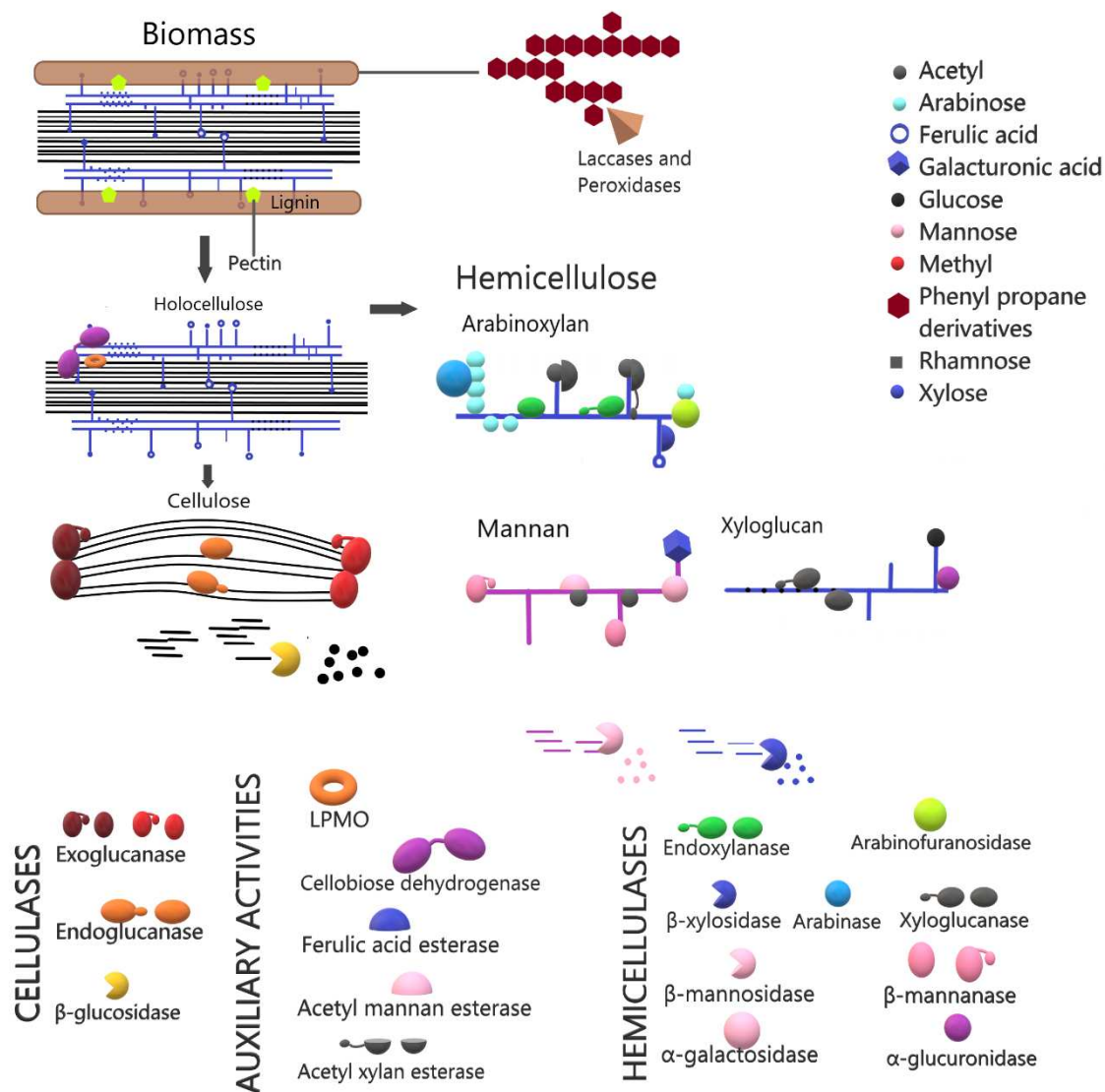


**Figure 5-1 Lignocellulosic biomass enzymatic lytic complex.**

Cellulosic lytic complex is responsible for the hydrolysis of the main polysaccharide in the cell wall, cellulose. Endo-1,4- $\beta$ -D-glucanase (EC 3.2.1.4) randomly hydrolyzes cellulose chains, releasing cellobiose, which is hydrolyzed to glucose by action of  $\beta$ -glucosidase or cellobiase (EC 3.2.1.21). Cellobiose dehydrogenase is an auxiliary enzyme that oxidizes cellobiose, and cellobiose phosphorylase catalyzes the reversible phosphorylation of cellobiose into  $\alpha$ -D-glucose-1-phosphate and D-glucose. These enzymes activate other hydrolytic cellulases by relieving their inhibition by cellobiose. Exoglucanases have an affinity for crystalline sites along external cellulose chains. They include the 1,4- $\beta$ -D-glucan glucohydrolase (EC 3.2.1.74),



which releases glucose from 1,4- $\beta$ -D-glucans and slowly hydrolyze cellobiose, and 1,4- $\beta$ -D-glucan cellobiohydrolase (CBH) (EC 3.2.1.91), which releases cellobiose from 1,4- $\beta$ -glucans from the reducing- (CBH I) or nonreducing ends (CBHII). Moreover, CBH can hydrolyze highly crystalline cellulose (Obeng et al., 2017; M. de L. T. M. Polizeli et al., 2017; Wyman et al., 2005a). LPMOs (lytic polysaccharide monooxygenases) also aid crystalline cellulose degradation. These enzymes are described as metalloenzymes (EC 1.14.99) and break glycosidic bonds by oxidizing C1 and C4 or both (Berrin et al., 2017; Monclaro & Filho, 2017).

Delignification of the phenylpropane polymeric structures involves laccases (EC 1.10.3.2), which oxidize phenolic compounds and aromatic amines. Lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) can oxidize lignin at the phenolic and non-phenolic aryl-ether positions, respectively (Crestini et al., 2011).

Mannans, which are hemicellulose polysaccharides, are degraded by the synergistic action of three enzymes: (1)  $\beta$ -mannanase (EC 3. 2. 1.78) acts on the main chain; (2)  $\beta$ -mannosidase (EC 3.2.1.25) releases mannose from the non-reducing ends; (3)  $\beta$ -glucosidase (EC 3.2.1.21) releases monosaccharides cleaving  $\beta$ -D-1,4-glucopyranose in the final non-reducing region of the oligosaccharides that are released from glucomannan and galactoglucomannan cleaved by (1)  $\beta$ -mannanase. Acetyl mannan esterase (EC 3.1.1.6) and  $\alpha$ -galactosidase (EC 3.2.1.22) cleave the acetyl group bonds (A. M. Lopes et al., 2018; Moreira et al., 2011).

Xylan, also a hemicellulose polysaccharide, has a complex and heterogeneous nature, so its enzymatic hydrolysis is more complicated as compared to the enzymatic hydrolysis of other polysaccharides. Different enzymes are required depending on the specific type of xylan and on the desired product. Arabinofuranosidases, esterases, endoxylanases, and xylosidases are among the enzymes involved in xylan depolymerization (Wyman et al., 2005b). However, as substituents are removed, xylan becomes less soluble and forms aggregates that hinder and further delay its degradation. The simultaneous removal of these substituents and cleavage of the glycosidic bonds of the main xylan chain increase the degradation rate. Therefore, mixtures of enzymes with synergistic action are desirable and have been the target of intense research to understand their mode of action and optimize enzymes loadings (Ávila, Cairo, Damasio, et al., 2020b; Wyman et al., 2005b).

Endo-1,4- $\beta$ -xylanases (EC 3.2.1.8) cleave glycosidic bonds between xylose units that constitute the main chain, releasing xylo-oligosaccharides. However, to improve xylan depolymerization, other enzymes that act on the side chains are needed: acetyl xylan esterase (EC 3.1.1.6) removes the O-acetyl groups from positions 2, 3, or both in  $\beta$ -D-xylopyranosyl

residues;  $\alpha$ -glucuronidase (EC 3.2.1.131) hydrolyzes  $\alpha$ -1,2 bonds between glucuronic acid and  $\beta$ -D-xylopyranosyl residues found in glucoxylans; arabinase removes L-arabinose from positions 2, 3, or both in  $\beta$ -D-xylopyranosyl residues; and ferulic acid esterase (EC 3.1.1.-) and p-coumaric acid esterase (EC 3.1.1.-) cleave ester bonds from the xylan side chains.  $\beta$ -1,4-D-xylosidases (EC 3.2.1.37) hydrolyze small xylo-oligosaccharides and xylobiose, releasing xylose (Biely et al., 1997; M. L. T. M. Polizeli et al., 2005).

The potential application of lignocellulosic enzymes (**Figure 5-1**) includes bioconversion of agro-industrial residues into biofuels and production of high value-added products, such as prebiotics (xylo-oligosaccharides) and sweeteners (xylitol); juice clarification; beverage consistency and animal feed digestibility improvements (Ávila, Cairo, Damasio, et al., 2020b; A. M. Lopes et al., 2018; Motta et al., 2013).

### 5.3. Heterologous expression

Enzymatic hydrolysis is crucial for biofuel production and lignocellulosic biomass conversion: it is a controlled and selective process that demands less energy and milder conditions than acid hydrolysis. However, costs for enzymes production are still a hurdle. Great effort has been made to increase efficiency of enzyme cocktails (A. M. Lopes et al., 2018).

To meet industrial needs, the employed enzymes must present certain properties such as stability over a wide range of pH and temperature, high specific activity, and resistance to cations. In addition, enzyme production needs to be efficient, environmentally friendly, and easily reproducible. Generally, native enzymes do not meet these demands because their production rates are low, which is incompatible with industrial processes. Therefore, gene expression tools are necessary to produce recombinant enzymes with better activity, selectivity, and stability (Potvin et al., 2012).

The data available from sequencing, crystallography, molecular dynamics, and computational design provide all the essential information to efficiently produce recombinant enzymes with distinct properties. In this way, a specific process can be optimized for a specific substrate, which is an important strategy to deconstruct the biomass completely and obtain the industrial products of interest while reducing costs and saving resources (A. M. Lopes et al., 2018; Motta et al., 2013).

As the most engineered prokaryote, *Escherichia coli* is the first host cell to be considered because it is well characterized, and its genetics is easy to manipulate. Nevertheless, expression in *E. coli* poses countless problems such as linker sequence degradation in multi-domain proteins, production of low levels of soluble proteins, and poor secretion ability, which

makes this microorganism unsuitable for large-scale production (Bill, 2014). *Zymomonas mobilis* is also an amenable host for expression of enzymes secreted to the periplasma or extracellularly. Just like *E. coli*, part of the expressed enzymes is intracellular. However, the fusion of signal peptides allows improved secretion into the media (Thirumalai Vasan et al., 2011).

Eukaryotic microorganisms that express heterologous enzymes are more attractive because they offer additional advantages over other expression systems: they allow post-translational modifications, grow under high cell densities, and provide high protein secretion into the medium. Furthermore, they are toxin-free and are generally recognized as safe (GRAS) organisms (S. Ahmed et al., 2009). The yeast secretion pathway generates favorable disulfide bridges and glycosylation, which increases enzyme stability and functionality. Glycosylation, which typically connects the catalytic domain with the carbohydrate-binding module region, offers protection against protease action and increases affinity for the substrate. This paves the way for more active and thermotolerant protein engineering (Lambertz et al., 2014).

*Saccharomyces cerevisiae* is the most engineered host due to high productivity, well established genetic tools and high tolerance to inhibitors, while *Kluyveromyces marxianus* possess the desirable thermotolerance (Lane & Morrissey, 2010), and *Saccharomyces pastorianus* the improved enzyme activity (Fitzpatrick et al., 2014). However, *Pichia pastoris* has become one of the most popular hosts for heterologous expression systems because this species produces structurally and functionally correct proteins (Çalık et al., 2015). *P. pastoris* grows rapidly in minimal medium and efficiently secretes recombinant protein, thereby allowing for savings with culture media and protein purification. Its genome is simple to manipulate, and commercial expression kits involving inductive or constitutive promoters are available (Potvin et al., 2012). Moreover, to optimize the downstream process, additional sequences, such as a poly-histidine or antibody recognition peptide sequence, may be inserted in the C-terminal peptide of the integrative plasmids used in the expression systems, so the recombinant protein can be detected and isolated with purification kits. These sequences do not affect the protein structure or biological function (Logez et al., 2012).

The success of the *Pichia pastoris* expression system was initially attributed to the inductive promoter methanol oxidase  $P_{AOX1}$ , the first isolated enzyme to be used for vector development and genetic manipulation protocols. Albeit effective, expression is induced only upon addition of methanol, which is flammable and poses serious health risks, especially when operated on a large scale. On the other hand, constitutive promoters enable protein synthesis to occur simultaneously to cell growth, since they are correlated to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene regulation. GAPDH (EC 1.2.1.12) is constantly expressed at

high levels because it participates in the glycolysis and gluconeogenesis pathways (Potvin et al., 2012; Waterham et al., 1997).

Given the recent advances in the development of expression systems, constitutive expression promoters are widely employed. They avoid the issues arising from the use of methanol as an expression inducer, simplifies the process, and reduces costs. These systems are stable and efficient, thus elevating protein production at the transcriptional level. (Çalık et al., 2015; Potvin et al., 2012; Waterham et al., 1997).

Not only thermal stability, but also high production and high catalytic performance are extremely relevant for the biotechnological and industrial application of enzymes. Many cellulosic and hemicellulosic enzyme genes produced by various fungi and bacteria have been cloned and expressed in *Pichia pastoris* (C. C. P. de Andrade, 2014; Cui et al., 2008; Özcan et al., 1991; Pérez & Eyzaguirre, 2016; J.-L. Xue et al., 2016). Also, from an industrial viewpoint, understanding enzymatic synergism is essential to accelerate biomass conversion and reduce the required amount of enzyme and hydrolysis time. These factors significantly affect production costs (A. M. Lopes et al., 2018).

Despite all the efforts to isolate and to express efficient enzyme genes, the costs associated with enzyme technologies remain a major obstacle (Lambertz et al., 2014). One strategy to overcome this issue is to design multifunctional enzymes with synergistic catalytic activities. Rational protein design enables the creation of polypeptides with multiple catalytic domains and specificity for two or more substrates (Diogo et al., 2015). Multifunctional enzymes have numerous advantages. First, they simplify the expression path, facilitating metabolic pathways. Second, several reactions are regulated simultaneously in a single polypeptide chain. Thus, chimeric proteins have been developed to reduce production costs and to improve the performance of catalytic domains (Diogo et al., 2015; Fan, Werkman, et al., 2009; Saadat, 2017).

#### **5.4. Chimeric proteins**

Three billion years of evolution have selected excellent catalytic proteins. However, these proteins are generally not optimal for an industrial application. Even though enzymes of extremophiles with appropriate activity for a specific process can be searched, the nature has selected these enzymes to act in a complex mixture of molecules, either inside the cells or in the environment where these organisms live, rather than in industrial bioreactors. To obtain all the desired properties, more advanced optimization is necessary (Minshull & Stemmer, 1999). In addition to the production of recombinant enzymes, another approach has been

used: proteins with different characteristics have been coupled by genetic fusion at the translational level. This approach generates multifunctional enzymes in a single polypeptide chain, which facilitates regulatory mechanisms and optimizes enzymatic performances (Elleuche, 2015).

Although mixtures of enzymes are widely employed in the industry, many studies have reported that chimeric enzymes perform better than enzyme cocktails. A chimera that catalyzes consecutive reactions can immediately transfer the intermediate to the next active site in the corresponding enzyme. Consequently, the intermediate is consumed before its distribution in the solution, which increases reaction efficiency (Fan, Werkman, et al., 2009; Guo et al., 2013; Saadat, 2017). Apart from their synergistic effect and easier production, two or more fused proteins may have their physical and chemical properties combined, such as temperature and pH profile. As a result, fused proteins have equally stable and active biocatalysis under the same operational conditions (Elleuche, 2015; Rizk et al., 2015).

Various multi-domain proteins, including cellulosomes, occur naturally (Fan, Wagschal, et al., 2009; K. Yu et al., 2015). The availability of sequential data has increased dramatically, which has heightened awareness of the complexity to which the mechanisms of genetic rearrangement have evolved. Consequently, multimodular proteins, the chimeras, make up a large proportion of an organism's protein complement (Henikoff et al., 1997). Inspired by nature, bioengineers have been developing methods that mimic natural processes to produce chimeric proteins by synthetic domain fusion (Ribeiro et al., 2011; Saadat, 2017; K. Yu et al., 2015). The proximity between the catalytic domains of fused enzymes improves enzymatic performance as compared to the activities of the parent enzymes. Chimeric proteins are built for several purposes, including greater stability, expression efficiency, affinity, and specificity. Creating bifunctional enzymes to act on lignocellulosic materials has proven to be a practical option to optimize enzymes that are involved in depolymerization (Ribeiro et al., 2011).

Although different strategies exist, some general rules must be followed to provide adequate functionality to the proteins. Regardless of the protein fusion procedure, these rules include translation efficiency; the order of the fused structures depending on molecular size, structure and thermostability; and fusion flexibility (Elleuche, 2015; Saadat, 2017). Furthermore, a key factor to succeed in creating chimeras is to achieve compatible catalytic activities in relation to the physical-chemical requirements (e.g. optimal pH and temperature) of the individual proteins (Ribeiro et al., 2011).

Recently, numerous chimeras have been developed to optimize lignocellulosic biomass hydrolysis. A laccase/glucanase chimera developed using domain insertion was 20%

more active on sugarcane bagasse as compared to the mixture of these enzymes (Furtado et al., 2013). An endoxylanase/endomannase chimera built by tandem fusion displayed synergistic activity in hemicellulose hydrolysis (Guo et al., 2013). A xylosidase/arabinofuranosidase chimera obtained by tandem fusion released 30% more xylose (Fan, Wagschal, et al., 2009). A laccase/endoxylanase built by domain insertion provided to the endoxylanase domain a significant increase in thermotolerance and resistance to proteolytic attack (Ribeiro et al., 2011). The fusion of two complementary enzymes, xylanase and xylosidase, resulted in a chimera with higher optimum temperature and thermotolerance for the xylosidase domain, which enhanced xylose production as compared to the parent enzymes (Diogo et al., 2015).

Synthetically fused proteins are widely applied for process optimization. Three strategies are used to promote domain fusion: tandem fusion (end-to-end), domain insertion, and post-translational conjugation. Computational prediction of the structures follows the progress of these strategies. Even though the strategies focus on the fusion of two proteins, they are also applicable for multicomponent systems.

#### **5.4.1. Tandem fusion**

Tandem fusion is the predominant method used to synthetically join domains to build chimeric proteins (**Figure 5.2**). In tandem fusion, two or more sequences that encode proteins (Open Reading Frames - ORFs) are connected end-to-end, with or without a linker sequence, to produce a single polypeptide chain with bi or multifunctional characteristics (Elleuche, 2015; Saadat, 2017). This method is preferable because it is the least dependent on the structural knowledge of proteins, so it requires the use of special equipment less frequently. Albeit simpler, this method demands that several parameters be met to adapt the structural arrangement and to ensure chimera functionality (Diogo et al., 2015; Saadat, 2017).

Factors that determine the success of protein fusion include spacing between modules, individual receptor structures and their correlation, and linker size and complexity. The first gene to be fused must be free of the stop codon, and the last gene must not contain the signal peptide or the ribosome binding site. The order in which genes are inserted is extremely important because it affects the chimera's conformation, structure, and physical-chemical properties (Elleuche, 2015). The N-terminal residue, referring to the free amine group ( $-NH_2$ ) at the start of the polypeptide chain, plays a decisive role in protein half-life. Therefore, the gene of the most thermostable protein should be placed in the N-terminal position to make the next protein more thermally stable (S. Zhang et al., 2010).

Direct fusion works for cases in which the N-terminal and C-terminal (the end of the polypeptide chain terminated by a free carboxyl group (-COOH)) of the ligand sequences are flexible and do not have a regular secondary structure or a catalytic function. Nevertheless, if these terminals are essential for catalytic function or are not flexible or long enough to avoid steric hindrance, a short amino acid sequence (the linker) must be added to bind the proteins and to improve conformation and bioactivity. An optimized linker improves the levels of expression, corrects conformation, minimizes steric influences due to proximity of the catalytic domains, and maximizes module flexibility (George & Heringa, 2002; Rizk et al., 2015).

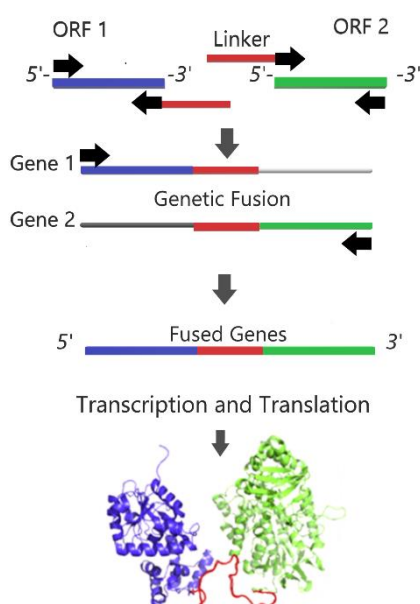


**Figure 5-2 Tandem fusion schematic illustration of the construction of a chimera by PCR overlap (black arrows indicate primer orientation)**

### ***Linker***

Natural multi-domain proteins consist of two or more domains that are joined by a linker sequence. This sequence of peptides connects the modules and helps to maintain inter-domain cooperative interaction and to preserve bioactivity. They can adopt varied secondary structures, such as  $\alpha$ -helix,  $\beta$ -strand, coil, and turns. Helix, which is rigid and stable, and coil, which is more flexible, are the most common structures. Knowledge about natural linkers is relevant to rationally design recombinant linkers to synthetically fuse recombinant enzymes (X. Chen et al., 2013; George & Heringa, 2002; K. Yu et al., 2015).

Rational linker design started with the development of the LINKER software (Crasto & Feng, 2000), which used an online server to automatically generate binding

sequences of determined length to link proteins. The program searches through a library of loops derived from the Brookhaven Protein Databank (PDB) and suggests the most favorable loop for the input. Subsequently, new automatic prediction tools from a database of sequences extracted from natural multi-domain proteins (e.g., LinkerDB) were developed to provide candidates that respected the protein sequence, length, secondary structure, and PDB code (George & Heringa, 2002), engendering the SynLinker, the most updated (<http://bioinfo.bti.a-star.edu.sg/synlinker>). In addition, many databases are available and can be accessed; for example, iGEM ([http://parts.igem.org/Protein\\_domains/Linker](http://parts.igem.org/Protein_domains/Linker)) and the Center for Integrative Bioinformatics at the University of Amsterdam (<http://www.ibi.vu.nl/programs/linkerdbwww/>).

The properties of natural linkers, like length, hydrophobicity, amino acids, and secondary structure, serve as a reference for the design of empirical linkers for recombinant proteins (X. Chen et al., 2013). Linkers are usually categorized by their length, amino acid composition, hydrophobicity, secondary structure, sensitivity to proteases, and solubility. However, the most comprehensive and prevalent way to classify linkers is based on flexibility, rigidity, and sensitivity to proteases (Saadat, 2017; K. Yu et al., 2015). Table 5-1 lists some examples of linkers that are used in chimeric hemicellulosic proteins produced by tandem fusion.

**Table 5-1: Examples of linkers and their purposes in hemicellulosic chimeric proteins**

| Example                        | Type              | Sequence                             | Function                          | Ref.                          |
|--------------------------------|-------------------|--------------------------------------|-----------------------------------|-------------------------------|
| Endoxyloglucanase              | Flexible          | (GGGG)                               | Flexibility                       | (Cota et al., 2013)           |
| Endoxylanase-xylosidase        | Natural, flexible | <i>Thermotoga petrophila</i><br>0899 | Steric hindrance<br>prevention    | (Diogo et al., 2015)          |
| Endoxylanase-mannase           | Rigid             | (EAAAK) <sub>3</sub>                 | Improves thermo-<br>stability     | (Guo et al., 2013)            |
| Endoxyloglucanase              | Flexible          | (GGSGG)                              | Flexibility                       | (Furtado et al., 2015)        |
| Xylosidase-arabinofuranosidase | Flexible          | (GGGGG...N)                          | Steric hindrance<br>prevention    | (Fan, Wagschal, et al., 2009) |
| Xylosidase-arabinosidase       | Flexible          | (SAGSSAAGSGSG)                       | Improve stability<br>and activity | (R. Wang et al., 2010)        |

### ***Flexible Linker***

Flexible linkers consist of small sequences that are rich in glycine and alanine. This type of linker is applied when the merged domains require more freedom of mobility or interaction. The presence of polar amino acids (such as serine, glutamic acid, and lysine) increases stability in aqueous solvents through hydrogen bond formation (X. Chen et al., 2013). The most often employed flexible linkers appear in the form of (Gly)<sub>n</sub> and (Gly-Gly-Gly-Gly-Ser)<sub>n</sub>, where “n” is the number of residues. This “n” is often lower than six, and the linkers form a loop (X. Chen et al., 2013; K. Yu et al., 2015). The use of linkers where n = 3 promotes greater catalytic



activity, and the Michaelis constant  $K_m$  value is lower as compared to a rigid linker (Guo et al., 2013). Short glycine-rich sequences allow greater mobility without disturbing enzyme conformation. In addition, short sequences make proteolytic attack less likely during protein expression and purification (Cota et al., 2013; Furtado et al., 2015).

Although glycine-rich sequences provide the necessary interdomain flexibility, monotonous ligands (GGGG or GGGGS or GGGGSGGGGS) can limit the catalytic activity. Furthermore, glycine-rich regions may decrease the level of expression by creating mRNA with stable secondary structure, and codons that are rich in guanidine (*G*) and cytosine (*C*) (*GGN* for glycine and *GCN* for alanine, where *N* can be any nucleotide base) may be unacceptable for some microorganisms (P. Lu & Feng, 2008).

However, some studies on the optimization of peptide ligands have suggested that addition of alanine and serine affects the flexibility of these ligands and can increase the enzymatic activity in different ways: alanine accelerates folding, and serine slows down unfolding (R. Wang et al., 2010). Combining small amino acids like alanine and serine with glycine-rich sequences and increasing the sequence length can overcome these problems, thereby maintaining flexibility and increasing catalytic activity. As noted in **Table 5-1**, proteins that require more distance between the domains to avoid steric hindrance and to increase catalytic activity were constructed with longer ligands. Nevertheless, proteases can cleave longer linkers more easily (J. Zhang et al., 2009).

In short, flexible linkers are sequences that are generally rich in small and/or polar amino acids. These linkers promote good flexibility and solubility and are suitable to fuse domains that require freedom of movement or interaction. Although these linkers do not have a rigid structure, they can passively maintain the distance between the domains, helping to adjust the length of the fused proteins and to elicit their adequate folding and biological activity (X. Chen et al., 2013).

### ***Rigid Linker***

While flexible linkers are advantageous because they passively connect the catalytic domains, hence allowing the modules to be somewhat mobile, the lack of rigidity or insufficient spacing between the domains can be a limitation. In these cases, establishing a fixed distance between the domains can ensure the maintenance of the independent activities of each enzyme (X. Chen et al., 2013). Numerous natural linkers have  $\alpha$ -helix structure (George & Heringa, 2002), which is stabilized by  $\text{Glu}^- \text{Lys}^+$  bridges. These linkers can act as rigid spacers

to inhibit interactions between the domains. This type of linker was discovered in proteases and pyruvate dehydrogenases (X. Chen et al., 2013).

On the basis of these natural ligands, empirical linkers A(EAAAK)<sub>n</sub> (where 2 < n < 5) have been developed and compared to flexible linkers of the same length, showing to separate the domains more efficiently (Arai et al., 2001). Another type of rigid linker bearing a proline-rich sequence, (XP)<sub>n</sub>, where X can be an alanine, a lysine, or a glutamine, has been developed. The presence of proline increases rigidity by favoring an  $\alpha$ -helix structure, the length of which is adjusted to achieve the ideal distance between the domains. Apart from having a circular and solid structure, proline contains an amide group that lacks hydrogen bonds, which prevents interactions with other amino acids (George & Heringa, 2002). Studies comparing (GGGS)<sub>n</sub> and (EAAAK)<sub>n</sub> linkers (where 2 < n < 4) have shown that the rigid linker provides greater thermostability (Guo et al., 2013) but lower catalytic activity (Guo et al., 2013; P. Lu & Feng, 2008).

### ***Cleavable Linker***

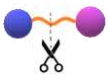
The two groups of linkers presented above consist of stable peptide sequences that preferably will not be cleaved. Stable ligands promote a longer half-life; however, as mentioned earlier, some problems must be overcome, including distance between the domains, reduced levels of expression, and decreased activity. Under these circumstances, ligands that can be cleaved by solvents or proteases can be introduced to release the fused domains *in vivo*, which eliminates the issue of steric impediment and increases protein bioactivity (X. Chen et al., 2013).

A well-studied process uses the reversible nature of disulfide bridges, reduced *in vivo*. After protein expression, this type of linker allows the domains to be separated and hence recover their individual characteristics, such as bioactivity (X. Chen et al., 2010). In this way, protease-sensitive sequences can be incorporated into linkers and then be cleaved by a specific protease, thus activating the fused protein at a specific site. MEROPS is a specific database of protease-sensitive sequences, so it is an important tool to design this type of linker (Rawlings et al., 2012). Unlike flexible and rigid linkers, there are no reports on the use of cleavable binders to construct lignocellulosic chimeras.

To sum up, linkers can adopt different structures and perform diverse functions to fulfill the requirements of the fused domains, as summarized in Table 5-2. Flexible linkers are generally rich in small or hydrophilic amino acids (e.g. glycine and serine), which promote interdomain flexibility. Rigid linkers are preferable when a defined spacing between the

domains is required. Both flexible and rigid linkers are stable and do not allow the joined proteins to separate, which contrasts with cleavable linkers, that are adopted when the recovery of the individual characteristics of the fused proteins is necessary. A rational choice should be based on the properties of the ligands and fused protein. The rapid development of protein biotechnology has made the design of linkers for protein fusion increasingly important. A better understanding of their structures and functions will facilitate the incorporation of linkers to construct stable bioactive recombinant fused proteins for various applications (X. Chen et al., 2013).

**Table 5-2: Summaries of linker classes and characteristics**

| <i>Linker</i>                                                                                           | <i>Advantage</i>                         | <i>Characteristics</i>                   | <i>Example</i>                                |
|---------------------------------------------------------------------------------------------------------|------------------------------------------|------------------------------------------|-----------------------------------------------|
| <b>Flexible</b><br>    | Interactions and spacing between domains | Rich in small or hydrophilic amino acids | (G) <sub>n</sub><br><br>(GGGGS) <sub>n</sub>  |
| <b>Rigid</b><br>     | Fixed distance between domains           | $\alpha$ -helix structure                | (EAAAK) <sub>n</sub><br><br>(XP) <sub>n</sub> |
| <b>Cleavable</b><br> | Domain separation                        | Cleaved by proteases or solvents         | Protease-sensitive sequences                  |

#### 5.4.2. Domain insertion

Domain insertion exists naturally in about 9% of multidomain proteins, especially those belonging to  $\alpha/\beta$  classes (K. Yu et al., 2015). The main advantage of domain insertion is that it allows the insertion of only the catalytic domain, and not the entire protein, in the genomic sequence of another enzyme. This method results in a molecule with lower weight when compared to the molecule that would be obtained by tandem fusion; also, reduces the metabolic stress generated by insertion of a heterologous gene in the host organism and enables the chimera to have more access and penetrability in complex substrates, such as the lignocellulosic biomass (Saadat, 2017).

This technique, illustrated in **Figure 5-3**, is more complicated than tandem fusion because it requires structural knowledge of the proteins to find where the domain can be inserted without interfering in the conformation and integrity (Furtado et al., 2013). In other words, the domain must be inserted in places (such as loops and turns) that are not directly involved with the enzymatic activity (Saadat, 2017; K. Yu et al., 2015). Computational methods that can

predict how the insertion of a segment can impact protein conformation are often used, e.g., GROMACS (Furtado et al., 2013; Ribeiro et al., 2011) and SCHEMA (Saadat, 2017). Basically, these programs read the protein structure to identify which interactions between residues may break upon segment insertion (K. Yu et al., 2015). However, conformational prediction does not avoid the issue of steric impediment resulted from the proximity of domains with the consequent decrease in catalytic activity (Elleuche, 2015; Saadat, 2017).

When this method was used for the first time, it involved inserting the domain of a  $\beta$ -lactamase into a protein binding to maltodextrin, which affected the active site of lactamase positively and increased its stability (Elleuche, 2015). Rational design based on computational methods allowed a xylanase to be inserted on the surface of a laccase loop, to yield a more accessible domain (Ribeiro et al., 2011). Improved stability at acid pH and temperature was achieved by combining the properties of an acidophilic and a thermophilic endoglucanase (Kufner & Lipps, 2013).

Besides the insertion of domains, other regions can be transferred to the target domain for physical-chemical improvements. Modules from members of the family 10 of the glycoside hydrolases (GH 10) have stood out for this purpose. When the homologous regions of endoxylanases that are responsible for binding with the substrate are known, the exchange of a module for another module from a member with more desirable characteristics, such as thermostolerance (Q. Wang & Xia, 2007) and alkali-tolerance (Nishimoto et al., 2002), can result in a chimera with improved properties with unaffected or improved activity.

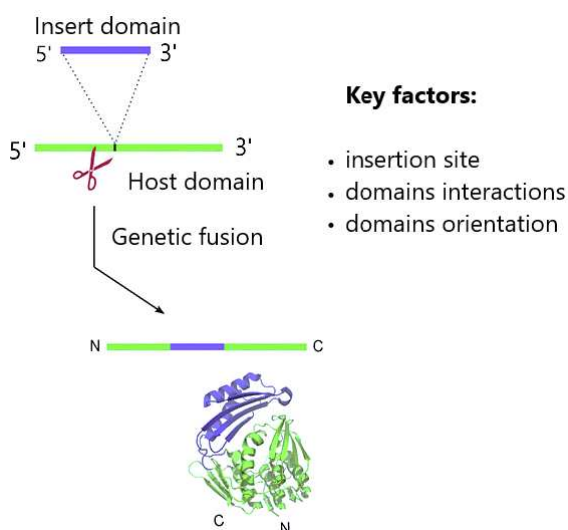


**Figure 5-3 Domain insertion schematic illustration**

### 5.4.3. Post-translational conjugation

Another strategy to create fused proteins is to join the individual proteins after their expression (**Figure 5-4**). Although the two strategies presented in the previous items represent the most popular methods to produce chimeric proteins, in some cases failures in expression or conformation may occur, impairing biological activity. Post-translational union allows the modules to be paired after they are expressed in optimized hosts, to bypass the limitation of the genetic fusion cloning steps (K. Yu et al., 2015).

This post-translational step is also called protein splicing, in analogy with the splicing of RNA introns, and it does not require any co-factor or auxiliary enzyme (Elleuche, 2015). Protein splicing can be modulated by mutation and converted into highly specific protein self-cleavage and binding reactions, which are useful tools in protein engineering (Paulus, 2000).

Proteins in their native conformations can be conjugated by means of a minimum binding tail, which avoids refolding steps. Such methods add small “tags” at the protein N- and C- terminal, in the same way as in the first stage of tandem fusion (amplification of the protein gene coupled to the linker sequence) (Witte et al., 2012). For example, sortase-catalyzed transacylation reactions, or “sortagging”, allows proteins to be labelled with small probes (<2kDa), then site-specific conjugation takes place (Popp et al., 2007). Chemical reactions based on non-specific crosslinking via amines and sulfhydryl also enable conjugation, but their products are heterogeneous because the reaction is not controlled (Witte et al., 2012).

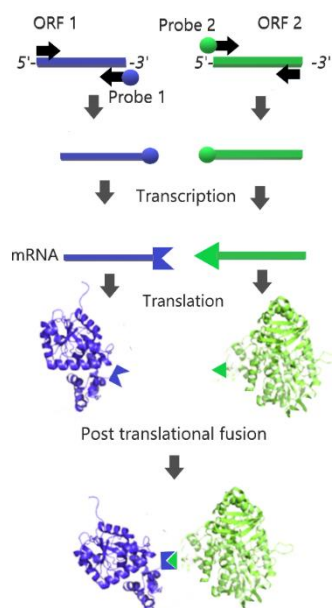


**Figure 5-4** Post-translational insertion schematic illustration (black arrows indicate primer orientation).

## 5.5. Structure prediction

Protein structures are more conserved than their sequences. A protein that presents 50% homology has 90% of its C $\alpha$  atoms positioned similarly, with a variation of 3 Å. This ensures successful structural homology modeling by means of bioinformatic tools (J. Zhang et al., 2009). A structure can be predicted by means of a known model structure and an alignment map of amino acid sequences (**Figure 5-5**). Bioinformatic tools like PSI-BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>) (Buchan & Jones, 2019) allow the target sequence to be aligned with a database of proteins of known structure, to provide the best candidates for modeling. One example is UCSF Chimera (<http://www.rbvi.ucsf.edu/chimera/>), a program for interactive structure visualization and analysis, including load density and sequence alignment. This tool has an interface for the MODELLER software (Webb & Sali, 2016), which is a three-dimensional prediction program of protein structures by homologous comparison based on the PDB file of the model sequence and the file of sequences alignment between the model and the target. Molecular dynamics simulations can be developed using the GROMACS algorithm (Hess et al., 2008) or GROMOS (Christen et al., 2005). Fusion Protein MODELLER (Chiang et al., 2006) is a tool that has been especially developed to model fused proteins, defining regions for flexible linkers between two or more rigid domains.

Predicting chimeric proteins constructed using domain insertion is cinematically more complicated than predicting chimeric proteins built using tandem fusion. The ROSETTA software is one of the tools that help to locate spatial conformational impediment and to identify hotspot residues in the protein-protein interface (Ribeiro et al., 2016). This method is applied for *de novo* predictions of the structure of proteins generated by domain insertion (K. Yu et al., 2015).

Unexpected consequences like failure of conformation, low level of expression, or impaired activity can happen mainly due to limited knowledge of the structure-function relationship in these proteins. Spatial orientation and interaction between the fused parts are still largely unknown and are often overlooked when new chimeric proteins are designed. Developing computer programs for specifically predicting structure and rational design is an imperative direction, as well as expanding a database of linkers containing new types of linkers with varied properties and more extensive study mechanisms about the linker functions on protein solubility, stability, and activity. Synthetic biology tools, including codon optimization, can be used to overcome problems of expression level. All these available tools contribute to a more rational

construction of proteins for the most diverse purposes (X. Chen et al., 2013; Guo et al., 2013; K. Yu et al., 2015).

## 5.6. Conclusion

The diverse industrial use of recombinant enzymes has led to the creation of relevant chimeric enzymes to reduce production costs and improve the characteristics of the enzymes. In all the methods discussed here, the most critical criteria are maintaining protein conformation and inhibiting overall interference, which in some cases can be achieved with the right choice of the linker sequence. Bioinformatic tools are important to the development of chimeras, the right choice of partners, structure prediction, and molecular simulations. Simplifying and improving large-scale processes involving synergic enzymatic reactions constitute one of the greatest challenges to overcome efficiency issues and production costs of bioenergy and value added biocompounds.

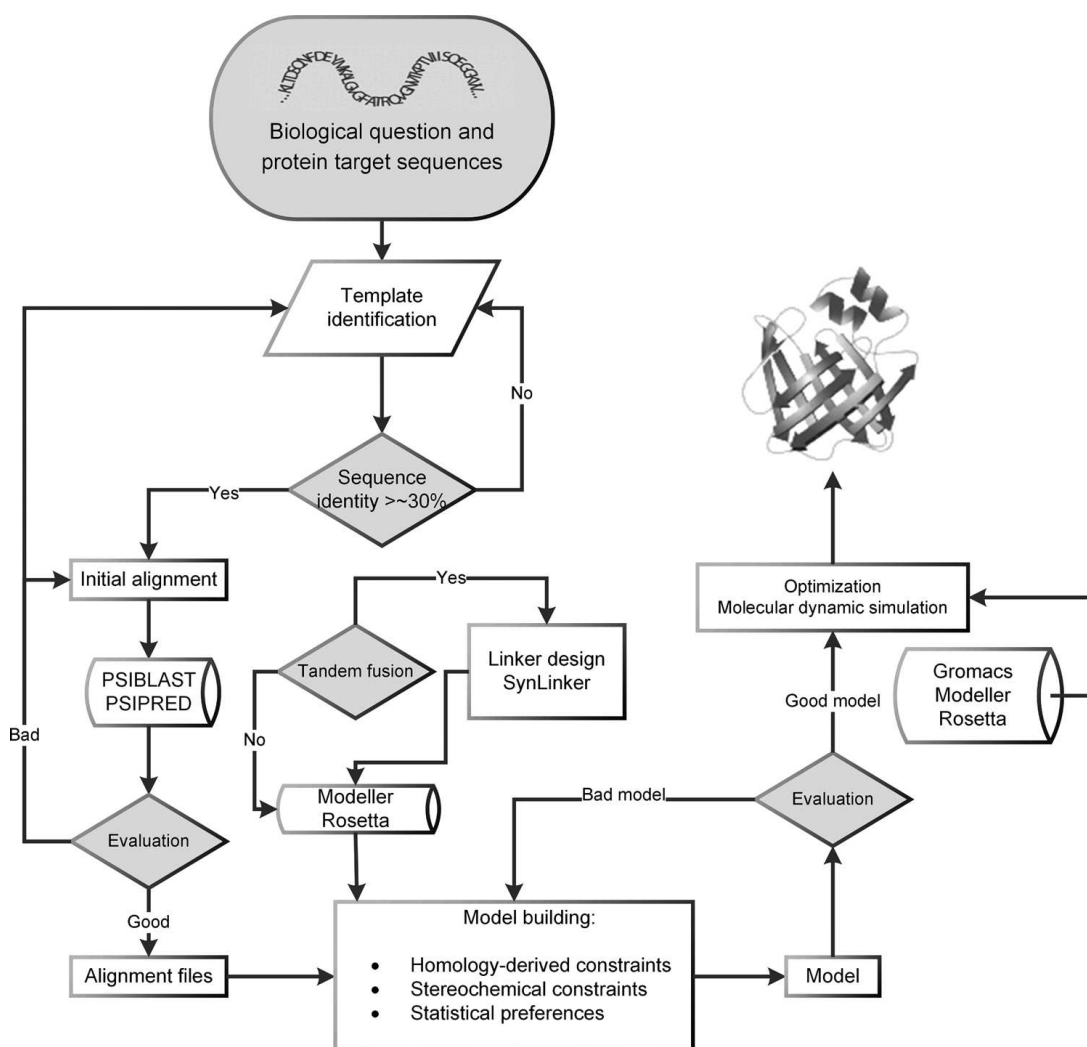


**Figure 5-5** Flowchart for protein modelling: scheme of the methods used for modeling a chimera

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## **CHAPTER 6.**

### **SYNERGISM FOR XYLOOLIGOSACCHARIDES, P-COUMARIC ACID AND FERULIC ACID PRODUCTION, AND THERMOSTABILITY MODULATION OF GH 62 A-ARABINOFURANOSIDASE**

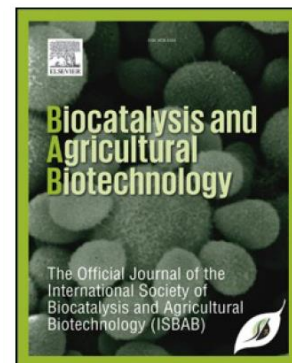
## Synergism for xylooligosaccharides, *p*-coumaric acid, ferulic acid, and thermostability modulation of GH 62 $\alpha$ -arabinofuranosidase

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### Stepping into the experimental results

Now let's understand how all that was presented before is connected. The first published scientific paper containing experimental results elucidates the role of GH 62  $\alpha$ -L-arabinofuranosidase for xylooligosaccharides, *p*-coumaric acid, and ferulic acid from agroindustrial wastes. Also, this pioneering investigation analyzed the thermodynamic and kinetic properties, structural predictions, and the impact of metal ions on the thermostability of a GH 62 arabinofuranosidase subfamily 1, which is the least studied, and little information about this subfamily is available in the literature. These findings hold significant promise for the development of economically viable enzymatic formulations tailored for industrial applications, particularly in the synthesis of prebiotic and antioxidant compounds within the food and pharmaceutical sectors, utilizing abundant sources rich in arabinoxylan such as agricultural byproducts like grains husks and peels. The synergism with GH 10 endoxylanase for XOS production showed that the simultaneous removal of arabinose side chains improves the catalytic efficiency, and this synergism could be further explored by designing a bi-functional chimeric enzyme focused on enhancing xylooligosaccharides production from arabinoxylan biomass. Therefore, this characterization of GH 62 arabinofuranosidase was essential prior the development of the chimeric enzyme in fusion with the GH 10 endoxylanase, introduced in **CHAPTER 4**.

(Supplementary material in **CHAPTER 12**)

### Abstract

GH 62  $\alpha$ -L-arabinofuranosidases are a group of enzymes that preferably hydrolyze L-arabinose residues of arabinoxylans. Albeit the least studied, these enzymes have gained attention because they exert synergistic action with other xylanolytic enzymes for arabinoxylan depolymerization. This is the first study on the thermodynamic and kinetic parameters, structural prediction, and metal ion effect on the thermostability of a GH 62 arabinofuranosidase subfamily 1. The optimum enzymatic conditions are pH 4.8 and 42 °C, and the presence of  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  ions enhanced thermostability and activity. The synergism of this arabinofuranosidase with a GH 10 endoxylanase increased the production of xylo-oligosaccharides 2.3 times with additional coproduction of 0.82 mM of ferulic acid and 0.09 mM of *p*-coumaric acid released from corn hull arabinoxylan (4% w/v). These results are relevant to developing an enzymatic formulation that is economically feasible for industrial applications, especially for prebiotic and antioxidant compounds production in the food and pharmaceutical sectors from sources rich in arabinoxylan, especially agro-industrial wastes of grains husks and peels.

**Keywords:** Arrhenius plot; Enzymatic hydrolysis; Homology modeling; Lignocellulosic biomass; Recombinant enzyme; Thermostability.

## 6.1. Introduction

Arabinoxylans (AX) are the main hemicellulosic group found in grasses and grains. They are composed of a xylose backbone, with  $\alpha$ -L-arabinofuranoside residues in the C (O)-3 or -2 positions of the xylose backbone, cross-linked to ferulic acid and *p*-coumaric acid via an ester linkage. The side chains form covalent bonds with cellulose and lignin, molding a complex that surrounds the cellulose fibers (Z. Zhang et al., 2014). Ferulic acid (FA) and *p*-coumaric acid (PCA) presence in arabinoxylans confers antioxidant properties and are used in the pharmaceutical and food industries due to their anti-inflammatory and antimicrobial properties, respectively (Cobs, 2020).

The enzymatic hydrolysis of arabinoxylan, especially those from agricultural wastes (grain husks, hulls, and peels) represents an economical and sustainable way for industrial applications (Yuwang et al., 2018), such as the production of xylo-oligosaccharides (XOS). XOS are xylose short-chains (with 2 to 6 xylose units) prebiotic oligosaccharides recognized as functional ingredients for food and pharmaceutical formulations. XOS obtainment from arabinoxylan enzymatic hydrolysis of agricultural wastes is appealing due to the positive effects on microbiota regulation, treating dysbiosis, preventing infections, reducing gut constipation, and facilitating digestion and nutrient absorption (V. Kumar et al., 2021), as well as antioxidant potential (Valls et al., 2018).

The L-arabinofuranosyl  $\alpha(1\rightarrow2)$  and  $\alpha(1\rightarrow3)$  bonds in non-reducing polysaccharides are cleaved by  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55). The GH 62 family solely contains arabinofuranosidases that preferably hydrolyze L-arabinose residues attached to the side chain of arabinoxylans, in contrast to most GH 43 and GH 51 enzymes, which show a preference for  $\alpha(1\rightarrow5)$  arabino-oligosaccharide bonds (Kaur et al., 2015). Furthermore, structural analysis of GH 62 arabinofuranosidase reported it potentially accommodates a feruloyl group attached to arabinose from the arabinoxylan side chains. This means GH 62 can release feruloyl L-arabinose residues (Tonozuka & Tanaka, 2017), which are efficient antioxidants that can be used in food, medicine and cosmetics (Lin et al., 2014). However, this family has been the least studied (Kaur et al., 2015).

Phylogenetic analysis has indicated that four GH 62 subfamilies exist, but information about whether this division represents distinctions in enzymatic properties is scarce. The first studies on GH 62 structural determination and characterization were only published in 2014 (Wilkens et al., 2017). To deepen our understanding of GH 62 enzymes, this study presents the thermodynamic and biochemical characterization of the GH 62  $\alpha$ -L-

arabinofuranosidase from *Aspergillus fumigatus*, and the synergism for enhancement in ferulic acid (FA), *p*-coumaric acid (PCA), and xylo-oligosaccharides (XOS) release. Our previous study reported the synergistic effect of GH 51 arabinofuranosidase and GH 10 endoxylanase for XOS production (Martins, Ávila, et al., 2020). To date, however, there are no previous studies on the application of GH 62 enzymes to improve hemicellulose fraction processing in the food industry.

## 6.2. Material and Methods

### 6.2.1. Material

Corn seed hull was donated by Milhão Ingredients (Goianira, Brazil). Composition characterization followed the NREL methodology (Sluiter et al., 2005, 2008) and is described in **Supplementary table 12-1**. Corn hull arabinoxylan was extracted with 5% KOH as described elsewhere (de Figueiredo et al., 2017). Wheat arabinoxylan was purchased from Megazyme Ltd. (Wicklow, Ireland). All other chemicals were reagent grade.

### 6.2.2. Protein prediction and bioinformatic analysis

The Afua\_2g12770 gene (Gene ID **3513529**) was analyzed by using the PSI-BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>), Clustal Omega tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), and InterProScan Sequence Search (<http://www.ebi.ac.uk/Tools/pfa/iprscan>). The putative *N*- and *O*-glycosylation sites were located by using NetNGlyc1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and NetOGlyc3.1 (<http://www.cbs.dtu.dk/services/NetOGlyc/>), respectively. The structural model by the comparative modeling used MODELLER9 v12 and was validated with PROCHECK. Polar hydrogens were added, and Gasteiger partial charges were assigned. Charges were computed using ANTECHAMBER Protein-ligand docking was carried out by using Autodock Vina and visualized with UCSF Chimera 1.14. The 3D structures of feruloyl arabinobiose (CID 6438898) and xylotetraose (CID 10230811) were downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov>). The docking grid box was centered on the conserved catalytic glutamic acid residue (85 × 80 × 60 Å), and the poses were ranked by their predicted binding energy and inspected based on the correct geometry between catalytic residues and substrates.

### 6.2.3. Expression of recombinant gene

The pGAPZαA vector (Invitrogen<sup>TM</sup>, USA) was used for cloning, sequencing, and expressing the Afua\_2g12770 α-L-arabinofuranosidase (Gene ID **3513529**). The Afua\_2g12770 recombinant gene (948 pb, without signal peptide and TAG codon) was



synthesized by GenOne (Brazil) and inserted between the *Eco*RI and *Xba*I sites. *Escherichia coli* DH5 $\alpha$  was heat shock-transformed and cultivated at 37 °C and 200 rpm in low-salt Luria-Bertani (LB) medium with 100  $\mu$ g/mL Zeocin (Thermo Fisher Scientific<sup>TM</sup>, USA), to propagate the recombinant plasmid. The plasmid containing the gene was extracted, linearized with the *Bsp*HI enzyme (5'...T<sup>^</sup>CATGA...3', Thermo Scientific<sup>TM</sup>, USA), and transformed into electro-competent *Komagataella pastoris* X-33 by electroporation (1500 V, 5 milliseconds). The transformants were selected for Zeocin resistance on YPDS plates containing 100  $\mu$ g/mL Zeocin according to the transformation protocol No. 4308 915.545 –03/2004 (Eppendorf). Gene insertion was confirmed by PCR, by using the 5' GAP forward and 3' AOX1 reverse primers recommended by Invitrogen (Invitrogen, 2010). The PCR conditions were 30s at 95°C, 30 cycles of 30s denaturation at 95°C, 60s hybridization at 53°C, and 90s elongation at 68°C, followed by a final 5 min extension at 68°C, using Taq DNA polymerase (Sinapse Inc. <sup>TM</sup>, Brazil).

#### **6.2.4. Enzyme production, purification, and activity**

Small-scale enzyme production was performed in 50 mL of YDP (in a 250-mL Erlenmeyer) incubated at 30 °C and 250 rpm for 96 h, as recommended by Invitrogen (Invitrogen, 2010). The culture supernatant containing the secreted enzyme was concentrated 100 times by ultrafiltration (Amicon Ultra-15, Merck KGaA, Germany) with a molecular weight cut-off of 10 kDa. Then, the enzyme was purified with the HisPur kit (Thermo Scientific<sup>TM</sup>, USA), as recommended (equilibration buffer: 10 mM imidazole; wash buffer: 25 mM imidazole; elution buffer: 250 mM imidazole. All buffers contained 10 mM PBS pH 7.4). The resin column was equilibrated with 2 resin-bed volumes of equilibration buffer, centrifuged at 700 g 2min. The sample was mixed to the equilibration buffer, so the total volume was 2 resin-bed volumes. The sample was incubated 30 min at 4 °C on an end-over-end rocking platform. The flow through was collect after centrifugation at 700 g 2 min 4 °C, and the resin was washed with 2 resin-bed volumes wash buffer twice, centrifuged at 700 g 2 min 4 °C. One resin-bed volume of elution buffer was added twice and centrifuged at 700 g 2 min 4 °C, and the histidine tagged protein was analyzed by SDS-PAGE. The protein content was determined by using bovine albumin (Sigma-Aldrich, USA) as the standard (Lowry et al., 1951). After purification, the buffer was exchanged by ultrafiltration (concentrated 20 times) and dilution in 500  $\mu$ L of 10 mM Tris-HCl buffer pH 7.4 and 150 mM NaCl, which afforded a final protein concentration of 1 mg/mL. The molecular mass under denaturing conditions was determined by SDS-PAGE gel electrophoresis with a gel system (Bio-Rad Laboratories), 4% acrylamide stacking gel (pH 6.8), and 12%

separating gel (pH 8.8). The proteins were visualized by staining with Coomassie brilliant blue G-250 (Thermo Scientific™, USA) (Laemmli, 1970).

The arabinofuranosidase activity was measured by adding 10  $\mu$ L of 10 mM *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (*p*NP-A) to 65  $\mu$ L of 100 mM McIlvaine buffer (variable pH), 20  $\mu$ L of water (or 50 mM  $\text{Ca}^{2+}$  or  $\text{Mn}^{2+}$ ), and 5  $\mu$ L of 0.01  $\mu\text{g}/\mu\text{L}$  purified enzyme at 45 °C for 30 min. The reaction was stopped with 50  $\mu$ L of 1 M  $\text{Na}_2\text{CO}_3$ , and the absorbance was read at 405 nm (Sakamoto & Kawasaki, 2003).

### 6.2.5. Biochemical characterization and kinetic parameters

A Central Composite Rotational Design (CCRD) with two factors, three repetitions in the central point condition, and four axial points, evaluated pH from 2.6 to 5.4 and temperature from 25.9 to 54.1 °C (**Supplementary table 12-3**). The statistical analysis was performed with the Protimiza Experimental Design software (Rodrigues & Iemma, 2014). The kinetic parameters ( $K_m$  and  $V_{max}$ ) were examined by incubating the diluted enzyme for 30 min in McIlvaine buffer containing 0 to 4 mg/mL wheat arabinoxylan (arabinose/xylose = 38:62) under the optimum temperature and pH conditions. The Graphpad Prism Software version 9 was used to compute the parameters. Enzyme stability at different pH was studied by incubating the enzyme in McIlvaine buffer at pH ranging from 3.0 to 8.0 at 4 °C for 24 h, and the relative activity was measured at the optimum conditions.

### 6.2.6. Thermostability, salt tolerance, and metal ion effects

The effect of metal ions and additives was evaluated by adding different metal ions and surfactants (1, 5, or 10 mM) to the enzymatic reaction. Salt tolerance was evaluated by incubating 0.1 mg/mL purified enzyme in McIlvaine buffer pH 4.8 containing 0 to 3 M NaCl at 4 °C overnight. The residual activity was measured under the optimum conditions.

The purified enzyme was thermally treated in a thermocycler at 50, 60, or 70 °C for 4 h; aliquots were removed every 20 min and immediately cooled at 4 °C for at least 1 h to ensure that all the samples had sufficient time for the reversibly denatured protein to be converted back to its native active form, while the irreversibly denatured form remained inactive. At the end of the thermal treatment, the enzymatic assay of the aliquots was carried out at 45 °C for 30 min, and the relative activities were expressed as a percentage of the original activities, to calculate the thermodynamic parameters (Saqib & Siddiqui, 2018), as explained below.

### Inactivation Constant and Half-Life of the Enzyme

The thermal inactivation constants ( $k_d$ ) were determined for each temperature at pH 4.8 (50, 60, and 70°C), along with the half-life ( $t_{1/2}$ ) as a function of temperature.

The  $k_d$  at each temperature was determined using the slope of the line from the natural logarithm of the relative activity ( $v/v_0$ ) plotted against time (minutes). The linear regression of the experimental data follows Equation 6.1:

$$\ln\left(\frac{v}{v_0}\right) = -k_d * t + C1 \quad (6.1)$$

Where:

- $v$  is the activity,
- $v_0$  is the initial activity,
- $k_d$  is the inactivation constant ( $\text{min}^{-1}$ ),
- $t$  is the time (min), and
- $C1$  is a constant.

The half-life ( $t_{1/2}$ ), defined as the time required for the enzyme to lose 50% of its initial activity, can be calculated using Equation 6.2. In this case,  $v$  is replaced with  $v_0/2$  in Equation 4.1:

$$t_{1/2} = -\ln(0.5)/k_d \quad (6.2)$$

Where:

- $t_{1/2}$  is the half-life of the enzyme (min), and
- $k_d$  is the denaturation constant ( $\text{min}^{-1}$ ).

### Calculation of Enzymatic Inactivation Energy

As in other chemical reactions, the rate of enzyme-catalyzed reactions increases with temperature. This effect is described by Arrhenius and represented in Equation 6.3:

$$k = A_0 * e^{(-Ea/RT)} \quad (6.3)$$

Where:

- $k$  is the rate constant ( $\text{min}^{-1}$ ),
- $A_0$  is the Arrhenius constant,
- $Ea$  is the activation energy ( $\text{kJ/mol}$ ),
- $R$  is the ideal gas constant ( $8.314 \times 10^{-3} \text{ kJ/mol}\cdot\text{K}$ ), and
- $T$  is the absolute temperature ( $\text{K}$ ).

The stability of the enzyme is also influenced by temperature, similarly to inactivation. Thus, by equating the rate constant to the inactivation constant ( $k_d$ ), we can obtain the value of enzymatic inactivation energy ( $E_d$ ) by linearizing the Arrhenius equation (Equation 6.4) through a plot of  $\ln(k_d)$  versus  $1/T$ . Using this value of  $E_d$  and substituting Equation 6.2 into Equation 6.3, it is possible to estimate the half-life of the enzyme as a function of temperature, as shown in Equation 6.5:

$$\ln(k_d) = \ln(A_0) - \frac{E_d}{R} * \frac{1}{T} \quad (6.4)$$

$$t_{1/2} = - \frac{\ln(0.5)}{A_0} * e^{E_d/RT} \quad (6.5)$$

#### Thermodynamic Parameters

The values for Gibbs free energy, enthalpy, and entropy were calculated using Equations 6.6, 6.7, and 6.8, respectively:

$$\Delta G = -RT * \ln \left( \frac{k_d * h}{k_B * T} \right) \quad (6.6)$$

$$\Delta H = E_d - RT \quad (6.7)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (6.8)$$

Where:

- $\Delta G$  is the Gibbs free energy (kJ/mol),
- $\Delta H$  is the enthalpy (kJ/mol),
- $\Delta S$  is the entropy (kJ/mol·K),
- $R$  is the ideal gas constant ( $8.314 \times 10^{-3}$  kJ/mol·K),
- $k_d$  is the inactivation constant ( $\text{min}^{-1}$ ) obtained from Equation 6.3,
- $k_B$  is the Boltzmann constant ( $1.38 \times 10^{-23}$  J/K), and
- $h$  is the Planck constant ( $11.04 \times 10^{-36}$  J·min),
- $E_d$  is the enzymatic inactivation energy (kJ/mol) obtained from Equation 6.4.

### 6.2.7. Circular Dichroism

The far-UV CD spectrum was measured between 190 and 260 nm in 20 mM phosphate buffer pH 6.0 at 25 °C on a spectrophotometer Jasco J-810 (Jasco Inc, Tokyo, Japan) equipped with a thermostatic Peltier system with constant nitrogen flow; a cuvette with 1-mm path length and protein concentration of 0.05 mg/mL were employed. The spectrum was measured at a rate of 1 nm/min four times, and ellipticity was reported as the mean residue molar ellipticity ( $\theta$ ,  $\text{deg}\cdot\text{cm}^2 \cdot \text{dmol}^{-1}$ ). The secondary structure was computed with BesStSel (<https://bestsel.elte.hu/index.php>).

### 6.2.8. Xylo-oligosaccharides and ferulic acid release

The synergistic cooperation degree of the equimolar mixture of Af\_afu62 and GH 10 *Papiliotrema flavescens*  $\beta$ -1,4-endoxylanase Xyn10cf (Martins, Ávila, et al., 2020) was calculated by the release of reducing sugars (Miller, 1959) and xylo-oligosaccharides production after incubation of 1% (w/v) wheat arabinoxylan (Megazyme, Ireland) with 10 U/ $\mu\text{L}$  Af\_afu62 and 30 U/ $\mu\text{L}$  Xyn10cf during 12 h at 40 °C and pH 5.0. The synergic degrees were calculated as the ratio between XOS content produced by the simultaneous activity of both enzymes and the sum of the activities of each enzyme separately (Gonçalves et al., 2012).

The XOS, PCA, and FA release were tested with corn hull arabinoxylan (4 % w/v) incubated with 10 U/ $\mu\text{L}$  Af\_afu62 and 30 U/ $\mu\text{L}$  Xyn10cf at pH 5.0 for 48 h at 40 °C. For control, the arabinoxylan was incubated under the same conditions except for the addition of enzymes. The hydrolysate was filtered (0.22  $\mu\text{m}$  membrane) and subjected to high-performance liquid chromatography (HPLC) sugar analysis. The XOS, PCA, and FA quantification followed the methodology described elsewhere (L. Brenelli et al., 2018; Martins, Ávila, et al., 2020).

### 6.3. Results and discussion

#### 6.3.1. Structural prediction of Af\_afu62

Sequence analysis confirmed the conserved domain of the GH 62\_1 family (pfam03664, cl14647 superfamily), characterized by the five-blade  $\beta$ -propeller structure illustrated in **Figure 6-1**. Af\_afu62 showed two conserved aspartic acid residues, D45 (general base) and D160 (pKa modulator), and one glutamic acid residue, E212 (general acid), which together form the catalytic triad. GH 62 enzymes have a calcium atom located in the center of the structure, held by a histidine residue (Tonozuka & Tanaka, 2017). The corresponding histidine in Af\_afu62 is H281, and it has been proposed as forming the catalytic nucleus together with the triad (Kaur et al., 2015).

From the alignment phylogeny (Table 6-1), we selected *Coprinopsis cinerea* (CcAbf62A) 5B6S, *Podospira anserina* 4N2Z (complexed with  $\alpha$ -L-arabinofuranose), and *Mycothermus thermophilus* 4PVI to model the Af\_afu62 structure. We computed interactions between the amino acid residues within a 4-Å distance (**Figure 6-1**). Cysteine residues 298 and 332 (C-terminal) are appropriately positioned for disulfide bridge formation. The residues of the catalytic triad D45, D160, and E212 are highlighted in yellow, and H281 is highlighted in blue (**Supplementary table 12-2**).

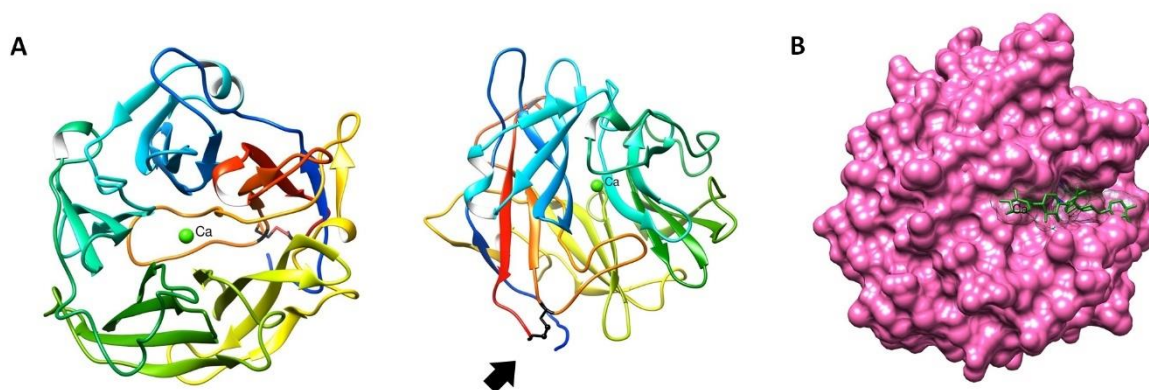
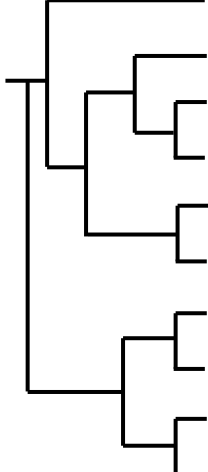


**Figure 6-1** Af\_afu62 structural homology model. A disulfide bridge is pointed by a black arrow and a calcium atom is shown in green (A); Enzyme surface illustrating the catalytic cleft and interaction with arabinoxylo-pentose (B).

Table 6-1 PSI-BLAST Homology of the Afua\_2g12770 with proteins from other species

| Phylogeny                                                                         | PDB ID       | Organism                              | Taxonomy          | Query Cover | E Value            | % Identity |
|-----------------------------------------------------------------------------------|--------------|---------------------------------------|-------------------|-------------|--------------------|------------|
|  | 4N1I         | <i>Ustilago maydis</i>                | Smut fungi        | 92%         | 3e <sup>-60</sup>  | 39.49%     |
|                                                                                   | 3WMY         | <i>Streptomyces coelicolor</i>        | Gram+ bacteria    | 93%         | 6e <sup>-67</sup>  | 41.27%     |
|                                                                                   | 6F1J         | <i>Talaromyces pinophilus</i>         | Ascomycete        | 94%         | 1e <sup>-72</sup>  | 43.89%     |
|                                                                                   | 6CC7         | <i>Thermothielavioides terrestris</i> | Ascomycete        | 94%         | 2e <sup>-65</sup>  | 40.13%     |
|                                                                                   | 4O8N         | <i>Streptomyces thermoviolaceus</i>   | Gram+ bacteria    | 94%         | 9e <sup>-72</sup>  | 40.75%     |
|                                                                                   | 5UBJ         | <i>Aspergillus nidulans</i>           | Ascomycete        | 97%         | 3e <sup>-65</sup>  | 42.73%     |
|                                                                                   | <b>Query</b> | <b><i>Aspergillus fumigatus</i></b>   | <b>Ascomycete</b> | -           | -                  | -          |
|                                                                                   | 5B6S         | <i>Coprinopsis cinerea</i>            | Basidiomycete     | 94%         | 7e <sup>-170</sup> | 69.94%     |
|                                                                                   | 4N2Z         | <i>Podospora anserina</i>             | Ascomycete        | 94%         | 3e <sup>-156</sup> | 64.35%     |
|                                                                                   | 4PVI         | <i>Mycothermus thermophilus</i>       | Ascomycete        | 92%         | 3e <sup>-142</sup> | 60.75%     |

The purified enzyme had a 7.4- fold purification factor and the purification yield was about 47 %. SDS-PAGE gel (**Figure 6-2a**) confirmed the molecular mass of ~37 kDa predicted for the purified enzyme, which was expected after the addition of the 6x-polyhistidine tag and post-translational modifications (*O*-glycosylation at positions 25, 26, 136, 143, 257, 265, and 271). Two peaks emerged in the far-UV CD spectrum (**Figure 6-2b**): a positive one at 195 nm and a negative one at 215 nm, typical of proteins rich in  $\beta$ -sheets (Sreerama & Woody, 2004). Secondary structure determination estimated an index of 44% extended antiparallel  $\beta$ -sheets and 11%  $\alpha$ -helixes, consistent with the predicted structure presented in **Figure 6-1**.

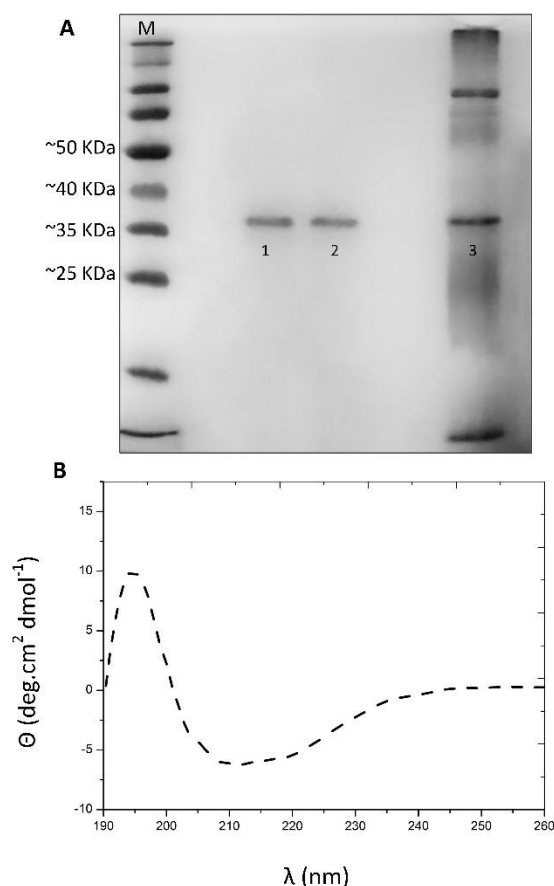


**Figure 6-2** SDS-page gel showing the molecular weight standard (M), the purified Af\_afu62 (lanes 1 and 2), and the culture supernatant of *P. pastoris* (lane 3) (A). Far-UV CD spectrum of purified Af\_afu62 (B).

According to the literature (Maehara et al., 2014),  $\alpha$ -L-arabinofuranose is accommodated by subsite -1, and catalytic cleavage occurs between subsites -1 and +1 (**Figure 6.3a**), which are very conserved among the GH 62 family of enzymes. Xylopentose interacts with subsites +4NR, +3NR, +2NR, +1R, and +2R, starting from the non-reducing (NR) to the reducing (R) terminal. Also, two tyrosine residues, Y 67 and Y 97 (highlighted in pink in **Supplementary table 12-2**) accommodate a ferulate residue between the arabinose C5-O5 atoms (Hashimoto et al., 2011; Tono-zuka & Tanaka, 2017). Two aspartic acid residues and one glutamic acid residue form the catalytic triad. A calcium ion is found on the central axis of the five blades, linked to a conserved histidine residue. This histidine residue appears to compose the catalytic nucleus together with the triad, and, according to the literature, the presence of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  causes small changes in the enzymatic activity (Tono-zuka & Tanaka, 2017).



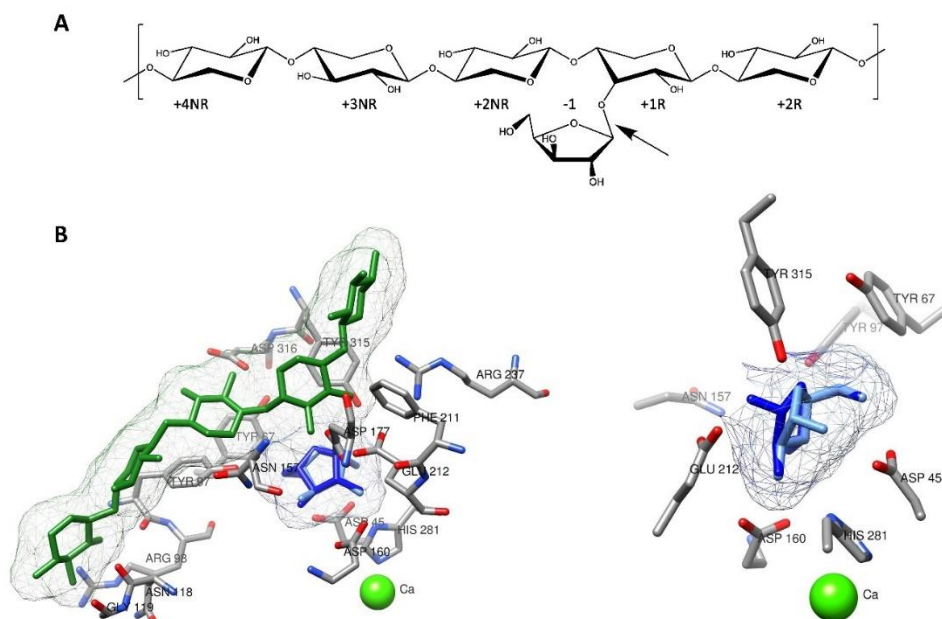


**Figure 6-3** Arabinofuranosidase cleavage site, indicated by a black arrow. The arabinoxylopolysaccharide molecular structure was built by using ChemDraw Professional 17.0 (A); Af\_afu62 catalytic cleft of accommodating a xylopentose, and catalytic pocket accommodating an  $\alpha$ -L-arabinofuranoside residue. Amino acid residues that make up the catalytic triad are shown (N45, E212, N160). H281 was identified as the residue corresponding to the calcium atom holder. Y67 and Y97 represent a potential site for ferulate. D157 possibly regulates activity against *p*-nitrophenyl- $\alpha$ -arabinofuranoside (B)

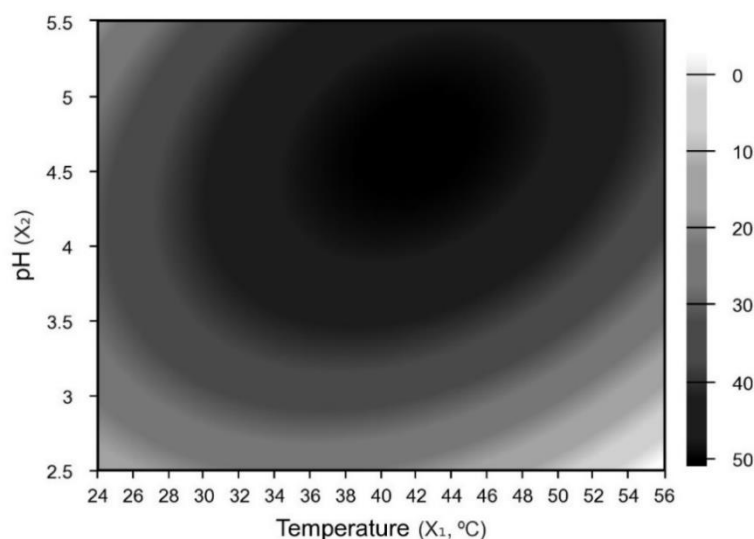


**Figure 6-4** Surface response from CCRD to determine the optimum temperature and pH range for Af\_afu62

### 6.3.2. Biochemical characterization

The optimum pH lies between 4.0 and 5.0, and the optimum temperature is between 37 and 45 °C (**Figure 6-4**). A similar result was reported by Pérez and Eyzaguirre (Pérez and Eyzaguirre, 2016), who also reported the low activity of GH 62 enzymes against p-nitrophenyl- $\alpha$ -arabinofuranoside substrate and high specificity for arabinoxylan. The positive coefficient of the interaction between pH and temperature indicated that, when these independent variables are combined, they affect the enzymatic activity depending on their value to generate a response (**Supplementary table 12-4**). We validated the model by setting the pH and temperature values to 4.8 and 42 °C, and the experimental value of  $70.487 \pm 1.813$  U/ $\mu$ L is within the predicted value range.

The enzymatic kinetics followed the model proposed by Michaelis-Menten. The purified enzyme has  $K_m$  and  $k_{cat}$  of  $1.37 \pm 0.3$  mg/mL and  $47.82 \pm 2.15$  s<sup>-1</sup>, respectively (at pH 4.8 and 42 °C). Despite little information about the kinetic parameters of GH 62 arabinofuranosidases, Table 6-2 gathers the parameters reported for wheat arabinoxylan. Af\_afu62 displays better catalytic efficiency than GH 62 from most of the cited bacteria and fungi, including *Mycothermus thermophilus* 4PVI, which is one of the models for Af\_afu62 structural prediction. We also observed lower  $k_{cat}$  and  $K_m$  as compared to *Aspergillus nidulans*. The catalytic efficiency ( $k_{cat}/K_m$ ) of Af\_afu62 was superior to all species, except for arabinofuranosidases from *Aspergillus nidulans*. The kinetic parameters of the 4PVI GH 62\_1 subfamily are the only ones available for this subfamily.

**Table 6-2 Comparison of the kinetic parameters and catalytic efficiencies ( $k_{cat} / K_m$  ratios) of GH 62 arabinofuranosidases from several microorganisms made by using wheat arabinoxylan as substrate**

| Organism                  |           | PDB  | Sub-family | Wheat arabinoxylan     |                                     |                                   | Reference                |
|---------------------------|-----------|------|------------|------------------------|-------------------------------------|-----------------------------------|--------------------------|
|                           |           |      |            | K <sub>m</sub> (mg/mL) | k <sub>cat</sub> (s <sup>-1</sup> ) | k <sub>cat</sub> / K <sub>m</sub> |                          |
| <i>A. fumigatus</i>       | Fungus    | -    | 62_1       | 1.37 ± 0.3             | 47.82 ± 2.15                        | 34.85                             | This study               |
| <i>A. nidulans</i>        | Fungus    | 5UBJ | 62_2       | 3.78 ± 1.05            | 361.74                              | 95.69                             | (Contesini et al., 2017) |
| <i>A. nidulans</i>        | Fungus    | -    | 62_2       | 2.3                    | 178                                 | 77.39                             | (Wilkens et al., 2016)   |
| <i>E. parvum</i>          | Fungus    | -    | 62_2       | 45.46 ± 2.44           | 42.04 ± 2.05                        | 0.92                              | (Long et al., 2020)      |
| <i>G. zeae</i>            | Fungus    | -    | 62_2       | 3.4 ± 0.3              | 27.7 ± 0.7                          | 8.2                               | (Sarch et al., 2019)     |
| <i>N. haematococca</i>    | Fungus    | -    | 62_2       | 9.2 ± 1.2              | 56.3                                | 6.1                               | (Sarch et al., 2019)     |
| <i>S. reilianum</i>       | Fungus    | -    | 62_2       | 4.0 ± 0.6              | 13 ± 0.5                            | 3.3                               | (Sarch et al., 2019)     |
| <i>S. coelicolor</i>      | Bacterium | 3WMY | 62_2       | 7.3                    | 18 ± 2                              | 2.5                               | (Maehara et al., 2014)   |
| <i>S. thermophilum</i>    | Fungus    | 4PVI | 62_1       | 3.66 ± 0.82            | 0.27                                | 0.07                              | (Kaur et al., 2015)      |
| <i>S. thermophilum</i>    | Fungus    |      | 62_2       | 9.38 ± 1.98            | 0.86                                | 0.09                              | (Kaur et al., 2015)      |
| <i>S. thermoviolaceus</i> | Bacterium | 4O8O | 62_2       | 12.0 ± 5.0             | 180.0 ± 65.0                        | 15.0                              | (W. Wang et al., 2014)   |

### 6.3.3. Influence of salt and pH on enzyme stability

The purified Af\_afu62 is stable at pH (**Figure 6-5a**) from 4.0 to 6.0, following the studies indicating that arabinofuranosidases from fungi have optimum pH in the acid region, usually between pH 4.0 and 5.0 (Patel et al., 2015; Terrone et al., 2020). Increasing pH to above 7.0 decreases activity by 30% probably because groups ionize, altering enzyme configuration. Af\_afu62 preserved activity after 24h incubation at pH from 4.0 to 6.0.

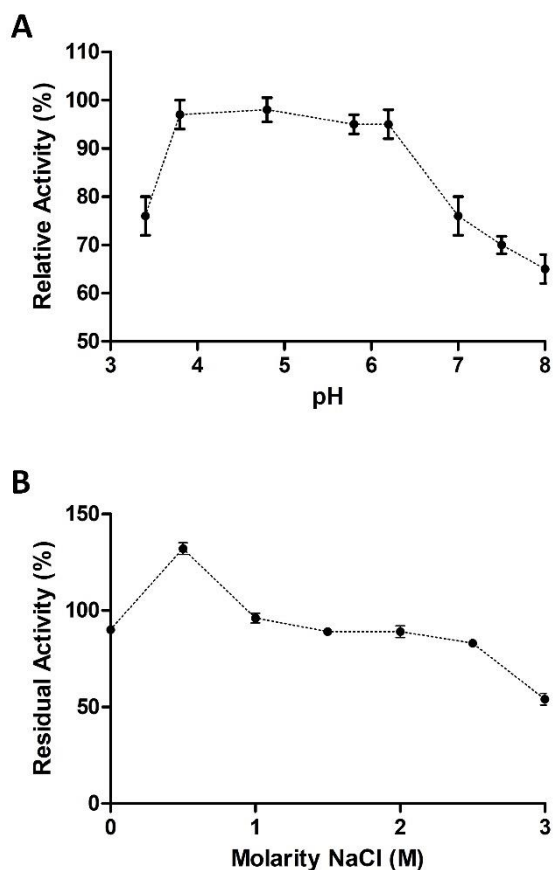


Figure 6-5 pH stability (A) and the effects of salt concentration and (B) after overnight incubation.

Incubation with 0.5 M NaCl activated the purified enzyme (140 % of control activity, **Figure 6-5b**). At 1 M and at 3 M NaCl, 92 and 54 % enzymatic activity is retained, suggesting that Af\_afu62 is a halotolerant enzyme. Good content of acidic amino acids, especially glutamate, may provide greater stability in the presence of higher salt concentrations (Oren, 2003; Terrone et al., 2020). The Af\_afu62 stability in acidic conditions and the presence of salt indicate that this enzyme presents desirable properties for lignocellulosic biomass hydrolysis. This characteristic is particularly relevant for bioprocesses to produce xylo-oligosaccharides from arabinoxylans, which are commonly extracted by alkaline methods in the presence of high NaCl concentrations (Brienzo et al., 2010).

### 6.3.4. Thermostability

GH 62 thermodynamics and how metal ions affect the GH 62 thermodynamic parameters have not been studied yet. However, Long et al. (2020) reported an increased thermostability of GH 62 from *Eupenicilium parvum* after they added calcium to the enzymatic reaction. Calcium-dependent thermostability has been reported for xylanases, glucosidases, and other arabinofuranosidases. This thermostability is partly explained by the catalytic domain structural stability provided by calcium. Studies have also reported that calcium, located in the central channel of the five-fold  $\beta$ -propeller structure, does not affect the specific GH 62 enzymatic activity (Long et al., 2020; Siguier et al., 2014; W. Wang et al., 2014). Nevertheless, herein  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  significantly increased the Af\_afu62 enzymatic activity. **Figure 6-6** illustrates the effects of other chemicals.

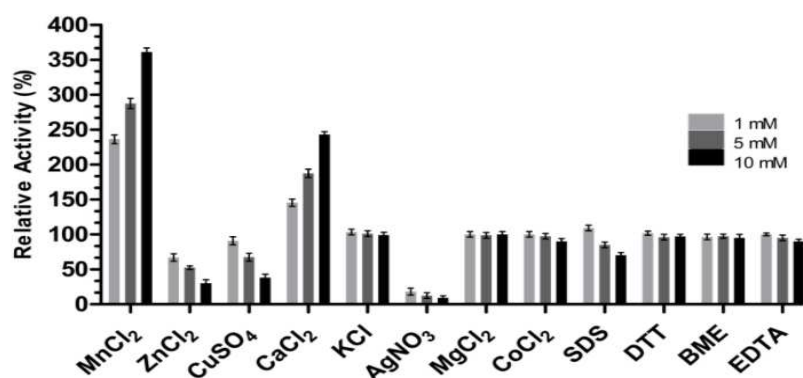


**Figure 6-6** Influence of metal ions or additives at 1, 5, or 10 mM on enzymatic activity

Cofactor activation enhances the catalytic power of enzymes, and the economic suitability of any enzyme for industrial applications is judged by its thermodynamic parameters (Abd El Aty et al., 2018). Table 6-3 shows that the cofactors  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$ , besides improving the activity, increased the residual activity (**Figure 6-7**), half-life time  $t_{1/2}$  from 78 to 210 min, at 50 °C. Over four-hour-exposure is necessary to reduce its initial activity to 10%, and the presence of the cofactors increased the D value from 258 to 697 min at 50 °C.

Large and positive values of both energies of deactivation  $E_d$  and enthalpy of denaturation  $\Delta H_d$  indicate high enzyme thermostability. The results in Table 6-3 also show that the  $E_d$  and  $\Delta H_d$  values increase in the presence of cofactors. Negative entropy of denaturation  $\Delta S_d$  values indicates a slightly less disordered transition state compared to the native enzyme, thereby highlighting reasonable thermostability at high temperatures (50, 60, and 70 °C) (Bisswanger, 2017; Soares da Silva et al., 2018). Negative values at all temperatures also indicate that aggregation takes place during thermal inactivation (Abd El Aty et al., 2018).

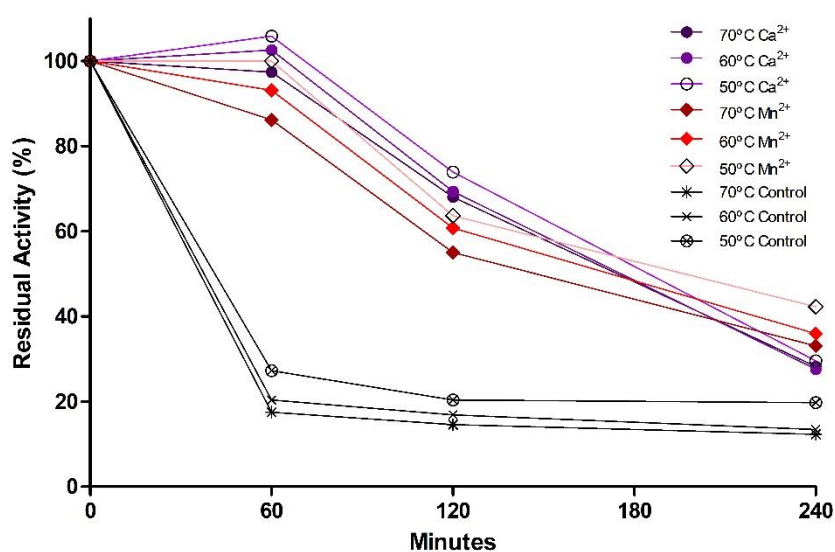


Figure 6-7 Residual activity of Af\_afu62 after thermal treatment at 50, 60, or 70 °C without cofactor (Control) and in the presence of 5 mM Ca<sup>2+</sup> or Mn<sup>2+</sup>.

The enzyme incubated with Ca<sup>2+</sup> or Mn<sup>2+</sup> has slightly increased Gibbs free energy of denaturation  $\Delta G_d$  at all the temperatures, along with a significant increase in  $t_{1/2}$ ,  $D$ ,  $E_d$ , and  $\Delta H_d$ . These results show that Ca<sup>2+</sup> and Mn<sup>2+</sup> enhance the Af\_afu62 thermostability at temperatures ranging from 50 to 70 °C. An increase in  $\Delta G_d$  means an increase in denaturation resistance and hence increased thermostability (Bisswanger, 2017; P. M. Souza et al., 2015).

Table 6-3 Af\_afu62 thermodynamic parameters

|                        | $k_d$<br>(min <sup>-1</sup> ) | $R^2$ | $t_{1/2}$<br>(min) | $D$ value<br>(min) | $\Delta G$<br>(kJ/mol) | $\Delta H$<br>(kJ/mol) | $\Delta S$<br>(J/mol.K) | $E_d$<br>(kJ/mol) |
|------------------------|-------------------------------|-------|--------------------|--------------------|------------------------|------------------------|-------------------------|-------------------|
| <b>Control</b>         |                               |       |                    |                    |                        |                        |                         |                   |
| 50 °C                  | 0.0089                        | 0.836 | 77.882             | 258.717            | 103.044                | 7.516                  | -295.616                | 10.203            |
| 60 °C                  | 0.0102                        | 0.850 | 67.956             | 225.744            | 105.940                | 7.433                  | -295.683                |                   |
| 70 °C                  | 0.0111                        | 0.835 | 62.446             | 207.440            | 108.963                | 7.350                  | -296.119                |                   |
| <b>Ca<sup>2+</sup></b> |                               |       |                    |                    |                        |                        |                         |                   |
| 50 °C                  | 0.0037                        | 0.933 | 187.337            | 622.320            | 105.403                | 10.330                 | -294.207                | 13.016            |
| 60 °C                  | 0.0046                        | 0.911 | 150.684            | 500.562            | 108.146                | 10.247                 | -293.859                |                   |
| 70 °C                  | 0.0049                        | 0.934 | 141.459            | 469.915            | 111.296                | 10.163                 | -294.718                |                   |
| <b>Mn<sup>2+</sup></b> |                               |       |                    |                    |                        |                        |                         |                   |
| 50 °C                  | 0.0033                        | 0.944 | 210.045            | 697.753            | 105.710                | 13.625                 | -284.962                | 16.311            |
| 60 °C                  | 0.0040                        | 0.975 | 173.287            | 575.646            | 108.533                | 13.541                 | -285.131                |                   |
| 70 °C                  | 0.0047                        | 0.985 | 147.478            | 489.912            | 111.415                | 13.458                 | -285.463                |                   |

### 6.3.5. Synergism for xylo-oligosaccharides and ferulic acid production

The release of reducing sugars was enhanced by 5% with the association of 30 U/ $\mu$ L Xyn10cf and 10 U/ $\mu$ L Af\_afu62, and total XOS (X2-X6) production increased by 42.6% (1058 mg/L) for wheat arabinoxylan 1% w/v (Table 6-4). The synergistic activity of Xyn10cf and Af\_afu62 on agricultural residue was tested for the ability to release XOS, PCA, and FA. Even without an esterase, the combination of both enzymes released higher concentrations of readily soluble PCA and FA from the alkaline extraction of arabinoxylan due to further degradation of the material since higher XOS concentration was also achieved (Z. Zhang et al., 2014). It was found that Xyn10cf (only) produced 2.43 g/L XOS, 0.62 mM FA (120 mg/L), and 0.06 mM PCA. The co-incubation of Xyn10cf with Af\_afu62 produced 5.67 g/L XOS, 0.82 mM (160 mg/L), and 0.09 mM PCA. GH 10 endoxylanase exhibits greater catalytic versatility than GH 11 endoxylanase, being less hampered by the presence of substituents along the xylan backbone. However, a high degree of branching along the backbone may impair xylan degradation by hindering the formation of the enzyme-substrate complex. Therefore, the simultaneous removal of arabinose residues improves the catalytic efficiency of Xyn10cf (Faulds et al., 2006). At the same time, the arabinose content was higher with co-incubation. Hence, arabinose-substituted xylo-oligosaccharides generated by Xyn10cf showed to be better substrates for Af\_afu62, being able to release more arabinose. Xylotetraose was not detected in this assay, similar to results from the literature (Brandt et al., 2020; Rahmani et al., 2019), where no X4 was found in the enzymatic reaction, indicating that either no X4 is formed during the reaction or, less likely, a very rapid turnover of X4 takes place.

These results highlight the functionality of the GH 62 enzymes for arabinoxylan debranching and xylo-oligosaccharide production, when these enzymes act synergistically with endoxylanases, with an improvement of PCA and FA release, whose ester linkages were cleavage by the alkaline pretreatment for the arabinoxylan extraction (Cobs, 2020).

**Table 6-4 Synergistic hydrolysis of wheat and corn hull arabinoxylan by Xyn10cf and Af\_afu62 for XOS and FA release.**

|                               | Af_afu62        | Xyn10cf         | Xyn+Abf          | Degree of synergy |
|-------------------------------|-----------------|-----------------|------------------|-------------------|
| <b>Wheat arabinoxylan</b>     |                 |                 |                  |                   |
| Reducing sugars               | 31.44 $\mu$ mol | 95.05 $\mu$ mol | 133.25 $\mu$ mol | 1.05              |
| Xylose (X1)                   | 0               | 0               | 0                |                   |
| Xylobiose (X2)                | 0               | 303.23          | 304.78           |                   |
| Xylotriose (X3)               | 0               | 318.88          | 393.6            |                   |
| Xylotetraose (X4)             | 0               | 0               | 0                |                   |
| Xylopentaose (X5)             | 0               | 99.07           | 253.89           |                   |
| Xylohexaose (X6)              | 0               | 20.71           | 105.72           |                   |
| Total XOS                     | 0               | 741.89 mg/L     | 1057.99 mg/L     | 1.43              |
| <b>Corn hull arabinoxylan</b> |                 |                 |                  | <b>Control</b>    |
| Arabinose                     | 1.31 g/L        | 0               | 3.37 g/L         | 0                 |
| Ferulic acid                  | 0.27 mM         | 0.62 mM         | 0.82 mM          | 0.22              |
| p-coumaric acid               | 0.02 mM         | 0.06 mM         | 0.09 mM          | 0                 |
| Total XOS                     | 0               | 2.43 g/L        | 5.67 g/L         | 0                 |

## 6.4. Conclusion

The Af\_afu62 structure reveals conserved residues in the catalytic site and suggests greater, if not exclusive, specificity for arabinoxylan. According to residual activity assays, the presence of the cofactors  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  improved the thermoresistance and increased the half-life time by 2.4 times. The synergism assays for xylo-oligosaccharides, p-coumaric acid, and ferulic acid production from alkali-treated agricultural residue demonstrated the biotechnological potential of using GH 62 for prebiotics and natural antioxidants production. These results provide a basis for further development of novel enzymatic routes for applications in the food and pharmaceutical industries.

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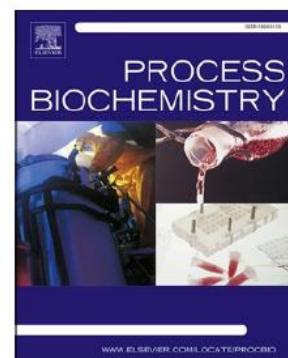
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**CHAPTER 7.**  
**NOVEL BI-FUNCTIONAL THERMOSTABLE CHIMERIC ENZYME**  
**FOR FEASIBLE XYLO-OLIGOSACCHARIDES PRODUCTION**  
**FROM AGRO-INDUSTRIAL WASTES**

## Novel bi-functional thermostable chimeric enzyme for feasible xylo-oligosaccharides production from agro-industrial wastes

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### Digging deeper into protein engineering

We are finally stepping into the chimeric enzyme study. After gathering all the results and information about the Xyn10cf GH 10 endoxylanase and the Af\_afu62 GH 62 arabinofuranosidase, these two enzymes were combined in a single polypeptide chain to generate a more efficient and stable enzyme, designed specifically for XOS production. This study used the arabinoxylan extracted from soy hulls and the corn husks for XOS production (the orange waste comes later, we will understand why). It is worth noting that this was the first chimeric enzyme designed for this sole purpose (XOS production). Of course, further improvements are always desired, so this scientific paper may be just the beginning of a whole new research to optimize hydrolysis conditions and explore the potential production of new bio compounds using this chimeric enzyme construction. An example of this continuous improvement is the next **CHAPTER 8** which describes the directed evolution of GH 62.

(Supplementary material in **CHAPTER 12**)

### **Abstract**

Arabinoxylan is a sustainable source for xylo-oligosaccharide production, as agro-industrial wastes are abundant and the industrial interest for these prebiotics is raising due to their bioactivities for food and cosmetics. However, enzyme production costs and thermostability still represent a drawback. The main enzymes required for hydrolysis are endoxylanase and arabinofuranosidase, where GH 10 endoxylanase presents great thermostability and activity, and GH 62 enzymes are the arabinofuranosidases with the highest specificity but poor thermostability. In this sense, the fusion of these enzymes can generate a more stable enzyme with multiple catalytic domains in a single batch. The present study designed a bifunctional enzyme combining a GH 10 endoxylanase and a GH 62 arabinofuranosidase to enhance activity and thermostability, thus increasing the feasibility of xylo-oligosaccharides production from wastes. The chimera showed superior specificity for arabinoxylan and halotolerance than the parental enzymes and higher efficiency than commercial cocktail. Thermostability improved especially for the arabinofuranosidase module, which increased 10 °C, enabling a process at higher temperatures and lower enzyme loads, which increases efficiency and decreases contamination. Calcium and manganese enhanced thermostability and activity. Xylobiose and xylotriose yields improved by 25%, and up to 7 g/L XOS was achieved from corn hull arabinoxylan hydrolysis.

**Keywords:** Arabinoxylan; Enzyme engineering; Lignocellulosic biomass; Prebiotics; Recombinant enzyme; Thermostability.

## 7.1. Introduction

Arabinoxylans (AX) are the principal group of hemicellulose found in cereal grains, located in the cell walls of starchy endosperm, aleurone, bran tissues, and in the husks, whose concentration varies depending on the tissue and grain. For example, arabinoxylan content in wheat bran is 20.9 %, whereas in rye grain is as much as 30 %. (Izydorczyk, 2021). They are composed of a chain of D-xylopyranosyl residues joined by  $\beta$ -1,4 glycosidic bonds, with  $\alpha$ -L-arabinofuranoside residues in the C (O)-3 or -2 positions of the xylose backbone (**Figure 7-1A**). The side groups form covalent bonds with cellulose and lignin, molding a complex that surrounds the cellulose fibers. These strong bonds with lignin and cellulose make their separation difficult while maintaining their integrity (Z. Zhang et al., 2014). Large quantities of grains are cultivated in the agroindustry and generate tons of agro-industrial residues. The particular interest in AX polymers is due to the potential to obtain prebiotic xylo-oligosaccharides (XOS) (Gonçalves et al., 2012).

Endo-1,4- $\beta$ -xylanases (EC 3.2.1.8) cleave the glycosidic bonds in the arabinoxylan (AX) backbone, releasing lower-grade xylose and XOS (**Figure 7-1B**). GH 10 endoxylanases require only two unsubstituted xylopyranosyl residues between branches. It results in higher shorter-chain XOS production, especially xylobiose and xylotriose, which shows the greatest prebiotic potential (Ávila, Martins, Costa, et al., 2020). However, to optimize the production of linear XOS, other enzymes that act on the AX side chains are necessary. The family GH 62 is composed of  $\alpha$ -L-arabinofuranosidases with almost exclusive specificity by  $\alpha$ (1 $\rightarrow$ 2) and  $\alpha$ (1 $\rightarrow$ 3) bonds of L-arabinofuranosyl residues of hemicellulose (Kaur et al., 2015). The simultaneous substituents removal and glycosidic bonds cleavage increases the degradation rate (**Figure 7-1B**). Thus, synergistic enzymes are desirable and under intense investigation to understand the mode of action and optimize enzyme mixtures and concentrations (Ávila, Cairo, Damasio, et al., 2020a; Wyman et al., 2005b). Several studies have already confirmed the synergistic effect of the mixture of endoxylanases and arabinofuranosidases for XOS production (Ávila, Martins, Costa, et al., 2020; Goldbeck et al., 2016; Gonçalves et al., 2012; Martins, Ávila, et al., 2020).

From an industrial point of view, the enzymatic synergism allows accelerating the activity for biomass conversion, reducing the amount of enzyme needed, the hydrolysis time, and production costs (A. M. Lopes et al., 2018). Although industries widely use enzymatic mixtures, several studies report chimeric enzymes present superior performance (Diogo et al., 2015). A common method to synthetically join catalytic domains is tandem fusion. Two or

more sequences encoding proteins connected end-to-end produce a single polypeptide chain with multiple functions. Short sequences compose the linkers among the domains to provide mobility freedom and avoid steric impediments. The most used linkers are in the form (Gly-Gly-Gly-Gly-Ser)<sub>n</sub>, where “n” is the number of residues (<6), forming a loop. Short and flexible linkers can promote higher catalytic activity, greater mobility without unsettling the conformation of enzymes, and decrease the probability of proteolytic attack during protein expression and purification (Martins, Dinamarco, et al., 2020).

In addition to the synergistic effect and greater ease of production, the catalysis of the fused enzymes presents similar stability and activity under the same conditions (Elleuche, 2015; Rizk et al., 2015). This is because the N-terminal position plays a decisive role in protein half-life and thermostability. Placing the most thermostable protein in the N-terminal position can increase the thermostability of the next linked protein. Additionally, the final structure of the whole molecule sets the stability under different pH and salt conditions to guarantee success in creating chimeric enzymes (Rizk et al., 2012; Suplatov et al., 2015). The proximity between the catalytic domains of fused enzymes often increases enzymatic performance compared to the activities of separate enzymes (Ribeiro et al., 2011).

Several chimeras were developed to enhance the hydrolysis efficiency of lignocellulosic biomass, but none was designed with the principal goal of optimizing XOS production. The GH 10 endoxylanase (EC 3.2.1.8) of *Papiliotrema flavescens* (Xyn10cf) showed high thermostability and synergy with arabinofuranosidases for short-chain XOS production, especially xylobiose and xylotriose (Martins, Ávila, et al., 2020). GH 62 arabinofuranosidase (ED 3.2.1.55) of *Aspergillus fumigatus* (Af\_afu62) presented high specificity for arabinoxylan and the synergism with GH 10 endoxylanase improved xylo-oligosaccharides production by 42.6%; however, its thermostability is still a drawback (Martins, Tramontina, et al., 2022). Thus, this study expressed a chimera by tandem fusion of a thermostable GH 10 endoxylanase of *P. flavescens* and GH 62 arabinofuranosidase of *A. fumigatus* using a flexible linker (GGGGS)<sub>3</sub> to obtain a bifunctional protein with enhanced hydrolytic power and thermotolerance. This strategy aimed to blend the biochemical characteristics and enhance the half-life of the less thermostable enzyme (Af\_afu62), increasing the catalytic performance. The results from biochemical characterization, thermostability, and synergic effects confirmed the goal of enhancing arabinoxylan specificity and the rate of catalysis to produce XOS from agro-industrial wastes.

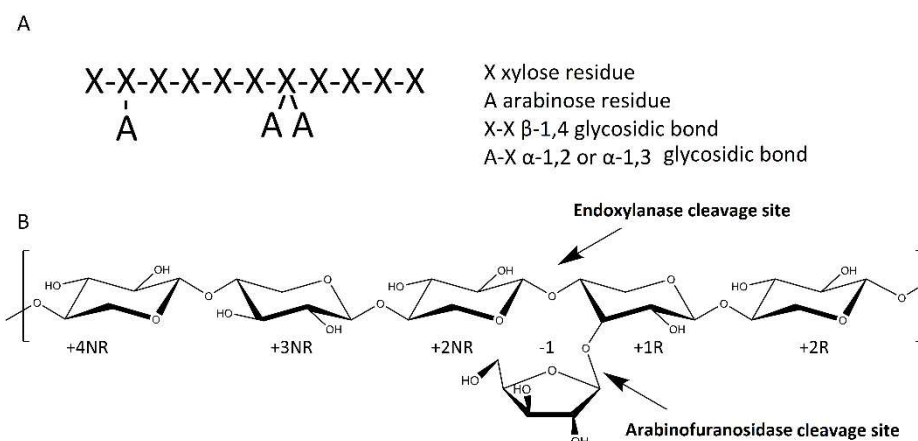


Figure 7-1 Schematic representation of arabinoxylan structure and cleavage sites of GH 10 endoxylanase and GH 62 arabinofuranosidase.

## 7.2. Material and Methods

### 7.2.1. *In silico* molecular modeling

The 3D structure of chimera Xyn10cf+Af\_afu62 was built by RoseTTAFold (Baek et al., 2021). The predicted structure was evaluated by the GDT score, Ramachandran's plot, and angstrom error estimate. These results were further evaluated by PROCHECK (Laskowski et al., 1993). The 3D structures of feruloyl arabinobiose (CID 6438898) and xylotetraose (CID 10230811) were downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). Polar hydrogens were added, and Gasteiger partial charges were assigned. Charges were computed using ANTECHAMBER (J. Wang et al., 2006). AutoDock Vina was used for protein–ligand docking, and the results were analyzed using UCSF Chimera (Pettersen et al., 2004; Trott & Olson, 2009).

### 7.2.2. Expression and purification of recombinant chimeric gene

The gene sequences of Xyn10cf GH 10 endoxylanase from *Papiliotrema flavescens* (GenBank Gene ID: Ahl20248.1, without a signal peptide and TAG codon), the flexible linker [GGGSGGGSGGGGS], Af\_afu62 GH 62 arabinofuranosidase from *Aspergillus fumigatus* (GenBank Gene ID: Afu\_2G12770, 948 pb, without a signal peptide and TAG codon) were optimized using the Codon Optimization Tool (<https://www.novoprolabs.com/tools/codon-optimization>), synthesized by GenOne (Rio de Janeiro, Brazil), and inserted into the pGAPZaA vector (Invitrogen, Carlsbad, CA, USA) between EcoRI and XbaI sites with 6x His-tag and  $\alpha$ -factor peptide signal. The synthetic gene sequence can be found in GenBank with the accession number OM867846.



*Escherichia coli* DH5 $\alpha$  was heat-shock transformed and cultivated at 37 °C and 200 RPM in low-salt Luria-Bertani (LB) medium with 100  $\mu$ g/ml Zeocin. The plasmid containing the chimeric gene Xyn10cf+Af\_afu62 was propagated in *E. coli*, extracted, and linearized with FastDigest PaeI (5'...T<sup>^</sup>CATGA...3', Thermo Scientific<sup>TM</sup>, USA) according to the instructions. Electrocompetent *Komagataella pastoris* X-33 was prepared according to the transformation protocol No. 4308 915.545 –03/2004 (Eppendorf) and transformed by electroporation (1500 V, 5 milliseconds). Transformants were selected for Zeocin resistance on YPDS plates containing 100  $\mu$ g/ml Zeocin. Ten colonies of resistant transformants were selected after three days of incubation at 30 °C and transferred to another YPDS plate with the same concentration of Zeocin. The presence of the insert was confirmed by PCR using the sequences of the primers 5' pGAP forward and 3' AOX1 reverse recommended by the Invitrogen cloning manual.

Small-scale enzyme production of the PCR-positive colonies was performed in 50 mL YDP (in 250 mL Erlenmeyer) at 30 °C and 250 RPM for 96 hours. The supernatant was concentrated by ultrafiltration (Amicon Ultra15, Merck KGaA, Germany) using a molecular weight cutoff of 10 kDa. The enzyme was purified using the HisPur kit (Thermo Scientific<sup>TM</sup>, USA), as recommended. Proteins were visualized on 12% SDS–PAGE for the separation gel and 4% for the stacking gel (Laemmli, 1970) and stained with Coomassie blue G-250 (Thermo Scientific<sup>TM</sup>, USA). Protein content was determined using bovine albumin (Sigma–Aldrich, USA) as a standard (Lowry et al., 1951). After purification, the protein was diluted to 1 mg/mL in 10 mM Tris-HCl buffer 150 mM NaCl pH 7.4. For enzymatic assays, the protein was diluted to a final concentration of 0.01 mg/mL.

The parental recombinant enzymes GH 10 endoxylanase and GH 62 arabinofuranosidase were also produced and purified according to the same methodology.

### 7.2.3. Circular dichroism (CD)

Far-UV CD spectrum was measured between 195 and 250 nm in phosphate buffer 20 mM pH 6.0 at 25 °C using a spectrophotometer Jasco J-810 (Jasco Inc, Tokyo, Japan) equipped with a thermostatic Peltier system with constant nitrogen flow, using a cuvette of 1 mm and protein concentration of 0.05 mg/mL and a rate of 1 nm/min. The ellipticity was reported as the mean residue molar ellipticity ( $\theta$ , deg.cm<sup>2</sup> dmol<sup>-1</sup>). The secondary structure was computed with BesStSel (<https://bestsel.elte.hu/index.php>) (Micsonai et al., 2018).

### 7.2.4. Biochemical characterization and kinetic parameters

The optimum temperature and pH were determined by a central composite rotational design (CCRD) with two factors, 3 repetitions in the central point condition and 4 axial

points (Rodrigues & Iemma, 2014), evaluating the enzymatic activity of endoxylanase and arabinofuranosidase separately using the substrates xylan of beechwood and  $\rho$ -nitrophenyl- $\alpha$ -L-arabinofuranoside, respectively. The temperature varied from 50 °C to 80 °C for endoxylanase and from 36 °C to 64 °C for arabinofuranosidase, and the pH varied from 3.4 to 6.2.

The kinetic parameters ( $K_m$  and  $V_{max}$ ) were examined by incubating the diluted enzyme for 30 min in McIlvaine buffer containing 0 to 4 mg/mL wheat arabinoxylan (Megazyme, Ireland) under the optimal conditions determined for temperature and pH. The parameters were calculated using GraphPad Prism Software version 9.

### **7.2.5. Effects of metal ions, inhibitors, pH, and salt concentration**

The effect of various metal ions ( $Na^+$ ,  $K^+$ ,  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Ni^{2+}$ ,  $Ag^{3+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ , and  $Co^{2+}$ ) and many inhibitors [EDTA, dimethyl sulfoxide (DMSO), Tween-20,  $\beta$ -mercaptoethanol, DTT, and sodium dodecyl sulfate (SDS)] was analyzed by incubating the purified chimera in 50 mM solutions of varying concentrations (1, 5, and 10 mM) for 1 h before the enzymatic assay.

The pH stability of the enzyme was studied by incubating in McIlvaine buffer pH ranging from 3.0 to 8.0 for 24 h at 4 °C. Salt tolerance was evaluated by incubating the enzyme with NaCl concentrations ranging from 0 to 3 M for 24 h at 4 °C. After 24 h, the relative activities were measured at the optimum conditions for enzymatic assays.

### **7.2.6. Thermostability and thermal shift assays**

Thermal treatment of the purified chimera was carried out in a thermocycler, scheduling each treatment at a controlled temperature of 50, 60, and 70 °C for 4 h. Aliquots were taken every 20 min and cooled at 4 °C for a minimum of 1 h to ensure that the reversibly denatured protein was converted back to its native active form, while the irreversibly denatured form remained inactive. At the end of the thermal treatment, the enzymatic assays of all the aliquots were carried out at 65 °C for 30 min, and the relative activities were expressed as a percentage of the original activities to calculate the thermodynamic parameters (Saqib & Siddiqui, 2018).

The melting temperatures of the chimera Xyn10cf+Af\_afu62 and the parental enzymes were assayed by differential scanning fluorimetry (DSF) using a real-time PCR instrument Eppendorf MasterCycler Realplex 4, 560-580 nm detection filter. Five microliters of enzyme preparations (1-5  $\mu$ g), 4  $\mu$ L of 50X stock solution of SYPRO® Orange (Supelco, Merck, S5692), 1, 5 and 10 mM  $CaCl_2$  or  $MnCl_2$ , and McIlvaine buffer to complete 12.5  $\mu$ L were pipetted into the wells of a qPCR plate and sealed. The temperature was raised from 25 °C to

95 °C at a step of 0.1 °C/s. The fluorescence was monitored by the qPCR system, and the  $T_m$  was calculated from the inflection points of the transition curve [Fluorescence =  $f(T)$ ], which was computed as the maximum of the first derivative  $[d(\text{Fluorescence})/d(T)]$ .

#### 7.2.7. Synergism for XOS production from agro-industrial wastes

The hydrolytic power of the chimera analyzed the XOS production from arabinoxylan extracted from soy and corn husks (see 0). Soluble concentrations of arabinoxylan (0.4 to 4.6% w/w) were incubated in McIlvaine buffer pH 5.0 with 10 µL/mL of diluted chimera (final protein concentration of 0.01 mg/mL) and 5 mM  $\text{MnCl}_2$ . The results were compared to the yields obtained from hydrolysis using 10 µL/mL of Shearzyme endoxylanase (NS50030- Novozymes®, final protein concentration at the saturation point of 0.165 mg/mL) under the optimum conditions previously determined (pH 5.0 and 50 °C, data not shown).

The synergic efficiency of the chimera and the parental enzyme mixture was estimated after 12 h hydrolysis of 1% wheat arabinoxylan (arabinose:xylose ratio 33.5:62, Megazyme, Ireland) at 40 °C and pH 5.0. The synergism was calculated by the ratio of xylo-oligosaccharides released by the chimera and the parental enzyme equimolar mixture, according to Equation 7.1.

$$D = \frac{Y_c}{Y_{mix}} \text{ or } \frac{Y_{mix}}{Y_x + Y_a} \quad (7.1)$$

Where D is the degree of synergy,  $Y_c$  is the total xylo-oligosaccharides produced by the chimera;  $Y_{mix}$  the total xylo-oligosaccharides produced by the equimolar mixture of the parental enzymes Af\_afu62 and Xyn10cf; and  $Y_x$  and  $Y_a$  are the total xylo-oligosaccharides produced by Xyn10cf and Af\_afu62, respectively.

#### 7.2.8. Analytical methods

##### *Enzymatic assays*

The endoxylanase activity was assayed with 25 µL of 4% wheat arabinoxylan or beechwood xylan (Megazyme, Ireland), 60 µL of 100 mM McIlvaine buffer (variable pH), 10 µL of Milli-q (or 50 mM  $\text{Ca}^{2+}$  or  $\text{Mn}^{2+}$ ) and 5 µL of the diluted enzyme (0.01 mg/mL). The tests were conducted at different temperatures for 10 min (Bailey et al., 1992). The reducing sugars were analyzed by DNS (Miller, 1959), with one xylanase unit (U/µL) being the amount of enzyme needed to produce 1 µmol of reducing sugars per minute.

The activity of arabinofuranosidase was measured with 10 µL of 10 mM  $p$ -nitrophenyl- $\alpha$ -L-arabinofuranoside ( $p$ NP-A) in 65 µL of 100 mM McIlvaine buffer, 20 µL of water or 50 mM cofactor  $\text{Ca}^{2+}$  or  $\text{Mn}^{2+}$ , and 5 µL of the diluted enzyme (0.01 mg/mL). After

incubation at 45 °C for 30 min, the reaction was stopped by adding 50 µL of 1 M Na<sub>2</sub>CO<sub>3</sub>. The concentration of p-nitrophenol was measured at 405 nm, with one unit (U/µL) of α-L-arabinofuranosidase defined as the amount of enzyme required to release 1 µmol of p-nitrophenol per min (Sakamoto & Kawasaki, 2003).

### ***Biomass characterization, hemicellulose extraction and XOS quantification***

Milhão Ingredients (Goianira, Goiás, Brazil) and Oleoplan S.A. (Veranópolis, Rio Grande do Sul, Brazil) kindly donated the corn and soy hulls, respectively. Corn hulls (pericarp fiber) was washed several times with hot distilled water to remove residual starch and then centrifuged. The starch and the corn hulls were dried at 50 °C overnight and weighed. Cellulose, hemicellulose, and lignin characterization followed the NREL/TP-510-42619 procedure (Sluiter et al., 2005, 2008). Protein was determined as the total nitrogen content by the Kjeldahl method using the N × 6.25 conversion factor according to the AOAC 984.13 method. Xylooligosaccharides were quantified as described elsewhere (Martins, Ávila, et al., 2020).

Xylan extraction used 5% KOH and a solid ratio of 1:10 at 121 °C for 30 min (de Figueiredo et al., 2017; Martins, Tramontina, et al., 2022). The samples were centrifuged at 5000×g for 20 min, and the supernatant was vacuum filtered, neutralized with acetic acid to pH 5.0-5.5, and precipitated with 3 volumes of cold ethanol. After an overnight precipitation at 4 °C, the solid fraction was separated by centrifugation at 5000 × g for 20 min, washed once with 50% ethanol 0.5% EDTA (Ávila, Martins, & Goldbeck, 2020; de Figueiredo et al., 2017), centrifuged, rinsed with Milli-Q®, centrifuged, dried at 50 °C overnight, and diluted in McIlvaine buffer pH 5.0.

### ***Statistical analysis and graphical software***

All experiments were conducted in triplicate, except CCDR. One-way ANOVA and Tukey post hoc tests were conducted using OriginPro 8.5 software. Graphics were developed using GraphPad Prism 5.

## **7.3. Results and discussion**

### **7.3.1. Structure prediction and molecular docking**

The quality of the predicted model of the chimera was evaluated based upon Ramachandran's plot, QMEAN score, and confidence based on the GDT score (1.0 good; 0.0 bad). The model presented a confidence of 0.84 and a span of 1<sup>-656</sup>. The 3D model showed > 95% residues in Ramachandran's favorable region and a QMEAN score lower than 2. The arabinofuranosidase domain displayed the typical 5-bladed β-propeller structure found in GH 62

enzymes, with the central axis occupied by a calcium ion (Tonozuka & Tanaka, 2017). The endoxylanase domain also accommodate 7  $\text{Ca}^{2+}$  ions in the central loops of helix-loop-helix structures, suggesting that calcium may contribute to GH 10 ( $\beta/\alpha$ )<sub>8</sub> stabilization (Abou-hachem et al., 2003) (**Figure 7-2**).

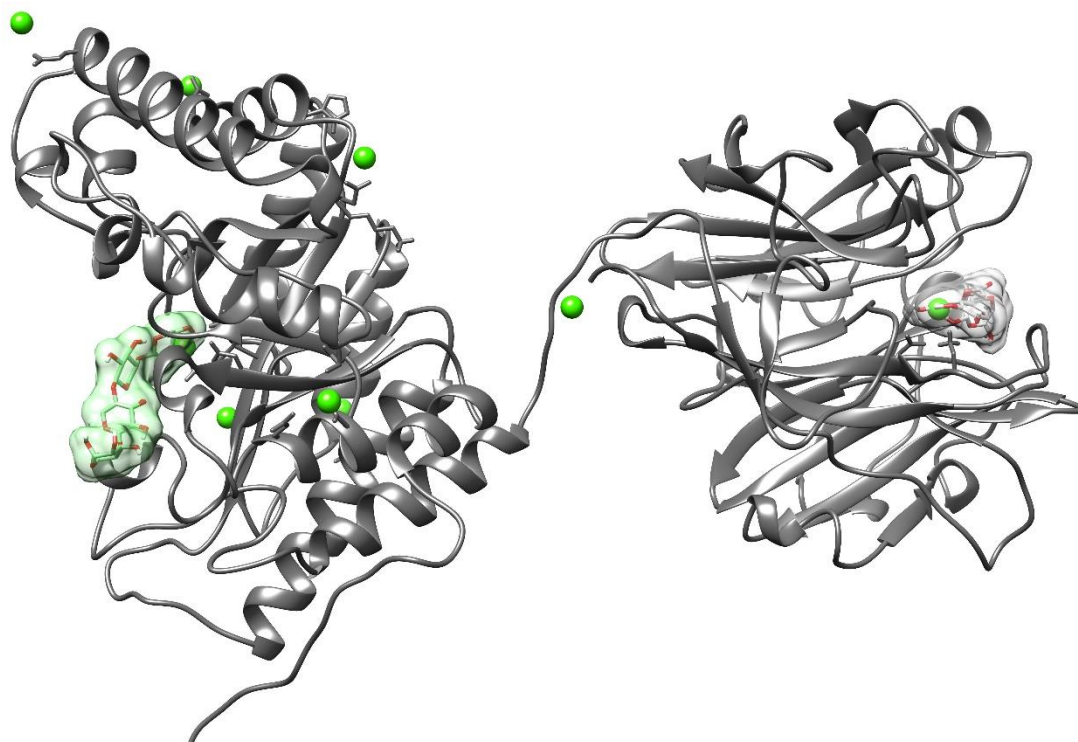


**Figure 7-2** Chimera structure and substrate binding sites. Endoxylanase Xyn10cf module on the left, docked with xylotetraose and arabinofuranosidase Af\_afu62 module on the right, docked with feruloyl arabinobiose. Calcium ions putative locations are represented as green balls

### 7.3.2. Recombinant expression and circular dichroism

The contours of the CD spectra of Xyn10cf, Afua\_2g12770, and the chimera Xyn10cf+Af\_afu62 are shown in **Figure 7-3A**. The CD spectra of Xyn10cf and Xyn10cf+Af\_afu62 exhibit double minima signatures at approximately 210 and 222 nm, which indicate a significant  $\alpha$ -helix content. The lower molar ellipticity values at these minima indicate that Xyn10cf presents a higher helical content than the chimera, which also contains significant amounts of other secondary structures. The CD spectrum of Af\_afu62 presents two main bands at 200 (positive) and 210 (negative) and lacks the "double minima signature", indicating that this protein contains a high amount of  $\beta$ -sheet content and poor helical content. These results are consistent with the expected structure of the ( $\alpha/\beta$ )<sub>8</sub> motif for GH 10 endoxylanase and the 5-bladed  $\beta$ -propeller motif for GH 62 arabinofuranosidase. The chimera

presented a helical content of 22.65%, consistent with the predicted structure. The parental Xyn10cf and Af\_afu62 presented a helical content of 42.5 % and 10.9 %, respectively.

SDS-page gel confirms the molecular mass of around 85 kDa (between 70 and 100 kDa bands), which is coherent with the sums of the molecular masses of parental Af\_afu62 (~37 kDa) and Xyn10cf (~48 kDa), poly-histidine tag, and flexible linker. The crude supernatant extract shows little degradation of the recombinant chimera (**Figure 7-3B**). The enzyme yield was of 10.4 mg/L after 96 h, while the cultures of the parental enzymes yielded 15 mg/L for Xyn10cf and 4 mg/L for Af\_afu62. Even though Xyn10cf separately resulted in higher enzyme concentration, the mixture of the cultures would result in dilution and improper proportion of the enzymes. Further research will be conducted to optimize fermentation conditions to scale up the production and improve protein yields.

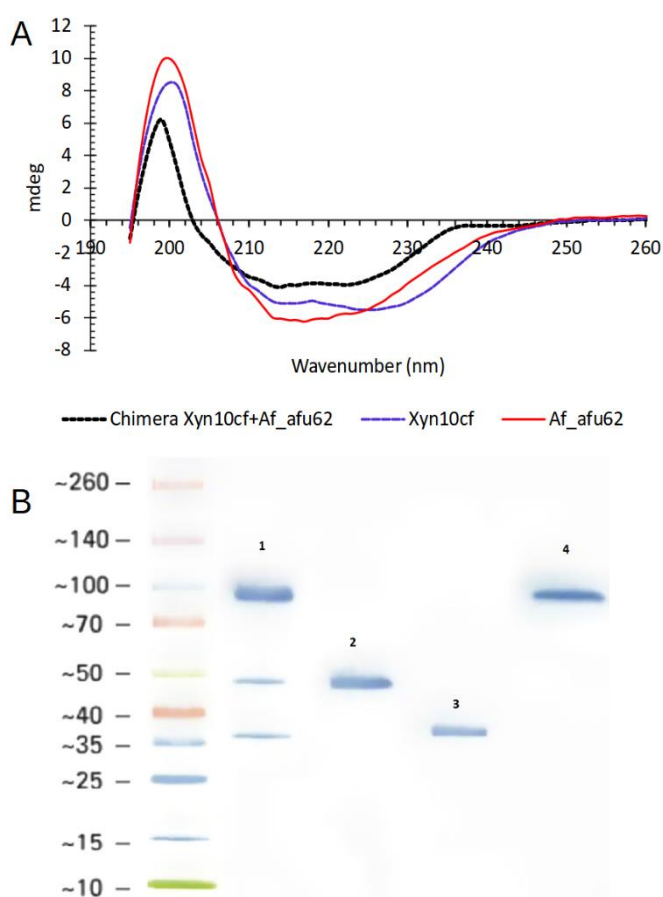


**Figure 7-3** (A) CD spectrum of Xyn10cf+Af\_afu62 and parental enzymes; (B) SDS page of crude supernatant of chimera enzyme (1), purified Xyn10cf (2), purified Af\_afu62 (3), purified chimera (4).

### 7.3.3. Biochemical characterization

CCRD showed good reproducibility under the central point conditions for both endoxylanase (65 °C and pH 4.8) and arabinofuranosidase activities (50 °C and pH 4.8). The analysis of the regression coefficients showed that the linear and quadratic terms for temperature and pH are significant at the 95% confidence level ( $p < 0.05$ ), except for the linear term for temperature (**Supplementary table 12-5 and Supplementary table 12-6**). Data analysis for multiple regression led to a second-order polynomial model, whose validity was verified by analysis of variance (ANOVA) for the response surface construction (**Figure 7-4**). The positive interaction coefficient of pH and temperature indicates that the combination of both independent variables influences enzymatic activity. The endoxylanase moiety presented optimum conditions between pH and temperature 4.8 – 6.0 and 60 - 78 °C, and the arabinofuranosidase was between 4.0 - 5.5 and 50 - 66 °C. The predicted values were validated at pH and temperature of 5.3 and 70 °C for endoxylanase activity and 4.8 and 55 °C for arabinofuranosidase. The experimental values of  $98.004 \pm 1.813$  U/ $\mu$ L and  $91.201 \pm 3.630$  U/ $\mu$ L, respectively, for endoxylanase and arabinofuranosidase, were within the predicted value range of the model.

The CCRD of both parental enzymes are presented in **Supplementary table 12-7** and **Supplementary table 12-8**. The parental Af\_afu62 enzyme showed a narrow optimum temperature range of 37 to 42 °C against the 50 to 66 °C displayed by the chimera Af\_afu62 module, with an average increase of 46%.

Regarding the optimum pH and temperature for the entire chimeric protein, an intersection area was observed in the response surface where both catalytic domains presented the highest activity at pH 4.5 and 5.0 and temperatures from 60 to 65 °C. Therefore, it can be assumed that the physicochemical characteristics of Xyn10cf and Af\_afu62 merged with the fusion. At the same time, the optimum temperature range of Xyn10cf did not decrease and maintained between 60 °C and 78 °C after fusion.

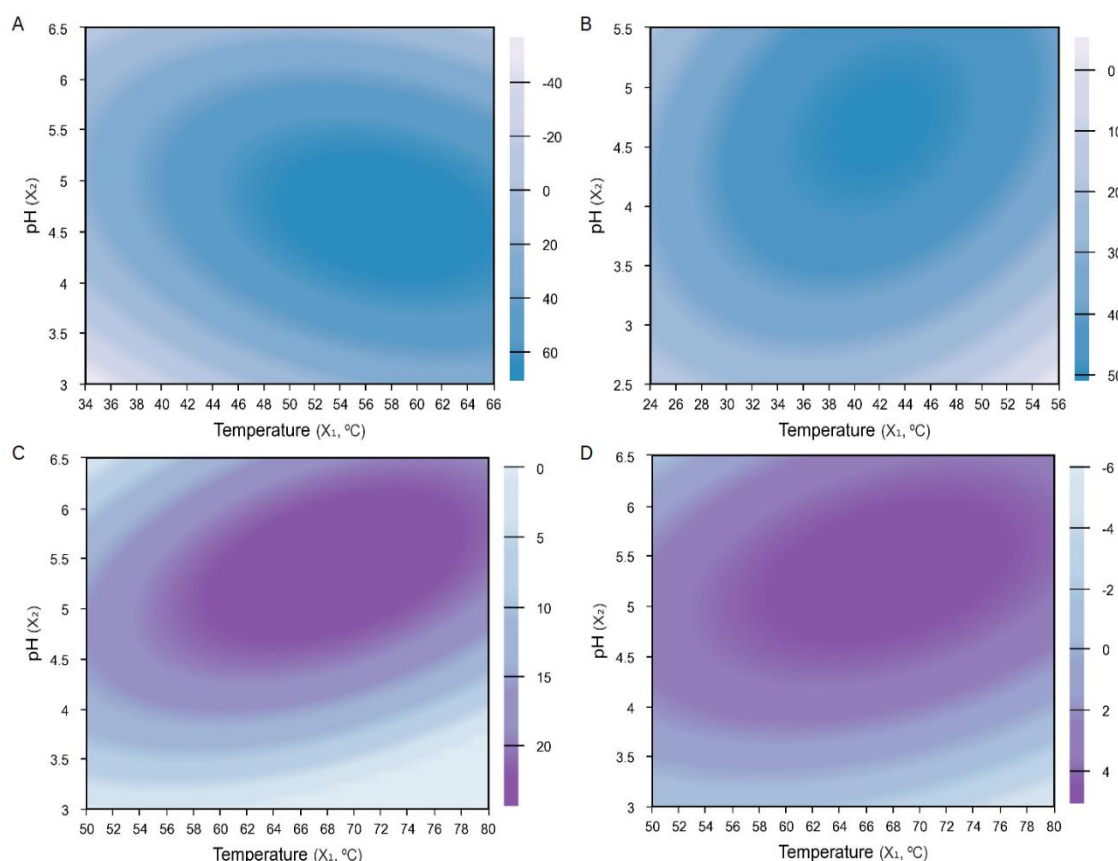


**Figure 7-4** Surface response to optimal conditions of pH and temperature determined for (A) chimeric arabinofuranosidase, (B) parental arabinofuranosidase, (C) chimeric endoxylanase, and (D) parental endoxylanase.

#### 7.3.4. Kinetic parameters of commercial arabinoxylan

The chimera Xyn10cf+Af\_afu62 presented a  $v_{\max}$  value 1.23 times higher than that of the parental Xyn10cf and 1.02 times higher than that of Af\_afu62. The  $k_m$  value was 45.77% and 85.02% lower than Af\_afu62 and Xyn10cf, respectively. The  $k_{\text{cat}}$  value was also enhanced, and therefore, the catalytic efficiency ( $k_{\text{cat}}/k_m$ ) of Xyn10cf+Af\_afu62 was 1.88 and 8.2 times higher than that of parental Af\_afu62 and Xyn10cf, respectively (Table 7-3). The catalytic efficiency of chimeras designed with short flexible linkers was also discussed by Guo et al. (Guo et al., 2013), where  $k_{\text{cat}}/k_m$  of the chimera designed with (GGGS)<sub>3</sub> linker enhanced 1.53 times.

#### 7.3.5. Effects of metal ions, chemicals, salt, and pH

The chimera Xyn10cf+Af\_afu62 displayed high relative activity in the range of pH 4.0 to 7.5, maintaining more than 90% maximum activity (**Figure 7-5**). Compared to the parental enzymes, the chimera displayed superior behavior at pH values above 7.5 than Af\_afu62 but was inferior to Xyn10cf, again confirming that the physicochemical characteristics merged. Also, Xyn10cf+Af\_afu62 displayed elevated halotolerance, maintaining 82% maximum



activity with 3 M NaCl (**Figure 7-5**). The stability in salty and alkali media is especially important for arabinoxylan hydrolysis since its extraction usually uses alkaline methods whose neutralization prior to ethanol precipitation produces elevated quantities of salts and acetate (Brienzo et al., 2010).

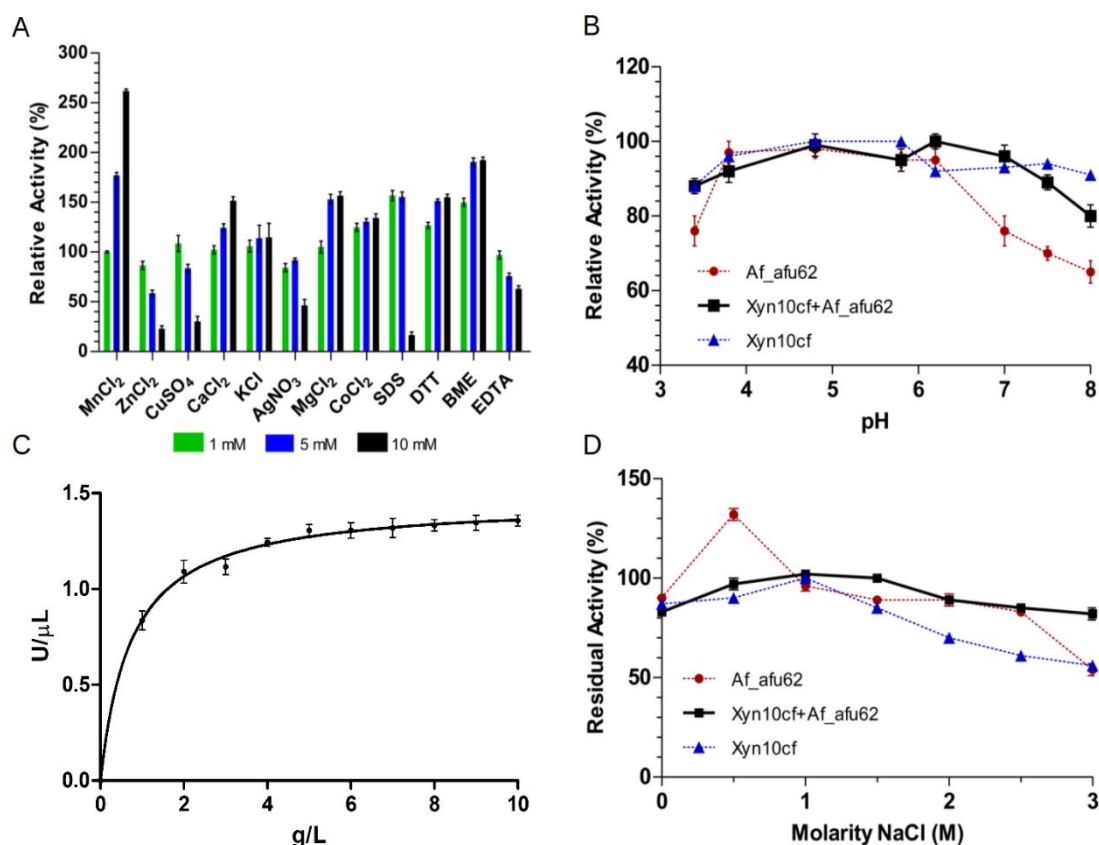


**Figure 7-5** Characterization of chimeric enzyme Xyn10cf+Af\_Afu62: (A) Metal and inhibitor effects, (B) pH stability, (C) kinetic parameters, and (D) salt tolerance

The effects of metal ions and chemicals on the enzymatic activity of the chimera and parental enzymes were also analyzed (**Figure 7-5**). The chimera Xyn10cf+Af\_afu62 and the parental Xyn10cf were significantly stimulated by  $\text{Mn}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Mg}^{2+}$ . BME and DTT, which are disulfide bond reducing agents known to prevent the oxidation of sulfhydryl groups, significantly enhanced their activity, indicating the presence of a reduced thiol group of cysteine. The activities of the chimera and both parental enzymes were suppressed by  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Ag}^{3+}$ , which complex with reactive protein groups, affecting the catalytic site. Lower concentrations of SDS (1 and 5 mM) enhanced the catalytic activity, but 10 mM SDS strongly inhibited the enzymes, suggesting that hydrophobic interactions are essential to maintaining the protein structures. EDTA suppressed the activities of the chimera Xyn10cf+Af\_afu62 and

parental enzymes, indicating that they require a cofactor for its activation (J. de C. Pereira et al., 2017).

Mn<sup>2+</sup> and Ca<sup>2+</sup> were the ions that displayed positive effects for both parental (endoxylanase and arabinofuranosidase) and chimeric enzymes. Several studies have reported the influence of these ions on the thermostability of many enzymes. GH 10 from *Thermotoga thermarum* retained nearly 100% of its residual activity after incubation at 85 °C for 2 h with 5 mM Ca<sup>2+</sup> and more than 50% after 90 °C for 1 h (Shi et al., 2013), and the calcium-binding site of GH 10 from *Rhodothermus marinus* was shown to play a role in thermostability (Abou-hachem et al., 2003). GH 62 arabinofuranosidase from *Eupenicilium parvum* also enhanced thermostability after the addition of Ca<sup>2+</sup> (Long et al., 2020).

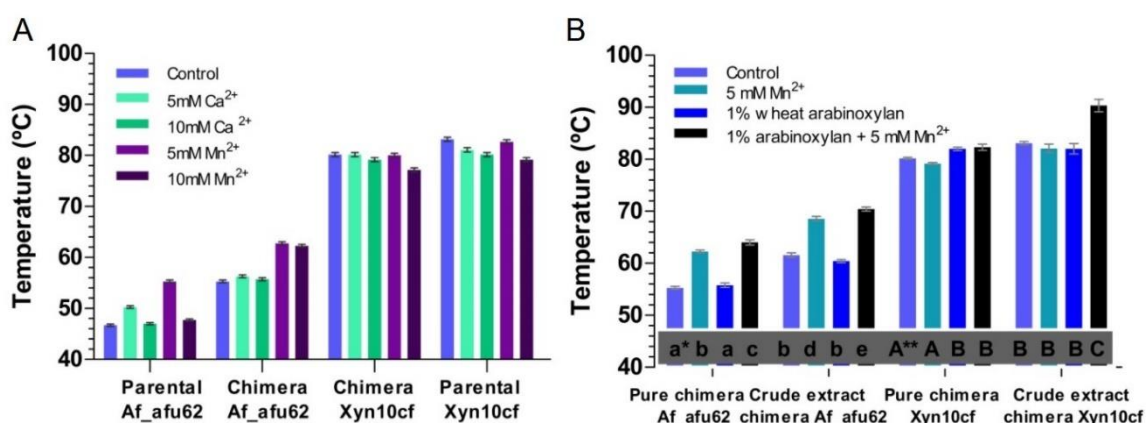


**Figure 7-6** Melting temperatures of the chimera and parental enzymes: cofactors Mn<sup>2+</sup> and Ca<sup>2+</sup> influence (A); arabinoxylan and Mn<sup>2+</sup> influence (B). (\*different letters mean significant differences among the Af\_afu62 melting temperatures; \*\*different capital letters mean significant differences among the Xyn10cf melting temperatures).

### 7.3.6. Thermal shift

Differential scanning fluorometry (DSF) was used to analyze the changes in thermostability of the chimeric and parental enzymes, as well as the effects of Ca<sup>2+</sup> and Mn<sup>2+</sup> ions. DSF is a useful technique to investigate multidomain proteins, and it is possible to visualize the transition state of each domain in the enzyme. The goal of this assay was to show the impact of fusing the catalytic domains on thermostability and investigate the cofactor influence (**Figure 7-6**). The chimera Xyn10cf+Af\_afu62 displayed two melting points, one related to the arabinofuranosidase domain and the second to the endoxylanase (the most thermostable). Researchers revealed that the integration order of the two enzymes results in different properties of the chimera, where the most thermostable should be N-terminal (Guo et al., 2013). A higher T<sub>m</sub> for

the arabinofuranosidase domain was observed in all the assays, with an increase of 10 °C, while the endoxylanase domain displayed a lower  $T_m$  than the parental Xyn10cf, with a reduction of 3 °C, probably due to protein aggregation (Abou-hachem et al., 2003). Additionally, the presence of 5 mM  $Ca^{2+}$  and especially  $Mn^{2+}$  enhanced  $T_m$  for Xyn10cf+Af\_afu62 and parental Af\_afu62 up to 6.8 °C. The  $T_m$  of the chimera endoxylanase domain did not suffer significant change with 5 mM  $Ca^{2+}$  or  $Mn^{2+}$ , while the parental Xyn10cf displayed 4 °C reduced  $T_m$  in the presence of the ions. The chimera showed a superior tolerance and affinity for the ions than the parental enzymes. Despite the small reduction in  $T_m$  for the endoxylanase module, the overall thermostability improvement for the arabinofuranosidase domain and cofactor concentration up to 5 mM overcame this drawback. The flexible linkers are often related to improved kinetic parameters but reduced thermostability, while rigid linkers may improve thermostability and reduce the substrate specificity (Guo et al., 2013). However, this present study showed the opposite as, although Xyn10cf didn't present significant changes in thermostability, Af\_afu62 thermostability improved 1.2 times.

Significant differences in  $T_m$  were found for the crude supernatant and purified chimeric enzyme, as presented in **Figure 7-6**. The crude supernatant presented  $T_m$  values for arabinofuranosidase and endoxylanase modules of 61.6 °C and 88.1 °C, respectively, against 55.3 °C and 83.2 °C from the purified chimeric enzyme. Additionally, the presence of 1% wheat arabinoxylan and 5 mM  $Mn^{2+}$  increased  $T_m$  to 70.4 °C and 90.3 °C (arabinofuranosidase and endoxylanase, respectively), since hydrophobic interactions of the substrate-binding site play a role in the thermostability of GH 10 (Aronsson et al., 2018; Imjongjairak et al., 2015; Y. Wang et al., 2022) and the  $\beta$ -propeller structure of GH 62 stabilizes the arabinofuranosyl substituent through hydrogen bonding and hydrophobic interactions (W. Wang et al., 2014).

### 7.3.7. Thermostability

Table 7-1 presents the thermodynamic parameters of the fused and parental enzymes under the influence of 5 mM  $Mn^{2+}$  or  $Ca^{2+}$ . Our previous study already reported the positive effects of  $Mn^{2+}$  and  $Ca^{2+}$  on Af\_afu62 thermostability (Martins, Tramontina, et al., 2022). The same treatment was therefore applied to the parental endoxylanase Xyn10cf and compared to the chimera results. The cofactors impaired the thermostability of parental Xyn10cf by decreasing half-life time ( $t_{1/2}$ ) from 770 to 247 min at 50 °C and energy of denaturation ( $E_d$ ) from 53 to 30 kJ/mol. This result is in accordance with the thermal shift analyses.

**Table 7-1 Thermodynamic parameters of chimera Xyn10cf+Af\_afu62 and parental enzymes in the presence of 5 mM  $Ca^{2+}$  or 5mM  $Mn^{2+}$  (no ion for control) after thermal treatment at 50, 60, and 70 °C.**

| <b>Chimera</b><br><b>Xyn10cf+Af_afu62</b> | <b>k<sub>d</sub></b><br><b>(min<sup>-1</sup>)</b> | <b>R<sup>2</sup></b> | <b>t<sub>1/2</sub></b><br><b>(min)</b> | <b>D-value</b><br><b>(min)</b> | <b>ΔG<sub>d</sub></b><br><b>(kJ/mol)</b> | <b>ΔH<sub>d</sub></b><br><b>(kJ/mol)</b> | <b>ΔS<sub>d</sub></b><br><b>(J/mol.K)</b> | <b>E<sub>d</sub></b><br><b>(kJ/mol)</b> |
|-------------------------------------------|---------------------------------------------------|----------------------|----------------------------------------|--------------------------------|------------------------------------------|------------------------------------------|-------------------------------------------|-----------------------------------------|
| <b>Control</b>                            |                                                   |                      |                                        |                                |                                          |                                          |                                           |                                         |
| 50 °C                                     | 0.0033                                            | 0.9515               | 222.260                                | 697.753                        | 105.710                                  | 34.378                                   | -220.740                                  | 37.065                                  |
| 60 °C                                     | 0.0042                                            | 0.9918               | 146.899                                | 548.235                        | 108.398                                  | 34.295                                   | -222.431                                  |                                         |
| 70 °C                                     | 0.0074                                            | 0.9968               | 99.463                                 | 311.160                        | 110.120                                  | 34.212                                   | -221.210                                  |                                         |
| <b>Ca<sup>2+</sup></b>                    |                                                   |                      |                                        |                                |                                          |                                          |                                           |                                         |
| 50 °C                                     | 0.0012                                            | 0.9993               | 577.623                                | 1918.821                       | 108.428                                  | 46.620                                   | -191.268                                  | 49.306                                  |
| 60 °C                                     | 0.0020                                            | 0.9897               | 346.574                                | 1151.293                       | 110.453                                  | 46.536                                   | -191.854                                  |                                         |
| 70 °C                                     | 0.0035                                            | 0.9861               | 198.042                                | 657.881                        | 112.256                                  | 46.453                                   | -191.761                                  |                                         |
| <b>Mn<sup>2+</sup></b>                    |                                                   |                      |                                        |                                |                                          |                                          |                                           |                                         |
| 50 °C                                     | 0.0012                                            | 0.9061               | 577.623                                | 1918.821                       | 108.428                                  | 42.576                                   | -203.780                                  | 45.263                                  |
| 60 °C                                     | 0.0021                                            | 0.9694               | 330.070                                | 1096.469                       | 110.318                                  | 42.493                                   | -203.585                                  |                                         |
| 70 °C                                     | 0.0032                                            | 0.9637               | 216.608                                | 719.558                        | 112.512                                  | 42.410                                   | -204.288                                  |                                         |
| <b>Parental Xyn10cf</b>                   | <b>k<sub>d</sub></b><br><b>(min<sup>-1</sup>)</b> | <b>R<sup>2</sup></b> | <b>t<sub>1/2</sub></b><br><b>(min)</b> | <b>D-value</b><br><b>(min)</b> | <b>ΔG<sub>d</sub></b><br><b>(kJ/mol)</b> | <b>ΔH<sub>d</sub></b><br><b>(kJ/mol)</b> | <b>ΔS<sub>d</sub></b><br><b>(J/mol.K)</b> | <b>E<sub>d</sub></b><br><b>(kJ/mol)</b> |
| <b>Control</b>                            |                                                   |                      |                                        |                                |                                          |                                          |                                           |                                         |
| 50 °C                                     | 0.0009                                            | 0.9479               | 770.164                                | 2558.428                       | 109.201                                  | 51.102                                   | -179.790                                  | 53.788                                  |
| 60 °C                                     | 0.0014                                            | 0.9950               | 495.105                                | 1644.704                       | 111.441                                  | 51.018                                   | -181.366                                  |                                         |
| 70 °C                                     | 0.0029                                            | 0.9923               | 239.016                                | 793.995                        | 112.792                                  | 50.935                                   | -180.262                                  |                                         |
| <b>Ca<sup>2+</sup></b>                    |                                                   |                      |                                        |                                |                                          |                                          |                                           |                                         |
| 50 °C                                     | 0.0028                                            | 0.9960               | 247.553                                | 822.352                        | 106.151                                  | 29.217                                   | -238.075                                  | 31.904                                  |
| 60 °C                                     | 0.0038                                            | 0.9764               | 182.407                                | 605.943                        | 108.675                                  | 29.134                                   | -238.753                                  |                                         |
| 70 °C                                     | 0.0056                                            | 0.9940               | 123.776                                | 411.176                        | 110.915                                  | 29.051                                   | -238.566                                  |                                         |
| <b>Mn<sup>2+</sup></b>                    |                                                   |                      |                                        |                                |                                          |                                          |                                           |                                         |
| 50 °C                                     | 0.0016                                            | 0.9818               | 433.217                                | 1439.116                       | 107.655                                  | 27.899                                   | -246.808                                  | 30.586                                  |
| 60 °C                                     | 0.0025                                            | 0.9595               | 277.259                                | 921.034                        | 109.835                                  | 27.816                                   | -246.192                                  |                                         |
| 70 °C                                     | 0.0031                                            | 0.9811               | 223.596                                | 742.769                        | 112.602                                  | 27.733                                   | -247.325                                  |                                         |

**k<sub>d</sub>** = first order rate denaturation constant; **t<sub>1/2</sub>** = half-life time; **D-value** = decimal reduction (time required to reduce residual activity to 10 %); **ΔH<sub>d</sub>** = activation enthalpy of denaturation; **ΔG<sub>d</sub>** = activation Gibbs free energy of denaturation; **ΔS<sub>d</sub>** = activation entropy of denaturation; **E<sub>d</sub>** = activation energy of denaturation

All thermodynamic parameters of the chimera increased in the three scenarios (control, 5 mM Ca<sup>2+</sup> and 5 mM Mn<sup>2+</sup>). The enthalpy of denaturation (ΔH<sub>d</sub>) expresses the total energy required to denature an enzyme, related to the E<sub>d</sub> value (energy of denaturation). Higher values of E<sub>d</sub> and ΔH<sub>d</sub> indicate higher thermostability. Additionally, high positive Gibbs free energy (ΔG<sub>d</sub>) values indicate an increase in denaturation resistance (Bisswanger, 2017; P. M. Souza et al., 2015). ΔG<sub>d</sub> computed a slight increase for the chimera Xyn10cf+Af\_afu62 and parental Af\_afu62 incubated with Ca<sup>2+</sup> and Mn<sup>2+</sup> at all temperatures, along with the significant

enhancement of  $t_{1/2}$ , D value,  $E_d$ , and  $\Delta H_d$ . On the other hand, there was an expression decrease in the  $\Delta G_d$  and  $\Delta H_d$  values of Xyn10cf incubated with  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$ . In conclusion,  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  enhanced the thermostability of Xyn10cf+Af\_afu62 in the temperature range of 50 - 70 °C.

Although the chimera displayed a lower  $E_d$  value than parental endoxylanase, the presence of  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  increased stability, represented by the increase in  $E_d$ , D-value, and  $t_{1/2}$  values. The chimera D-value (time required to decrease residual activity to 10 %) at 50 °C increased from 697 to 1918 min with 5 mM  $\text{Ca}^{2+}$ , increasing  $E_d$  from 37.1 to 49.3 kJ/mol, closer to the Xyn10cf control value of 53.4 kJ/mol. Therefore, the drop in thermostability from the increase in protein flexibility and  $\beta$ -sheet content after fusion may be overcome with cofactors in the hydrolysis reaction.

### 7.3.8. Synergism analysis and XOS production from natural substrates

The synergism assay of parental enzymes and chimera was carried out at 40 °C, a temperature at which the three enzymes (the two parental enzymes and the chimera enzyme) are fully stable. The degree of synergism consisted of the ratio between the chimera activity and the sum of activities of the parental enzymes' equimolar mixtures, as shown in Table 7-3. Despite the small increase of 1% in total XOS production, xylobiose and xylotriose yields, which present higher prebiotic potential, improved 15.5% and 33%, respectively.

**Table 7-2** presents the composition of each biomass. Soy hull hemicellulose is constituted mainly by arabinose and xylose units (~84%), followed by glucuronic acid and acetyl groups. Corn hull presented a higher hemicellulosic content; ~73% of its hemicellulose was characterized as arabinose and xylose polymers, and the remaining 27% constituted galactose, mannose, acetyl groups, and uronic acid units. Corn hull presented a higher arabinose content, 11.5%, and soy hull, 5.6%.

**Table 7-2 Chemical composition of corn and soy hulls agro-industrial wastes**

| %                    | Corn hull    | Soy hull     |
|----------------------|--------------|--------------|
| <b>Cellulose</b>     | 15.06 ± 2.90 | 41.72 ± 0.90 |
| <b>Hemicellulose</b> | 40.33 ± 2.31 | 29.36 ± 2.31 |
| Xylose               | 17.83 ± 2.10 | 19.71 ± 2.10 |
| Arabinose            | 11.5 ± 0.20  | 5.65 ± 0.20  |
| <b>Lignin</b>        | 2.76 ± 1.30  | 9.88 ± 1.30  |
| <b>Extractives</b>   | 22.92 ± 2.90 | 9.67 ± 0.22  |
| <b>Starch</b>        | 15.03 ± 3.40 | -            |
| <b>Protein</b>       | 2.61 ± 0.30  | 12.10 ± 1.00 |
| <b>Ashes</b>         | 0.88 ± 0.10  | 0.47 ± 0.10  |

The extraction with 5% KOH resulted in an arabinoxylan purity (based only on the content of xylose and arabinose) of 73.4% for soy hull and 75.1% for corn hull. Increasing the

alkali concentration increased the degradation of arabinoxylan, which reduced the purity of the extracted material. Additionally, a higher alkali concentration increases the extraction of glucans from corn hull that goes along arabinoxylan, which is also classified as 4-O-methyl-glucuronoarabino-xylan acetate (de Mattos et al., 2019).

The positive interaction between substrate concentration and temperature revealed the best conditions for the efficient conversion of arabinoxylan into xylooligosaccharides. Higher conversion yields (arabinoxylan to XOS) were obtained for low substrate loads: 50% conversion was obtained with a 0.4% solid load (**Figure 7-7**). However, higher XOS concentrations were obtained using a higher substrate load: 6.97 g/L and 3.33 g/L XOS were obtained at 60 °C with 4% arabinoxylan from corn and soy hulls, respectively. These results are comparable to the recent literature: 5.3 g/L XOS were obtained from 10% wheat bran by a recombinant endoxylanase (Liu et al., 2022), 4.1 g/L XOS from sugarcane straw were produced by the synergic mixture of Xyn10cf and a commercial GH 51 arabinofuranosidase (*Aspergillus niger*) (Martins, Ávila, et al., 2020), and 11 g/L XOS were obtained from hydrothermal treatment of corn stover, which required additional purification steps to remove impurities generated due to biomass degradation (Buruiana et al., 2017).

Comparing the yields of the bifunctional chimera and the Shearzyme xylanase at a 1% substrate load, 2.40 g/L (from corn) and 1.37 g/L (from soy) XOS were obtained at 60 °C using the bifunctional chimera, against 0.46 g/L (from corn) and 0.84 g/L (from soy) using Shearzyme xylanase at 50 °C. The higher amount of arabinose in corn hull may directly influence the hydrolysis yields. The low yield obtained with Shearzyme xylanase indicates the hindrance brought by the arabinose side chains, impairing xylanase activity. However, the chimeric enzyme was able to increase the hydrolysis yield 5.22 times, proving the synergy between arabinofuranosidase and endoxylanase for the efficient depolymerization of arabinoxylan from lignocellulosic biomass.

A potential drawback for the industrial production of chimeric enzymes is the cleavage of the linker, leading to protein degradation and reduction of catalysis power, or loss of activity in one of the domains due to improper conformation (Malgas et al., 2020). Also, the ratio between the enzymes required to improve the yields of XOS in different biomass may differ from an equimolar mixture (Fonseca-Maldonado et al., 2014). However, both the equimolar mixture and the chimeric enzyme proved the synergy between Xyn10cf and Af\_afu62. Based on the literature (Cattaneo et al., 2018; Nath et al., 2019; Ribeiro et al., 2016), producing chimeric enzymes could be more advantageous than producing single enzymes separately due

to the kinetic advantages presented by chimeras over a simple mixture of parental enzymes, representing a positive influence on the cost of enzymes (Malgas et al., 2020).

**Table 7-3** Kinect parameters and degree of synergy for XOS production, where “Mix” stands for the equimolar mixture of the parental enzymes Af\_afu62 and Xyn10cf.

|                                      | <b>Af_afu62</b> | <b>Xyn10cf</b> | <b>Mix</b> | <b>Chimera</b> |
|--------------------------------------|-----------------|----------------|------------|----------------|
| <b>k<sub>m</sub></b>                 | 1.372           | 4.969          | -          | 0.745          |
| <b>v<sub>max</sub></b>               | 1.435           | 1.190          | -          | 1.462          |
| <b>k<sub>cat</sub>/k<sub>m</sub></b> | 209.111         | 47.917         | -          | 392.483        |
| Xylose (X1)                          | 0               | 0              | 0          | 44.54          |
| Xylobiose (X2)                       | 0               | 303.23         | 304.78     | 351.06         |
| Xylotriose (X3)                      | 0               | 318.88         | 393.6      | 523.23         |
| Xylopentaose (X4)                    | 0               | 0              | 0          | 0              |
| Xylopentose (X5)                     | 0               | 99.07          | 253.89     | 94.46          |
| Xylohexaose (X6)                     | 0               | 20.71          | 105.72     | 99.35          |
| Total XOS (mg/L)                     | 0               | 741.89         | 1057.99    | 1068.1         |
| Arabinose (mg/L)                     | 471.64          | 0              | 940.76     | 997.20         |
| Degree of synergy                    |                 |                | 1.43       | 1.01           |

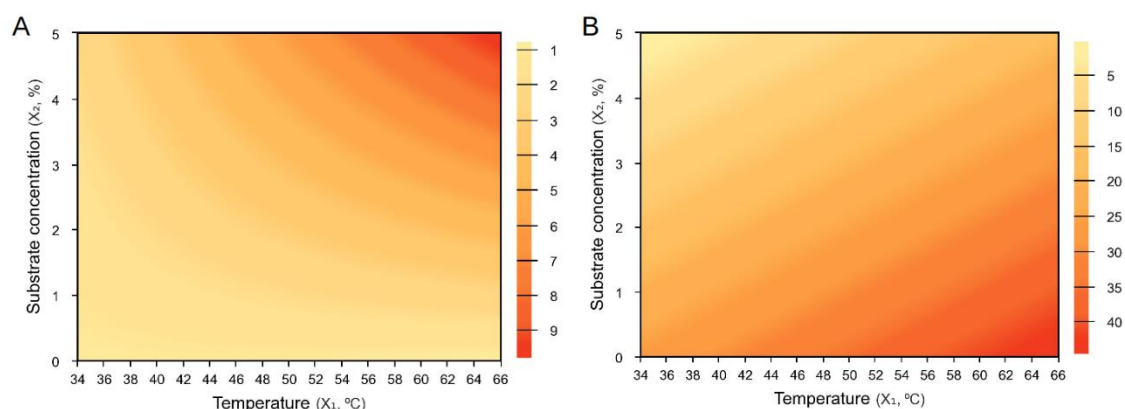


**Figure 7-7** Effect of temperature and corn hull arabinoxylan concentration (1–5% w/v) on (A) total xylooligosaccharides production (g/L) and (B) arabinoxylan conversion yields into xylooligosaccharides after 48 h hydrolysis.

## 7.4. Conclusion

To reduce the costs of enzyme production for XOS production, this novel bifunctional chimera combining the GH families 10 and 62 presented good thermostability and higher

substrate specificity in comparison to the parental enzymes. The cofactors  $Mn^{2+}$  and  $Ca^{2+}$  improved the activity and thermostability. The synergism of the endoxylanase and arabinofuranosidase domains improved the production of xylobiose and xylotriose, and up to 7 g/L XOS was obtained after hydrolysis of arabinoxylan from agro-industrial wastes at 60 °C. The molecular fusion of synergic enzymes is a promising strategy to produce more efficient and thermostable enzymes and reduce operational costs.

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**CHAPTER 8.**  
**THERMOSTABILITY ENHANCEMENT OF GH 62**  
**A-L-ARABINOFURANOSIDASE BY DIRECTED**  
**EVOLUTION AND RATIONAL DESIGN**

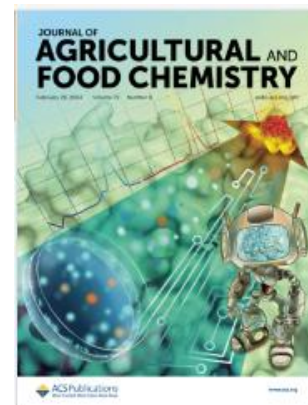
## Thermostability enhancement of GH 62 $\alpha$ -L-arabinofuranosidase by directed evolution and rational design.

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### Still in protein engineering (but not for long)

The attentive reader noticed that although the fusion of GH 10 and GH 62 brought significant improvements for catalytic efficiency and thermostability of the chimeric enzyme, there is still a gap between the melting temperature of the modules, where GH 62 stands way behind the high melting temperature of GH 10. For an enzymatic reaction occurring at lower temperatures that may not be a problem but ensuring that both modules present similar biochemical characteristics is highly important for proper large-scale applications. Therefore, the present work, developed during the **sandwich period** at **Johns Hopkins University**, focused solely on the GH 62 module for rationally analyzing its structure and determine the drivers for thermostability. We aimed to enhance the thermostability of GH 62  $\alpha$ -L-arabinofuranosidase through a combination of directed evolution and rational design by generation variants using error-prone PCR and selecting mutants with improved thermostability. Molecular dynamics (MD) simulations showed the impact of point mutations on the enzyme's active site, and computational analysis provided insights into the molecular mechanisms underlying enhanced thermostability, including potential impact on substrate binding, catalytic cavity modulation, and protein stability. By integrating experimental and computational approaches, this study aimed to better understand the effects of point mutations on GH 62  $\alpha$ -L-arabinofuranosidase's stability and functional properties. The results contribute to the development of improved enzymes for industrial applications and provide valuable insights into the predictability of computational modeling in protein engineering studies (Supplementary material in **CHAPTER 12**).

### Abstract

GH 62 arabinofuranosidases are known for their excellent specificity for arabinoxylan of agroindustrial residues and their synergism with endoxylanases and other hemicellulases. However, low thermostability of some GH enzymes hampers potential industrial applications. Protein engineering research highly desires mutations that can enhance thermostability. Therefore, we employed directed evolution using one round of error-prone PCR and site-saturation mutagenesis for thermostability enhancement of GH 62 arabinofuranosidase from *Aspergillus fumigatus*. Single mutants with enhanced thermostability showed significant  $\Delta\Delta G$  changes ( $< -2.5$  kcal/mol) and improvements in perplexity scores from evolutionary scale modeling inverse folding (ESM-IF1). The best mutant, G205K, increased the melting temperature by 5 °C and the energy of denaturation by 41.3%. We discussed the functional mechanisms for improved stability. Analyzing the adjustments in  $\alpha$ -helices,  $\beta$ -sheets, and loops resulting from point mutations, we have obtained significant knowledge regarding the potential impacts on protein stability, folding, and overall structural integrity.

**Keywords:** Arabinoxylan; error-prone PCR; protein evolution; site-directed mutagenesis

## 8.1. Introduction

In the agroindustrial field,  $\alpha$ -L-arabinofuranosidases (E.C.3.2.1.5.) synergistically play a prominent role with other hemicellulases to ensure an effective degradation of hemicellulose to obtain a wide range of bioproducts including medicinal substances, dietary fiber, ferulic acid, and fermentable sugars for bioethanol. GH 62  $\alpha$ -L-arabinofuranosidases catalyze the hydrolysis of the terminal non-reducing  $\alpha$ -1,2, and  $\alpha$ -1,3-L-arabinofuranoside substitution of  $\alpha$ -L-arabinofuranoside such as arabinoxylan, the primary hemicellulosic group in grain and grasses (Poria et al., 2020; Wilkens et al., 2017). The synergistic effect of GH 62 arabinofuranosidase and endoxylanase is a sustainable route for obtaining xylooligosaccharides from agroindustrial residues. We recently designed a bi-functional chimera to improve arabinoxylan specificity and degree of synergy to enhance xylooligosaccharides production from corn and soy husks (Martins, Silva, et al., 2022; Martins, Tramontina, et al., 2022).

Despite the high specificity and synergy for arabinoxylan depolymerization, GH 62 arabinofuranosidases present a drawback for industrial applications. Their high flexibility and low  $\alpha$ -helix content compared to other hemicellulases, such as GH 10 endoxylanase (Martins, Silva, et al., 2022), result in lower thermostability and half-life at temperatures above 50°C, which could hamper industrial processes where higher hydrolysis temperatures are necessary to speed up reaction rate, reduce microbial contamination, and increase substrate solubility (Y. Yang et al., 2016). One solution to outdo this drawback is engineering novel enzymes with enhanced thermostability by directed evolution or rational design. To this date, there are no reports of a GH 62 re-engineered specifically to improve thermostability.

Directed evolution is an approach that simulates Darwinian evolution by molecular biology techniques to rapidly evolve peptides and proteins into new forms with improved properties for specific applications. We can create millions of mutants in a single experiment and subject them to high-throughput screening to identify those with enhanced desired properties. Researchers have successfully employed this approach to improve the catalytic power of GH51 arabinofuranosidases by one-round error-prone PCR (Sürmeli et al., 2019) and site-directed mutagenesis (Y. Yang et al., 2016). Random mutagenesis by error-prone PCR has the advantage of enhanced probability of finding unexpected beneficial mutations in different regions of a protein, while the primary benefit of site-directed mutagenesis is that the diversity in a library is focused on a location of the protein known or thought to be essential for function or stability. Furthermore, we can explore mutations not likely to occur by error-prone PCR random mutagenesis by site-directed mutagenesis (Hida et al., 2007; Lehmann & Wyss, 2001).

On the other hand, rational design based on protein structure helps identify hotspots for mutation by predicting the effect of mutations on structural stability, reducing workload. One strategy for identifying thermostabilizing mutations involves the comparison of more thermostable proteins with less stable ones and identifying amino acid sequence patterns that could correlate with improved thermostability. Another approach for engineering thermostability lies in the hypothesis that at a given position in a sequence alignment of homologous proteins, a consensus amino acid contributes more than average to the protein stability than a no consensus one. Therefore, substituting no consensus amino acids for consensus ones may be an approach to improving protein thermostability. However, there is still room for improvement of rational design algorithms' accuracy because the predictive power is usually limited, and the thermostabilizing mutations need to be assessed individually by site-directed mutagenesis, which limits the sequence space amenable to testing (Lehmann & Wyss, 2001; Xing et al., 2021).

In our study, we aimed to enhance the thermostability of GH 62  $\alpha$ -L-arabinofuranosidase through a combination of directed evolution and rational design. We generated variants using error-prone PCR and selected mutants with improved thermostability based on predicted hotspots identified by sequence consensus. Additionally, we employed *in silico* perplexity calculation using the ESM-IF1 algorithm for site-directed mutagenesis. Through molecular dynamics simulations, we investigated the impact of point mutations on the plasticity and functional characteristics of the enzyme's active site. The computational analysis provided insights into the molecular mechanisms underlying enhanced thermostability, including potential impact on substrate binding, catalytic cavity modulation, and protein stability.

By integrating experimental and computational approaches, our study aimed to better understand the effects of point mutations on GH 62  $\alpha$ -L-arabinofuranosidase's stability and functional properties. The results contribute to the development of improved enzymes for industrial applications and provide valuable insights into the predictability of computational modeling in protein engineering studies.

## **8.2. Material and Methods**

### **8.2.1. Subcloning and expression of Af\_afu62**

We optimized the codon of the Af\_afu62 gene (Genbank ID Afu\_2G12770, 948 pb, without a signal peptide and TAG codon) (Martins, Tramontina, et al., 2022) from *Aspergillus fumigatus* and M5 *Bacillus subtilis* signal peptide (MNDLNDFLKTISLSFGFFLLLSLPT-VAEA) (Jonet et al., 2012) for *E. coli* expression. We synthesized the gene and the artificial



recognition sites EcoRI and XhoI. To clone, the synthesized DNA (signal peptide and full-length Af\_afu62) and pET21a(+) were digested with EcoRI and XhoI and ligated together. The recombinant plasmid had an N-terminal 6-His tag. We propagated the plasmid in *E. coli* DH5 $\alpha$  by electroporation and grew it in LB medium supplemented with ampicillin (100  $\mu$ g/mL). Then, we extracted and transformed the recombinant plasmids into electrocompetent *E. coli* NiCo21(DE3). The transformation was confirmed by PCR and sequencing, using the forward primer (5'-CTAGGGCGCTGGCAAG-3') and reverse primer (5'-CCGATCTTCCCCATCGG-3').

For extracellular expression Af\_afu62 (wild-type and its variants), we transferred single colonies of NiCo21(DE3)/pET21a+Af\_afu62 into 96-well plates having 500  $\mu$ L LB medium supplemented with ampicillin (100  $\mu$ g/mL) per well, cultured overnight (37  $^{\circ}$ C, 250 rpm). Subsequently, we transferred 5  $\mu$ L culture suspension (1:50) to a new microplate having 250  $\mu$ L fresh LB supplemented with ampicillin (100  $\mu$ g/mL), grown for 2 h to reach an OD<sub>600</sub> = 0.4 - 0.5 (early exponential phase), and cooled down on ice. Then, we added 250  $\mu$ L of LB media supplemented with ampicillin (100  $\mu$ g/mL), 2.5 mM isopropyl  $\beta$ -d-1-thiogalactopyranoside (IPTG), and 10 mM glycine into each well and induced them at 30  $^{\circ}$ C for 24h. We added glycine to the medium to retard the formation of inclusion bodies in the periplasm and increase the extracellular production of the recombinant proteins (Yamani & Baktir, 2017). Then, we separated the cells by centrifugation at 4,000 g for 10 min and stored the supernatant at 4  $^{\circ}$ C for further studies.

### 8.2.2. Error-prone PCR

We first propagated the plasmid pET21a(+)-Af\_afu62 and extracted it for the error-prone PCR. The extracted plasmid was amplified by PCR over 30 cycles using the forward primer (5'-CTAGGGCGCTGGCAAG-3') and reverse primer (5'-CCGATCTTCCCCATCGG-3'), Q5® High Fidelity polymerase (New England Biolabs Inc, MA, USA), and GC enhancer (PCR conditions: 98 °C for 3min, 30 cycles of denaturation at 98 °C for 10s, annealing at 65 °C for 30s, extension at 72 °C for 45s, and a final extension at 72 °C for 2 min). The amplified fragment was the template for the error-prone PCR.

Then, we generated the Af\_afu62 gene variants by one round of error-prone PCR (Cadwell & Joyce, 1992) using Taq polymerase and the forward primer (5'- ACAG-CAAATGGGTCGCGGATCCGAATTC -3', EcoRI site underlined) and reverse primer (5'- TCAGTGGTGGTGGTGGTGGTGCTCGAG - 3', XhoI site underlined). We built mutagenic

conditions using 10  $\mu$ M each primer, 1:10 Mutagenic PCR Buffer (70 mM  $\text{MgCl}_2$ , 500 mM KCl, 100 mM Tris pH 8.3, 160  $\mu\text{g}/\mu\text{L}$  BSA), 1:10 Mutagenic dNTP mix (2 mM dATP, 2 mM dGTP and 5 mM dCTP, 5 mM dTTP), and 50  $\mu$ M  $\text{MnCl}_2$ , following the conditions: initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30s, annealing at 62 °C for 45s, extension at 72 °C for 60s, and a final extension at 72 °C for 10 min. PCR products were purified from agarose gel, digested, and ligated into the pET21a(+) expression vector.

### 8.2.3. Site-directed mutagenesis

We constructed mutagenic primers for the target regions of the protein to introduce mutations by inverse PCR to obtain a library of variants with a new distribution of amino acids at a chosen position that contributes to thermostability (we performed this after error-prone PCR screening and variants sequencing).

The primers with the NNK codon usage for the site-directed mutagenesis are in Table 8-1. We replaced the selected residues using inverse PCR. 1  $\mu\text{L}$  (< 5ng) pET21a+Af\_afu62 plasmid (wild-type) was extracted and amplified over 30 cycles of PCR using the Q5 ® High Fidelity polymerase (New England Biolabs Inc, MA, USA), and GC enhancer (PCR conditions: 98 °C for 3min, 30 cycles of denaturation at 98 °C for 10s, annealing for 30s (variable temperatures), extension at 72 °C for 150s, and a final extension at 72 °C for 5 min). The annealing temperature for each primer pair is listed in Table 8-1. We digested the amplified fragments using DpnI at 37 °C for 1.5 h. Successful PCR reactions were purified using the Purelink Quick Gel Extraction and Zymo Cleanup and phosphorylated. The phosphorylation reaction used about 400ng DNA, 2  $\mu\text{L}$  T4 DNA Ligase and 1  $\mu\text{L}$  T4 PNK, incubated 1h at 37 °C. Then, the DNA was purified (Zymo Cleanup), ligated, and transformed to NiCo21(DE3) for screening.

### 8.2.4. Purification of high-activity variants

We overexpressed the high-activity variants in a 10 mL LB medium and obtained the soluble extracts as described above. The supernatant was concentrated 20 times by ultrafiltration (Amicon Ultra-15, Merck KGaA, Germany) with a molecular weight cut-off of 10 kDa and loaded onto HisPur™ Ni-NTA Spin Columns (Thermo Scientific™, USA), following the recommended protocol (equilibration buffer: 10 mM imidazole; wash buffer: 25 mM imidazole; elution buffer: 250 mM imidazole. All buffers had 10 mM sodium phosphate pH 7.4 and 300 mM NaCl). We dialyzed the fractions using 20 mM Tris–HCl (pH 7.5) 150 mM NaCl buffer to remove imidazole. The enzyme concentration was determined using the DC Protein assay (Bio-

Rad Labs Inc, USA) and bovine serum albumin (BSA) as standard. The molecular mass under denaturing conditions was determined by SDS-PAGE gel electrophoresis, using NuPAGE™ 4-12%, Bis-Tris gel, and MOPS SDS Running Buffer (Thermo Scientific™, USA). The proteins were visualized by staining with Coomassie brilliant blue R-250.

### 8.2.5. Biochemical assays

Detecting of arabinofuranosidase activity in the crude supernatant was determined by enzymatic hydrolysis of wheat arabinoxylan (Megazyme International Ireland, Co., Wicklow, Ireland). In short, 10 µL of culture supernatant was added to a 90 µL reaction volume containing 1% (w/v) wheat arabinoxylan and 5 mM MnCl<sub>2</sub> (Martins, Tramontina, et al., 2022) in McIlvaine buffer pH 5.0 (unless otherwise specified), in triplicate. The released arabinose was detected using L-Arabinose/D-Galactose Assay Kit (Megazyme International Ireland, Co., Wicklow, Ireland).

We evaluated the thermostability of mutants by incubating the samples in the absence of substrate at a determined temperature for various periods. The initial activity at 42 °C was considered 100 % activity. Next, we assessed residual activity at 42 °C after thermal treatment from 45 °C to 65 °C and pH 5.0 using a thermocycler gradient (Mastercycler® nexus X2 - Eppendorf SE). We aliquoted every 30 min for 4 h and immediately cooled them at 4 °C for at least 1 h to ensure sufficient time for the reversibly denatured protein to convert back to its native active form while the irreversibly denatured remained inactive. At the end of the thermal treatment, we conducted the enzymatic assay of the aliquots at 42 °C for 30 min. We computed the relative activities as a percentage of the original activities to calculate the thermodynamic parameters (Saqib & Siddiqui, 2018).

$T_{50}^{60}$  refers to the temperature at which the enzyme activity drops to 50% of its initial after an incubation time of 60 min. We quantified the enzymatic activity of the mutants and WT by measuring the residual activity of the enzyme after incubation at different temperatures for 60 min.

The enzyme half-life ( $t_{1/2}$ ) was calculated as  $\ln(2)/k_{inact}$  from linear plots of  $\ln(\text{residual activity})$  versus time (min) to obtain the inactivation rate constant  $k_{inact}$  (min<sup>-1</sup>). The half-life was the elapsed time for the enzyme activity to decline to half of its initial level at 50°C ( $t_{1/2}^{50}$ ) and 60°C ( $t_{1/2}^{60}$ ).

We calculated the energy of denaturation  $E_d$  (kJ/mol) from the linearization of the Arrhenius plot ( $\ln(k_{inact})$  versus  $1/T$ ), according to Equation 1:

$$\ln(k_{inact}) = \ln(A_0) - \frac{E_d}{R} * \frac{1}{T} \quad (1)$$

where  $k_{inact}$  is the inactivation rate constant,  $A_0$  is the Arrhenius constant,  $E_d$  is the energy of denaturation,  $R$  is  $8.314 \times 10^3$ , and  $T$  is the absolute temperature (Kelvin).

### 8.2.6. Determination of $k_{cat}$ and $K_M$

For determination of the Michaelis-Menten constants  $K_M$  and  $k_{cat}$ , the activity assay was performed at 45 °C pH 5.0 for 15 min using substrate concentrations from 0 to 20  $\mu$ M wheat arabinoxylan and  $1 \times 10^{-8}$   $\mu$ M of enzyme concentration. The reported values are the average of triplicate experiments.

### 8.2.7. Enzyme denaturation

We assayed the melting temperatures of the thermoresistant variants by differential scanning fluorimetry (DSF) using the Protein Thermal Shift™ Dye Kit (ThermoFisher Scientific Inc, USA). We pipetted 12.5  $\mu$ L of enzymes preparations (1-5  $\mu$ g), 2.5  $\mu$ L of Diluted Protein Thermal Shift™ Dye (8×), and 5  $\mu$ L Protein Thermal Shift™ Buffer into the wells of a qPCR plate and sealed, raising the temperature from 25 °C to 95 °C at the step of 0.1 °C/s. We computed the  $T_m$  from the inflection points of the transition curve [Fluorescence =  $f(T)$ ] as the maximum of the first derivative [ $d(\text{Fluorescence})/d(T)$ ].

We determined the denaturation temperatures of the variants by differential scanning calorimetry (N-DSC II instrument, Calorimetry Sciences, Provo, UT). The purified enzyme samples (0.5 mg/mL) were dialyzed against 100 mM NaCl, 50 mM Tris/HCl buffer pH 7.0, and the dialysis buffer was used as a reference solution. Samples were analyzed at a heating rate of 1 °C per minute over a temperature range of 30–90 °C. The denaturation temperature was defined as the temperature at maximum molar heat capacity. Data analysis was carried out with GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

### 8.2.8. Bioinformatic analysis

We compared the deduced amino acid sequence from nucleotide sequencing to the wild-type (WT) sequence using Clustal  $\Omega$  and BLAST algorithms. We aligned the amino acid sequences of Af\_afu62 and templates from the PDB library using UCSF Chimera (Pettersen et al., 2004).

We used the Hotspot Wizard 3.0 server (<https://loschmidt.chemi.muni.cz/hotspot-wizard>) to find hotspots for thermostability enhancement and undertake calculations of  $\Delta\Delta G$ , which indicates the change in stability caused by a point mutation. We calculated the hotspots

by sequence consensus, and the  $\Delta\Delta G$  as the difference between the minimal  $\Delta G$  of the WT structure and the minimal  $\Delta G$  of the mutant. Negative  $\Delta\Delta G$  values indicate increased stability (Alford et al., 2017).

Then, we used the ESM-IF1 (Evolutionary Scale Modeling) language to calculate the inverse folding to predict the functional effects of sequence variation. The ESM-IF1 inverse folding model predicts protein sequences from their backbone atom coordinates. For mutational effect predictions with ESM-IF1, we scored mutations using the masked-marginal likelihood ratio between the mutant and wild-type amino acids (C. Hsu et al., 2022).

### 8.2.9. Computational Simulations

We performed molecular dynamics (MD) simulations using the Amber20 package (Salomon-Ferrer et al., 2013). We used the crystal structure of GH 62 from *Coreopsis cinerea* (PDB code: 5B6S, resolution: 1.7 Å, chain: A) (Tonozuka & Tanaka, 2017) as the basis for constructing six different systems using MODELLER (Webb & Sali, 2016). These systems included the wild-type (1) Arabinofuranosidase GH 62 from *Aspergillus fumigatus* (Af\_afu62) and five single-mutants' molecular systems: (2) S127G, (3) S188F, (4) G205K, (5) T256G, and (6) Q271T. Before the MD simulations, we determined the protonation states of the residues at a pH of 5.0 using the empirical propKa program (Olsson et al., 2011). The systems were parameterized using the all-atom force fields CHARMM36m (Huang et al., 2017) and solvated using the TIP3P explicit solvation model in a truncated octahedral water box (Jorgensen et al., 1996). A cut-off distance of 20.0 Å was employed to establish the separation between the simulation box wall and the solvated atoms within the system. We added calcium and chloride ions for electro-neutralization. The minimization process involved five steps, during which the water molecules, hydrogens, ions, and the entire protein structure were subjected to minimization using the steepest descent and conjugate gradient methods. This approach, as described previously (Hestenes & Stiefel, 1952), also included the gradual relaxation of restraints. Subsequently, the entire system underwent a series of 14 heating steps. We conducted the initial heating phase under constant volume conditions for 5 picoseconds (0 to 5 Kelvin). Then, from the 2nd to the 7th, we achieved a gradual temperature increase from 5 to 313.5 Kelvin throughout 50 picoseconds. Upon reaching the 7th heating stage, the temperature was 313.5 K until the 14th step. We conducted a further 2 nanoseconds of MD to achieve density equilibration of the system under 1 bar pressure while maintaining a temperature of 313.5 K. All covalent bonds involving hydrogen atoms were kept at fixed length using SHAKE (Andersen, 1983). We

implemented a distance constraint to limit the separation between histidine, crystallographic water, and calcium ions during the equilibration stage. We initially set it at approximately 15.0 kcal and gradually reduced it. The purpose of this constraint was to ensure the proper coordination of calcium in the GH 62 active site.

We conducted a total of 200 nanoseconds of MD for each system using the isothermal-isobaric (NPT) ensemble. To ensure the accuracy and consistency of our results, we replicated our MD simulation systems. We analyzed the RMSD values of wild-type (WT) and mutant structures over a trajectory of 200 ns, focusing on the heavy atom's backbone of the enzyme. Additionally, the RMSF, cavity analysis, and secondary structure analysis were all performed for the last 50 ns of the simulation for a more accurate comparison between the systems after equilibration. We analyzed the volume variation of the catalytic pocket using MDpocket (Schmidtke et al., 2011). The initial detection used Fpocket, which served as a benchmark for generating the required input pocket file for the subsequent analysis of MD trajectories under MDpocket. The primary objective of our investigation was to analyze the effects of point mutations in the proximity of the calcium coordination region adjacent to the active site, which is widely recognized for its importance to the protein conformation, particularly the catalytic pockets (Kaur et al., 2015). We performed a secondary structure analysis using the Define Secondary Structure of Proteins (DSSP) algorithm (Kabsch & Sander, 1983) to examine the changes in the secondary structural components within the systems.

**Table 8-1 Primers for site-directed mutagenesis library**

| <i>Position</i> |         | <i>5' – 3'</i>                 | <i>T<sub>m</sub> (°C)</i> | <i>Anneal (°C)</i> |
|-----------------|---------|--------------------------------|---------------------------|--------------------|
| <i>S127</i>     | Forward | (N)(N)(N)ATGCCGTCTATCATCGCG    | 64-71                     |                    |
|                 | Reverse | AGAGAAGAAGTTTTTCGGTTTA-GACC    | 64                        | 65                 |
| <i>S188</i>     | Forward | (N)(N)(N)AACAA-AAACGCGCTGTTCG  | 64-71                     |                    |
|                 | Reverse | GTCAGACAGCGCGATAACG            | 66                        | 65                 |
| <i>G205</i>     | Forward | (N)(N)(N)GAATACCTGCTGCTGG      | 61-69                     |                    |
|                 | Reverse | GTTACCGGTGTGGTAAACGTTAG        | 65                        | 62                 |
| <i>T256</i>     | Forward | (N)(N)(N)CCGTGGACCAAATCTA-TCTC | 63-70                     |                    |
|                 | Reverse | ACCAGAGAACGCAACGTTGTTAG        | 67                        | 64                 |
| <i>Q271</i>     | Forward | (N)(N)(N)ACCGACCAGACCATG       | 60-69                     |                    |
|                 | Reverse | GGTACGGATCAGTTCACCG            | 65                        | 61                 |

### 8.2.10. Statistical Analysis

All data represent the mean  $\pm$  standard deviation of three independent replicates. We performed unpaired and two-tailed t-tests ( $p < 5\%$ ) using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). We computed Pearson and Spearman's coefficients

from the correlation between the experimental data (melting temperature) and ESM-IF1 final scores. Pearson's coefficient measures the linear relation between 2 variables, where -1, 0, and 1 indicate perfect negative, none, and perfect positive correlation, respectively. Spearman's rank measures the monotonic relationship between the two variables, capturing non-linear correlation not computed by Pearson, where -1 indicates perfect negative correlation, 0 indicates no relation, and 1 indicates perfect positive correlation.

### 8.3. Results

#### 8.3.1. Directed evolution for selecting thermostable mutants.

Typically, low mutation frequencies are better to avoid deleterious mutations masking the effects of beneficial ones (Lehmann & Wyss, 2001). Consequently, we conducted one round of error-prone PCR to introduce, on average, 1-2 mutations per kb in the Af\_afu62 gene. Three mutant libraries using error-prone PCR were generated. Low initial DNA concentration (less than 100ng) resulted in high mutation frequency (9-16 mutations/kb), including deletions and insertions causing frame shifts, precluding the proper expression of the enzyme. Decreasing  $Mn^{2+}$  concentration to 50  $\mu M$  and increasing initial target DNA concentration to 500 ng displayed less than 50% inactive mutants and an overall mutation rate of 1.8/kb.

We cloned the variants into NiCo21(DE3) and cultivated individual colonies in 96-well microplates. After induction of secretion of Af\_afu62 with 2.5 mM IPTG at 30 °C for 24h, we separated the cells by centrifugation and used the crude supernatant of the cultures to quickly evaluate the thermostability of mutants exhibiting higher arabinofuranosidase activity than the wild-type (WT). After screening a total of 350 colonies, the mutants with initial activity higher than  $0.5(\mu - \sigma)_{WT}$  after 30 min incubation at 50 °C were sequenced, where  $\mu_{WT}$  and  $\sigma_{WT}$  are the average and standard error of WT activity. Notably, beneficial mutations were at non-conserved residues (with high mutability probability) in loops at the surface of the enzyme: S127A (tct→gct), S188Y (tct→tat), G205D (ggt→gat); T256S (acc→tcc); and Q271H (cag→cat) (**Supplementary table 12-10**). Solvent-exposed residues or residues far away from the active cleft were the top candidates for stabilizing mutations to avoid triggering allosteric effects that might alter the entire protein structure and substrate specificity.

Then, we analyzed consensus sequence hotspots using Hotspot Wizard 3.0 server. We considered mutants with a predicted  $\Delta\Delta G$  (folding free energy)  $\leq -2.5$  kcal/mol as significant (Bednar et al., 2015). The five positions identified by the error-prone PCR experiment were among the 19 calculated hotspots (**Supplementary table 12-9**). These positions presented

a calculated  $\Delta\Delta G < -2.5$  kcal/mol. Consequently, we selected these positions for the site saturation experiments to analyze if other amino acids at these locations might yield higher stability. Degenerate NNK mutagenic primers were constructed for each position to create a site-saturation library by inverse PCR. We screened 70 colonies from each of the five positions for thermostability-enhanced mutants to cover the theoretically possible variants (**Figure 8-1**).

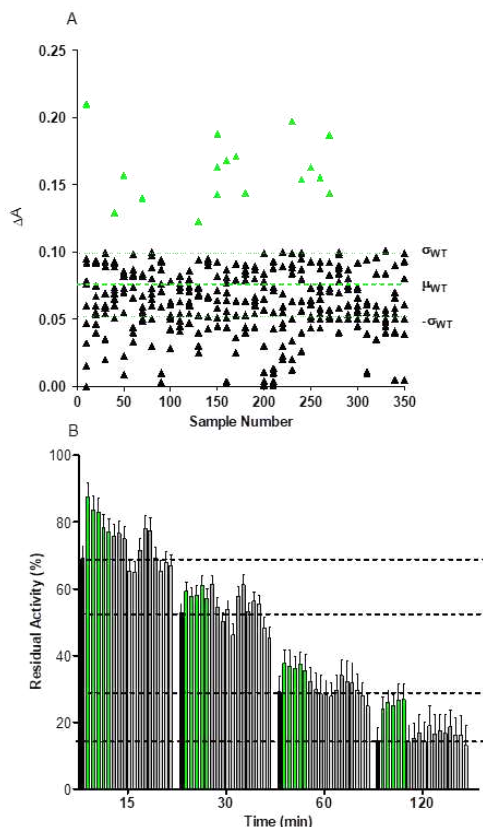


**Figure 8-1** Screening results of the site-saturation mutagenesis library. (A) the initial activity of 350 clones at 50 °C for 30 min. The bold dashed line represents the average and standard error of the WT initial activity. Mutants activities in green were above  $0.5(\mu - \sigma)_{WT}$  and were selected for the second step (B), where mutants with residual activity above  $1.5(\mu - \sigma)_{WT}$  were sequenced.

### 8.3.2. Site-directed saturation library analysis

The process consisted of initial enzymatic activity without pre-exposure to heat treatment and residual activities after incubation at different temperatures for up to 120 min. We only selected mutants with initial activity higher than  $0.5(\mu - \sigma)_{WT}$  for heat treatment and residual activity assay after 120 min exposure at 55 °C (**Figure 8-1**), where  $\mu_{WT}$  and  $\sigma_{WT}$  are the average and standard error of WT activity. Mutants with residual activity above  $1.5(\mu - \sigma)_{WT}$  were overexpressed for further biochemical characterization.



### 8.3.3. Characterization of Thermostability-Enhanced Mutants

We purified high activity-displaying variants for enzyme characterization to understand the structure-stability relation. We verified the purity of Af\_afu62 and its variants by SDS-Page analysis (**Supplementary figure 12-1**). The molecular weights of the protein samples were around 37 kDa, as expected based on previous characterization (Martins, Tramontina, et al., 2022). Af\_afu62 and its variants presented similar purity profiles and protein concentrations. The mutations S127G, S188F, G205K, T256G, and Q271T consistently improved thermostability as measured when wheat arabinoxylan was the substrate. G205K had significantly more residual activity at 50 °C and 60 °C and  $T_{50}^{60}$  (temperature at which the enzyme activity drops to 50% after 60 min) increased to 64.7 °C, as presented in Table 8-2 and **Supplementary figure 12-3**. G205K doubled the decimal-reduction time at 50 °C and improved 89.9 % half-life time at 50 °C. The energy of denaturation increased by 41.3 %. As a comparison, the GH 62 from *G. zeae* and *N. haematococca* lost 78% and 99% activity, respectively, after 45 min incubation at 50 °C (Sarch et al., 2019). Similarly, SthAbf62A from *Streptomyces thermoviolaceus* presented optimum temperature at 55 °C but lost 60% activity after 60 min incubation at 60 °C (W. Wang et al., 2014).

All variants presented higher optimal reaction temperatures than WT, which showed optimal temperatures below 45 °C (pH 5.0). The results showed that the mutants had optimal activities around 50 °C except for G205K and S188F, which showed optimal results at higher temperatures (53 °C) (**Figure 8-2, Table 8-2**). Per the  $T_{50}^{60}$  values, all mutants showed a significant reduction in activity at temperatures above 56 °C. Concerning the main significant improvements, the mutants exhibit a wide range of optimal pH values (4–6), differing from the WT with optimum pH of 4.6 (**Figure 8-2B**).

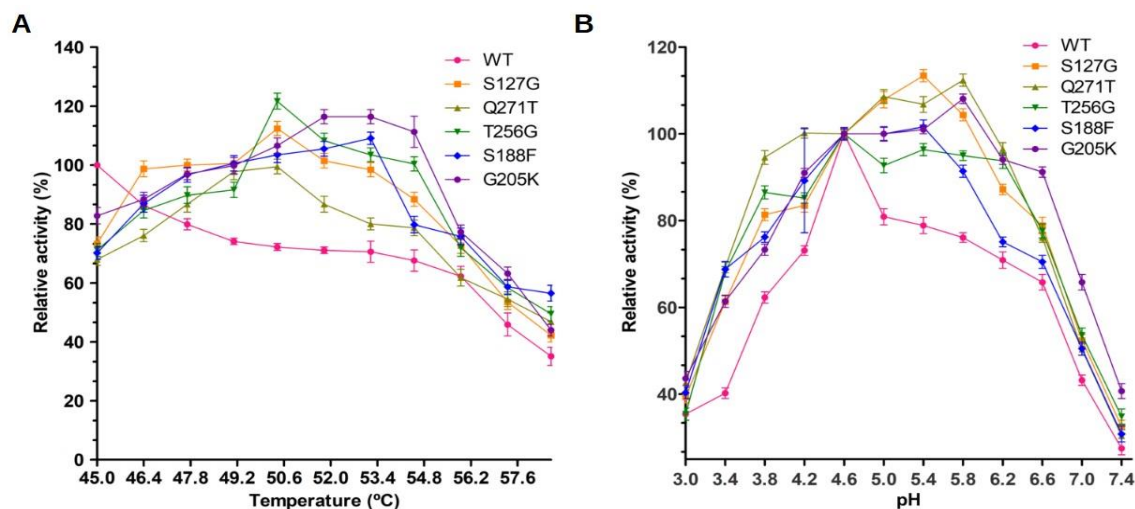


**Figure 8-2 (A) The optimal reaction temperature for the WT and selected mutants; (B) Optimal reaction pH for the WT and selected variants**

The mutants showed a significant increase ( $p < 0.05$ ) of 12 % in the arabinose content after 48 h incubation at 42 °C than WT (which released 3.39 mg/mL arabinose). The mutants at S127G (3.79 mg/mL arabinose) and T256G (3.63 mg/mL arabinose) also presented significant differences. The variants, therefore, showed more versatility than WT, working at optimal activity over a wide range of operating conditions and being able to further hydrolyze wheat arabinoxylan over 48 h incubation (**Figure 8-3A**).

Table 8-2 displays the kinetic parameters of WT and its variants at 45 °C pH 5.0. The  $K_M$  values of variants were lower than the WT, suggesting heightened substrate affinity for the variants. Nevertheless, upon considering the 95 % confidence interval and standard error, we found no significant difference between  $K_M$  and  $v_{max}$  values of WT and variants ( $p < 0.05$ ). Af\_afu62 showcases superior catalytic efficiency of other GH 62 enzymes, even in the absence of comprehensive kinetic parameter data for GH 62 arabinofuranosidases, specially for GH 62 subfamily 1. This is evident when contrasting Af\_afu62 with GH 62 subfamilies 1 and 2 across various bacteria and fungi, including *Mycothermus thermophilus*, which presented optimum activity at 50 °C and pH 5.0. Notably, the kinetic parameters for the GH 62\_1 subfamily from *Scytalidium thermophilus* represent the sole available data for this specific subfamily (**Supplementary table 12-13**). For an in-depth analysis of the kinetic parameters of Af\_afu62 and other GH 62 enzymes, readers are encouraged to refer to our previous work (Martins, Tramontina, et al., 2022).

The catalytic efficiency ( $k_{cat}/K_M$ ) of Af\_afu62 outperformed that of all species, except for AnAbf62A from *Aspergillus nidulans*, which contains a carbohydrate binding

module and optimum temperature between 60 and 65 °C (Contesini et al., 2017; Wilkens et al., 2016) . However, AnAbf62A also presented its melting temperature at 60 °C and no residual activity data was available for comparison.

Regarding the thermodynamic parameters, the mutants showed significantly higher energy of denaturation ( $E_d$ ) than WT, indicating enhanced thermoresistance. The increase in  $D_{50}$  and  $t_{1/2}$  is consistent with the melting temperatures presented in **Figure 8-3**. T256G showed the lowest improvement in thermodynamic parameters and did not present a significant improvement in melting temperature determined by differential scanning fluorimetry (DSF) compared to WT. S127G, S188F, and Q271T melting temperatures were statistically higher than WT (**Figure 8-4D**). The G205K variant demonstrated noteworthy enhancements in both experimental assays compared to other variants and the wild-type (WT), evidenced by a substantial increase in the melting temperature from 49 to 58 °C. These findings align well with the denaturation temperatures determined through differential scanning calorimetry (DSC), as illustrated in **Supplementary figure 12-4**. It is essential to highlight that the denaturation temperatures determined by DSF tend to underestimate actual melting temperatures due to the influence of substrate and cofactors present in the medium. Notably, the thermostability of the  $\beta$ -propeller structure in GH 62 is intricately tied to hydrophobic interactions within the substrate-binding site. This structural stability is further reinforced by hydrogen bonding and hydrophobic interactions facilitated by the arabinofuranosyl substituent (W. Wang et al., 2014).

**Table 8-2 Thermodynamic and kinetic parameters of Af\_afu62 wild-type (WT) and variants for wheat arabinoxylan at 45°C pH 5.0 for 15 min.**

|              | $T_{50}^{60}$    | $R^2$ | $K_M$<br>( $\mu\text{M}$ ) | $k_{CAT}$<br>( $\times 10^{10} \text{ min}^{-1}$ ) | $R^2$ | $k_{CAT}/K_M$<br>( $\times 10^9 \mu\text{M}^{-1} \text{ min}^{-1}$ ) |
|--------------|------------------|-------|----------------------------|----------------------------------------------------|-------|----------------------------------------------------------------------|
| <b>WT</b>    | 59.42 $\pm$ 0.20 | 0.993 | 23.22 $\pm$ 6.02           | 5.95                                               | 0.954 | 2.41                                                                 |
| <b>S127G</b> | 63.19 $\pm$ 0.47 | 0.986 | 16.33 $\pm$ 5.69           | 4.58                                               | 0.963 | 2.81                                                                 |
| <b>S188F</b> | 61.80 $\pm$ 0.56 | 0.979 | 13.27 $\pm$ 4.77           | 3.52                                               | 0.952 | 2.65                                                                 |
| <b>G205K</b> | 64.72 $\pm$ 0.76 | 0.993 | 15.90 $\pm$ 5.08           | 4.55                                               | 0.967 | 2.86                                                                 |
| <b>T256G</b> | 61.43 $\pm$ 0.77 | 0.977 | 15.32 $\pm$ 4.83           | 3.75                                               | 0.967 | 2.45                                                                 |
| <b>Q271T</b> | 63.21 $\pm$ 0.56 | 0.984 | 16.61 $\pm$ 5.95           | 4.08                                               | 0.961 | 2.46                                                                 |

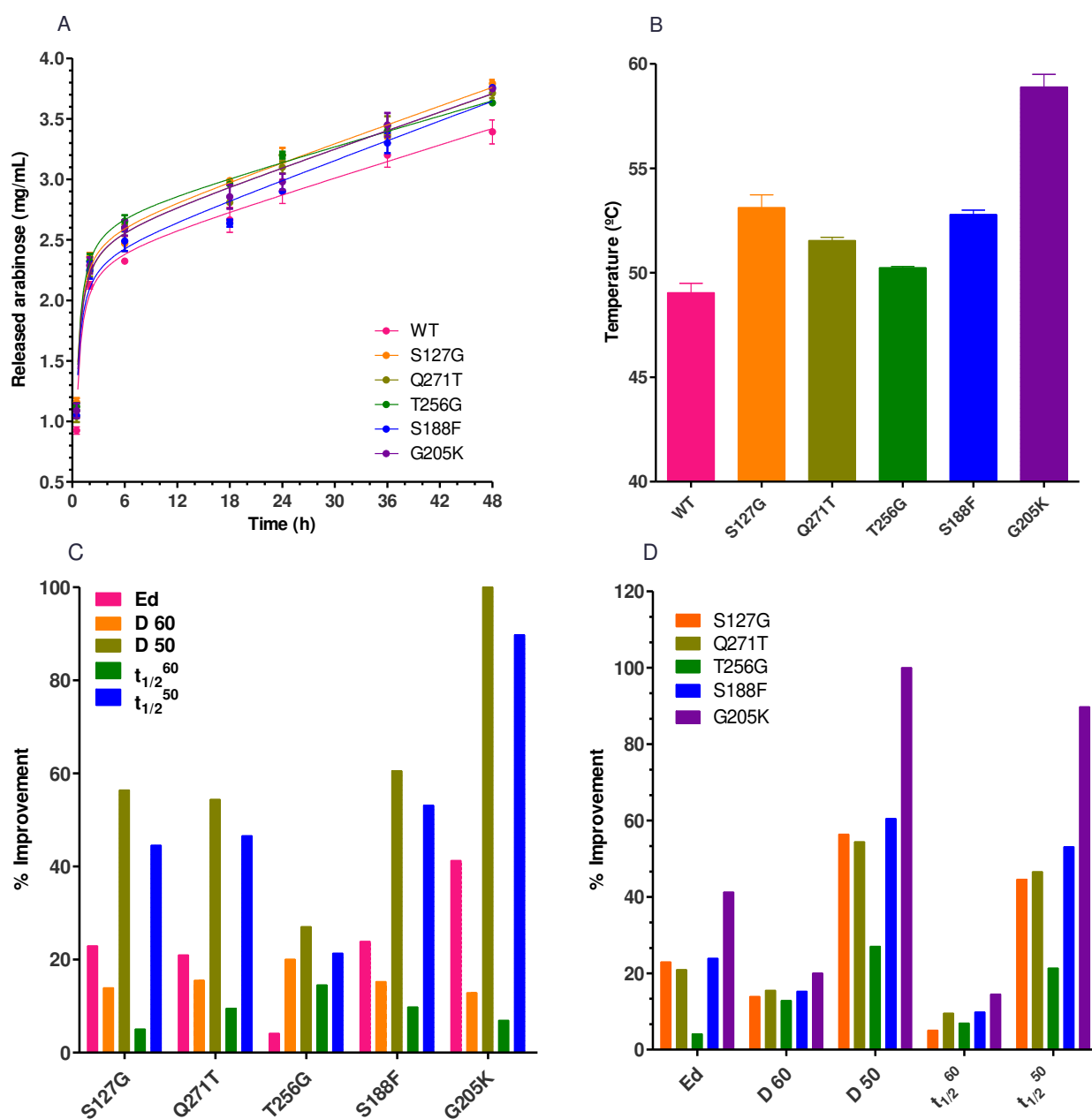


**Figure 8-3** (A) Released arabinose content after 48h hydrolysis at 42 °C. (B) Melting temperatures for purified WT and variants. The first derivative of the fluorescence emission is a function of temperature. (C) Thermodynamic parameters of WT and its variants: Ed (kJ/mol), D60 (min), D50 (min), t<sub>1/2</sub><sup>60</sup> (min), t<sub>1/2</sub><sup>50</sup> (min). (D) Percentage of improvements in thermodynamic parameters compared to WT.

### 8.3.4. Mutational Landscape Analysis

We computed the frequency of the amino acids based on 200 sequences comparison and the mutational landscape of each mutated position at Hotspot Wizard 3.0 server, a tool designed for interactive selection of protein engineering targets (**Figure 8-4A**). This analysis

helped compare with the experimental finds, revealing that glycine showed higher occurrence in position 127, coherent with the mutation S127G. The occurrence of aspartic acid at position 205 is 10%, and we identified the mutation G205D in the random mutagenesis library. On the other hand, the occurrence of lysine is 2% at position 205 (G205K). Threonine accounts for 4% of occurrences in position 271 (Q271T). We also identified the mutations S188T, T256A, and G205N in the site-saturated mutagenesis library. These mutations exhibit the highest occurrence rates at their respective positions, with percentages of 37%, 54%, and 55% respectively. We did not characterize them due to their comparatively lower level of improvement in comparison to S188F, T256G, and G205K.

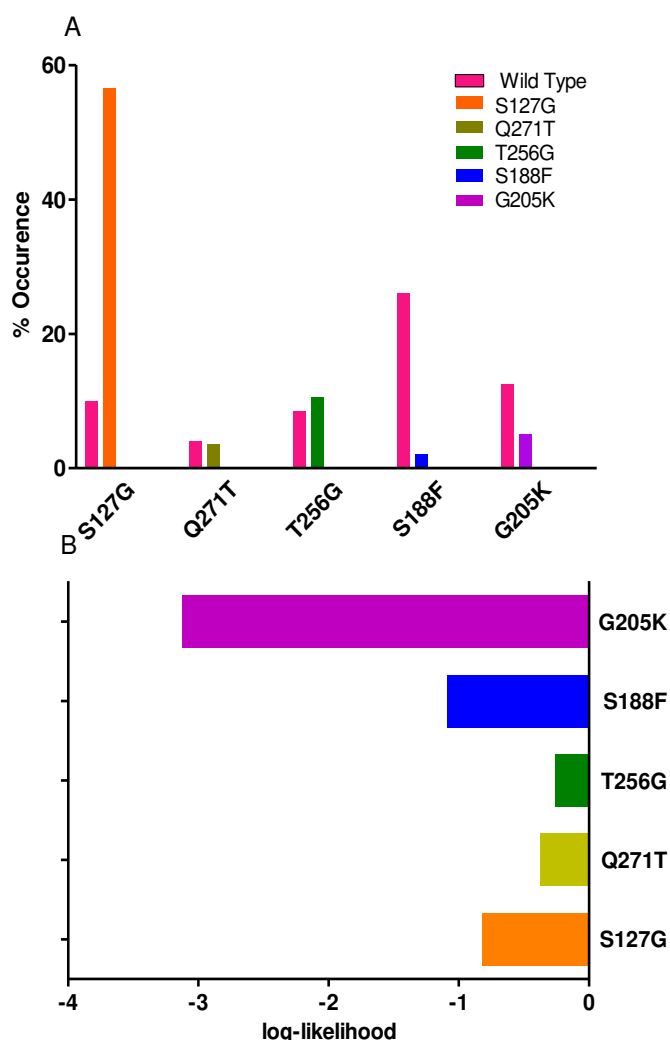


**Figure 8-4** (A) Probability of occurrence of amino acids at the selected residues, where the pink column represent the wild type aminoacid at each selected residue. (B) The score of log-likelihood for mutant sequences according to the wild type Af\_afu62 structure.

Thermophilic proteins have more hydrophobic and charged amino acids and fewer uncharged polar residues (Xu et al., 2020). In our study, G205K presented the highest melting temperature and improvements in thermodynamic parameters. One interpretation is that higher hydrophobicity and positive charge of the lysine (K) residue enabled a more compact configuration. Moreover, G205K, which replaced a smaller amino acid (glycine, G) with a bulkier residue (lysine, K), might build up a steric hindrance effect, increasing the interaction between residues and improving enzyme stabilization. Similarly, upon mutation of serine 188 to phenylalanine (S188F), this hydrophobic residue contains a benzene ring and could also introduce a bulkier side chain. A similar functional mechanism could be attributed to the S188T mutation since threonine also presents a bulkier side chain than serine and is less hydrophilic (Zouari Ayadi et al., 2015). As expected, the most negative log-likelihood from ESM-IF1 scores were computed for G205K (-3.12) and S188F (-1.08), indicating improved stability (**Figure 8-4B**).

We calculated the correlation values between the mutant melting temperatures and the ESM-IF1 scores. The data showed a Pearson correlation coefficient of -0.98 ( $p = 0.0007$ ) and a Spearman correlation coefficient of -0.90 ( $p = 0.0833$ ). This strong negative correlation indicates that lower ESM-IF values should correspond to higher melting temperatures. Although a medium/strong negative correlation was substantial, which indicates an adequate fit between the ESM-IF values and the experimental data, further investigation is needed due to lack of statistical significance for nonparametric correlation (**Supplementary figure 12-2**).

In this study, the mutations S127G (log-likelihood -0.82) and T256G (log-likelihood -0.26, the lowest stability improvement) also showed improved thermostability, even though T256G did not significantly improve melting temperature compared to the WT. Since glycine is more flexible regarding adopting backbone torsion angles, these substitutions for glycine may have improved thermostability by releasing steric strains from unfavored dihedral angles (Z. Lu et al., 2022). Threonine's hydrophilic nature and displays the presence of an OH group on its side chain may contribute to additional hydrogen bond formation with surrounding residues. The mutation Q271T might result in the formation of an additional hydrogen bond, between residues T271 and T272, and residues R269 and T272. Also, Q271T (log-likelihood -0.38) mutation increases the possibility of additional hydrogen bonds with water molecules (S. Zhang et al., 2014). Despite the significant insights acquired from the computational and experimental analyses described previously, the details regarding the molecular intricacies arising from these mutations in the dynamics of protein structure were further investigated with molecular dynamics (MD) simulations.

### 8.3.5. Molecular Dynamic (MD) Simulations

We investigated the behavior of six distinct systems by 250 ns MD simulations. All systems exhibited similar RMSD time histories throughout the simulations (**Supplementary figure 12-5 and Supplementary table 12-11**) albeit with minor variations. The RMSF also behaved similarly regarding the overall fluctuation profile. However, we observed notable differences in the fluctuation patterns of specific regions and residues among the systems. The RMSF profiles (**Figure 8-5A**) underscore these differences. Consistently observed fluctuations emerged around residues F99 and L286 in all mutations, which were absent in the WT system. These fluctuations occurred within the  $\beta 6$ - $\beta 7$  (Blade II) and  $\beta 19$  (Blade V) regions, respectively.

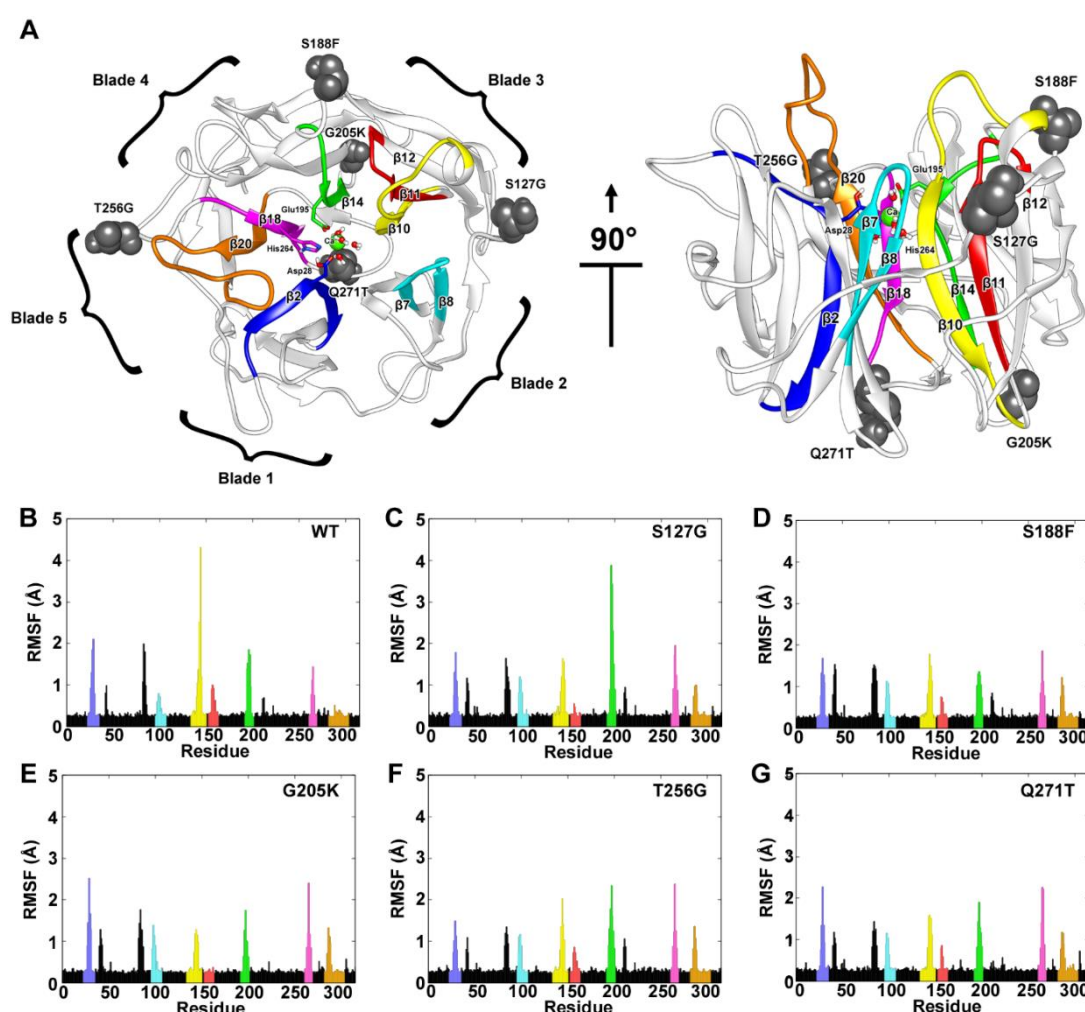


**Figure 8-5** Location of the point mutations and the impacts on the protein dynamics. (A) Representation of Af\_afu62 enzyme and the location of the point mutations. The RMSF of the (B) Wild-type, (C) S127G, (D) S188F, (E) G205K, (F) T256G, and (G) Q271T were calculated based on the enzyme backbones over the last 50 ns simulations at 300 K.

The  $\beta 6$ - $\beta 7$  (Blade II) region, previously identified as a xylotriase subsite (Kaur et al., 2015), plays a role in restricting a curved cylindrical sub-cavity that leads to the active site of the enzyme. Furthermore, the movement of  $\beta 19$ , which crosses the inner portion of the enzyme, impacts both the catalytic cavity and the calcium coordination. This region encompasses a known disulfide bond linking  $\beta 18$  and  $\beta 19$ , while a connecting loop obstructs the lower part of the tunnel (Siguier et al., 2014).

Notable fluctuations characterized the G205K system, with residue D28 within the  $\beta 2$  region (Blade I), one of the residues forming the catalytic triad, exhibiting the highest fluctuation. Additionally, both the wild-type and Q271T systems displayed fluctuations above exceeding  $2\text{\AA}$  within the  $\beta 2$  region. The residue H264 exhibited the highest fluctuation in the S188F, T256G, and Q271T mutations, with a common fluctuation observed in S157 across these systems. Among all systems, the residue E195 in the S127G mutant showed the largest fluctuations. Contrastingly, the wild-type system exhibited more fluctuation at residue D143, located between  $\beta 8$  and  $\beta 9$  (**Figure 8-5B**).

An intriguing observation is the occurrence of these fluctuations in the protein regions distant from the mutation sites. This orthogonal effect occurs at locations common to various mutations or unique to a specific mutation. Such differences in fluctuation patterns may be associated with the distinct thermostability profiles observed experimentally. It also suggests that the impact of point mutations extends beyond their immediate vicinity, potentially influencing the overall conformational dynamics and protein stability (Weinkam et al., 2013).

### *Secondary Structure analysis*

The results of the MD simulations indicate that the point mutations did not induce significant alterations in protein folding or disrupt local hydrogen bonding within the protein. The overall fold of a five-bladed  $\beta$ -propeller, consisting of four antiparallel  $\beta$ -strands in each blade, characteristic of GH 62 enzymes, remained preserved (Kaur et al., 2015; Siguier et al., 2014). Nonetheless, we observed subtle variations in secondary structure in specific regions, as depicted in **Figure 8-6A**. These differences are attributed to the influence of the mutations on local conformational dynamics and may have implications for the functional properties of the enzyme. Although the global structural fold remains intact, these localized variations in secondary structure highlight the potential impact of the mutations on specific regions of the enzyme.



We observed that certain regions exhibited extended secondary structures compared to the WT system. During the MD simulations, we noticed changes in secondary structures depending on the specific mutation (**Figure 8-6A**). This finding aligns with the observed subtle fluctuations in the RMSF analysis, as some motifs correlate with these fluctuations. To further investigate differences in the behavior of secondary structures, we examined their characteristics during the last 50 ns of the trajectories (**Figure 8-6A and B**). In some systems, we observed a loss of configurations associated with beta-sheet regions. Notably, in the S127G system, we observed an overall increase of approximately 5% in the secondary structure of the enzyme, with 4.1% attributed to beta-sheet motifs (**Figure 8-6B**). We observed proportional reductions in turn and coil regions, particularly with a more notable decrease in turn regions. This behavior was consistently seen across all mutations, with G205K exhibiting the fewest turn regions among the systems. A higher proportion of secondary structure content has been associated with enhanced thermal stability for other enzymes (Z. Ahmed et al., 2022; S. Kumar & Deshpande, 2021).

Analysis of the secondary structure patterns during the last 50 ns of the trajectories indicates that all mutations exhibited a higher percentage of secondary structure motifs than the percentage of turns and coil regions of the WT enzyme (**Figure 8-6B, Supplementary figure 12-8**). This suggests that the point mutations induced a shift towards a more structured conformation characterized by a higher proportion of secondary structure elements.

The higher number of secondary structure motifs observed in the mutations may indicate improved thermostability, as corroborated by experimental evidence. Furthermore, the observed changes in secondary structure could correlate to the subtle fluctuations observed during the MD simulations, particularly that of the  $\beta 19$  and  $\beta 20$  regions surrounding residue D143. These findings suggest a potential connection between alterations in secondary structure, local residue fluctuations, and overall enzymatic stability.

Regarding the residues fluctuations associated with differences in secondary structure, we identified four regions of interest within the enzyme. These regions include residues D28, F99, H264, and L286. The first, notably involving D28, part of the catalytic triad, does not exhibit significant fluctuations in the S127G, S188F, and T256G mutations. This region corresponds to  $\beta 2$  (Blade II). The second region, involving H264, is situated between  $\beta 17$  and  $\beta 18$ , representing a loop region that connects Blade IV to Blade V.

The fluctuations observed around residues F99 and L286, which are consistently higher in all mutations compared to the wild type, are particularly interesting. These residues

are in regions involved in forming the entrance to the binding site, specifically the  $\beta$ 6-7 (Blade II) and  $\beta$ 19 (Blade V) regions. Notably, the loop between  $\beta$ 19 and  $\beta$ 20 encompasses a small helix, and our analysis suggests that the increased fluctuation observed in this region may be associated with its ability to modulate the size of the substrate-binding cavity.

### ***Cavity Analysis***

To assess the potential impact of system dynamics on the catalytic site, we performed cavity analysis on the last 50 ns of the simulations. Our analysis unveiled significant changes in the size of the catalytic cavity across all mutations, with volumes exceeding those of the wild-type system (**Figure 8-6C**). Despite variances in the catalytic pocket's dynamics among the systems, the G205K mutation exhibited larger volume expansion relative to the WT. Considering the cavity volume fluctuations, the S127G, S188F, and G205K mutations displayed similar behaviors characterized by elevated volume fluctuations (**Supplementary figure 12-6 and Supplementary table 12-12**). Particularly, the S127G mutation exhibited the greatest mean deviation, indicating increased flexibility between closed and open states during the MD simulations. Conversely, the T256G and Q271T mutations demonstrated comparable behavior to the wild-type system.

Among all systems analyzed, only the Q271T mutation exhibited reduced flexibility in the cavity, suggesting a more rigid substrate binding site. In contrast, the other systems displayed greater mobility in the opening and closing movements of the enzyme's binding site than the wild-type system. This increased mobility implies a potentially more dynamic and adaptable nature of the binding site in these systems, allowing for conformational changes that facilitate substrate binding and catalysis.

Although the volume of the cavities provides valuable insights for distinguishing between these systems, it is crucial to emphasize the distinctions in cavity shapes (**Supplementary figure 12-7**). The variations in shape and size of the cavities could be attributed to the  $\alpha$ -helix formed between  $\beta$ 19 and  $\beta$ 20 and varying degrees of flexibility of this region. This loop is associated with the opening and closing movements of the catalytic cavity. Cavity shapes that are more elongated or deeper may indicate better substrate adjustments within the catalytic site, even at higher temperatures. Therefore, computational analysis of MD simulations provides insights into the dynamic behavior of the mutated systems. These findings shed light on the

dynamic nature of these regions and their potential implications for substrate binding and catalytic cavity modulation.

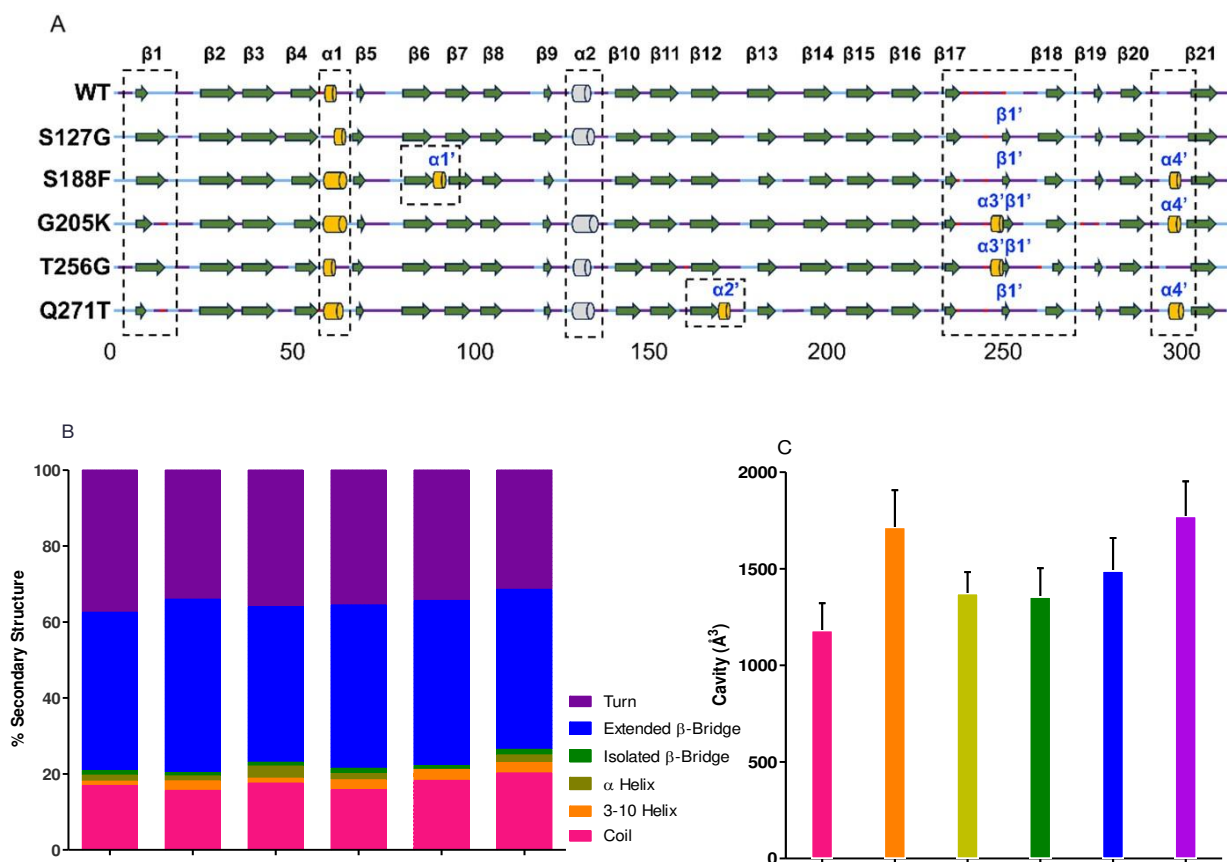


**Figure 8-6** Secondary Structure and Cavity analysis obtained from the last 50ns of MD simulation of wild type and mutations. (A) Alterations in secondary structures obtained from the mean structure (B) DSSP analysis of trajectories with values are expressed as a percentage of the distributions of all the residues (C) Cavity analysis of the catalytic site

## 8.4. Discussion

We identified five key residues affecting the thermostability of GH 62 arabinofuranosidase from *Aspergillus fumigatus* by directed evolution. G205K amino acid substitution showed the best improvement and ESM-IF1 score, increasing melting temperature from 49 to 58 °C, and improving the energy of denaturation by more than 40%. We identified conservative mutations in the selected mutants, which indicate their evolutionary importance. The selected variants can be templates for new directed evolution rounds to combine and find new potential sites that play a role in enzyme stability. Also, ESM-IF1 inverse folding modeling can predict residues for mutation, saving time and labor in rounds of library screening from direct evolution experiments. The effects caused by other mutations such as Q271T, S127G, T256G, and S188F may be related to the creation of additional hydrogen bonds, released steric strains, and

increased hydrophobicity. Further detailed insights obtained through molecular dynamics (MD) simulations revealed significant regional and residue-based differences in fluctuation patterns of the enzyme. The residues D28, F99, H264, and L286, exhibited distinct fluctuation behaviors, which could impact substrate binding and catalytic cavity modulation. Simultaneously, the catalytic cavity in all mutated systems showed significant changes in size compared to the WT. Also, despite the point mutations not causing significant alterations in the overall protein fold, there were subtle variations in secondary structures that could impact the functional properties of the enzyme. These findings contribute to our understanding of how point mutations influence the structural dynamics and stability of the enzyme, shedding light on their functional implications.

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### Supporting Information statement

Additional results details, including tables of aminoacid sequence analysis, RMSDs, and cavity analysis; supplementary figures of mutant screening and MD simulations, DSSP analysis, and cavity analysis.

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## **CHAPTER 9.**

### **INTEGRATED BIOREFINERY FOR XYLOOLIGOSACCHARIDES, PECTIN, AND BIOENERGY PRODUCTION FROM ORANGE WASTE**

## Integrated biorefinery for xylooligosaccharides, pectin, and bioenergy production from orange wastes

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### A slight change of subject

As explained in the general introduction of this thesis, the waste from orange processing underwent a distinct biorefinery approach utilizing a commercial cocktail rich in endoxylanase activity for economic viability assessment instead of the beloved chimeric enzyme developed and characterized throughout this thesis work. The biochemical characterization of this biomass (introduced in **CHAPTER 4**) showed that the (small) portion of hemicellulose was not constituted by arabinoxylan, therefore it wouldn't make sense to use the chimeric enzyme for its depolymerization and XOS production. However, the huge amount of waste generated per year in Brazil was a great incentive for this study in an attempt to find a better way to reduce the organic pollution and aggregate value to this biomass. The success of this work stems from its integrated use. All streams were explored for bioproducts and bioenergy generation, along with solvents recyclability, which was a critical factor for economic viability. The cost and energy efficiencies were assessed to develop a process to utilize orange processing wastes for XOS, pectin, and bioenergy, showing the profitability and the environmental properties of the proposed biorefinery. It is worth mentioning that this economical assessment study follows a **patent** deposit, presented in **Appendix IV**, and garnered some **awards** to our team along our trajectory (see Doctoral Period Memorial in **CHAPTER 1**).

(The supplementary material is presented in **CHAPTER 12**)



### **Abstract**

Orange waste is one of the most underutilized and available biowaste. This waste accounts for 50% of the fruit weight after juice extraction, presenting an environmental problem due to inappropriate disposal. Industrial waste exploitation is a sustainable approach to obtaining bioproducts and bioenergy. However, the efficiency of chemical treatments and enzymatic cocktails is still a bottleneck in these processes. One potential route to reducing costs is to design consecutive extractions and hydrolysis stages to yield more products from different fractions of the same feedstock, reducing time and bulk material usage. This study assessed the economic performance of OPW biorefinery for xylooligosaccharides (XOS), pectin, and bioenergy, involving three aspects: (1) experimental procedures, (2) simulation approach, and (3) optimization tools. The optimization of hydrothermal treatment for pectin extraction decreased material costs by >60% and preserved the xylan content for xylooligosaccharides production via enzymatic hydrolysis. This study analyzed different biorefinery capacities, and solid waste exploitation for heat and steam generation, improving sustainability. The production cost of high-purity pectin and xylooligosaccharides is 3.67 \$/kg with an efficient solvent recovery and self-sufficiency for standard power usage. These results proved the economic feasibility and low environmental impacts of OPW biorefinery.

**Keywords:** Antioxidant potential; Bioeconomy; Circular economy; Energy analysis; SuperPro Designer; Prebiotics.

## 9.1. Introduction

Orange processing waste (OPW), composed of fruit peels and bagasse (~50 % of the fruit wet weight), is a prospective feedstock for the bioenergy and bioactive compounds co-production toward integrated biorefinery (Clark et al., 2013; Ortiz-Sanchez et al., 2021; Tsouko et al., 2020). The biggest citrus plants, such as the *CitroSuco* in Brazil, can process more than sixty million boxes (2.4 million tons) of fruit in one season (USDA, 2022). The large amounts of OPW induce soil contamination since the traditional management involves landfill disposal and incineration, which may cause leachate production and serious organic pollution due to the high demand for biological oxygen for its decomposition (Balu et al., 2012b; Ortiz-Sanchez et al., 2021). In the last years, studies have focused on new processes for OPW valorization through the production of biofuels and high-value compounds, such as essential oil, cellulose, pectin, lactic acid, xylooligosaccharides (XOS), and phenolic compounds (Balu et al., 2012a; Fazzino et al., 2021; Ortiz-Sanchez et al., 2021; Praveen et al., 2017; Teigiserova et al., 2022; Tsouko et al., 2020).

Global Market Insights reported that citrus fruits had been the prime source of hydrocolloid pectin because of their active phytochemical content, high degree of esterification, gelling properties, and elastics characteristics at elevated temperatures. Even though the food industry demands a high production of pectin for its use as a gelling agent and as an additive in bio-packing manufacturing, industrial pectin production is quite energy-consuming due to high quantities of water, ethanol, and utility requirements (electricity, heating, cooling). Also, pectin manufacturers are taking initiatives to offer clean-label ingredients for food manufacturers since natural ingredients are perceived to be healthier and better for the planet. Consumers lean toward plant-based ingredients instead of animal-based products with less processed ingredients, thus driving the market's growth. The pectin global market is forecasted to reach approximately 103,990 tons and USD 957.70 million in 2023 to USD 1,261.20 million by 2028, at a CAGR of 5.66% during the forecast period (2023-2028) (Ahuja & Malkani, 2020; Mordor Intelligence, 2023a; Tsouko et al., 2020).

Likewise, the CAGR projection for the prebiotics market is 9.71% over the next five years. The rising popularity of functional foods and beverages comes from the increasing health consciousness of consumers, which drives the demand for prebiotics as sugar substitutes and probiotics growth stimulators. The ban on antibiotics in the animal feed industry also influences this market sector (Mordor Intelligence, 2023b). Xylooligosaccharides (XOS) present a remarkable potential as a functional food ingredient due to superior benefits than other

prebiotics. XOS also has temperature and pH stabilities and favorable organoleptic properties. The global XOS market expectation is to achieve ~\$130 million by 2025 (L. B. Brenelli et al., 2022). The enzymatic hydrolysis of xylan (hemicellulose) is the most sustainable way to produce XOS with high purity and prebiotic potential (Ávila, Martins, Costa, et al., 2020). However, XOS production is still more expensive than other prebiotics because of the costs related to alkalis used during pretreatments (generally NaOH or KOH) and enzymatic cocktails. Industries are developing various approaches using renewable feedstocks and integrated processes for biorefineries (Amorim et al., 2019).

The increasing consumers' awareness of the harms of artificial ingredients and processed and unnatural food products, along with regulations from governmental bodies favoring the use of natural additives in food, is propelling the growth of the market of natural ingredients like pectin and xylooligosaccharides (Mordor Intelligence, 2023a). Understanding the economic effectiveness and naming the key drivers of the production cost is essential to evaluate industrial capacities and innovative technologies to supply commercial demands. Software for process simulation and techno-economic analysis as SuperPro Designer has successfully used developed and improved innovative technologies (Achinas et al., 2019; Koulouris & Petrides, 2020). This software uses mass and energy balances to decide equipment sizing and estimate the capital investment, unit cost, and revenues, as well as the environmental impacts, bottlenecks, and potential areas for improvement.

Despite the vast literature reporting the OPW use as a source of bioproducts, most studies are still at an early stage of development. There is still a lack of studies assessing their economic feasibility on a large scale, especially for XOS production because of the low amount of xylan in OPW. Other authors reported the techno-economic analysis of OPW biorefineries for pectin and biogas production (Ortiz-Sanchez et al., 2021) and the profitability of XOS production from different types of industrial wastes (Lan et al., 2021a).

A critical factor for the better profitability of the OPW biorefinery is the market prices of value-added products, such as XOS. Scientific papers did not report yet the integrated use of OPW for XOS and other high-value products that can help the viability of the biorefinery, nor did other research identify the drivers for production cost and OPW biorefinery's financial performance under varied configurations (treatment conditions, product purities, and solvent recycling). To address this gap, this study assessed the energy and cost efficiency to develop a process to utilize OPW for pectin, XOS, and biogas through process optimization and simulation models. We organized this study into (1) determination of material composition; (2)

experimental design for pectin and xylan extraction; (3) xylan enzymatic hydrolysis for XOS production; (4) scenarios analysis using SuperPro Designer software.

## **9.2. Material and methods**

### **9.2.1. Raw material composition and processing**

Vita Citrus orange juice industry (Ribeirão Preto, SP, Brazil) donated the orange processing waste (OPW), which we dried in an air-circulating oven at 50 °C for 24 h and grounded. We removed the free sugars by washing twice for 1 h at 50 °C using a solid concentration of 50 g/L. Cellulose, hemicellulose, ash, extractives, and lignin determination followed the National Renewable Energy Laboratory (NREL) procedures (Sluiter et al., 2005, 2008).

Pectin characterization followed the spectrophotometric method (Ibarz et al., 2006). Solutions were freshly prepared before the experiments: m-Hydroxydiphenyl: A 0.15% solution of m-hydroxydiphenyl (Sigma Chemical, St. Louis, MO, USA) in 0.5% NaOH, protected from light by wrapping with foil; Sodium tetraborate 0.0125 M in concentrated sulphuric acid; D-Galacturonic acid (Sigma Chemical, St. Louis, MO, USA) for standard curve. See section 9.2.4 for details.

### **9.2.2. Pectin extraction**

This study extracted pectin from the free-sugar OPW, conserving the hemicellulosic integrity in the residual solids, analyzing the conditions of pectin extraction by a central composite rotatable design (CCRD) with 3 pivotal points and 2 axial points, 2 % w/v citric acid (pH  $2.0 \pm 0.2$ ), and a solid ratio of 1:40 w/v. The independent variables were temperature (70 - 130 °C) and incubation time (1 - 70 min). We used the Protimiza Experimental Design tool (Rodrigues & Iemma, 2014) for statistical analysis. After extraction, we centrifuged the acidic suspension (9000 g, 10 min, 4 °C) and filtered the supernatant. We treated the filtrate with 1 volume of ethanol, adjusted the pH to pH 3.5 (pH of minimum pectin solubility) with KOH, and incubated overnight at 4 °C for pectin precipitation. We collected the pectin-rich extract by centrifugation (9000 g, 10 min) and dried and expressed the yield of the pectin-rich extract as its weight per 100 g of sugar-free OPW. A rotary evaporator recovered the ethanol (at 50 °C), and HPLC determined the chemical composition of the ethanol-free liquid fraction (Martins, Ávila, et al., 2020). Pectin characterization followed the spectrophotometric method (Ibarz et al., 2006).

### 9.2.3. Xylan extraction and enzymatic hydrolysis

We washed the remaining solids from pectin extraction with water to remove the citric acid and centrifuged (9000 g, 10 min). The experiments for xylan extraction analyzed KOH concentration between 2.5 % and 15 % and temperatures between 90 °C and 120 °C during 30 - 120 min using a solid ratio of 1:10 w/v. We filtered the material (Whatman paper #1) and neutralized it with 0.1 volume of acetic acid to pH ~5.5 (color change seen). We precipitated xylan with 1 volume of ethanol overnight at 4 °C. Then, we centrifuged the xylan (9000 g, 20 min), washed it with 50 % ethanol + 0.5 % EDTA, centrifuged it again, and dried it overnight at 50 °C (Ávila, Martins, & Goldbeck, 2020). Enzymatic hydrolysis occurred in acetate buffer pH 5.0 with 1 % v/v Shearzyme endoxylanase (NS50030, Novozymes®, final protein concentration of 0.165 mg/mL) at 50 °C for 48 h, followed by heat inactivation at 98 °C for 5 min. Then, we filtered the samples (0.22 µm PVDF membrane, Millex) and analyzed them for XOS composition and antioxidant potential.

### 9.2.4. Analytical procedures

We evaluated the antioxidant capacity of XOS by DPPH and phosphate-molybdenum free-radical capture methods, using Trolox as standard (Bondet et al., 1997; Prieto et al., 1999). We quantified the phenolic compounds using the Folin-ciocalteau method as described elsewhere, with gallic acid as standard (dos Santos et al., 2020). XOS quantification followed the methodology described elsewhere (Martins, Ávila, et al., 2020). This study used the reducing sugar method to measure the endoxylanase activity (Bailey et al., 1992; Miller, 1959) and the Lowry method for protein quantification (Lowry et al., 1951).

Pectin characterization followed the spectrophotometric method (Ibarz et al., 2006): 0.5 mL of pectin sample and 0.5 mL of distilled water were added followed by the addition of 5 mL of the sulphuric/tetraborate solution and placed in a water-ice bath. The mixture was shaken in a vortex mixer to homogenize the sulphuric solution. The cold bath avoids the temperature rising excessively, allowing a higher control of the time of the hot sulphuric reaction. The tube is then placed in a water bath at 80 °C for 6 min and, then immediately placed again in the water-ice bath until reaching room temperature. A 0.1 mL of m-hydroxydiphenyl solution was added and, after a vortex stirring to homogenize the mixture, spectrophotometric absorbance at 520 nm was carried out, with absorbance readings being taken at different times until maximum absorbance. In order to obtain results with the maximum reliability, it is proposed that the m-hydroxydiphenyl should be added to each test tube and then to wait until

spectrophotometer gives the highest absorbance reading, and this is the reading to be considered. The simultaneous addition of m-hydroxydiphenyl to all the samples was therefore avoided. To check the reliability of the readings, the D-galacturonic acid content of a sample was determined in each standard calibration series. The sample was treated as the standard D-galacturonic acid concentration tubes and the spectrophotometric reading was also taken at the highest absorbance.

All experiments were carried out by quadruplicate

### 9.2.5. Biorefinery simulation

The software SuperPro Designer v11 (Intelligen, USA) simulated bioenergy generation, pectin extraction, and XOS production concerning technical and economic parameters and environmental assessment. The software provided the international cost of the equipment (**Supplementary table 12-14**), assuming that the plant operates 200 batches per year. The process has four main areas, namely (1) OPW feedstock washing and CHP plant; (2) pectin extraction; (3) pectin precipitation; (4) xylan extraction and enzymatic hydrolysis (**Supplementary figure 12-15, Supplementary figure 12-16, Supplementary figure 12-17, Supplementary figure 12-18**).

#### *OPW Feedstock washing and CHP plant.*

A typical capacity of an extractor for medium-sized fruit in an industry of orange juice is 5 to 7 tons of fruit per hour, corresponding to about 2,500 L of juice and ~2.5-3.5 tons of OPW generated per hour. Therefore, one extractor generates at least 60-84 tons of OPW in 24 hours (Hui, 2004; I. B. T. Pereira et al., 2018). Based on the average amount of OPW generated by medium-sized plants, this study adopted an amount of 100 tons of OPW as input.

The first step is to reduce the particle size and separate the free sugars. After grinding, we washed the OPW with warm water (50 °C). The first screw-pressing machine collected the first fraction of free sugars. Then, the material is washed with warm water and pressed again. One batch generates 67.3 tons of sugar-free OPW (85 % humidity) and ~13 tons of sugars. The sugars go to the anaerobic digester in the CHP plant, and evaporation recovers 19.7 m<sup>3</sup> of water (**Supplementary figure 12-15**). The evaporator returns the water at feverish temperatures, which heats the washing water. This heat exchange provides warm water for the washing steps without needing an electric heater, thus saving energy.

The CHP plant runs 7800 hours/year in continuous mode. The organic waste streams from the processes go to the reactor for biogas production in an anaerobic digester,

performed at mesophilic conditions of 35 °C in a semi-continuous flow stirred reactor for 600 h and 0.01 kW/m<sup>3</sup> of power for heating and stirring. The reaction conversion efficiency is 90 % for proteins and sugars and 50 % for cellulose, hemicellulose, pectin, and lignin (D. Kumar & Murthy, 2011). After digestion, the biogas is compressed and burned in a gas-turbine generator. The turbine propels the compressor and the generator to produce power. During electricity generation, steam generation equipment captures the heat from the exhaust stream (450 °C) via an input of air and water. The plant can use or sell the produced power and heat. 4.5 tons/batch of digestate can be sold as fertilizer (Achinas et al., 2019).

### ***Pectin extraction***

Washed and grounded OPW (85 % humidity) is mixed with citric acid (2 % w/v final acid concentration) at 99 °C for 70 min (10 min setup, 80 m<sup>3</sup> working volume). Solids are separated by vacuum filtration and washed with water. Then, reverse osmosis recovers the water. Impurities are removed by a granular activated carbon adsorber (GAC, 18 min bed contact, binding capacity of 9.14 mg/g), removing HMF, furfurals, and some flavonoids. Then, the column is washed with NaOH and water. Two columns are operating in a staggered mode so there'd always be one column being loaded while the other would be washing. Based on the chemical composition of pectin in Table 3, the following yields were adopted: 70 % solubilization efficiency; 10 % degradation of hemicellulose, cellulose, and pectin into furfural, HMF, and formic acid, respectively; and 5 % conversion of hemicellulose into XOS. This step produces 2.85 tons of soluble pectin (**Supplementary figure 12-16**) and 11 tons of lignocellulosic biomass (with 40 % moisture).

### ***Pectin precipitation***

Four scenarios evaluated the energy requirements and ethanol consumption. These scenarios involved four concentrations of the pectin-rich stream (0 %, 30 %, 50 %, and 70 % evaporation). After evaporation, pectin solids are precipitated with 50 % v/v ethanol for 12 h and separated by vacuum filtration. A drum dryer separates the ethanol from the remaining monosaccharides, XOS, and citric acid. Each batch produces 3 tons of 95 % pectin solids, and 99 % of the consumed alcohol is recovered by distillation, along with 48 m<sup>3</sup> of water (**Supplementary figure 12-17**).

We computed citric acid recovery by precipitation and crystallization. Purification starts by adding Ca (OH)<sub>2</sub> for every 3 flow volumes to precipitate calcium citrate, separated by vacuum filtration. The filtrate goes to XOS purification, and the calcium citrate is acidified with

dilute sulfuric acid to precipitate calcium sulfate (gypsum). Another filter separates the gypsum from the citric acid solution, which is crystallized using an evaporator/crystallizer. Then, filtration separates the crystals (Carmichael & Petrides, 2020).

### *Xylan extraction and enzymatic hydrolysis*

11 tons of lignocellulosic biomass (40 % moisture) is mixed with 2.5 % KOH at 99 °C for 120 min and 10 % solid concentration in a stirred reactor (80 m<sup>3</sup> working volume). Then, vacuum filtration separates the solids, and 4.68 tons of cake (rich in cellulose) goes to the CHP plant. An evaporator concentrates the filtrate (rich in xylan). Here, we analyzed four scenarios to reduce the usage of ethanol and energy: 0 %, 30 %, 50 %, and 70 % evaporation. In scenario 1, the xylan precipitates without prior evaporation. Scenario 1 has no evaporator but requires an additional vacuum filter, a drum dryer, and a vertical tank. Scenario 2 (30 %) and scenario 3 (50 %) need an evaporator system and an additional drum dryer. Scenario 4 (70 %) requires only the evaporator.

After evaporation, KOH is neutralized with an equimolar concentration of acetic acid, forming CH<sub>3</sub>COOK. The extract is transferred to a tank and mixed with 1 volume of ethanol for 12 hours. Then, a solution of 50 % ethanol and 0.5 % EDTA washes ~1 ton of xylan. The xylan is then washed with water and stored. A distillation system recovers the solvents (ethanol and water).

Enzymatic hydrolysis used 2 % xylan (w/v) in acetate buffer pH 5.0 at 50 °C for 48 h using 1 % v/v Shearzyme endoxylanase (NS50030, Novozymes®). After hydrolysis, filtration removes the solids. The hydrolysate is mixed with the stream containing the filtrate from pectin extraction (74 kg XOS) and stored in a tank (600 m<sup>3</sup>). 900 L/h of hydrolysate is concentrated ten times by ultrafiltration using a Dft RO membrane, which removes monosaccharides too. Then, a PBA column equipped with dextran resin removes the buffer, enzyme, and impurities. We set the loading flow rate to 12-bed volumes/min and 2000 g/L dextran resin's binding capacity. The PBA columns process 1570 L/batch. Finally, XOS is dried using a rotary dryer to achieve 98.84 % purity and 0.5 % moisture, representing 825 kg/batch. Reverse osmosis recovers 54 m<sup>3</sup> of water (**Supplementary figure 12-18**).

### *CIP (cleaning in place)*

The simulations also computed the “cleaning in place” (CIP) to wash the equipment. It happens in a closed circuit of detergents and rinsing solutions, removing residues and bacteria. The operating temperature varies based on the type of waste and the equipment.



Prewash uses cold water to remove coarse residues. Then, an alkaline cleaning solution (2.0 % NaOH) removes residues such as proteins and fats, followed by a 30-minute acid cleaning to remove mineral residues and rinsing with water to remove the chemicals. We did not compute CIP water in the water recovery system.

### ***Economic and environmental analysis***

For the economic assessment, the plant cost (PC) is the sum of the total plant indirect cost (TPIC, engineering, and construction) and the total plant direct cost (TPDC, equipment buys costs, installation costs, process piping, instrumentation, electrical, building, yard improvement, and auxiliary facilities costs). The contractor's fee and contingency are calculated as 5 % and 8 % of the PC and constitute the direct fixed capital cost of the plant. The biorefinery operates 200 batches a year. The main operational costs include labor, facility, and utility (**Supplementary table 12-15**). We acquired the data from the SuperPro Designer database and literature (Achinas et al., 2019; R. D. G. Ferreira et al., 2018; Lan et al., 2021b; Teigiserova et al., 2022). We calculated the environmental impacts through emissions from inputs, products, and waste streams.

## **9.3. Results and discussion**

Brazil is the leading nation concerning citrus fruit processing, accounting for 50% of the world's total. Among the citrus fruits, oranges correspond to 80% of the total processed fruits (Surdesignated2022). In 2023, Brazilian production is estimated at 16.75 billion tons of fruits, 80% destined to juice production. São Paulo state accounts for around 80% of the national production and harvesting happens all year round (Instituto Brasileiro de Geografia e Estatística, 2023). The “Pera Rio” orange (*Citrus sinensis* (L.) Osbeck) is the most cultivated type, ideal for juices due to its sweet taste and few seeds. The primary season for harvesting is from May to October. The off-season produces a small quantity of “Pera Rio” oranges and other species to suppress the market need, such as “Charmute” orange and “Natal” oranges (Ana Silvia Martins Franco, 2016; Instituto Brasileiro de Geografia e Estatística, 2023). This guarantees the orange supply all year round for continuous industrial processing and exportation, which also means an ongoing generation of orange waste. The waste has no commercial value unless dried, texturized, and sold as fertilizer or animal feed (Rezzadori et al., 2012).

Studies proposed alternatives to achieve more sustainable industrial processing of OPW, focusing on analyzing economic and environmental aspects related to OPW biorefinery and others immersed in novel green technologies to reduce the eco-footprint of OPW upgrading.

**Table 9-1** compares the present proposal and recent studies from 2017-2023. This proposal estimated energy reduction and reuse of reagents and investigated different economic scenarios. Other research reported the feasibility of OPW for pectin and biogas production (Ortiz-Sanchez et al., 2021) and the techno-economic analysis of producing xylo-oligosaccharides industrial wastes (Lan et al., 2021a). Here, the integrated use of OPW for pectin, XOS, and bioenergy was analyzed to add value and reduce the eco-footprint of OPW biorefinery.

### 9.3.1. OPW composition

The chemical characterization showed that  $81 \pm 1.9$  % of OPW wet weight is moisture, while  $50 \pm 3.4$  % of OPW dry weight is free sugars. The free sugars are composed of glucose (38.5 %), fructose (37.2 %), and sucrose (22.3 %). > 90 % of the total free sugars were separated in the first washing. **Table 9-2** presents the chemical composition of washed and dried OPW, ascertaining that OPW is adequate for pectin extraction, whose reminiscent solids are poor in lignin and rich in cellulose and hemicellulose. Nevertheless, chemical variations may occur due to fruit maturity, growth conditions, and equipment adjustments for orange juice extraction (Ortiz-Sanchez et al., 2021).

### 9.3.2. Pectin extraction

We assayed pectin extractions according to Table 9-3. We adopted the solid ratio of 1:40 w/v according to preliminary assays (data not shown) and literature (Tsouko et al., 2020). Although higher ratios may favor pectin extraction due to lower viscosity, the excessive use of solvents leads to higher processing costs and autohydrolysis due to over-dilution (Tsouko et al., 2020). In this study, the integrity of the remaining solids was the main factor for its further use for XOS production.

**Table 9-1 Studies on the use of orange processing waste using different technologies and main products.**

| <b>Products</b>                           | <b>Technologies</b>                                                                       | <b>Feasibility Study and Cost Estimate</b>                                                                                                                                                                                                                                                                                                                                                        | <b>Eco-Footprint</b>                                                                                                                                                                                                 | <b>Ref</b>                   |
|-------------------------------------------|-------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------|
| <b>XOS, Pectin, Power, Steam</b>          | Citric acid extraction, KOH extraction, Enzymatic hydrolysis, CHP plant                   | The use of residual solids and free sugars for biogas production circumvents the need for pretreatment before anaerobic digestion. The integrated process reduced the net unit cost of pectin and XOS production. Optimization of the scenarios for pectin and xylan extraction reduced more than 50% of bulk material cost. XOS production is workable only if associated with other bioproducts | Recyclability of solvents and reduction in all environmental impact categories were successfully achieved.                                                                                                           | This study                   |
| <b>Essential Oil, Pectin, Phenols</b>     | Solar hydro-distillation of essential oils and phenols, HCl extraction                    | The distillation time was reduced by >30%, with no need for an added source of energy. The investment cost is 28.5% higher. However, the solar system does not need other expenses                                                                                                                                                                                                                | There was no significant difference between the oils' quality of solar extraction (green solution) and conventional methods.                                                                                         | (Hilali et al., 2019)        |
| <b>Essential Oil, Pectin, Biogas</b>      | Steam distillation, Citric acid extraction, Anaerobic digestion                           | Only high scales can be applied in a biorefinery context. The energy assessment of the proposed OPW biorefinery demonstrates the need to reduce the thermal needs of the process. Thus, a deep heat integration study must be performed.                                                                                                                                                          | Not discussed                                                                                                                                                                                                        | (Ortiz-Sanchez et al., 2021) |
| <b>Mucic Acid, Biogas, Digestate</b>      | Anaerobic digestion; pectin extraction with citric acid, Mucic acid production simulation | Biogas and mucic acid production costs were reduced by 36 % increasing the OPW flow. Mucic acid production demands 42 % of the capital investment of the OPW biorefinery.                                                                                                                                                                                                                         | The liquid releases of mucic acid production present high negative impacts and should be studied carefully                                                                                                           | (Ortiz-Sanchez et al., 2020) |
| <b>Limonene</b>                           | Solvent extraction                                                                        | Not discussed                                                                                                                                                                                                                                                                                                                                                                                     | Bio-based CPME and 2-MeTHF are promising alternatives for replacing hexane in the extraction of limonene. Recyclability of solvents conducted.                                                                       | (Ozturk et al., 2019)        |
| <b>Citric Acid, Limonene, Animal Feed</b> | Microwave extraction, Solid state fermentation                                            | Not discussed                                                                                                                                                                                                                                                                                                                                                                                     | The sensible solution would be only the limonene extraction. The essential oil extraction can be realized as an add-on in current juice facilities, while dried residual OPW should be directly used as animal feed. | (Teigiserova et al., 2022)   |

Assay 8 (100 °C and 70 min) showed the highest purity (81 %) and lowest material degradation, releasing 0.7 mg/mL XOS (Table 9-3). This assay showed superior efficiency compared to other studies (Benassi et al., 2021), with a pectin recovery of 58.5 %. Assays 2 and 4 supplied 58 % pectin and released 0.54 and 1300 mg/mL XOS, respectively. The higher XOS concentration in assay 4 is due to the acidic autohydrolysis of the hemicellulose (70.35 % conversion). However, higher temperatures (> 120 °C) and longer incubation times resulted in more

acid pH due to intense biomass degradation and production of formic acid, HMF, furfural, and acetic acid, as presented in Table 9-3. We obtained the mathematical models through second-order polynomial equations ( $p < 0.05$ ). **Figure 9-1** shows the effects of temperature and incubation times for pectin extraction (a) and xylan recovery (b), with  $R^2$  values of 0.98 and 0.95, respectively. Xylan recovery decreased at temperatures above 105 °C and incubation time above 50 min. **Table 9-2** presents the hemicellulose content in residual solids. No studies analyzed the liquid fraction (which holds XOS) and residual solids (lignocellulose) that remain after pectin extraction for comparison with these results. It is worth mentioning that citric acid is cheaper than other organic acids for pectin extraction (Valladares-Diestra et al., 2022). Citric acid improves economic feasibility through recycling and presents a low environmental footprint (Pinheiro et al., 2008).

**Table 9-2: Composition of OPW fractions**

| %                 | Sugar-free OPW | Extracted pectin* | Residue from pectin extraction | Extracted xylan | Residue from xylan extraction |
|-------------------|----------------|-------------------|--------------------------------|-----------------|-------------------------------|
| Pectin            | 40.5±2.5       | 81.2±1.4          | 11.2±1.0                       | 10.0±0.8        | 15.4±0.8                      |
| Hemicellulose     | 14.8±0.2       | 4.9±0.2           | 30.1±2.9                       | 69.0±1.2        | 8.0±0.1                       |
| Cellulose+ starch | 39.0±2.4       | 14.3±0.3          | 56.7±3.2                       | 21.0±1.0        | 76.6±1.1                      |
| Ashes             | 1.0±0.0        | -                 | -                              | -               | -                             |
| Lignin            | 1.2±0.0        | -                 | -                              | -               | -                             |
| Protein           | 3.2±0.1        | -                 | -                              | -               | -                             |
| Fats              | 2.3±0.1        | -                 | -                              | -               | -                             |
| Total             | 102.5±2.6      | 100.4±0.7         | 97.1±3.1                       | 101.0±1.0       | 101.0±1.0                     |

\*Under optimized conditions.

### 9.3.3. Xylan extraction and enzymatic hydrolysis

We obtained the purest xylan with 2.5 % KOH at 100 °C for 120 min. Xylose was the main monosaccharide in the alkali-extracted material (>1 g/L). We detected glucose, galactose, and mannose with > 5% KOH (~0.005 g/L); arabinose content increased with increasing KOH concentrations (0.04 g/L with 5 % KOH and 0.07 g/L with 10 % KOH). Higher KOH concentrations did not increase the extracted weight mass (~30 % of the first mass). The remaining solids presented 76 % cellulose and 8 % hemicellulose. These results are per the literature: Pereira et al., (2022) reported that the largest xylan solubilization (80.81 % for the orange bagasse waste) occurred with KOH compared to NaOH and hydrogen peroxide. The characteristics of each biomass, especially chemical composition, influence the best hemicellulose solubilization conditions.

We neutralized the extracted material with acetic acid before the addition of ethanol. Some methodologies recommend the addition of 10 volumes (Zilliox & Debeire, 1998) or 3 volumes (Khair et al., 2021) to precipitate xylan. However, higher ethanol volumes reduced the purity from 69 % (1 ethanol volume) to 15 % (3 ethanol volumes) due to increased arabinose content in the material.

Later, the enzymatic hydrolysis yielded 9.16 g/L of XOS, which stands for a conversion of 72 % (Table 9-5). The hydrolysis product contained 0.32 g/L of monosaccharides. The main oligosaccharide was xylotetraose (x4), ~ 74 % of the total XOS. The hydrolysate with higher XOS concentration presented higher antioxidant activity. A considerable fraction of phenolic compounds ( $\mu\text{g}$  gallic acid equivalent) was also detected (Table 9-4) and contributed to the antioxidant potential of the material.

**Table 9-3 Pectin yields, hemicellulose recovery, and components of hydrothermal assays (CCRD matrix)**

| Assay                                 | 1     | 2     | 3     | 4     | 5     | 6     | 7    | 8           | 9     | 10    | 11    |
|---------------------------------------|-------|-------|-------|-------|-------|-------|------|-------------|-------|-------|-------|
| °C                                    | 80    | 120   | 80    | 120   | 70    | 130   | 100  | 100         | 100   | 100   | 100   |
| Min                                   | 10    | 10    | 60    | 60    | 35    | 35    | 1    | 70          | 35    | 35    | 35    |
| Final pH                              | 2.42  | 2.36  | 2.42  | 2.35  | 2.40  | 2.33  | 2.4  | 2.43        | 2.4   | 2.4   | 2.4   |
| Pectin yield (%)                      | 6     | 12    | 13.0  | 42.0  | 7.0   | 39.0  | 12.0 | <b>42.0</b> | 32.0  | 34.0  | 33.0  |
| Pectin purity (%)                     | 40    | 58.0  | 40.0  | 58.0  | 35.0  | 57.0  | 20.0 | <b>81.0</b> | 61.0  | 60.0  | 59.0  |
| Pectin recovery (%)                   | 4.20  | 12.1  | 9.0   | 42.3  | 4.2   | 38.1  | 4.0  | <b>58.5</b> | 33.9  | 34.9  | 33.8  |
| Pectin in remaining solids (%)        | 21.0  | 23.0  | 22.0  | 9.0   | 25.0  | 11.0  | 24.0 | 13.0        | 16.0  | 16.0  | 16.0  |
| Hemicellulose in remaining solids (%) | 10.1  | 11.9  | 10.7  | 7.6   | 8.6   | 12.5  | 16.7 | <b>15.9</b> | 13.7  | 14.1  | 14.0  |
| Xylobiose (g/L)                       | 0.327 | 0.275 | 0.339 | 0.446 | 0.431 | 0.453 | -    | 0.347       | 0.341 | 0.341 | 0.344 |
| Xylotriose (g/L)                      | 0.334 | 0.266 | 0.343 | 0.398 | 0.414 | 0.436 | -    | 0.349       | 0.339 | 0.340 | 0.339 |
| Xylotetraose (g/L)                    | -     | -     | -     | 0.200 | -     | -     | -    | -           | -     | -     | -     |
| Xylopentaose (g/L)                    | -     | -     | -     | 0.133 | -     | -     | -    | -           | -     | -     | -     |
| Xylohexaose (g/L)                     | -     | -     | -     | 0.125 | -     | -     | -    | -           | -     | -     | -     |
| Total XOS (g/L)                       | 0.660 | 0.541 | 0.683 | 1.301 | 0.845 | 0.889 | 0    | 0.695       | 0.680 | 0.681 | 0.681 |
| Arabinose (g/L)                       | 0     | 0     | 0.025 | 0.342 | 0     | 0.107 | 0    | 0.122       | 0.015 | 0.015 | 0.015 |
| HMF (g/L)                             | 0     | 0.007 | 0     | 0.01  | 0     | 0.009 | 0    | 0.007       | 0     | 0     | 0     |
| Furfural (g/L)                        | 0     | 0     | 0     | 0.019 | 0     | 0.015 | 0    | 0           | 0     | 0     | 0     |
| Acetic acid (g/L)                     | 0.03  | 0.034 | 0.034 | 0.03  | 0.043 | 0.03  | 0    | 0.033       | 0.03  | 0.03  | 0.03  |
| Formic acid (g/L)                     | 0     | 0.021 | 0.02  | 0.03  | 0     | 0.022 | 0    | 0.016       | 0     | 0     | 0     |

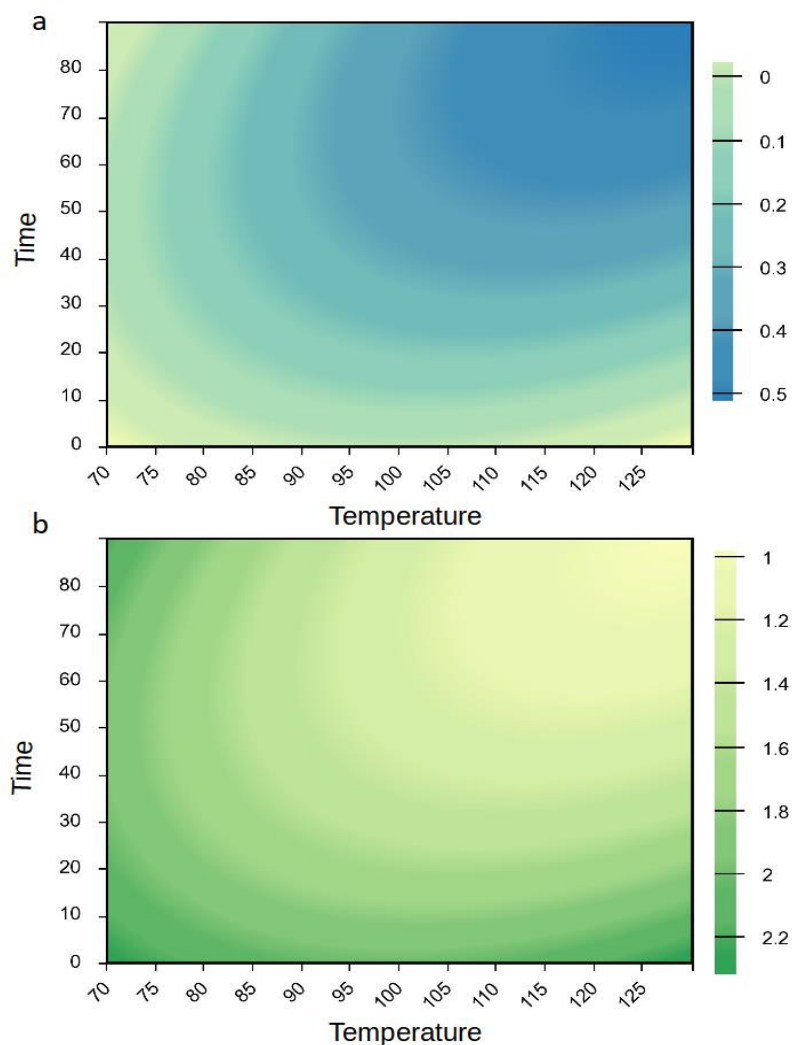


**Figure 9-1** Response surface plots of CCRD for pectin extraction (a) and xylan recovery (b) on the temperature and time of extraction.

**Table 9-4** XOS composition from xylan enzymatic hydrolysis, antioxidant ability, and total phenols

| <b>Solid concentration<br/>(w/v)</b> | <b>1 %</b> | <b>2 %</b> | <b>3 %</b> | <b>4 %</b> |
|--------------------------------------|------------|------------|------------|------------|
| <b>Xylose</b>                        | 0.343      | 0.349      | 0.348      | 0.352      |
| <b>Xylobiose (x2)</b>                | 0.319      | 0.334      | 0.329      | 0.339      |
| <b>Xylotriose (x3)</b>               | 0.320      | 0.326      | 0.309      | 0.341      |
| <b>Xylotetraose (x4)</b>             | 3.522      | 6.690      | 1.820      | 1.976      |
| <b>Xylopentaose (x5)</b>             | 0.117      | -          | -          | -          |
| <b>Xylohexaose (x6)</b>              | 1.524      | 1.807      | 0.497      | 1.736      |
| <b>Total XOS (g/L)</b>               | 5.802      | 9.157      | 2.955      | 4.391      |
| <b>XOS (mg/mg xylan)</b>             | 0.840      | 0.663      | 0.107      | 0.212      |
| <b>% Conversion</b>                  | 84.10%     | 72.68%     | 21.23%     | 10.72%     |
| <b>µg gallic acid eq</b>             | 52.33      | 104.62     | 154.32     | 193.80     |
| <b>µM Trolox eq</b>                  | 3,803      | 20,279     | 11,587     | 15,279     |

### 9.3.4. Process simulation

**Figure 9-2** shows the flowchart of the biorefinery, where 13 tons of free sugars are collected from 100 tons OPW (wet basis). Each batch produces 2.9 tons of 95 % pectin and 825 kg of XOS (from 1 ton of extracted xylan). The remaining solids from all stages go to the CHP plant, and a new batch begins every 38 hours (**Supplementary figure 12-19**).

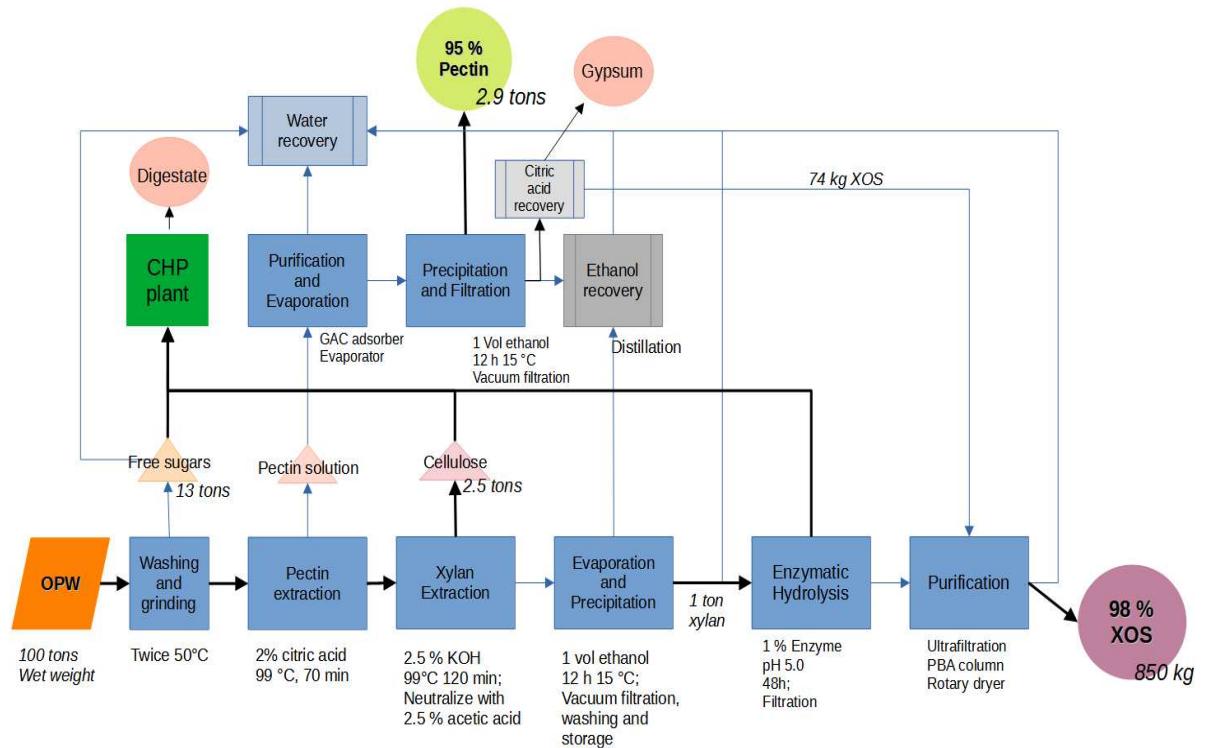


**Figure 9-2 Biorefinery flowchart**

**Figure 9-3a** shows that scenario 1 for pectin precipitation, with no evaporation, resulted in the highest usage of ethanol, steam, and chilled water. In Scenario 1, a cooling system is necessary before precipitation because of the elevated inlet temperature. Scenario 2 (30 % evaporation) resulted in the lowest power consumption, and scenario 3 (50 % evaporation) resulted in the highest power and cooling water consumption. Scenario 4 (70 % evaporation) resulted in the lowest ethanol, steam, and cooling water consumption but presented the topmost equipment investment and payback time. Also, evaporation above 50 % led to a viscous solution, which would be difficult to process in an industrial operation. The total capital investments calculation adopted scenario 2, based on its energy and ethanol consumption.

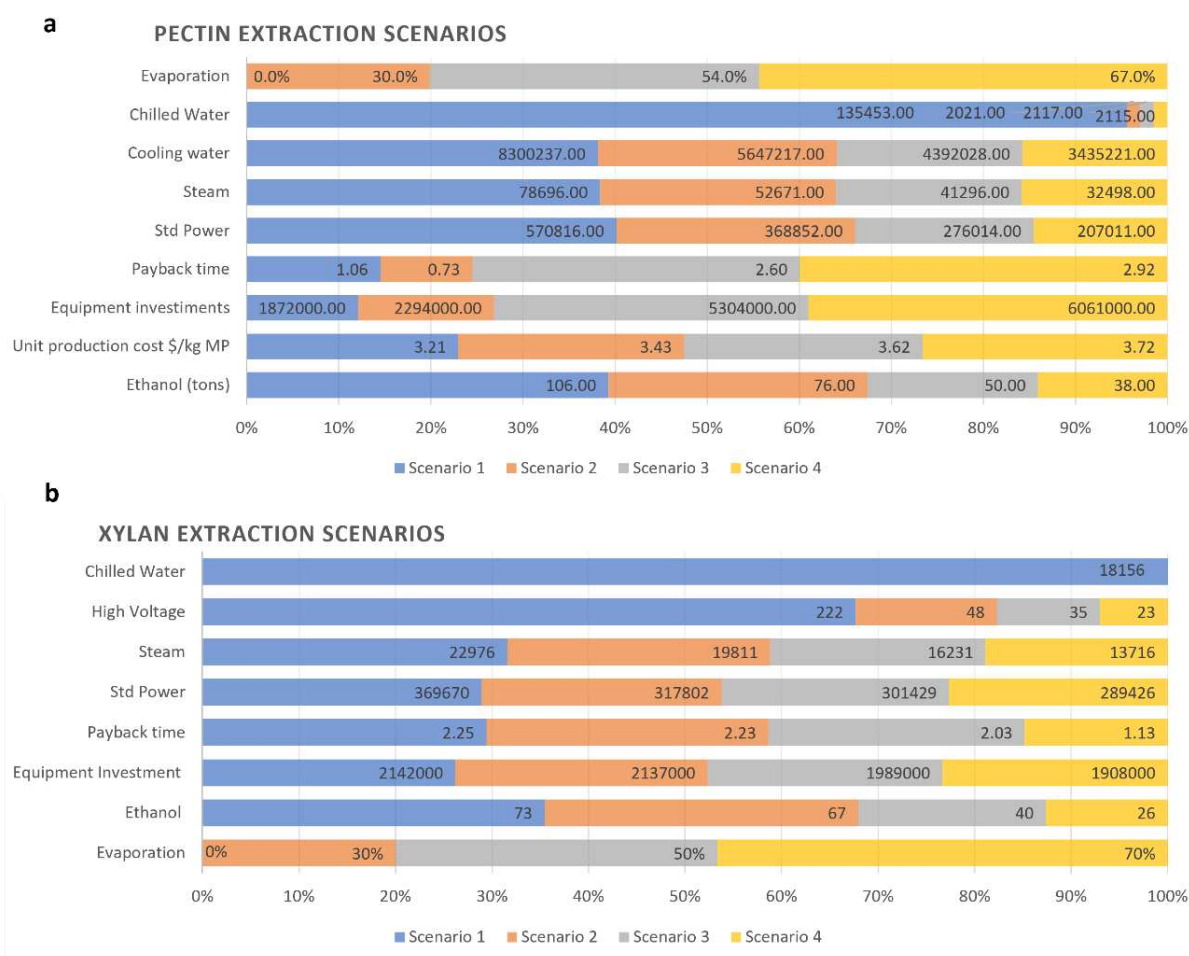


**Figure 9-3 Economic scenarios for precipitation of pectin (a) and xylan (b) on solvent evaporation before ethanol precipitation.**

When considering citric acid recovery, 92 % of the citric acid can be recycled. This process also generates 158.38 kg of gypsum, a soft mineral filler that has applications as fertilizer, a constituent of drywalls, and to produce bioplastics (Murariu et al., 2022). However, the citric acid recovery increased the final unit production cost by \$ 0.40 per kg.

For xylan precipitation, **Figure 9-3b** presents the four scenarios analyzed to reduce the consumption of ethanol and energy. Scenario 4 (70 % evaporation) showed the lowest investment, payback time, ethanol, and energy consumption. Unlike pectin, the xylan-rich extract can be evaporated up to 70 % without disturbing the following operations due to high viscosity.



Table 9-5 Power and solvents consumption and recovery; environment properties, wastes, and emissions

| Power Consumption    | Total Cost    | CHP Plant Revenue | Final Cost     | Savings |
|----------------------|---------------|-------------------|----------------|---------|
| Std Power            | \$ 75,400.00  | \$ 403,540.00     | \$ -328,270.00 |         |
| Steam                | \$ 102,756.00 | \$ 14,516.00      | \$ 88,240.00   | 14.13%  |
| Cooling Water        | \$ 262,196.00 |                   |                |         |
| Chilled Water        | \$ 644.00     |                   |                |         |
| High Voltage         | \$ 1,374.00   |                   |                |         |
| Solvents recovery    | Ethanol       | Citric Acid       | Water          |         |
| Consumed             | 19641358.00   | 23282.00          | 38914146.00    | kg/yr   |
| Recovered            | 19122780.35   | 20661.81          | 33428184.50    | kg/yr   |
| Recovery yield       | 97.36%        | 88.75%            | 85.90%         |         |
| Total consumption    | 518577.65     | 2620.19           | 5485961.50     | kg/yr   |
| Total cost           | \$ 114.09     | \$ 1179.08        | \$ 10,971.92   |         |
| Savings              | \$ 4,207.01   | \$9,297.82        | \$ 66,856.37   |         |
| Environment property | In (kg/yr)    | Out (kg/yr)       | % Reduction    |         |
| TOC                  | 3963358       | 3663278           | 7.57%          |         |
| COD                  | 14076171      | 13314481          | 5.41%          |         |
| BOD5                 | 1440397       | 1166563           | 19.01%         |         |
| TKN                  | 22959         | 22959             | 0.00%          |         |
| TDS                  | 1991781       | 1476244           | 25.88%         |         |
| VDS                  | 1580563       | 1072947           | 32.12%         |         |
| Aqueous waste        | kg/batch      | kg/yr             | %              |         |
| HMF                  | 0.53          | 105               | 0.00%          |         |
| Impurities           | 181.56        | 36386             | 0.12%          |         |
| NaOH                 | 2287.03       | 457406            | 1.54%          |         |
| Phosphoric acid      | 30.5          | 6099              | 0.02%          |         |
| Sulfuric acid        | 3.76          | 753               | 0.00%          |         |
| H2O                  | 145692        | 29138398          | 98.30%         |         |
| Total                | 148206.1      | 29641295          | 100.00%        |         |
| Emissions            | kg/batch      | kg/yr             | %              |         |
| CO <sub>2</sub>      | 27328.92      | 5465784           | 6.18%          |         |
| N <sub>2</sub>       | 313509.9      | 62702700          | 70.94%         |         |
| O <sub>2</sub>       | 63987.95      | 12797808          | 14.48%         |         |
| H <sub>2</sub> O     | 37134.87      | 7427266           | 8.40%          |         |
| Acetic acid          | 0.89          | 159               | 0.00%          |         |
| Sulfuric acid        | 1.64          | 329               | 0.00%          |         |
| Total                | 441961.6      | 88393558          | 100.00%        |         |

### 9.3.5. CHP plant and environment analysis

Anaerobic digestion is one of the most promising techniques for biofuel production because of its easy implementation, versatility, low investment, and appealing alternative for waste management, minimizing the environmental impacts of inappropriate disposal. Therefore, one of the worthwhile applications of OPW is anaerobic digestion (AD) and biogas production due to its high energy content, abundance, and availability. Nevertheless, pretreatment is necessary before AD to reduce the antioxidant compounds, d-limonene, and other components that reduce biogas yields (Jiménez-Castro et al., 2020). This study circumvented the need for pretreatment since it used waste solids (free sugars and cellulose) from the biorefinery stages, improving profitability and sustainability.

The gas turbine generator runs continuously and can produce 4,035,402 kW-h/yr., saving 403,540 \$/yr. (0,10 \$/kW-hr) along with 14,516 \$/yr. from the steam generation (**Supplementary figure 12-15**). Also, 4.5 tons/batch of digestate can be sold at 70 \$/ton, generating an annual credit of 70,000 \$/yr. Using digestate as a biofertilizer in orange crops can reduce the environmental impact of orange production and agrochemicals. It is another way to increase the sustainability of food processing (Mariana et al., 2021). Table 5 shows the total power consumption and savings Table 9-5. The CHP plant supplies 14.13 % of savings related to steam costs, and there is a credit of \$ 328,270 from power generation. Therefore, this biorefinery may present self-sustainability for standard power usage. Table 9-5 also shows that the plant can recover 85.90 % water, 97.36 % ethanol, and 88.75 % citric acid.

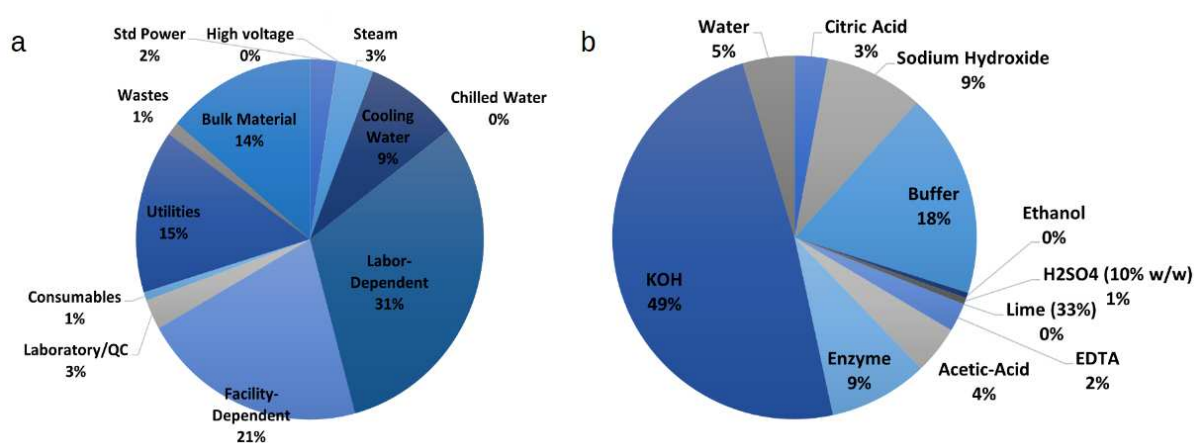


Figure 9-4 Biorefinery cost composition (a) Bulk material cost composition (b).

The designed process showed reductions in all environmental properties, as presented in Table 9-5, decreasing the organic pollution that may accrue from OPW's inadequate disposal. CO<sub>2</sub> emissions correspond to 6 % of total emissions (27 metric tons of CO<sub>2</sub> per 100 tons OPW, or 6 kg per kg of products). This value is below the reported in the literature for pectin production from fruit wastes, accounted for 9.69 kg CO<sub>2</sub> per kg pectin (Nadar et al., 2022), and supports the low environmental impact of the proposed process.

The environmental impact is directly proportional to the biorefinery complexity, but even more complex biorefineries showed better environmental performance than the disposition of OPW in landfills (Mariana et al., 2021). The valorization of OPW contributes to reducing greenhouse gas emissions, water releases, and fossil fuel use since the impact categories related to these aspects are the lowest in the biorefinery (Jiménez-Castro et al., 2020; Mariana et al., 2021). Future experiments must implement a sensitivity analysis to confirm if the extraction of essential oil and phenolic compounds from the washed OPW (before the pectin extraction stage) is also feasible.

**Table 9-6 Executive summary of biorefinery**

| <b>Executive summary</b>                   |               |           |
|--------------------------------------------|---------------|-----------|
| Total Capital Investment                   | 20,005,000.00 | \$        |
| Charged Capital Investment                 | 10,142,000.00 | \$        |
| Net Operating Cost                         | 2,793,918.00  | \$/yr     |
| Total Revenues                             | 16,031,000.00 | \$/yr     |
| Total Credits/savings                      | 93,027.00     | \$/yr     |
| Batch Size                                 | 3,810.81      | kg MP     |
| Cost Basis Annual Rate                     | 762,160.00    | kg MP/yr  |
| Net Unit Production Cost                   | 3.67          | \$/kg MP  |
| Unit Production Revenue                    | 55.00         | \$/kg MP* |
| Gross Margin                               | 82.67         | %         |
| Return On Investment                       | 80.60         | %         |
| Payback Time                               | 1.24          | yr        |
| IRR (After Taxes)                          | 38.01         | %         |
| Net present Value (NPV) (at 7.0% Interest) | 15,217,750.00 | \$        |

\* 15 \$/kg pectin and 40 \$/kg XOS

### 9.3.6. Techno-economic evaluation

**Figure 9-4** presents the cost composition, where bulk materials account for 13%. Since SuperPro pricing models estimated the facility-dependent and utility costs, based on the US market proportional to the purchased equipment cost, these may be overestimated since no adjustment factor to the prices provided by the software was applied (R. D. G. Ferreira et al., 2018). KOH accounts for 49 % of the material cost, and the enzyme accounts for 9 %. Strategies to reduce KOH and enzyme load were crucial to decrease the production cost, even though KOH still represents half the total bulk material cost.

The overall net unit production cost is 3.67 \$/kg of products (**Table 9-6**). **Figure 9-5** shows the cost analysis for different XOS purities, considering or not the citric acid recovery. 98 % purity XOS without citric acid recovery showed an 88.3 % investment return. 80 % XOS showed the lowest production cost, \$3.19/kg; however, it has the lowest selling price and investment return (64.66 %). Nevertheless, these results are superior to recent techno-economic studies, which showed a production cost of 9.80 \$/kg pectin (Nadar et al., 2022) and 3.10 \$/kg 95 % pure XOS (Lan et al., 2021b). The current market price for pectin is \$15/kg (Manhongo et al., 2021) and \$25–\$50/kg for XOS (80-98% purity) (L. B. Brenelli et al., 2022). These values prove the economic feasibility and profitability of the proposed biorefinery.

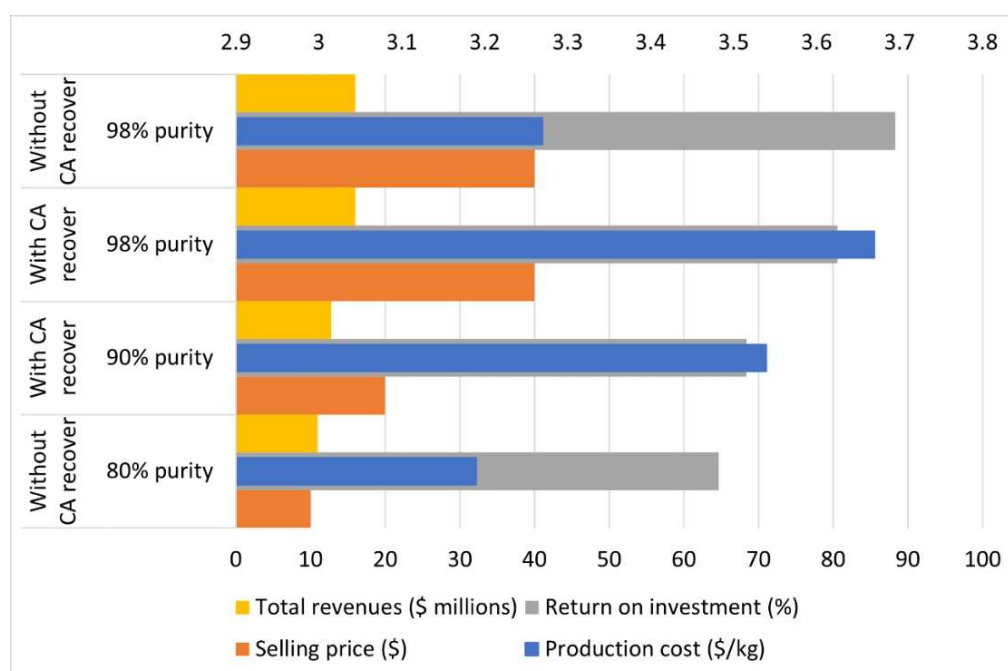


**Figure 9-5** Cost analysis and the investment return according to XOS purity and citric acid recovery.

## 9.4. Conclusion

The presented analysis of the factors affecting the cost on a large scale contributes to understanding the potential use of OPW in the food industry in an energy-self-sufficient and low-environment-impact manner. Process design reduced energy and solvent requirements by more than 60 %. These results are especially relevant for orange processing industries to increase profitability. Future research should investigate the impact of biomass seasonality and the implementation of the proposed biorefinery into existing orange processing industries to reduce initial capital investment. In short, researchers are diversifying OPW exploitation to detour landfilling disposal and its environmental damage while increasing the marketability of bioproducts and other value-added compounds. In this sense, the studies should consider all OPW fractions to obtain a wider diversity of products, creating integrated biorefineries to end up with food ingredients, materials, and bioenergy. In all cases, solvent and water recycling and reuse are critical to reduce environmental footprint and production costs.

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## **CHAPTER 10.**

### **GENERAL DISCUSSION**

## General Discussion

This thesis primary objective was to harness the potential of agroindustrial waste rich in arabinoxylan to produce short-chain xylooligosaccharides (XOS), since XOS are garnering significant attention due to their versatile applications, ranging from their use as prebiotics in the food and feed industries to potential therapeutic benefits, such as promoting gut health (H. H. Chen et al., 2012; McDonagh, 2021).

In a coherent sequence, **CHAPTER 2** and **CHAPTER 3** presented XOS as a functional ingredient, emphasizing the significance of enzymatic hydrolysis of xylan as an efficient and environmentally friendly method for production from agroindustrial wastes, aligning with the overarching theme of this thesis. Chapters 4 and 5 provided the reader with an understanding of the features of lignocellulosic biomass and the biochemical methods employed for its conversion into high-value products, including designing novel multi-functional chimeric enzymes. In **CHAPTER 5**, the concept of chimeric enzymes was introduced, highlighting their importance in enzymatic hydrolysis for the valorization of lignocellulose waste. The chapter emphasized the necessity for a more cost-effective process for enzyme production and the development of more efficient enzymatic cocktails.

As concluded in **CHAPTER 3**, the current state of xylooligosaccharides (XOS) production methods faces challenges, with enzymatic hydrolysis being a greener but complex process requiring biomass pretreatment. XOS-related products are not widely available in the market, urging more research focused on optimizing enzymatic hydrolysis and purification processes within a biorefinery framework for lignocellulosic biomass valorization. To increase market presence, there's a need for the development of XOS-containing products in food, medicines, feed, and health items, while utilizing agro-industrial wastes as an economical source.

The conclusion from the bibliometric analysis revealed that XOS research is a globally expanding field with established concepts and emerging technologies. Enzymatic hydrolysis of agroindustrial wastes is one of the key focus areas, with ongoing efforts to improve purity and reduce costs. The most cited documents highlight the significance of enzyme engineering in a biorefinery concept, offering potential cost reductions. Against this backdrop, this study identified three major wastes generated annually in Brazil to design a bioprocess for XOS and other value-added bioproducts through enzymatic hydrolysis: soy hulls, corn husks, and orange processing waste (Araújo et al., 2019; B. S. Pereira, de Freitas, Vieira, et al., 2022). Despite the relatively low xylan concentrations in orange processing waste, its substantial annual

generation rendered it a suitable candidate when under optimized extractions and enzymatic conditions, which were developed in this thesis (**CHAPTER 9**).

As presented in this thesis, arabinoxylan is a major component of plant cell walls, especially agroindustrial wastes, being composed of a backbone of xylose units linked by  $\beta$ -1,4-glycosidic bonds, with arabinose side chains branching off. To convert arabinoxylan into XOS, the removal of arabinose residues is essential. The enzyme GH 62, with its pronounced affinity for the specific  $\alpha$ -1,2 bonds present in arabinoxylan, plays a pivotal role in catalyzing this process (Izydorczyk, 2021). Despite this essential role and almost exclusive affinity to arabinoxylan, the current body of research on this enzyme family, especially the subfamily 1, remains limited. Our study aims to bridge this gap by conducting a comprehensive exploration of GH 62 enzyme (**CHAPTER 6**, **CHAPTER 7**, and **CHAPTER 8**).

Therefore, the development of an efficient chimeric enzyme initiated with the characterization of a novel GH 62 arabinofuranosidase, as outlined in **CHAPTER 6**. This enzyme demonstrated synergism with endoxylanase for XOS production and enhanced the release of p-coumaric acid and ferulic acid from corn husks. The influence of metal ions on thermostability played a crucial role in the development of the chimeric enzyme, ensuring that both enzymatic modules would have the highest stability under similar conditions since cofactors that improved the stability of the parental arabinofuranosidase also enhanced the activity and thermostability of the chimeric enzyme. The chimera exhibited superior affinity to the substrate and higher degree of synergy, showcased halotolerance surpassing that of the parental enzymes, and demonstrated higher efficiency compared to a commercial cocktail. The thermodynamic characterization of both parental enzymes revealed the enhancements in activity and thermostability achieved through the fusion of enzymes, as presented in **CHAPTER 7**.

Notably, thermostability, particularly in the arabinofuranosidase module, increased by 10°C, enabling operations at higher temperatures with lower enzyme loads. This improvement contributes to increased efficiency and reduced contamination risk. Through the hydrolysis of arabinoxylan derived from agro-industrial wastes at 60°C, the combined action of these enzymes resulted in the obtainment of up to 7 g/L of XOS. The molecular fusion of these synergistic enzymes, as presented in the study, proves to be a promising strategy for generating more efficient and thermostable enzymes while reducing operational costs.

Despite the superior thermostability exhibited by the chimeric enzyme, there existed a notable disparity in the melting temperatures of the enzymatic modules derived from endoxylanase and arabinofuranosidase. To decrease this discrepancy, specific point mutations

were introduced to further enhance thermostability of the GH 62 arabinofuranosidase module. **CHAPTER 8** of the study not only examined regions within the enzyme accountable for thermostability but also facilitated the design of experiments using bioinformatics to avoid laborious time in the lab screening thousands of mutants. This approach is aimed at enhancing the overall stability of the targeted protein, allowing scientists to employ a combinatorial predictive method for the creation of a smaller site saturation library. The data presented in this chapter served to validate the effectiveness and applicability of such a predictive approach in designing a library for systematically improving enzyme thermostability.

The primary objective of this research was to design a distinctive process for xylan extraction and enzymatic hydrolysis using the designed chimeric enzyme tailored for the release of XOS from arabinoxylan substrates. However, the chemical characterization of orange processing waste revealed that, contrary to expectations, the hemicellulose was not constituted by arabinoxylan but by simpler chains of arabinans and xylan. This discovery rendered the use of a chimeric enzyme unnecessary. Consequently, the biorefinery of orange waste was treated as a separate study, detailed in **CHAPTER 9**, wherein a commercial endoxylanase was employed instead of the designed chimeric enzyme for proper costs evaluation.

Furthermore, we demonstrated the technical-economic feasibility of the orange waste biorefinery to obtain not only XOS but also pectin and bioenergy through co-generation of heat and power. The analysis of cost factors on a large scale provides insights into the potential utilization of orange processing waste (OPW) in the food industry, emphasizing self-sufficiency in energy and minimizing environmental impact. Through process design, there has been a notable reduction of over 60% in energy and material requirements, offering significant implications for enhancing profitability in orange processing industries.

## **CHAPTER 11.**

### **GENERAL CONCLUSION AND FUTURE STUDIES**

## General Conclusion

To wrap it up, this thesis has pursued the overarching goal of unlocking the potential of agroindustrial waste for the main purpose of producing xylooligosaccharides (XOS). The identification of three major wastes in Brazil paved the way for the design of a bioprocess for XOS and other value-added bioproducts through enzymatic hydrolysis. XOS, recognized for their diverse applications in various industries, from food and feed to potential therapeutic uses, have become a subject of increasing interest. The exploration was unfolded through these chapters, each contributing a unique perspective to the overarching theme.

In this thesis, we aimed to unravel the properties of GH 62 arabinofuranosidase, its substrate specificity, and potential synergies for efficient arabinose removal in the context of XOS production from agroindustrial waste. By gaining a deeper understanding of GH 62 enzymes, we aim to contribute not only to advancements in bioprocessing technologies but also to the sustainable utilization of agricultural residues for valuable bio-based products.

Additionally, this research explored enzyme engineering, specifically in the context of chimeras and mutants designed to enhance thermostability. By addressing the disparities in melting temperatures between enzymatic modules of the chimera, this thesis contributes to the understanding of GH 62 enzymes and to the broader field of enzyme design and optimization. Chapter 8 not only examined regions within the GH 62 arabinofuranosidase responsible for thermostability but also introduced a novel approach using bioinformatics for designing experiments to avoid extensive and time-consuming laboratory screenings. This demonstrated the potential for systematically improving enzyme thermostability, providing insights for future studies in enzyme engineering and biocatalyst design.

The startling biorefinery of orange waste, treated as a parallel study, showcased not only the production of XOS but also the technical-economic feasibility of obtaining pectin and bioenergy, emphasizing sustainability and profitability. The findings underscore the importance of future initiatives that explore the influence of biomass seasonality and the integration of the proposed biorefinery into existing orange processing plants, aiming to mitigate initial capital investment. Researchers and industries are increasingly diversifying OPW exploitation, steering away from landfill disposal and associated environmental damage. Instead, their focus is on elevating the marketability of bioproducts and other value-added compounds.

To achieve this, studies should comprehensively consider all OPW fractions to yield a broader range of products. Integrated biorefineries are recommended to produce diverse

outputs such as food ingredients, materials, and bioenergy. Throughout these processes, solvent and water recycling and reuse is emphasized to not only reduce the environmental footprint but also cut down on production costs.

In conclusion, this research journey has not only uncovered novel insights into xylooligosaccharides production but has also ventured into enzyme engineering, introducing innovative strategies for enhancing the thermostability and catalytic power of enzymatic cocktails. This dual-focus underscores the multifaceted contributions of this thesis to the fields of biotechnology and bioengineering, offering both practical applications and insights for future research endeavors.

### **Future studies**

From the results of the present thesis, other gaps emerged that can be further explored in the future:

- I. Perform an economic evaluation of orange waste biorefining including the exploitation of other products such as phenolic compounds.
- II. Combine different point mutations in arabinofuranosidase to evaluate further effects on activity and thermostability.
- III. Build a library of linkers and improve signal peptide of chimeric enzyme to evaluate overall enzyme conformation and secretion.
- IV. Optimize fermentation conditions to improve yields of chimera enzyme production.

**CHAPTER 12.**  
**GENERAL REFERENCES, SUPPLEMENTARY**  
**MATERIALS, AND APPENDICES**



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## **SUPPLEMENTARY MATERIAL**

## Supplementary material of CHAPTER 6

Synergism for xylo-oligosaccharides, *p*-coumaric and ferulic acid production and thermostability modulation of GH 62  $\alpha$ -L-arabinofuranosidase

Supplementary table 12-1: Lignocellulosic composition of corn seed hulls

| <i>%</i>             | <b>Corn hull</b> |
|----------------------|------------------|
| <b>Cellulose</b>     | 15.06 $\pm$ 2.90 |
| <b>Hemicellulose</b> | 40.33 $\pm$ 2.31 |
| Xylose               | 17.83 $\pm$ 2.10 |
| Arabinose            | 11.5 $\pm$ 0.20  |
| <b>Lignin</b>        | 2.76 $\pm$ 1.30  |
| <b>Extractives</b>   | 22.92 $\pm$ 2.90 |
| <b>Starch</b>        | 15.03 $\pm$ 3.40 |
| <b>Protein</b>       | 2.61 $\pm$ 0.30  |
| <b>Ashes</b>         | 0.88 $\pm$ 0.10  |

Supplementary table 12-2 Clustal  $\Omega$  multiple alignment of arabinofuranosidase sequences from *A. fumigatus* and other arabinofuranosidases produced by other species (4N1I *Ustilago maydis*; 3WMY *Streptomyces coelicolor*; 6F1J *Talaromyces pinophilus*; 6CC7 *Thermothielavioides terrestres*; 4O8N *Streptomyces thermoviolaceus*; 5UBJ *Aspergillus nidulans*; 5B6S *Coprinopsis cinerea*; 4N2Z *Podospora anserina*; 4PVI *Mycothermus thermophilus*)

CLUSTAL Ω (1.2.4) multiple sequence alignment

Signal Peptide

Query MKLFLLLLSALAAAAALPSSFKWSSSGPLVEPKKDGRNIAAIKDPISIVKVDGTYHFVAS 60  
5B6S\_A:1-316 -----LPPSFRWSSSGPLIGPKSDSRRIGKIDPSVVYHDGRWHVFAS 43  
4N2Z\_A:22-337 -----LPTTWQWTSITGSLVGPKNDRGIAGIKDPTIILINGTHHFVAS 43  
4PVI\_A:22-342 -----SSWKWTSITGSLVLPFKNDERNIAGIKDPTAVLINGTYHFVAS 41  
4N1I\_A:9-302 -----SCALPTTYYWSSAPLAQPKD---GWVSLKDFTHVPYNGQHLVYAS 43  
3WMY\_A:139-435 -----CALPSTYRWSSTGVLQAPKS---GWALKDFTTVTTHNGRHLVYGS 42  
6F1J\_A:24-323 -----SGCALPSTYKWTSTGPLASPKS---GLVALRDYSHVIYNGQHLVYGS 44  
6CC7\_A:17-316 -----AMTCDLPSTYKWTSTGALQAPKN---GWTSLKDFTHVPYNGQHLVYGS 45  
408N\_A:81-381 -----ASCELPSTYKWTSTGALAQPRS---GWVSLKDFTVVPYNGQHLVYAT 44  
5UBJ\_A:13-326 -FAALLWSAPRVLAQCGLPSTYSWTSGLPAEPKD---GWASLKDFTAVPYNGQHLVYAT 56  
::: \*: . \* \*:. :\*: : : \* \* \*:.:

Query TATKSG---NLVYFNFKDFSQANQATFHYLDQTPIGSGYRAAPQVFFFKPHNLWYLVFN 118  
5B6S\_A:1-316 TAKTEG---NLVYISFTDWAQAGSASFYYLDQAPLGTGYRAAPQVFYFAPQRLWYLVFN 101  
4N2Z\_A:22-337 TAQSAG---NLVYFTFADWADAPNATFYLDQAPLGTGYRAAPQVFWAPHKLWYLVFN 101  
4PVI\_A:22-342 TAKSEG---NVMVYFNFTDFAEANNAPFYLDQAPLGVGYRAAPQVFYFEPHKLWYLVFN 99  
4N1I\_A:9-302 YHD-STKYGSMASFPEKHWADMATATQTGMTQA-----AVAPTIFYFTPKKLWFLVFN 96  
3WMY\_A:139-435 TS-SGSSYSGSMVFSPTTNWSDMATAGQANMAQA-----AVAPTIFYFAPKNILWYLVFN 95  
6F1J\_A:24-323 TANTAGSYGSMNFGFLFSDWSEMSSAQNTMTSG-----AVAPTIFYFAPKSVWILAVFN 98  
6CC7\_A:17-316 MV-SGSSYSGSMNFGFLFDWNGMGSVSQNKMSQA-----AVAPTIFYFAPKKVWILAVFN 98  
408N\_A:81-381 YHDTGTRWGSMMNFEFGDWSQMATARQANMNSP-----TVAPTIFYFAPKDIWLVAVFN 98  
5UBJ\_A:13-326 YHDTGTSWGSMMNFGFLFSNWSDMATASQNAQTQS-----TVAPTIFYFEPKDVWILAVFN 110  
.: : \* : . : : : \*\* : \* : \* : \* : \* :

Query GNAAY--STNPNIANPAGWSKPKNFFS-SMPSIIAQNKGNQVWDMWTICDS-----SN 169  
5B6S\_A:1-316 GNAAY--STNPDINNPGWTAPTRTFYS-GMPAIIIRNNINGQVWDMWVICDS-----SL 152  
4N2Z\_A:22-337 GNAAY--STNPDINNPGWTAAPKVFYFPGDMPKIIEQNIGEGYVWDMWVICDS-----AS 153  
4PVI\_A:22-342 GNAAY--STNPDINDPSKWTAPAEVFPYNGMPKIIADNIGNGYVWDMWVCDDEEDPNKAL 157  
4N1I\_A:9-302 GSAPFTYRTSDTPTKVNWSAPQLFTGKVADSGT-----GPDQTVIADG-----RK 144  
3WMY\_A:139-435 GSWPFYIYRTSDPTDPNGWSAPQLFTGSGISGSDT-----GPDQTLIADG-----QN 143  
6F1J\_A:24-323 GPYAFSRYTSDTPSNANGWSSPQLFTGTIGSGST-----GVIDQTVIGDS-----EN 146  
6CC7\_A:17-316 GPTSFSYRTSDPTNPNGWSSPQLFTGTIGSGST-----GVIDQTVIGDD-----TN 146  
408N\_A:81-381 GGSAFSRYTSDPTDPNGWSSQQLFTGSIADSAT-----GPDQTLIIGD-----TH 146  
5UBJ\_A:13-326 GPATFSYLTSDPTDPANGWSSQQLFTSGSISDSDT-----GVIDQTVIGDS-----TT 158  
\* : \* : . : \* : : : : : \* : \* : \*

Query CYLFSSDNGHLHYSQTSLANFPNGMGNT-VIALSDSN-KNALFEASNYYHT----GNGE 223  
5B6S\_A:1-316 CHLFSSDNGHLHYSQTSLSNFPNGMGNEP-VIAMQDSN-KNRLFEEANVYRID---GSNE 207  
4N2Z\_A:22-337 CHLFSSDNGHLHYSQTSLEQFPNGMSQP-VIAMQDNRN---DLFEACAVYSLPD---GK 206  
4PVI\_A:22-342 CHLFSSDNGHLHYSQTTLAQFPNGMSEP-EIVLQQTNYIALAEACIYRKGAEGTQK 216  
4N1I\_A:9-302 VYLFVFAVNGKYVYRTSMAIGDFFANFGTASEVILSDTQ---AKLFEAVQVYTVAGQ---NQ 199  
3WMY\_A:139-435 MYLFFAGDNGKHYRASMPIGNFPGNFGSSYTTIMSDTK---ANLFEAGVQVYKVGQ---NQ 198  
6F1J\_A:24-323 MYLFFAGDNGHYRASMPIGNDFPGSFGSASTIVLSDST---NNLFEAVEVYTVGEQ---NQ 201  
6CC7\_A:17-316 MYLFFAGDNGKHYRASMPIGNFPGSFGSSSTVVMDSST---NNLFEAVQVYKLQGG---TK 201  
408N\_A:81-381 MYLFFAGDNGKHYRASMPIGNDFPGSFGSTATSESNPFGMSDTR---NNLFEAPQVYKLQGG---NR 201  
5UBJ\_A:13-326 MYLFFAGDNGRIYRASMPIDQFPDGTSEVIELSDER---NNLFEAVQVYTVSGQ---SKD 215  
:\*\* \*\*\*\*\*: : \* : \* : \* : \* : \*

Query YLLLVAEIGSDGQRYFSWTSNSLTGWSKGLANTEANPFARSNVAVFSGTPWTKSISHGE 283  
5B6S\_A:1-316 YLLLVHAEIGSDGRRWFSWTSSTSIAGPWRALADQESNPFARANNVAFGTGNAWTRDISHGE 267  
4N2Z\_A:22-337 YLLLVHAEIGTDGHRWFSWTDASIRGPWQGLANTEQNPWARSNNVQFDGVDWTKSISHGE 266  
4PVI\_A:22-342 YLLLVHAEIGQGHRYFSWTSDRIDGQWPLADTEANPWAGEANVVEFGQVWTKSISHGE 276  
4N1I\_A:9-302 YLMIVAEAQGTNG-RYFSFTANSLDGEWKVQAGSEAPFAGKAN---SGASWTNDVSHGD 255  
3WMY\_A:139-435 YLMIVAEAMGANG-RYFSFTASSLGGSWTPQAASEGNPFAGKAN---SGATWTDNISHGD 254  
6F1J\_A:24-323 YLMIVAEIAGANG-RYFSFTASSLGGTWTQAASTENPFAFAGKAN---SGATWTDNISSGD 257  
6CC7\_A:17-316 YLMIVAEIGSQG-RYFSFTATSLGGSWTPNAATESPPFAGKAN---SGATWTDNISHGD 257  
408N\_A:81-381 YLMIVAEIGAQQRYFSFTATSLDGEWTPQATSESNPFAGKAN---SGATWTDNISHGE 258  
5UBJ\_A:13-326 YLMIVAEIGAQQ-RYFSFTADSLGGSWTPQAATESAPFAGKAN---SGATWTDNISHGD 271  
\*\*\*: \* : \* : \* : \* : \* : \* : \* : \* : \*

Query LIRTTDQTM-T-ISPCKLQYLYGLSPSA-GGDYNLLPWKLGLITQNTNSC 332  
5B6S\_A:1-316 LIRSGNDQTL-T-ISPCLRYLYQGLDPNS-GGDYNLPLPWKLGLITQNTNSAC 316  
4N2Z\_A:22-337 IIRDTGVDEKLLIDPCNIRFMYQGMPSA-GGEYNALPWRLGFIAHNNPAC 316  
4PVI\_A:22-342 VIRTLTDQTLTLDLSEPIQLFYQGVDPNA-QTEYNALPWRLGLITQ----- 321  
4N1I\_A:9-302 LIRSNPDQTM-LIDPCRLQLLYQGRDKNPSSVDLAPRY----- 294  
3WMY\_A:139-435 LVRNDPDQTM-T-VDPCLNLQFLYQGSFSPA-GGDYNLPLPWRPGVLT----- 297  
6F1J\_A:24-323 LVRNTNDQTM-T-IDACNLQFLYQGRSTSS-GGDYNLPLPYQPGLLT----- 300  
6CC7\_A:17-316 LVRTPDQTM-T-VDPCLNLQFLYQGRAPNS-GGSYNLPLPYRPGLLT----- 300  
408N\_A:81-381 LIRTTADQTM-T-VDPCLNLQFLYQGRDPGS-GGTYDLLPYRPGLLT----- 301  
5UBJ\_A:13-326 LVRSTPDQTM-S-IDPCNLQLLYQGRDPSL-NPGYDLLPYRPGLLT----- 314  
: : \* : \* : \* : \* : \* : \* : \* : \*

Asterisks (\*) indicate conserved residues; yellow residues indicate the active site; green residues indicate the substrate binding site; red residues indicate the calcium binding site; and the blue residue is responsible for holding the central calcium atom.

Supplementary table 12-3 CCRD matrix and enzymatic activity

| Assay | Real values |     | Coded values |       | Activity<br>(U/μL) |
|-------|-------------|-----|--------------|-------|--------------------|
|       | T (°C)      | pH  | T (°C)       | pH    |                    |
| 1     | 30          | 3.8 | -1           | -1    | 40.335             |
| 2     | 50          | 3.8 | 1            | -1    | 33.216             |
| 3     | 30          | 5.8 | -1           | 1     | 54.155             |
| 4     | 50          | 5.8 | 1            | 1     | 68.393             |
| 5     | 25.9        | 4.8 | -1.41        | 0     | 44.942             |
| 6     | 54.1        | 4.8 | 1.41         | 0     | 43.685             |
| 7     | 40          | 3.4 | 0            | -1.41 | 31.960             |
| 8     | 40          | 6.2 | 0            | 1.41  | 57.924             |
| 9     | 40          | 4.8 | 0            | 0     | 64.624             |
| 10    | 40          | 4.8 | 0            | 0     | 64.205             |
| 11    | 40          | 4.8 | 0            | 0     | 65.043             |

Supplementary table 12-4 ANOVA of enzymatic activity ( $p < 0.05$ ) for model prediction of optimum temperature ( $x_1$ ) and pH ( $x_2$ ) conditions and experimental validation of the modeled equation.

| Source of variation                                            | Sum of quads | Degree of freedom | Mean square         | f-calculated        | p-value |
|----------------------------------------------------------------|--------------|-------------------|---------------------|---------------------|---------|
| Regression                                                     | 970          | 4                 | 242                 | 37                  | 0.00024 |
| Residues                                                       | 40           | 6                 | 7                   |                     |         |
| Lack of adjustment                                             | 40           | 4                 | 10                  | 101.2               | 0.00981 |
| Pure error                                                     | 0.2          | 2                 | 0.1                 |                     |         |
| Total                                                          | 1,009.8      | 10                |                     |                     |         |
| R <sup>2</sup>                                                 |              |                   |                     |                     | 96.03%  |
| $Y_1 = 48.47 - 6.79 x_1^2 + 8.04 x_2 - 6.56 x_2^2 + 4 x_1 x_2$ |              |                   |                     |                     |         |
| Experimental validation                                        |              | Predicted value   |                     | Experimental value  |         |
| pH                                                             |              | 4.8               |                     |                     |         |
| Temperature                                                    |              | 42 °C             | 68.093 ± 2.413 U/μL | 70.487 ± 1.813 U/μL |         |

**Supplementary material of CHAPTER 7**

**Novel bi-functional thermostable chimeric enzyme for feasible xylo-oligo-saccharides production from agro-industrial wastes**

Supplementary table 12-5 CCRD matrix for enzymatic activity of chimeric enzyme

| <b>Endoxylanase</b>         | <b>Real values</b> |           | <b>Coded values</b> |           | <b>Activity<br/>(U/μL)</b> |
|-----------------------------|--------------------|-----------|---------------------|-----------|----------------------------|
|                             | <b>T (°C)</b>      | <b>pH</b> | <b>T (°C)</b>       | <b>pH</b> |                            |
| 1                           | 55.0               | 3.8       | -1                  | -1        | 55.989                     |
| 2                           | 75.0               | 3.8       | +1                  | -1        | 26.174                     |
| 3                           | 55.0               | 5.8       | -1                  | +1        | 68.949                     |
| 4                           | 75.0               | 5.8       | +1                  | +1        | 93.937                     |
| 5                           | 50.9               | 4.8       | -1.41               | 0         | 64.629                     |
| 6                           | 79.1               | 4.8       | +1.41               | 0         | 65.053                     |
| 7                           | 65.0               | 3.4       | 0                   | -1.41     | 20.837                     |
| 8                           | 65.0               | 6.2       | 0                   | +1.41     | 77.081                     |
| 9                           | 65.0               | 4.8       | 0                   | 0         | 91.819                     |
| 10                          | 65.0               | 4.8       | 0                   | 0         | 91.734                     |
| 11                          | 65.0               | 4.8       | 0                   | 0         | 90.040                     |
| <b>Arabino-furanosidase</b> | <b>Real values</b> |           | <b>Coded values</b> |           | <b>Activity<br/>(U/μL)</b> |
|                             | <b>T (°C)</b>      | <b>pH</b> | <b>T (°C)</b>       | <b>pH</b> |                            |
| 1                           | 40.0               | 3.8       | -1                  | -1        | 33.216                     |
| 2                           | 60.0               | 3.8       | +1                  | -1        | 79.281                     |
| 3                           | 40.0               | 5.8       | -1                  | +1        | 45.361                     |
| 4                           | 60.0               | 5.8       | +1                  | +1        | 53.736                     |
| 5                           | 36.0               | 4.8       | -1.41               | 0         | 30.703                     |
| 6                           | 64.0               | 4.8       | +1.41               | 0         | 91.844                     |
| 7                           | 50.0               | 3.4       | 0                   | -1.41     | 46.617                     |
| 8                           | 50.0               | 6.2       | 0                   | +1.41     | 34.472                     |
| 9                           | 50.0               | 4.8       | 0                   | 0         | 87.657                     |
| 10                          | 50.0               | 4.8       | 0                   | 0         | 88.075                     |
| 11                          | 50.0               | 4.8       | 0                   | 0         | 88.075                     |

Supplementary table 12-6 ANOVA of pH (x<sub>1</sub>) and temperature (x<sub>2</sub>) effects on enzymatic activity and experimental validation of the generated model for endoxylanase and arabinofuranosidase modules

| Endoxylanase                                                                                                                                                                  |              |                     |             |                     |         |  |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|---------------------|-------------|---------------------|---------|--|
| Source of variation                                                                                                                                                           | Sum of quads | Degree of freedom   | Mean square | f-calculated        | p-value |  |
| Regression                                                                                                                                                                    | 404          | 4                   | 101         | 210                 | 0.00002 |  |
| Residues                                                                                                                                                                      | 3            | 6                   | 1           |                     |         |  |
| Lack of adjustment                                                                                                                                                            | 3            | 4                   | 1           | 11.2                | 0.08368 |  |
| Pure error                                                                                                                                                                    | 0.1          | 2                   | 0.1         |                     |         |  |
| Total                                                                                                                                                                         | 407          | 10                  |             |                     |         |  |
| R²                                                                                                                                                                            |              |                     |             |                     | 99.26%  |  |
| Y <sub>1</sub> = 22,80 - 0,13 x <sub>1</sub> - 3,02 x <sub>1</sub> <sup>2</sup> + 5,01 x <sub>2</sub> - 5,01 x <sub>2</sub> <sup>2</sup> + 3,43 x <sub>1</sub> x <sub>2</sub> |              |                     |             |                     |         |  |
| Experimental validation                                                                                                                                                       |              | Predicted Value     |             | Experimental Value  |         |  |
| pH                                                                                                                                                                            | 5.3          | 96.680 ± 1.76 U/μL  |             | 98.004 ± 1.813 U/μL |         |  |
| Temperature                                                                                                                                                                   | 70 °C        |                     |             |                     |         |  |
| Arabinofuranosidase                                                                                                                                                           |              |                     |             |                     |         |  |
| Source of variation                                                                                                                                                           | Sum of quads | Degree of freedom   | Mean square | f-calculated        | p-value |  |
| Regression                                                                                                                                                                    | 3,431        | 4                   | 858         | 36                  | 0.00025 |  |
| Residues                                                                                                                                                                      | 143          | 6                   | 24          |                     |         |  |
| Lack of adjustment                                                                                                                                                            | 143          | 4                   | 36          | 1083.1              | 0.00092 |  |
| Pure error                                                                                                                                                                    | 0.2          | 2                   | 0           |                     |         |  |
| Total                                                                                                                                                                         | 3,574        | 10                  |             |                     |         |  |
| R²                                                                                                                                                                            |              |                     |             |                     | 95.99%  |  |
| Y <sub>1</sub> = 65,95 + 13,21 x <sub>1</sub> - 9,63 x <sub>1</sub> <sup>2</sup> - 17,40 x <sub>2</sub> <sup>2</sup> + 7,07 x <sub>1</sub> x <sub>2</sub>                     |              |                     |             |                     |         |  |
| Experimental validation                                                                                                                                                       |              | Predicted Value     |             | Experimental Value  |         |  |
| pH                                                                                                                                                                            | 4.8          | 93.427 ± 4.000 U/μL |             | 91.201 ± 3.630 U/μL |         |  |
| Temperature                                                                                                                                                                   | 55 °C        |                     |             |                     |         |  |

Supplementary table 12-7 CCRD matrix for enzymatic activity of the parental enzymes endoxylanase and arabinofuranosidase

| <b>Endoxylanase<br/>parental</b>              | <b>Real values</b>                |           | <b>Coded values</b>               |           | <b>Activity<br/>(U/<math>\mu</math>L)</b> |
|-----------------------------------------------|-----------------------------------|-----------|-----------------------------------|-----------|-------------------------------------------|
|                                               | <b>T (<math>^{\circ}</math>C)</b> | <b>pH</b> | <b>T (<math>^{\circ}</math>C)</b> | <b>pH</b> |                                           |
| 1                                             | 55.0                              | 3.8       | -1                                | -1        | 2.310                                     |
| 2                                             | 75.0                              | 3.8       | +1                                | -1        | 0.260                                     |
| 3                                             | 55.0                              | 5.8       | -1                                | +1        | 3.270                                     |
| 4                                             | 75.0                              | 5.8       | +1                                | +1        | 4.370                                     |
| 5                                             | 50.9                              | 4.8       | -1.41                             | 0         | 3.060                                     |
| 6                                             | 79.1                              | 4.8       | +1.41                             | 0         | 3.150                                     |
| 7                                             | 65.0                              | 3.4       | 0                                 | -1.41     | 0.090                                     |
| 8                                             | 65.0                              | 6.2       | 0                                 | +1.41     | 3.850                                     |
| 9                                             | 65.0                              | 4.8       | 0                                 | 0         | 4.710                                     |
| 10                                            | 65.0                              | 4.8       | 0                                 | 0         | 4.790                                     |
| 11                                            | 65.0                              | 4.8       | 0                                 | 0         | 4.740                                     |
| <b>Arabino-<br/>furanosidase<br/>parental</b> | <b>Real values</b>                |           | <b>Coded values</b>               |           | <b>Activity<br/>(U/<math>\mu</math>L)</b> |
|                                               | <b>T (<math>^{\circ}</math>C)</b> | <b>pH</b> | <b>T (<math>^{\circ}</math>C)</b> | <b>pH</b> |                                           |
| 1                                             | 40.0                              | 3.8       | -1                                | -1        | 40.335                                    |
| 2                                             | 60.0                              | 3.8       | +1                                | -1        | 33.216                                    |
| 3                                             | 40.0                              | 5.8       | -1                                | +1        | 54.155                                    |
| 4                                             | 60.0                              | 5.8       | +1                                | +1        | 68.393                                    |
| 5                                             | 36.0                              | 4.8       | -1.41                             | 0         | 44.942                                    |
| 6                                             | 64.0                              | 4.8       | +1.41                             | 0         | 43.685                                    |
| 7                                             | 50.0                              | 3.4       | 0                                 | -1.41     | 31.960                                    |
| 8                                             | 50.0                              | 6.2       | 0                                 | +1.41     | 57.924                                    |
| 9                                             | 50.0                              | 4.8       | 0                                 | 0         | 64.624                                    |
| 10                                            | 50.0                              | 4.8       | 0                                 | 0         | 64.205                                    |
| 11                                            | 50.0                              | 4.8       | 0                                 | 0         | 65.043                                    |



Supplementary table 12-8 ANOVA of pH (x1) and temperature (x2) effects on enzymatic activity and experimental validation of the generated model for parental endoxylanase and arabinofuranosidase enzymes

| Endoxylanase (pa-<br>rental)                                                                                                                           |              |                   |                   |              |                    |  |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|-------------------|-------------------|--------------|--------------------|--|
| Source of variation                                                                                                                                    | Sum of quads | Degree of freedom | Mean<br>square    | f-calculated | p-value            |  |
| Regression                                                                                                                                             | 27.8         | 4                 | 7                 | 173.2        | 0.000              |  |
| Residues                                                                                                                                               | 0.2          | 6                 | 0                 |              |                    |  |
| Lack of adjustment                                                                                                                                     | 0.2          | 4                 | 0.1               | 36.4         | 0.027              |  |
| Pure error                                                                                                                                             | 0.00         | 2                 | 0                 |              |                    |  |
| Total                                                                                                                                                  | 28.10        | 10                |                   |              |                    |  |
| R <sup>2</sup>                                                                                                                                         |              |                   |                   |              | 99.14%             |  |
| Y <sub>1</sub> = 4,75 - 0,82 x <sub>1</sub> <sup>2</sup> + 1,30 x <sub>2</sub> - 1,38 x <sub>2</sub> <sup>2</sup> + 0,79 x <sub>1</sub> x <sub>2</sub> |              |                   |                   |              |                    |  |
| Experimental validation                                                                                                                                |              |                   | Predicted Value   |              | Experimental Value |  |
| pH                                                                                                                                                     | 5.3          |                   | 5.04 ± 0.13 U/μL  |              | 4.94 ± 0.18 U/μL   |  |
| Temperature                                                                                                                                            | 70 °C        |                   |                   |              |                    |  |
| Arabinofuranosidase<br>(parental)                                                                                                                      |              |                   |                   |              |                    |  |
| Source of variation                                                                                                                                    | Sum of quads | Degree of freedom | Mean<br>square    | f-calculated | p-value            |  |
| Regression                                                                                                                                             | 970          | 4                 | 242               | 37           | 0.00024            |  |
| Residues                                                                                                                                               | 40           | 6                 | 7                 |              |                    |  |
| Lack of adjustment                                                                                                                                     | 40           | 4                 | 10                | 101.2        | 0.00981            |  |
| Pure error                                                                                                                                             | 0.2          | 2                 | 0.1               |              |                    |  |
| Total                                                                                                                                                  | 1,009.8      | 10                |                   |              |                    |  |
| R <sup>2</sup>                                                                                                                                         |              |                   |                   |              | 96.03%             |  |
| Y <sub>1</sub> = 48,47 - 6,79 x <sub>1</sub> <sup>2</sup> + 8,04 x <sub>2</sub> - 6,56 x <sub>2</sub> <sup>2</sup> + 4 x <sub>1</sub> x <sub>2</sub>   |              |                   |                   |              |                    |  |
| Experimental validation                                                                                                                                |              |                   | Predicted Value   |              | Experimental Value |  |
| pH                                                                                                                                                     | 4.5          |                   | 50.94 ± 1.58 U/μL |              | 49.78 ± 1.63 U/μL  |  |
| Temperature                                                                                                                                            | 40 °C        |                   |                   |              |                    |  |

Supplementary material of CHAPTER 8

Thermostability enhancement of GH 62  $\alpha$ -L-arabinofuranosidase by directed evolution and rational design.

Supplementary table 12-9 Hotspots identified by sequence consensus strategy and calculated  $\Delta\Delta G$  (kcal/mol) of respective mutations using Hotspot wizard server 3.0.  $\Delta\Delta G \leq -2.5$  kcal/mol was labeled in red. The symbol \* indicates the variants found in the epPCR screening, and the symbol • indicate the variants selected after site-saturation mutagenesis experiments.

|       | A      | R     | N    | D      | C     | Q     | E    | G       | H       | I    | K      | L     | M    | F      | P     | S      | T       | V    | W     | Y      |
|-------|--------|-------|------|--------|-------|-------|------|---------|---------|------|--------|-------|------|--------|-------|--------|---------|------|-------|--------|
| S127  | -4.9 * | -11.5 | -5.9 | -2.4   | -0.4  | -5.1  | -3   | -3.3 •  | -6.1    | 6.6  | -1.5   | -4    | -7.4 | -1.1   | 119.3 | -      | -6.5    | 9.4  | -7    | -11.8  |
| G205  | -0.1   | 0.3   | 0    | -2.5 * | 0.5   | -3.2  | -0.1 | -       | 0.6     | 2.4  | -8.5 • | -0.7  | -1.4 | -1.5   | 88.6  | 0.1    | 1       | -5.2 | -0.1  | -0.7   |
| Q271  | -0.4   | 2.8   | 0.5  | 1.7    | -1    | -     | 1.2  | 2       | -12.6 * | -1   | -3.4   | -10.5 | -0.6 | 0.8    | 123.2 | -0.3   | -11.5 • | 0.2  | -3.2  | 1.3    |
| T256  | 4.6    | 5.9   | 9.9  | 11     | -15.3 | 6.4   | 10.9 | -18.4 • | 3.1     | 10.7 | 11     | 4.2   | 10.8 | 6.9    | 6.4   | -9.3 * | -       | 6.4  | 8.1   | 4.3    |
| S188  | 0.1    | 0.2   | -0.3 | 0.1    | 2.1   | -0.1  | 0.1  | -5.7    | -4.8    | 0.9  | 0.1    | -6    | -6.1 | -5.4 • | 1.4   | -      | 0.5     | 0.8  | -3.2  | -6.1 * |
| D297  | -6.8   | 2.6   | -2.2 | -      | -9.8  | -5.1  | -2.9 | -1.5    | -6.8    | -0.4 | -1.2   | 0.6   | -1.4 | -10.9  | -0.8  | 3      | 1.8     | -1.6 | -0.4  | -0.7   |
| V14   | -6.3   | 10.1  | -5.1 | 4      | 6.8   | -1    | 0.3  | 3.5     | 1.6     | -9.5 | 10.2   | -0.6  | 2.6  | 15.5   | 183.6 | 4.6    | 1.4     | -    | 2.5   | 14.5   |
| K120  | -3.7   | 0.4   | 0.1  | 0.7    | -1.8  | 0     | 0.6  | 0       | 0.2     | 0.6  | -      | 10.8  | 0.8  | -2.9   | 4.4   | -3.7   | -2.1    | 4.7  | 7     | -0.4   |
| N204  | -1.2   | 4.7   | -    | -0.1   | 0.8   | 0.2   | -0.4 | -4      | 0.8     | 5.8  | -0.7   | 0.5   | 1.1  | -7.4   | 53.9  | -7.9   | 4.8     | 1    | -6.5  | -7.8   |
| N239  | -0.4   | -0.9  | -    | -12.9  | -3.4  | -3.9  | 1.2  | -3.6    | -2.7    | 5.6  | -1.2   | -3.3  | 9.2  | -5.6   | 27    | -0.5   | -1.6    | 6.5  | -11.1 | -5.3   |
| N140  | -6.1   | 1.9   | -    | 5.9    | 1.3   | -2.3  | -0.9 | 0.4     | 1.4     | 6.5  | 6.5    | 1.2   | -1.9 | 0.3    | 138.9 | 4.7    | 0.9     | 0.7  | 0.7   | -0.8   |
| N228  | 5.2    | 4.7   | -    | -9.5   | 7.1   | 4.3   | -1.7 | -8.4    | -9.4    | 5    | 4.5    | 4.1   | 4.4  | 0.2    | 0.7   | -1.8   | 0       | 5    | -2.7  | -0.4   |
| M128  | 0.1    | 2.9   | 2.7  | 2.5    | -8.3  | 1.6   | 1.9  | -3.6    | -0.2    | -12  | -11.1  | 6.8   | -    | -6.4   | 3.3   | 2.6    | 0.1     | 0.2  | 0.7   | 4.2    |
| S10   | -5.4   | 0.8   | 3.6  | 4.4    | 2.8   | 6.3   | 0.9  | 1.4     | -5.7    | 10.3 | 2.3    | 3.1   | 2.6  | 5.8    | 152.4 | -      | 2.3     | 8.5  | 5     | -9.4   |
| N101  | -3.2   | -4    | -    | -11.7  | -2.6  | -10.1 | -1.3 | -2.7    | 2.3     | -2.4 | -2.2   | 0.5   | -5   | 3.7    | 122.1 | -4     | 1.9     | -3.9 | -2.2  | -2.7   |
| N111  | 2.8    | 1.5   | -    | -1.7   | 3.6   | -5    | 2.3  | 1.9     | -4.9    | -4.7 | -4.6   | 0.7   | 1.7  | -4.9   | 44.7  | 2.1    | -4.3    | -0.3 | -4    | -1.9   |
| P12   | 0.7    | 3.9   | 3.2  | 8.7    | -2.5  | 3.4   | 1.3  | 3.4     | 72      | -0.3 | 1.5    | 12.6  | -2.7 | 77.1   | -     | 4.7    | -3.2    | 0.9  | -4.4  | 77.1   |
| **K27 | -1     | -2.4  | -0.2 | 2.3    | 0.6   | -3.3  | 0.5  | -2.3    | 6.7     | 2.1  | -      | 1.5   | -2   | 32.9   | 133   | -2.1   | -5.7    | 3    | 8.9   | 33.4   |
| **I76 | -6.9   | 12.4  | 1.4  | 3.8    | -1    | -12.1 | -3.9 | -2.7    | 2.8     | -    | 3.1    | -6.3  | -4.7 | 5      | 248.3 | 0.8    | -5.1    | 1.7  | 55.8  | 17.2   |

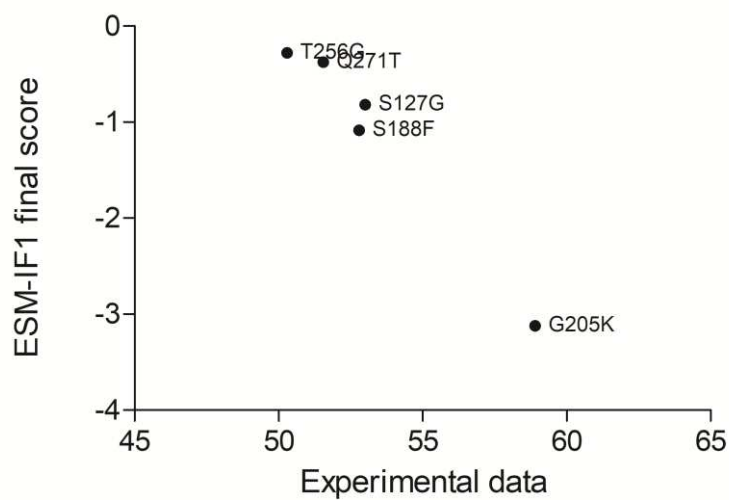


Supplementary figure 12-1 SDS-page of Wild type (WT) and variants purification

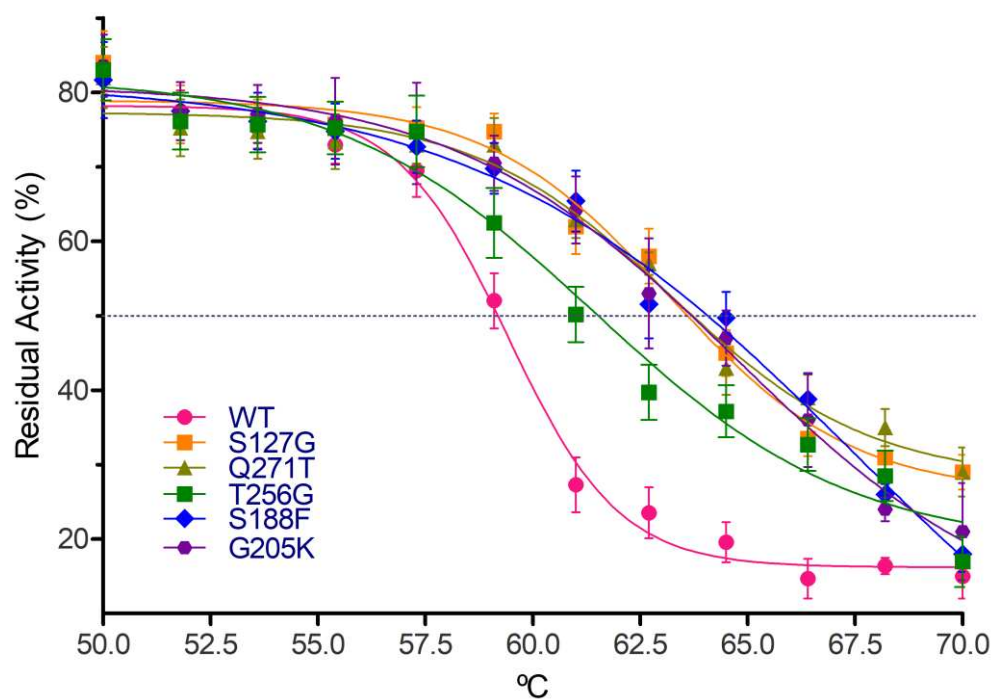
**Supplementary table 12-10 Clustal  $\Omega$  multiple alignment of arabinofuranosidase sequences from *A. fumigatus* and other arabinofuranosidases produced by other species (4N1I *Ustilago maydis*; 3WMY *Streptomyces coelicolor*; 6F1J *Talaromyces pinophilus*; 6CC7 *Thermothielavioides terrestres*; 4O8N *Streptomyces thermoviolaceus*; 5UBJ *Aspergillus nidulans*; 5B6S *Coprinopsis cinerea*; 4N2Z *Podospora anserina*; 4PVI *Mycothermus thermophilus*)**

| CLUSTAL $\Omega$ (1.2.4) multiple sequence alignment |                                                                |     |
|------------------------------------------------------|----------------------------------------------------------------|-----|
| Signal Peptide                                       |                                                                |     |
| Query                                                | MKLFLLLLISALAAAAALPSSFKWSGSLVPEKKDGRNIAAIKDPSTVKVDGTYYHFAS     | 60  |
| 5B6S_A:1-316                                         | -----LPSSFWRSSSGPLIGPKSDSRRIGQIKDPSVYHDGRWHVFAS                | 43  |
| 4N2Z_A:22-337                                        | -----LPTTQWMTSTGLVGPKNDRGRIAGIKDPTIILINGTHHFAS                 | 43  |
| 4PVI_A:22-342                                        | -----SSWKWVSTGLVFPKNDERNIAGIKDPTAVLINGTYHFAS                   | 41  |
| 4N1I_A:9-302                                         | -----SCALPTTYRWTSAPLAQPKD---GWVSLKDFTHVPYNGQHLVYAS             | 43  |
| 3WMY_A:139-435                                       | -----CALPSTYRWSSSTGLAQPKS---GVALKDFTTVTHNGRHLVYGS              | 42  |
| 6F1J_A:24-323                                        | -----SGCALPSTYKWTSTGLASPKS---GLVALRDYSHVIYNGQHLVYGS            | 44  |
| 6CC7_A:17-316                                        | -----AMTCDLPTTYKWNSTGALAQPKN---GWTSKDFTHVPYNGQHLVYGS           | 45  |
| 4O8N_A:81-381                                        | -----ASCCLPSTYRWTSGLAQPKN---GWVSLKDFTVVPYNGQHLVYAT             | 44  |
| 5UBJ_A:13-326                                        | -FAALLWSAPRVLAQCGLPSTYSWTSTGLAEPKD---GWASLKDFTAVPYNGQVLYVAT    | 56  |
| ::: * * . * * . . : * : : * * * : :                  |                                                                |     |
| Query                                                | TATKSG--YNLVYFNKDFDSQANQATFHYLDQTPIGSGYRAAPQVFFFKPHNLWYLVFQN   | 118 |
| 5B6S_A:1-316                                         | TAKTEG--YNLVYISFTDWAQAGSASFYLDQAPLGTGYRAAPQVYFAPQRLWYLVYQN     | 101 |
| 4N2Z_A:22-337                                        | TAQSAG--YNLVYFTFADWADAPNATFYLDQAPLGTGYRAAPQVFWFAPHKLWYLVYQN    | 101 |
| 4PVI_A:22-342                                        | TAKSEG--YNMVYFNFTDFAEANNAPFYLDQAPLGVYRAAPQVYFEPHKLWYLVYQN      | 99  |
| 4N1I_A:9-302                                         | YHD-STKYGSMASFSPKHWADMATATQTGMTQA-----AVAPTIFYFAPKNIWLYVQW     | 96  |
| 3WMY_A:139-435                                       | TS-SGSSYSGSMVFSPFTNWSMDASAGQNAMNQ-----AVAPTIFYFAPKNIWLYVQW     | 95  |
| 6F1J_A:24-323                                        | TANTAGSYGSMNFGFLFSDWSMESSASQNTMTSG-----AVAPTIFYFAPKNIWLYVQW    | 98  |
| 6CC7_A:17-316                                        | MV-SGGSYSGSMNFGFLTDWNGMGVSQNKMSQA-----AVAPTIFYFAPKNIWLYVQW     | 98  |
| 4O8N_A:81-381                                        | THDTGTWGSMMNFEPPGDSQMATARQNAMNSP-----TVAPTIFYFAPKNIWLYVQW      | 98  |
| 5UBJ_A:13-326                                        | YHDTGTWGSMMNFGFLFSDWSMATASQNAMTQS-----TVAPTIFYFAPKNIWLYVQW     | 110 |
| : : : * : . . : : : * * : * * : * * * *              |                                                                |     |
| Query                                                | GNAAY--STNPNIANPAGWSKPKNFFS-SMPSIIAQNKGNWDMWTICDS-----SN       | 169 |
| 5B6S_A:1-316                                         | GNAAY--STNPDIINNPAGWTAPRTFYS-GMPAIIRNNIINGHWDMMVICDS-----SL    | 152 |
| 4N2Z_A:22-337                                        | GNAAY--STNPDIINNPAGWTAPRTFYS-GMPAIIRNNIINGHWDMMVICDS-----SL    | 153 |
| 4PVI_A:22-342                                        | GNAAY--STNPDIINNPAGWTAPRTFYS-GMPAIIRNNIINGHWDMMVICDS-----SL    | 157 |
| 4N1I_A:9-302                                         | GSAPFTYRTSTDPKVNWSAPQPLFTGKVADSGT-----GPIDQTVIADD-----RK       | 144 |
| 3WMY_A:139-435                                       | GSWPFYIRTSSDPTDNPWSAPQPLFTGKISGSDT-----GPIDQTVIADD-----RK      | 143 |
| 6F1J_A:24-323                                        | GPYAFSYRTSTDPNANGWSSPQPLFTGTISGSST-----GVIDQTVIGDS-----EN      | 146 |
| 6CC7_A:17-316                                        | GPTSFSYRTSSDPTDNPWSAPQPLFTGTISGSST-----GVIDQTVIGDS-----EN      | 146 |
| 4O8N_A:81-381                                        | GGSAFSYRTSHDPTDNPWSSEQVLFSGSISADSAT-----GPIDQTVIGDS-----TH     | 146 |
| 5UBJ_A:13-326                                        | GPTAFSYLTSSDPTDANGWSSPQPLFSGSISDSST-----GVIDQTVIGDS-----TT     | 158 |
| * : * . . * : . : : : * : *                          |                                                                |     |
| Query                                                | CYLFSSDDNGHLYRSQTSLSNFPNGFNFP-VIAMQDSN-KNRLFEEANVYID---GNGE    | 223 |
| 5B6S_A:1-316                                         | CHLFSSDDNGHLYRSQTSLSNFPNGFNFP-VIAMQDSN-KNRLFEEANVYID---GNSE    | 207 |
| 4N2Z_A:22-337                                        | CHLFSSDDNGHLYRSQTSLSNFPNGFNFP-VIAMQDSN-KNRLFEEANVYID---GNSE    | 206 |
| 4PVI_A:22-342                                        | CHLFSSDDNGHLYRSQTSLSNFPNGFNFP-VIAMQDSN-KNRLFEEANVYID---GNSE    | 216 |
| 4N1I_A:9-302                                         | VYLFVADNGKYYRTSMAIGDFPANFGTASEVILSDQT---AKLFEAVQVYTVAGQ---NQ   | 199 |
| 3WMY_A:139-435                                       | MYLFFAGDNGKIYRASMPIGNFPGFSGSYTTIMSDTK---ANLFEAVQVYTVAGQ---NQ   | 198 |
| 6F1J_A:24-323                                        | MYLFFAGDNGKIYRASMPIGNFPGFSGSYTTIMSDTK---ANLFEAVQVYTVAGQ---NQ   | 201 |
| 6CC7_A:17-316                                        | MYLFFAGDNGKIYRASMPIGNFPGFSGSYTTIMSDTK---ANLFEAVQVYTVAGQ---NQ   | 201 |
| 4O8N_A:81-381                                        | MYLFFAGDNGKIYRASMPIGNFPGFSGSYTTIMSDTK---ANLFEAVQVYTVAGQ---NR   | 201 |
| 5UBJ_A:13-326                                        | MYLFFAGDNGKIYRASMPIGNFPGFSGSYTTIMSDTK---ANLFEAVQVYTVAGQ---SKDT | 215 |
| : * * : * * : : * * : : * * : *                      |                                                                |     |
| Query                                                | YLLLVEAIGSDGQRYFRSWTSNSLTGSKWGLANTEANPFARSNNVAFSGTPWTKSISHGE   | 283 |
| 5B6S_A:1-316                                         | YLLLHEAIGSDGRRWFRSWTSTSIAGPWRLADQESNPFARANNVAFSGTAWTRDISHGE    | 267 |
| 4N2Z_A:22-337                                        | YLLLVEAIGTDGHRWFRSWTADSIKGPWQGLANTEQNPWARSNNVAFSGDVTWTKSISHGE  | 266 |
| 4PVI_A:22-342                                        | YLLLVEAIGQEGHRYFRSWTSDRIDGQWIPADTEANPWAGEANVVFEGQKWTKSISHGE    | 276 |
| 4N1I_A:9-302                                         | YLMIVEAQGTNG-RYFRSFTANSLDGEWKVQAGSEAPFAGKAN---SGASWTNDVSHGD    | 255 |
| 3WMY_A:139-435                                       | YLMIVEAMGANG-RYFRSFTASSLGSWTPQAASEGNPFAGKAN---SGATWTNDISHGD    | 254 |
| 6F1J_A:24-323                                        | YLMIVEAIGANG-RYFRSFTASSLGGTWTQAATESNPFAGKAN---SGATWTNDISSGD    | 257 |
| 6CC7_A:17-316                                        | YLMIVEAIGSQG-RYFRSFTATSLGGSWTPQAATESNPFAGKAN---SGATWTNDISHGD   | 257 |
| 4O8N_A:81-381                                        | YLMIVEAIGAQQRYFRSFTATSLDGEWTPQAATESNPFAGKAN---SGATWTNDISHGE    | 258 |
| 5UBJ_A:13-326                                        | YLMIVEAIGAQQ-RYFRSFTADSLGGSWTPQAATESNPFAGKAN---SGATWTNDISHGD   | 271 |
| * * : * * * : * * * : * * * * * * * * * * * * * *    |                                                                |     |
| Query                                                | LIRTSNDQTLT-ISPCKLQYLYQGLSPSA-GGDYDLLPWKLGLITQTSNCS            | 332 |
| 5B6S_A:1-316                                         | LIRSGNDQTLT-ISPCKLQYLYQGLSPSA-GGDYNSLPWKLGLITQTSNCS            | 316 |
| 4N2Z_A:22-337                                        | LIRSGNDQTLT-ISPCKLQYLYQGLSPSA-GGDYNSLPWKLGLITQTSNCS            | 316 |
| 4PVI_A:22-342                                        | VIRTLTDQTLTLDLSEPIQFLYQGVDPNA-QTEYNALPWRLGLITQ-----            | 321 |
| 4N1I_A:9-302                                         | LIRSNPDQTM-ITDPCRLQLLYQGRDNKVPSSYDLAPYR-----                   | 294 |
| 3WMY_A:139-435                                       | LVRDNDQTM-ITDPCRLQLLYQGRDNKVPSSYDLAPYR-----                    | 297 |
| 6F1J_A:24-323                                        | LVRTNDQTM-ITDPCRLQLLYQGRDNKVPSSYDLAPYR-----                    | 300 |
| 6CC7_A:17-316                                        | LIRVTNDQTM-ITDPCRLQLLYQGRDNKVPSSYDLAPYR-----                   | 300 |
| 4O8N_A:81-381                                        | LIRVTNDQTM-ITDPCRLQLLYQGRDNKVPSSYDLAPYR-----                   | 301 |
| 5UBJ_A:13-326                                        | LVRSTPDQTM-ITDPCRLQLLYQGRDNKVPSSYDLAPYR-----                   | 314 |
| : * : : : * * * : * : * * : *                        |                                                                |     |

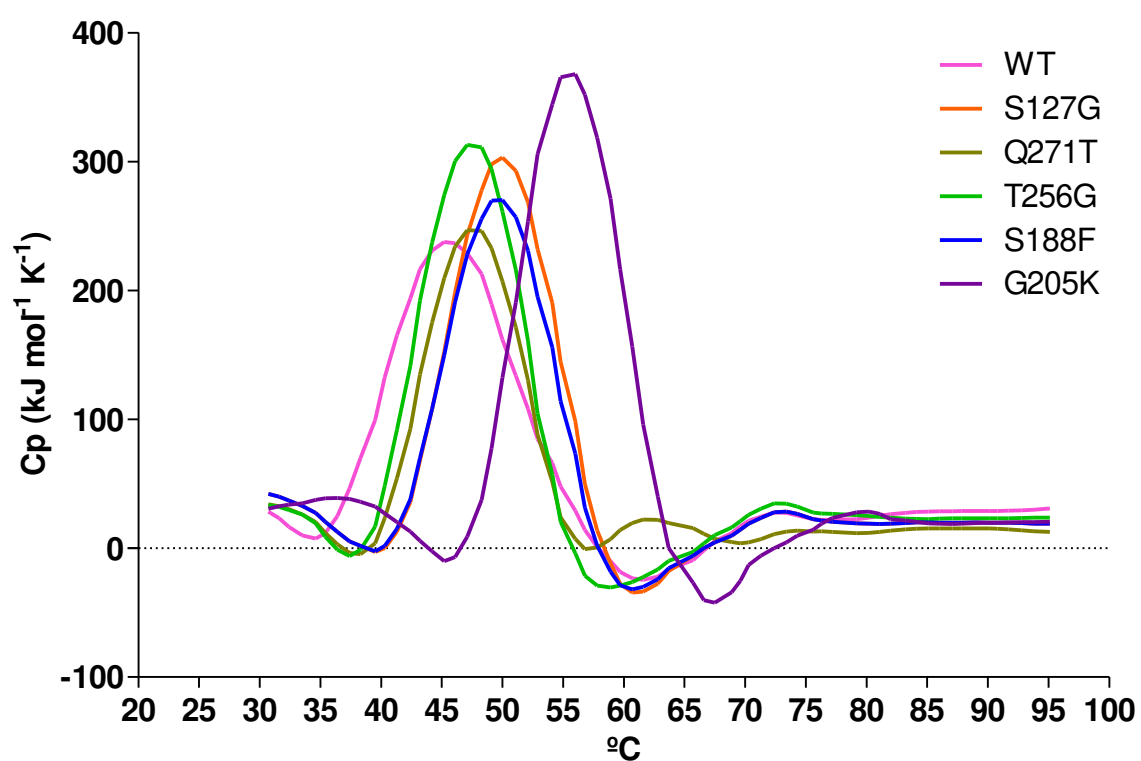
Asterisks (\*) indicate conserved residues.



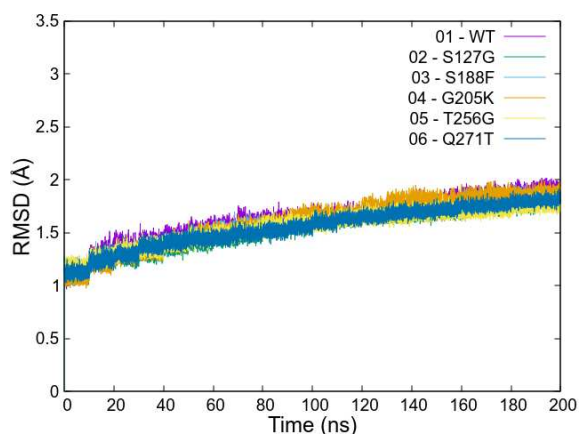
Supplementary figure 12-2 Correlation between ESM-IF1 final scores and melting temperatures of selected mutants.



Supplementary figure 12-3 Characterization of variants: enzymes were incubated at 50°C for 60 min and assayed for residual activities at 42°C. The activity of each enzyme (WT or mutants) measured without exposure to the heat treatment was considered as 100%.  $T_{50}^{60}$  value was determined using the inflection point of a sigmoidal fit of residual activities.



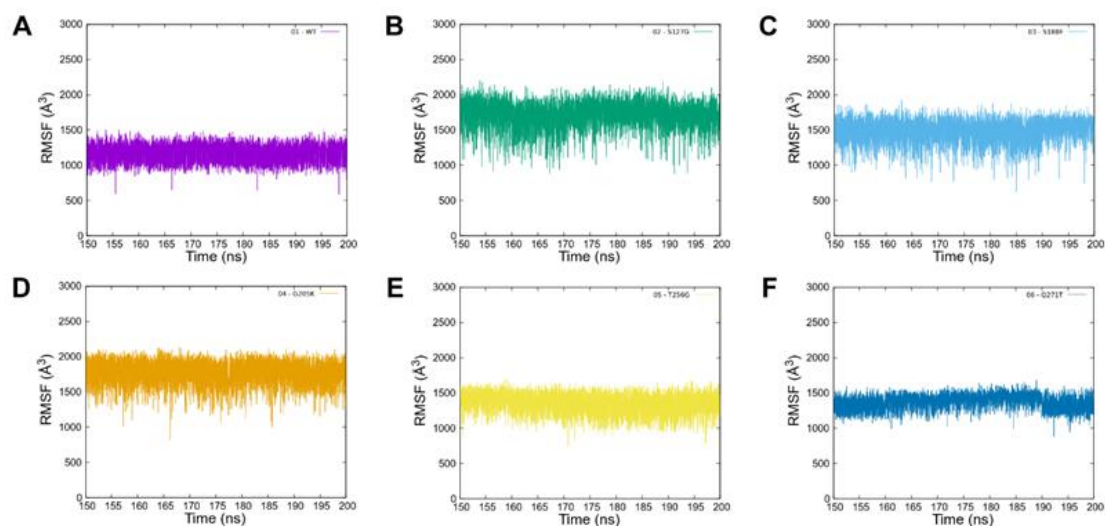
Supplementary figure 12-4 Denaturation heat capacity curve obtained by differential scanning microcalorimetry (DSC).



Supplementary figure 12-5 RMSD Analysis of different systems relative to their average structure.

Supplementary table 12-11 RMSDs for wild type and mutations (Standard Deviations in parentheses).

| System | RMSD Mean (in Å) |
|--------|------------------|
| WT     | 1.61 (0.21)      |
| S127G  | 1.54 (0.21)      |
| S188F  | 1.61 (0.21)      |
| G205K  | 1.61 (0.21)      |
| T256G  | 1.54 (0.14)      |
| Q271T  | 1.54 (0.21)      |



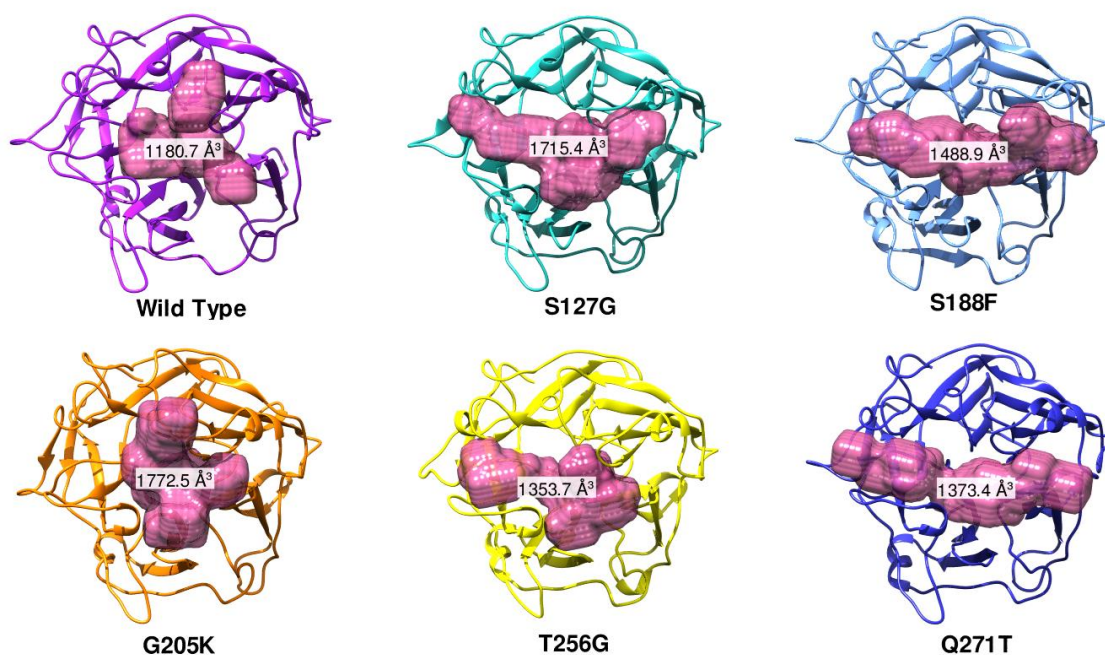
Supplementary figure 12-6 Cavity analysis of the last 50ns of Molecular Dynamics (MD) Simulation for A) wild type and mutations B) S127G, C) S188F, D) G205K, E) T256G, and F) Q271T.

Supplementary table 12-12 Cavity analysis of the catalytic site with standard deviation.

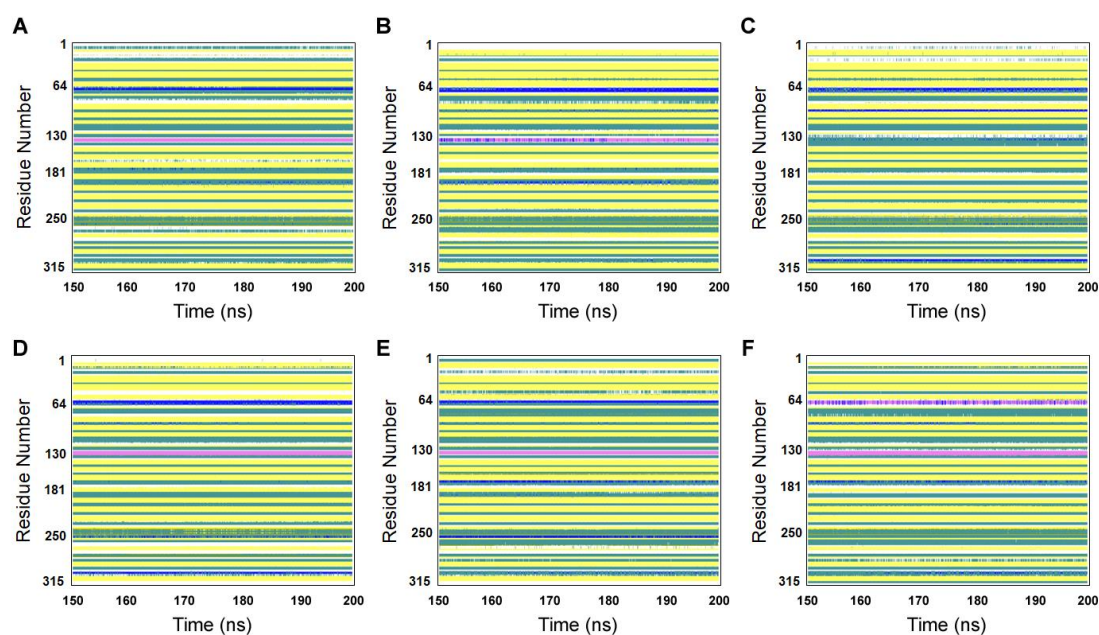
| System | Cavity (Å <sup>3</sup> ) |
|--------|--------------------------|
| WT     | 1180.7 ± 141.4           |
| S127G  | 1715.4 ± 192.0           |
| S188F  | 1488.9 ± 170.8           |
| G205K  | 1772.5 ± 180.7           |
| T256G  | 1353.7 ± 149.7           |
| Q271T  | 1373.4 ± 109.5           |

**Supplementary table 12-13 Comparison of the kinetic parameters and catalytic efficiencies (kcat / Km) of GH 62 arabinofuranosidases from several microorganisms made by using wheat arabinoxylan as substrate**

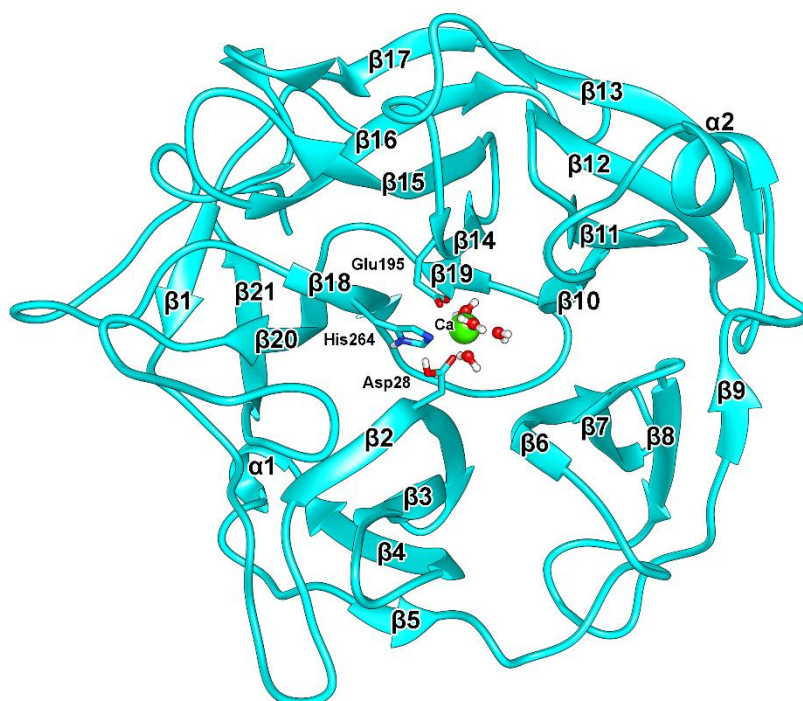
| Organism                  |           | Optimum conditions      | Sub-family | Wheat arabinoxylan |              |           | Ref                      |
|---------------------------|-----------|-------------------------|------------|--------------------|--------------|-----------|--------------------------|
|                           |           |                         |            | Km (mg/mL)         | kcat (s-1)   | kcat / Km |                          |
| <i>A. fumigatus</i>       | Fungus    | pH 4.5–5.0 and 42–45 °C | 62_1       | 1.37 ± 0.3         | 47.82 ± 2.15 | 34.85     | This study               |
| <i>A. nidulans</i>        | Fungus    | pH 5.5 and 60–65 °C     | 62_2       | 3.78 ± 1.05        | 361.74       | 95.69     | (Contesini et al., 2017) |
| <i>A. nidulans</i>        | Fungus    | pH 4.0–6.0 and 55 °C    | 62_2       | 2.3                | 178          | 77.39     | (Wilkens et al., 2016)   |
| <i>E. parvum</i>          | Fungus    | pH 4.5 and 65 °C        | 62_2       | 45.46 ± 2.44       | 42.04 ± 2.05 | 0.92      | (Long et al., 2020)      |
| <i>G. zeae</i>            | Fungus    | pH 6.5 and 40 °C        | 62_2       | 3.4 ± 0.3          | 27.7 ± 0.7   | 8.2       | (Sarch et al., 2019)     |
| <i>N. haematococca</i>    | Fungus    | pH 6.5 and 40 °C        | 62_2       | 9.2 ± 1.2          | 56.3         | 6.1       | (Sarch et al., 2019)     |
| <i>S. reilianum</i>       | Fungus    | pH 6.5 and 40 °C        | 62_2       | 4.0 ± 0.6          | 13 ± 0.5     | 3.3       | (Sarch et al., 2019)     |
| <i>S. coelicolor</i>      | Bacterium | pH 5.5 and 37 °C        | 62_2       | 7.3                | 18 ± 2       | 2.5       | (Maehara et al., 2014)   |
| <i>S. thermophilum</i>    | Fungus    | pH 5.0–6.5 and 50 °C    | 62_1       | 3.66 ± 0.82        | 0.27         | 0.07      | (Kaur et al., 2015)      |
| <i>S. thermoviolaceus</i> | Bacterium | pH 7.0 and 55 °C        | 62_2       | 12.0 ± 5.0         | 180.0 ± 65.0 | 15.0      | (W. Wang et al., 2014)   |



**Supplementary figure 12-7 Average shape of the Cavity of the last 50ns of MD simulation**

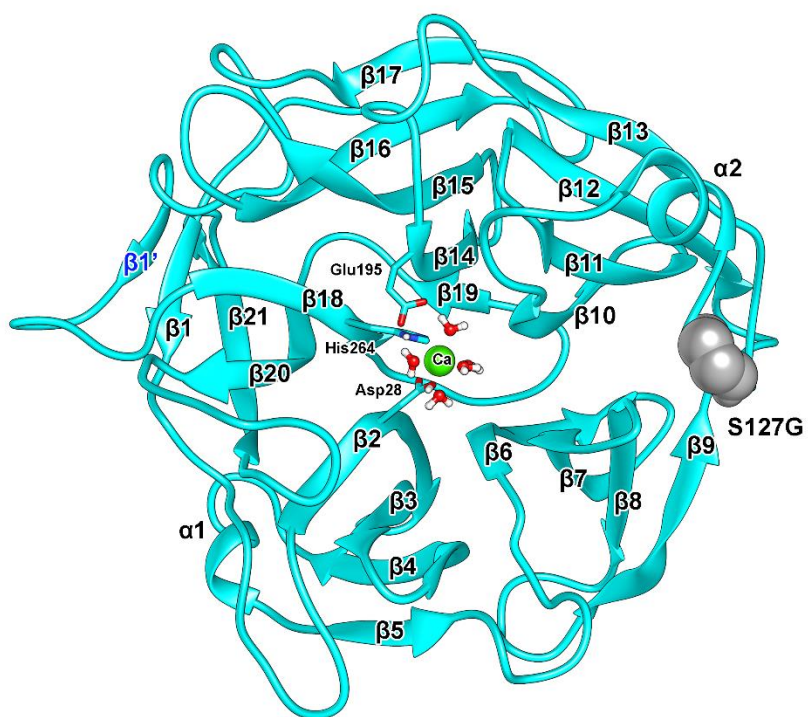


Supplementary figure 12-8 DSSP analysis of trajectories from the last 50ns of MD simulations for A) wild type and mutations B) S127G, C) S188F, D) G205K, E) T256G, and F) Q271T.

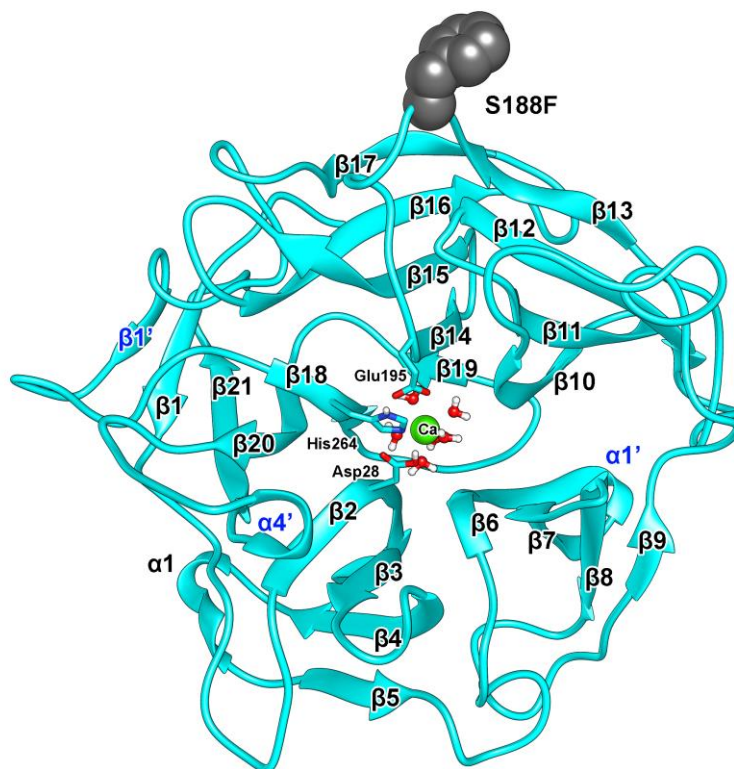


Supplementary figure 12-9 Last point structure of the Wild Type enzyme obtained after 250ns of MD simulation.

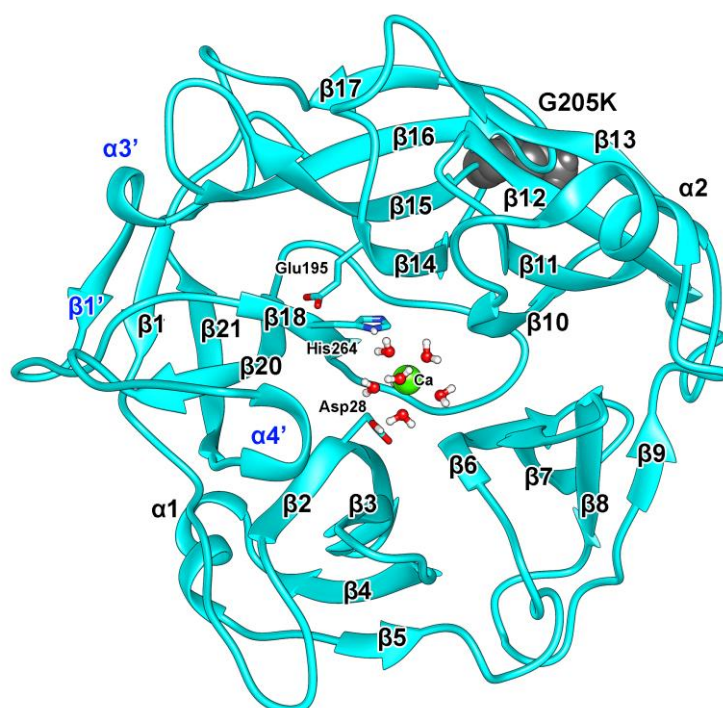




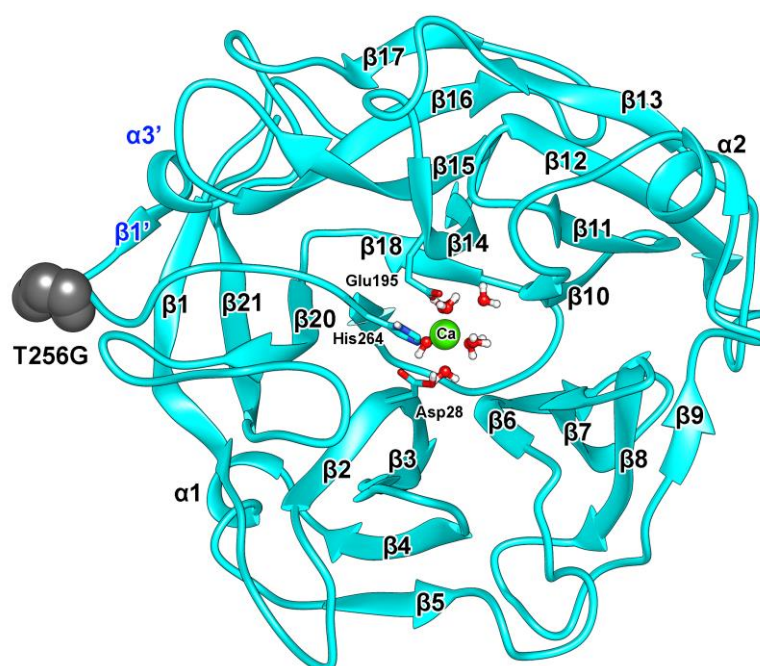
Supplementary figure 12-10 Last point structure of the S127G mutated enzyme obtained after 250ns of MD simulation.



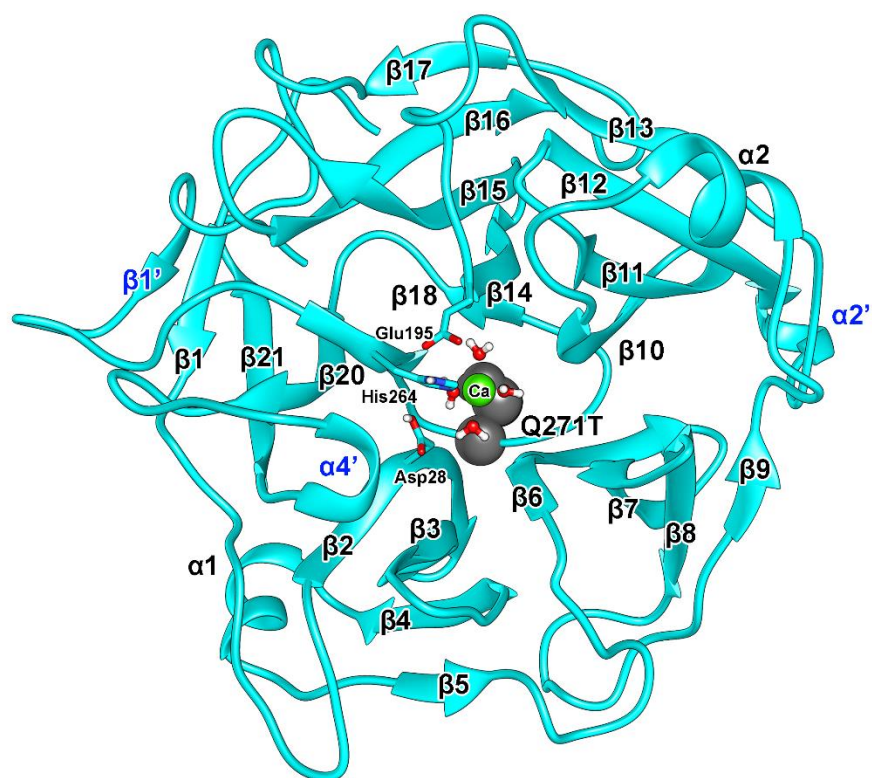
Supplementary figure 12-11 Last point structure of the S188F mutated enzyme obtained after 250ns of MD simulation



Supplementary figure 12-12 Last point structure of the G205K mutated enzyme obtained after 250ns of MD simulation.



Supplementary figure 12-13 Last point structure of the T256G mutated enzyme obtained after 250ns of MD simulation.



Supplementary figure 12-14 Last point structure of the Q271T mutated enzyme obtained after 250ns of MD simulation

**Supplementary material of CHAPTER 9**

**Integrated biorefinery for xylooligosaccharides, pectin, and bioenergy production from orange wastes**

**Supplementary table 12-14 Economic assumptions for biorefinery**

|                            |                        |
|----------------------------|------------------------|
| <b>Material costs</b>      |                        |
| Acetic acid                | 0.05 \$/kg             |
| Buffer                     | 0.01 \$/L              |
| Citric Acid                | 0.45 \$/kg             |
| Enzyme                     | 11.40 \$/L             |
| Ethanol                    | 0.04 \$/m <sup>3</sup> |
| KOH                        | 476.00 \$/ton          |
| Lime 33 %                  | 0.03 \$/kg             |
| NaOH                       | 0.50 \$/kg             |
| Sulfuric Acid              | 0.27 \$/kg             |
| Water                      | 0.02 \$/m <sup>3</sup> |
| <b>Utility costs</b>       |                        |
| Standard power             | 0.10 \$/KW-h           |
| High voltage               | 54.03 \$/MWh           |
| Steam                      | 1.50 \$/MT             |
| Cooling water              | 0.05 \$/MT             |
| <b>Labor</b>               |                        |
| Operator 1                 | 69 \$/h                |
| Operator 2                 | 18.40 \$/h             |
| Quality control            | 5 % total labor cost   |
| <b>Facility costs</b>      |                        |
| Maintenance                | 0.02 % TPDC            |
| Depreciation               | Straight-line method   |
| Insurance                  | 1.00 % TPDC            |
| Taxes                      | 0.02 % TPDC            |
| Factory expenses           | 2.50 % TPDC            |
| <b>Economic Parameters</b> |                        |
| Construction period        | 30 months              |
| Startup period             | 4 months               |
| Project lifetime           | 15 years               |
| Inflation                  | 4 %                    |
| NPV interest               | 7 %                    |
| Income taxes               | 25 %                   |

Supplementary table 12-15 Economic Evaluation Report

| Main Equipment                            |         |                                    |                |           |
|-------------------------------------------|---------|------------------------------------|----------------|-----------|
| Quantity/<br>Standby/<br>Staggered        | Name    | Description                        | Unit Cost (\$) | Cost (\$) |
| 1 / 0 / 0                                 | AD-101  | Anaerobic Digester                 | 400.000        | 400.000   |
| Vessel Volume = 636704,95 L               |         |                                    |                |           |
| 1 / 0 / 0                                 | EV-101  | Multi-Effect Evaporator            | 106.000        | 106.000   |
| Evaporation Area = 276,90 m <sup>2</sup>  |         |                                    |                |           |
| 1 / 0 / 0                                 | ET-101  | Extraction Steam Turbine-Generator | 90.000         | 90.000    |
| Turbine Delivered Shaft Power 58,88kW     |         |                                    |                |           |
| 1 / 0 / 0                                 | SR-101  | Shredder                           | 82.000         | 82.000    |
| Rated Throughput = 50,00 MT/h             |         |                                    |                |           |
| 1 / 0 / 0                                 | GT-101  | Gas Turbine-Generator              | 65.000         | 65.000    |
| Electric Power = 464,37 kW                |         |                                    |                |           |
| 1 / 0 / 0                                 | HX-104  | Heat Exchanger                     | 54.000         | 54.000    |
| Heat Exchange Area = 88,18 m <sup>2</sup> |         |                                    |                |           |
| 1 / 0 / 0                                 | SP-101  | Screw Press                        | 39.000         | 39.000    |
| Throughput = 129,34 ton/h                 |         |                                    |                |           |
| 1 / 0 / 0                                 | SP-102  | Screw Press                        | 39.000         | 39.000    |
| Throughput = 101,00 MT/h                  |         |                                    |                |           |
| 1 / 0 / 0                                 | HX-102  | Condenser                          | 15.000         | 15.000    |
| Condensation Area = 327,10 m <sup>2</sup> |         |                                    |                |           |
| 1 / 0 / 0                                 | PM-101  | Centrifugal Pump                   | 14.000         | 14.000    |
| Pump Power = 0,42 kW                      |         |                                    |                |           |
| 1 / 0 / 0                                 | SG-101  | Steam Generator                    | 12.000         | 12.000    |
| Throughput = 930,50 kg/h                  |         |                                    |                |           |
| 1 / 0 / 0                                 | HX-103  | Heat Exchanger                     | 12.000         | 12.000    |
| Heat Exchange Area = 10,63 m <sup>2</sup> |         |                                    |                |           |
| 1 / 0 / 0                                 | HX-105  | Heat Exchanger                     | 9.000          | 9.000     |
| Heat Exchange Area = 6,80 m <sup>2</sup>  |         |                                    |                |           |
| 1 / 0 / 0                                 | V-101   | Blending Tank                      | 9.000          | 9.000     |
| Vessel Volume = 21,80 m <sup>3</sup>      |         |                                    |                |           |
| 1 / 0 / 0                                 | V-104   | Blending Tank                      | 9.000          | 9.000     |
| Vessel Volume = 372,03 m <sup>3</sup>     |         |                                    |                |           |
| 1 / 0 / 0                                 | RVF-101 | Rotary Vacuum Filter               | 58.000         | 58.000    |
| Filter Area = 143,16 m <sup>2</sup>       |         |                                    |                |           |
| 1 / 0 / 0                                 | GAC-101 | GAC Adsorber (for Liquid Streams)  | 52.000         | 52.000    |
| Column Volume = 71,93 m <sup>3</sup>      |         |                                    |                |           |
| 1 / 0 / 0                                 | R-101   | Stirred Reactor                    | 38.000         | 38.000    |
| Vessel Volume = 80,46 m <sup>3</sup>      |         |                                    |                |           |
| 1 / 0 / 0                                 | V-107   | Horizontal Tank                    | 32.000         | 32.000    |
| Vessel Volume = 39,96 m <sup>3</sup>      |         |                                    |                |           |
| 1 / 0 / 0                                 | HX-102  | Heat Exchanger                     | 26.000         | 26.000    |
| Heat Exchange Area = 31,45 m <sup>2</sup> |         |                                    |                |           |
| 1 / 0 / 0                                 | RO-102  | Reverse Osmosis Filter             | 12.000         | 12.000    |
| Membrane Area = 8,75 m <sup>2</sup>       |         |                                    |                |           |
| 1 / 0 / 0                                 | HX-101  | Heat Exchanger                     | 4.000          | 4.000     |
| Heat Exchange Area = 1,93 m <sup>2</sup>  |         |                                    |                |           |
| 1 / 0 / 0                                 | PFR-101 | Plug Flow Reactor                  | 234 000        | 234 000   |
| Vessel Volume = 181381,05 L               |         |                                    |                |           |
| 1 / 0 / 0                                 | CR-101  | Crystallizer                       | 195 000        | 195 000   |
| Vessel Volume = 0,24 m <sup>3</sup>       |         |                                    |                |           |
| 1 / 0 / 0                                 | EV-102  | Multi-Effect Evaporator            | 147 000        | 147 000   |
| Evaporation Area = 42,93 m <sup>2</sup>   |         |                                    |                |           |
| 1 / 0 / 0                                 | DDR-101 | Drum Dryer                         | 103 000        | 103 000   |
| Drum Area = 264,76 m <sup>2</sup>         |         |                                    |                |           |

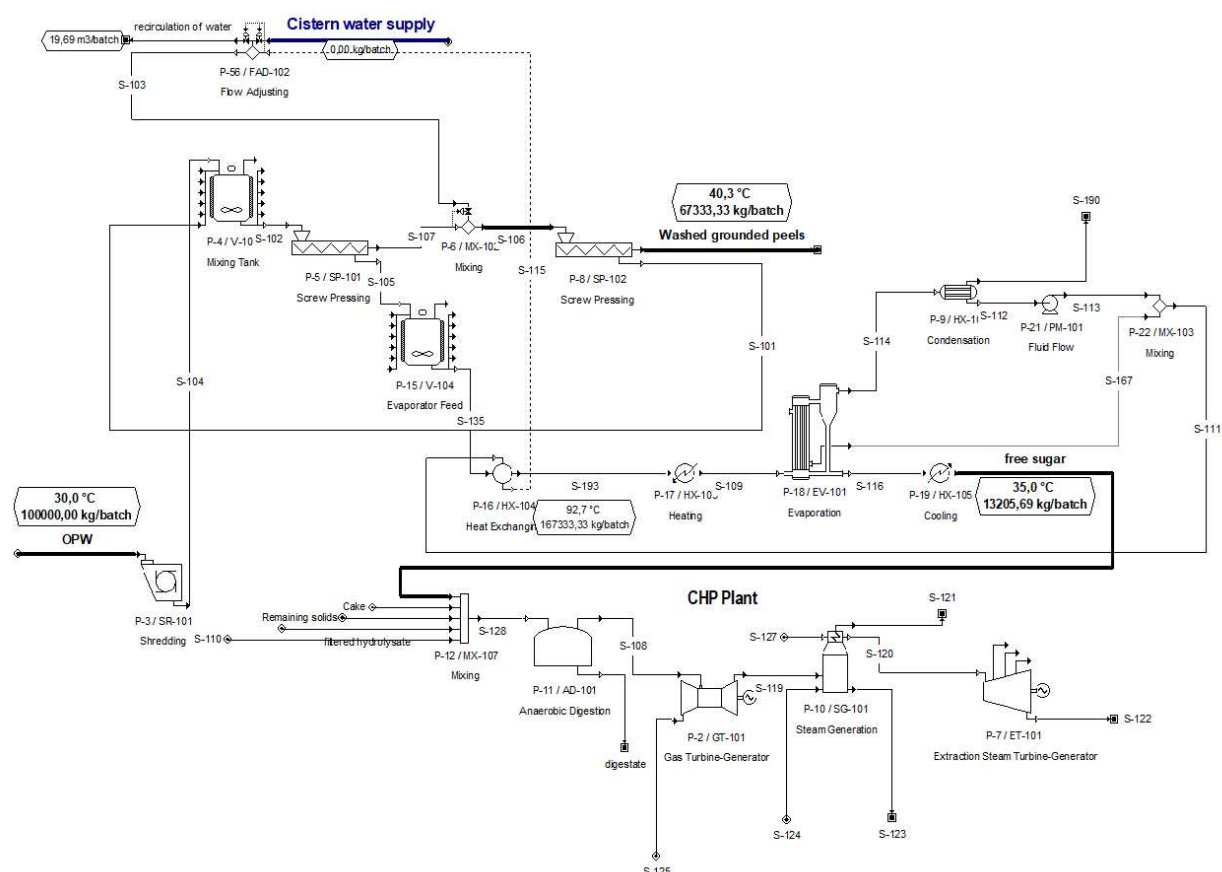
|                                           |         |                      |        |        |
|-------------------------------------------|---------|----------------------|--------|--------|
| 1 / 0 / 0                                 | RVF-103 | Rotary Vacuum Filter | 59 000 | 59 000 |
| Filter Area = 2,73 m <sup>2</sup>         |         |                      |        |        |
| 1 / 0 / 0                                 | RVF-101 | Rotary Vacuum Filter | 58 000 | 58 000 |
| Filter Area = 108,42 m <sup>2</sup>       |         |                      |        |        |
| 1 / 0 / 0                                 | HX-114  | Heat Exchanger       | 51 000 | 51 000 |
| Heat Exchange Area = 81,14 m <sup>2</sup> |         |                      |        |        |
| 1 / 0 / 0                                 | HX-115  | Heat Exchanger       | 49 000 | 49 000 |
| Heat Exchange Area = 78,50 m <sup>2</sup> |         |                      |        |        |
| 1 / 0 / 0                                 | V-106   | Neutralizer          | 41 000 | 41 000 |
| Vessel Volume = 2,01 m <sup>3</sup>       |         |                      |        |        |
| 1 / 0 / 0                                 | HX-112  | Condenser            | 40 000 | 40 000 |
| Condensation Area = 373,01 m <sup>2</sup> |         |                      |        |        |
| 1 / 0 / 0                                 | RVF-105 | Rotary Vacuum Filter | 39 000 | 39 000 |
| Filter Area = 0,12 m <sup>2</sup>         |         |                      |        |        |
| 1 / 0 / 0                                 | RVF-104 | Rotary Vacuum Filter | 39 000 | 39 000 |
| Filter Area = 0,06 m <sup>2</sup>         |         |                      |        |        |
| 1 / 0 / 0                                 | HX-111  | Condenser            | 38 000 | 38 000 |
| Condensation Area = 55,59 m <sup>2</sup>  |         |                      |        |        |
| 1 / 0 / 0                                 | V-109   | Receiver Tank        | 38 000 | 38 000 |
| Vessel Volume = 106,38 m <sup>3</sup>     |         |                      |        |        |
| 1 / 0 / 0                                 | V-107   | Neutralizer          | 35 000 | 35 000 |
| Vessel Volume = 0,03 m <sup>3</sup>       |         |                      |        |        |
| 1 / 0 / 0                                 | V-110   | Horizontal Tank      | 32 000 | 32 000 |
| Vessel Volume = 164,76 m <sup>3</sup>     |         |                      |        |        |
| 1 / 0 / 0                                 | C-101   | Distillation Column  | 30 000 | 30 000 |
| Column Volume = 545,03 L                  |         |                      |        |        |
| 1 / 0 / 0                                 | PM-102  | Centrifugal Pump     | 13 000 | 13 000 |
| Pump Power = 0,37 kW                      |         |                      |        |        |
| 1 / 0 / 0                                 | HX-113  | Heat Exchanger       | 9 000  | 9 000  |
| Heat Exchange Area = 0,91 m <sup>2</sup>  |         |                      |        |        |
| 1 / 0 / 0                                 | HX-110  | Heat Exchanger       | 3 000  | 3 000  |
| Heat Exchange Area = 0,79 m <sup>2</sup>  |         |                      |        |        |

|                                                |               |                         |         |                  |
|------------------------------------------------|---------------|-------------------------|---------|------------------|
| 1 / 0 / 0                                      | C-101         | PBA Column              | 620.000 | 620.000          |
| Column Volume = 421,17 L                       |               |                         |         |                  |
| 1 / 0 / 0                                      | RDR-101       | Rotary Dryer            | 149.000 | 149.000          |
| Drying Area = 23,01 m <sup>2</sup>             |               |                         |         |                  |
| 1 / 0 / 0                                      | EV-101        | Multi-Effect Evaporator | 147.000 | 147.000          |
| Mean Heat Transfer Area = 20,01 m <sup>2</sup> |               |                         |         |                  |
| 2 / 0 / 0                                      | UF-101        | Ultrafilter             | 142.000 | 284.000          |
| Membrane Area = 75,19 m <sup>2</sup>           |               |                         |         |                  |
| 1 / 0 / 0                                      | DDR-101       | Drum Dryer              | 106.000 | 106.000          |
| Drum Area = 49,67 m <sup>2</sup>               |               |                         |         |                  |
| 1 / 0 / 0                                      | V-101         | Vertical-On-Legs Tank   | 90.000  | 90.000           |
| Vessel Volume = 62,96 m <sup>3</sup>           |               |                         |         |                  |
| 1 / 0 / 0                                      | R-102         | Stirred Reactor         | 55.000  | 55.000           |
| Vessel Volume = 57,02 m <sup>3</sup>           |               |                         |         |                  |
| 1 / 0 / 0                                      | Reactor       | Stirred Reactor         | 55.000  | 55.000           |
| Vessel Volume = 76,71 m <sup>3</sup>           |               |                         |         |                  |
| 1 / 0 / 0                                      | SB-101        | Silo                    | 49.000  | 49.000           |
| Vessel Volume = 100,61 m <sup>3</sup>          |               |                         |         |                  |
| 1 / 0 / 0                                      | Filter        | Rotary Vacuum Filter    | 39.000  | 39.000           |
| Filter Area = 58,45 m <sup>2</sup>             |               |                         |         |                  |
| 1 / 0 / 0                                      | RVF-103       | Rotary Vacuum Filter    | 39.000  | 39.000           |
| Filter Area = 104,47 m <sup>2</sup>            |               |                         |         |                  |
| 1 / 0 / 0                                      | vacuum filter | Rotary Vacuum Filter    | 39.000  | 39.000           |
| Filter Area = 138,08 m <sup>2</sup>            |               |                         |         |                  |
| 1 / 0 / 0                                      | V-102         | Blending Tank           | 38.000  | 38.000           |
| Vessel Volume = 594,07 m <sup>3</sup>          |               |                         |         |                  |
| 1 / 0 / 0                                      | AF-101        | Air Filter              | 8.000   | 8.000            |
| Rated Throughput = 944526,79 L/h               |               |                         |         |                  |
| Unlisted Equipment                             |               |                         |         | 461.000          |
|                                                |               |                         |         |                  |
|                                                |               |                         |         |                  |
| <b>TOTAL</b>                                   |               |                         |         | <b>4.605.000</b> |

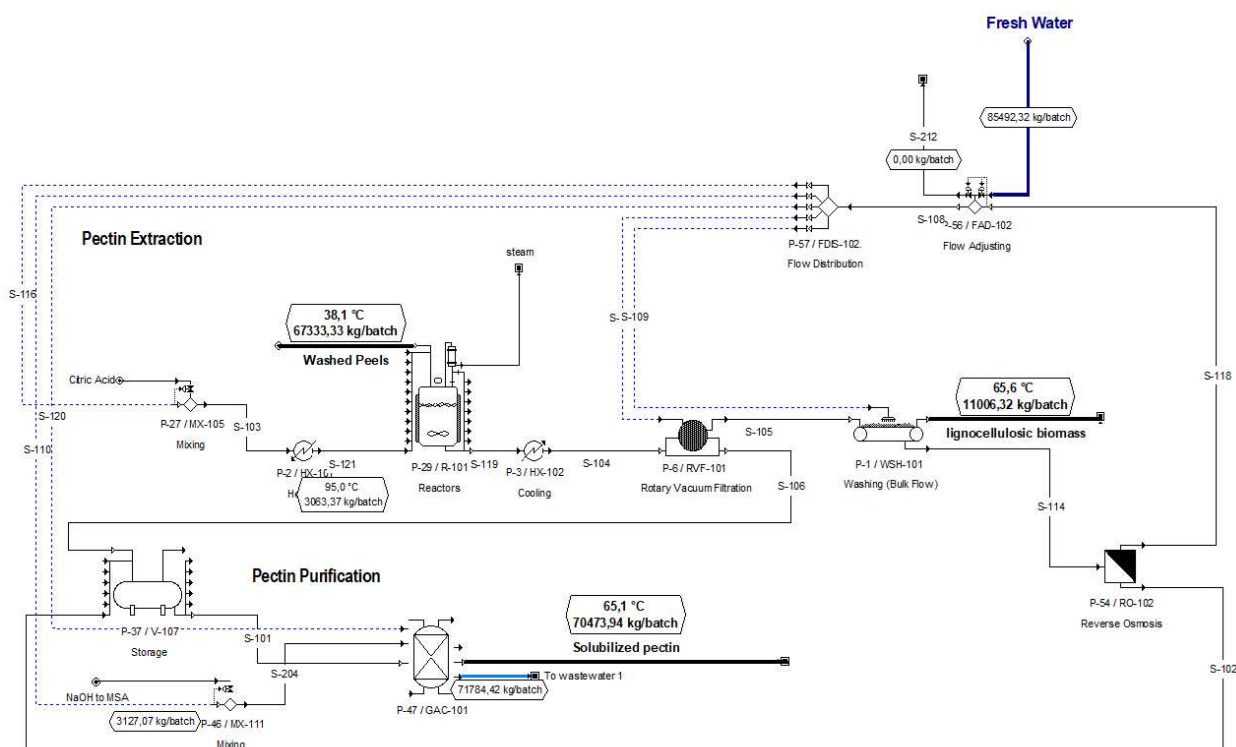
## FIXED CAPITAL ESTIMATE SUMMARY (2022 prices in \$)

|                                                           |               |
|-----------------------------------------------------------|---------------|
| <b>3A. Total Plant Direct Cost (TPDC) (physical cost)</b> |               |
| 1. Equipment Purchase Cost                                | 4,605,000.00  |
| 2. Installation                                           | 1,331,000.00  |
| 3. Process Piping                                         | 921,000.00    |
| 4. Instrumentation                                        | 921,000.00    |
| 5. Insulation                                             | 138,000.00    |
| 6. Electrical                                             | 461,000.00    |
| 7. Buildings                                              | 921,000.00    |
| 8. Yard Improvement                                       | 691,000.00    |
| 9. Auxiliary Facilities                                   | 921,000.00    |
| TPDC                                                      | 6,911,000.00  |
| <b>3B. Total Plant Indirect Cost (TPIC)</b>               |               |
| 10. Engineering                                           | 2,727,000.00  |
| 11. Construction                                          | 3,819,000.00  |
| TPIC                                                      | 6,547,000.00  |
| <b>3C. Total Plant Cost (TPC = TPDC+TPIC)</b>             |               |
| TPC                                                       | 17,457,000.00 |
| <b>3D. Contractor's Fee &amp; Contingency (CFC)</b>       |               |
| 12. Contractor's Fee                                      | 873,000.00    |
| 13. Contingency                                           | 1,398,000.00  |
| CFC = 12+13                                               | 2,270,000.00  |
| <b>3E. Direct Fixed Capital Cost (DFC = TPC+CFC)</b>      |               |
| DFC                                                       | 19,727,000.00 |

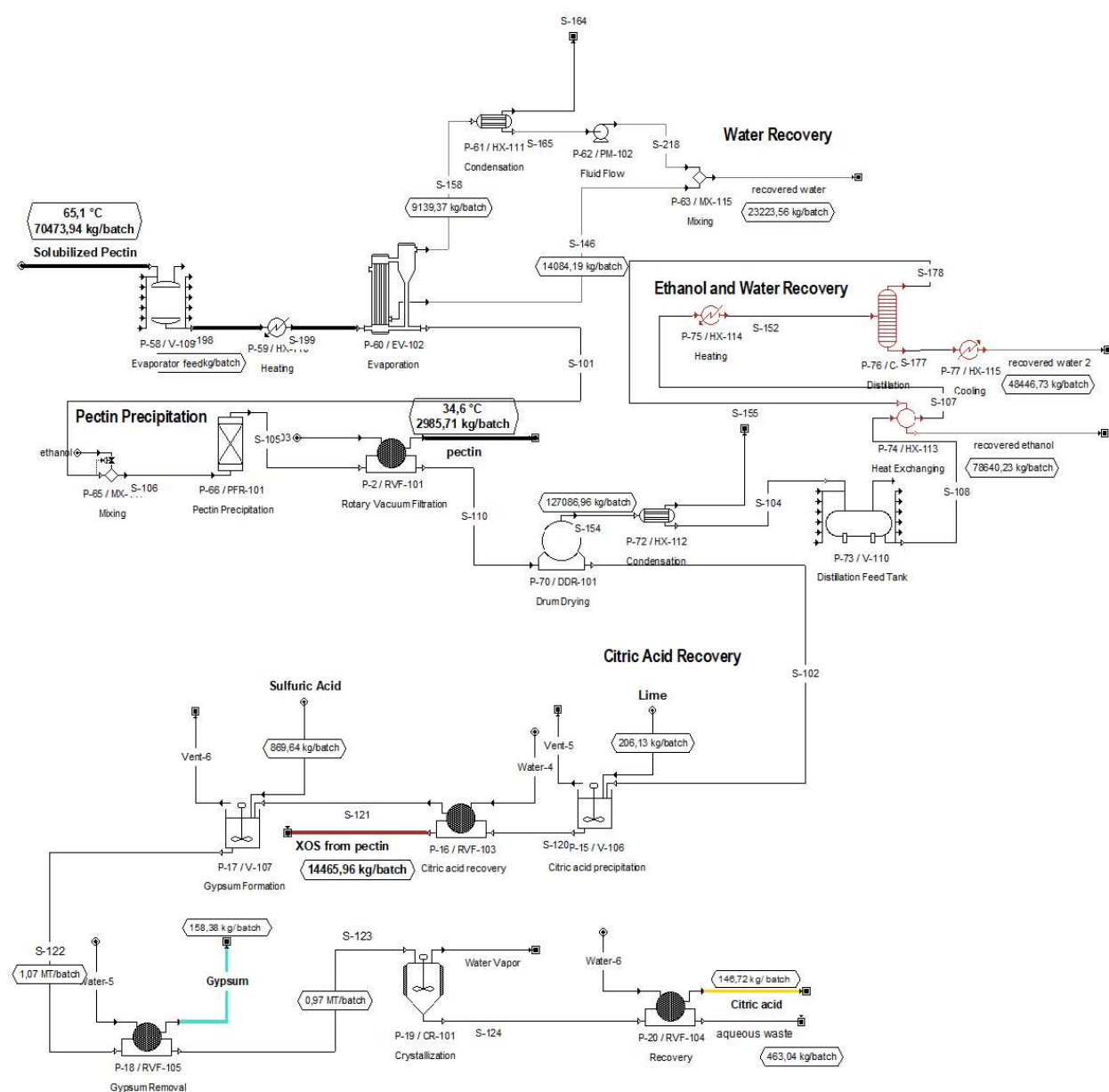




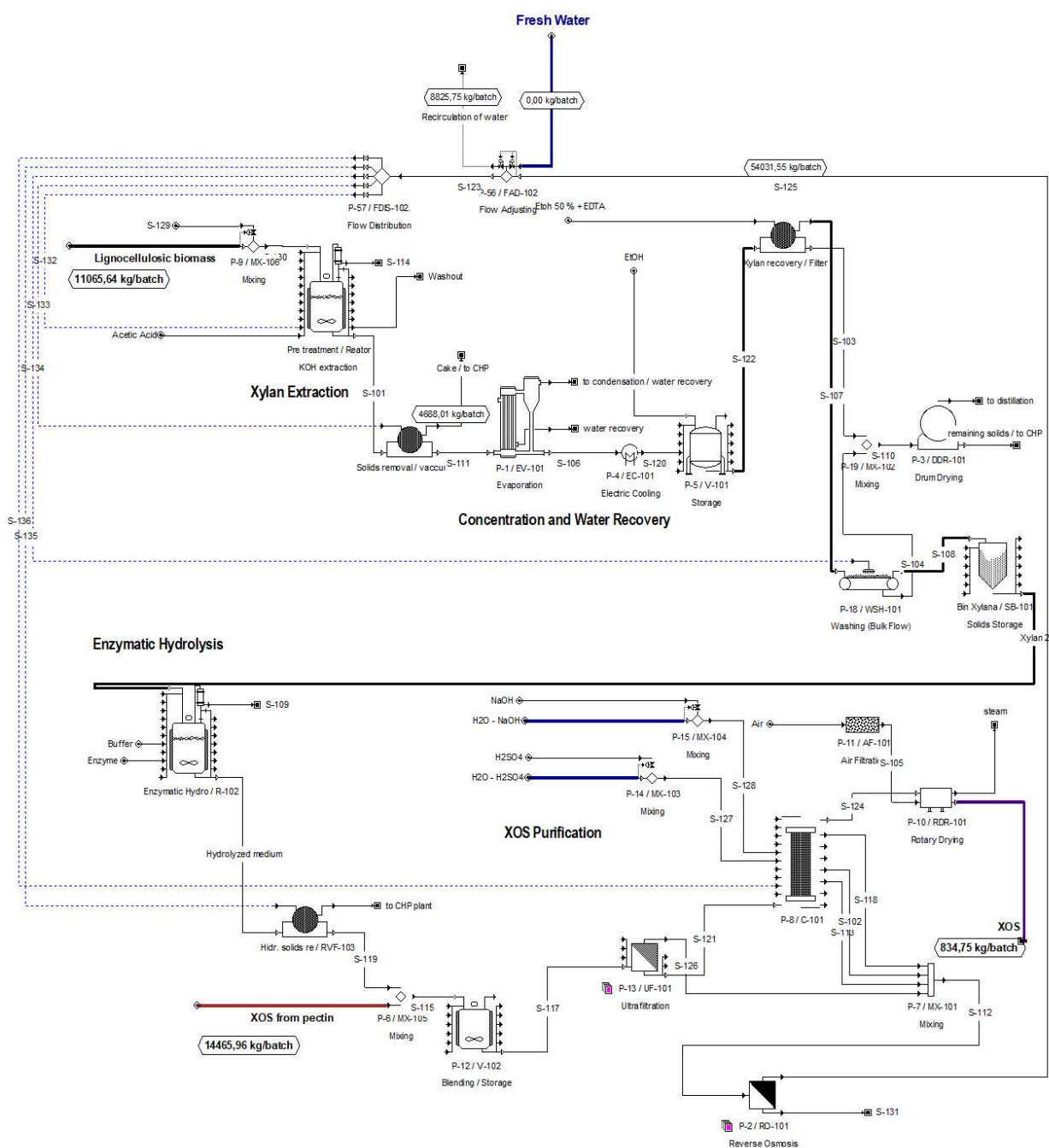
Supplementary figure 12-15 Sugar washing and proposed CPH plant



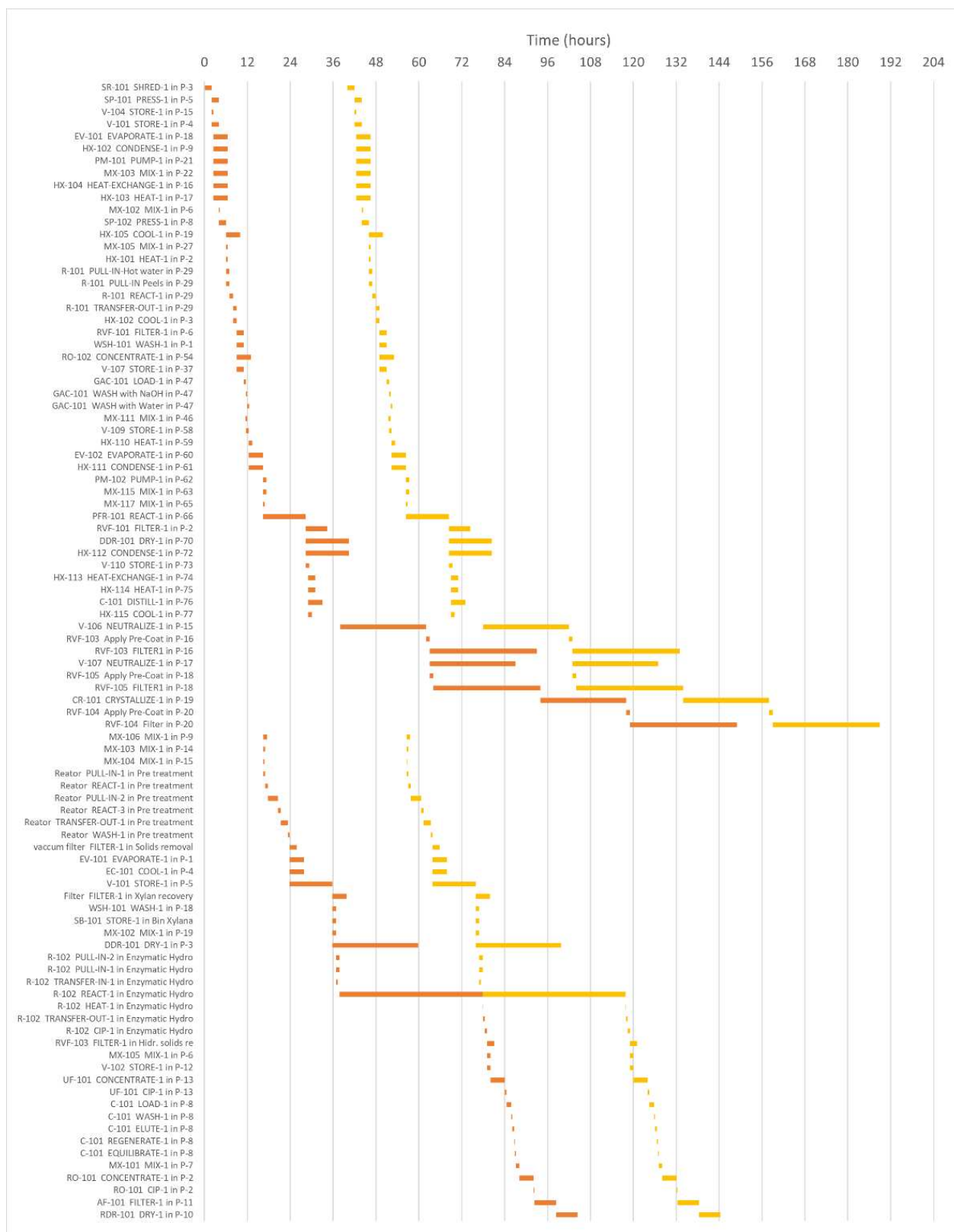
Supplementary figure 12-16 Pectin extraction section



Supplementary figure 12-17 Pectin precipitation section (with citric acid recovery system)



Supplementary figure 12-18 Xylan extraction, enzymatic hydrolysis, and xylooligosaccharides purification



Supplementary figure 12-19 38 hours schedule batch chart (orange: first batch; yellow: second batch)

**APPENDICES:**

**Book chapters, patent file, publishers authorizations,  
and original files**

## **Appendix I: Lignocellulose Bioconversion through Chemical Methods, Platform Chemicals, and New Chemicals**

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## I.1. Introduction

The modern concept of biorefinery of producing valuable bioproduct taking over the traditional petrochemical route is recently launched and observed the future direction for industrialization in terms of enhanced carbohydrate-based products. The sustainable production of commercially valuable chemicals and biofuels using lignocellulose-based processes has been considered one of the hot areas and needs innovation of new emerging technologies (Patel et al., 2019). This has the potential to decrease greenhouse gas emissions, bringing benefits to rural economies and promoting energy security (Bhatia et al., 2020). Lignocellulosic biomass is an inexpensive renewable source that can be used to produce biofuels and bioproducts. It is composed of three major interwoven components (cellulose, hemicellulose, and lignin) (Bhatia et al., 2018). However, its recalcitrant nature presents a technical challenge for releasing fermentable sugars, and a major hurdle in its use in biorefinery (Bhatia et al., 2020; Sindhu et al., 2017).

Pretreatment of lignocellulosic biomass is the initial step in exposing the cellulose and hemicellulose content for hydrolysis using enzymatic or chemical methods to increase the surface area and provide easily accessible binding sites for enzyme (Bhatia et al., 2020). Lignin content, hemicellulose chemistry, and acetyl groups affect the enzymatic conversion of cellulose into glucose. These pretreatments include mechanical pretreatment, biological pretreatment, alkaline pretreatment, dilute acid hydrolysis, ammonia fiber explosion pretreatment, hydrothermal treatment (steam explosion and hot water pretreatment), and novel green processes (ionic liquids and sub/supercritical fluids) (Agbor et al., 2011; Brodeur et al., 2011; Renu Singh et al., 2014).

Enzymatic hydrolysis is mainly focused for biomass degradation with various potentials (Goldbeck et al., 2014), and it has endless capacity to improve productivity and reduce costs (Ávila et al., 2018). The discovery and production of new enzymes through bio-prospection, genetic engineering and strain improvements have led to the identification of thousands of enzymes. A better understanding of which enzymes and proportions that are essential for lignocellulosic degradation could lead to rational design of more efficient and less expensive mixtures (Ávila et al., 2020). This chapter provides an update on recent reports and an overview of the advancements in biochemical methods and strategies for lignocellulosic biomass conversion in bioethanol and other chemical products.

## **I.2. Lignocellulose Biomass**

Lignocellulosic biomass is the most abundant plant material, available in much higher quantities and at lower cost than starch and sucrose-based materials. This biomass makes up the cell walls of woody plants such as trees, shrubs and grasses. Its industrial use can come from various sources, such as agricultural, forest residues, and municipal wastes. The use of agricultural residues, e.g. cereal straws and corn cobs, as biorefinery feedstock makes it possible to produce food and fuels using the same land (Bhatia et al., 2018). However, the structural complexity has become a technical challenge for releasing fermentable sugars from the biomass, and a major hurdle in its use in biorefinery (Bhatia et al., 2020). The aspects about chemical composition and recalcitrance structure properties are covered in subtopics 2.1 and 2.2 below.

### **I.2.1. Chemical composition of lignocellulosic biomass**

Lignocellulose biomass is composed of carbohydrate polymers of cellulose (40–50%), hemicellulose (20–30%), lignin 10–25%, small amounts of pectin, proteins, and extrac-tives (chlorophyll, waxes, and nonstructural sugars) (Bhatia et al., 2020). The composition of lignocellulosic biomass varies with plant species, age, stage of growth, and season (Sindhu et al., 2017).

#### ***I.2.1.1. Cellulose***

Cellulose is a linear polymer composed of D-glucose units linked together by  $\beta$ -(1–4) glycosidic bonds (Wyman et al., 2005). The degree of polymerization (DP) is approximately 4000–6000 in woody biomass. Polymers of cellulose are interlinked through hydrogen, and van der Waals bonds to form a microfibril, and present crystalline and amorphous forms. Microfibrils are covered by hemicellulose and lignin. Crystalline cellulose fibers attach to each other by non-covalent hydrogen bonding, which provides 3–30 times lower degradability as compared to the amorphous part. Cellulase is readily able to hydrolyze amorphous cellulose but is not effective at degrading the less accessible crystalline portion (Taherzadeh & Karimi, 2008)

#### ***I.2.1.2. Hemicellulose***

Hemicellulose is the second largest polysaccharides in the lignocellulosic biomass. It supports cellulose microfibrils by hydrogen bonding and also binds to lignin by covalent bonds. Unlike cellulose, hemicellulose is a heterogeneous and amorphous polysaccharide with DP around 200 composed of pentoses (xylose and arabinose), hexoses (glucose, galactose and mannose) and sugar acids such as D-glucuronic acid, 4-Omethyl-D-glucuronic acid and D-galacturonic acid. The composition and structure of hemicellulose vary depending on the



species. Moreover, considerable differences of hemicellulose composition exist between the stem, branches, roots and barks. Xylan is the main component of hemicellulose in grasses and hardwood, while glucomannan is the dominant hemicellulose component in softwood (Saha, 2003).

### ***1.2.1.3. Lignin***

Lignin is the most complex amorphous polyphenolics polymer composed of three o-methoxylated p-hydroxyphenyl propanoid units, variable depending on the source (Fengel and Wenger, 2011). These monomer units give rise to p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) subunits when incorporated into a lignin polymer (Bhatia et al., 2020). Gymnosperms (softwood) and fern lignin are generally composed of G, followed by a small H content. Contrastingly, in angiosperm (hardwood) plants, lignin is mainly composed of S units followed by G units; in herbaceous crops, G followed by H and S units. Various monomer units are linked through ( $\beta$ -O-4) aryl ether bonds (Brandt et al., 2013). Lignin acts as glue around cellulose and hemicellulose, providing mechanical strength and resistance against microbial attack. It makes biomass recalcitrant by restricting the accessibility of cellulase and by preventing the deactivation of enzymes by various lignin-derived compounds (Bichot et al., 2018).

### **1.2.2. Biomass types and recalcitrance properties**

Biomass recalcitrance to bioprocessing is directly related to the inherent properties of the biomass source. Lignin content, cellulose accessibility to cellulase, and cellulose crystallinity determine the overall digestibility of the biomass. The complexity of a given biomass type is reflected in the relationship between its structural and carbohydrate components (Bhatia et al., 2020). The factors that contribute to recalcitrance include crystallinity and degree of polymerization of cellulose; accessible surface area (or porosity); protection of cellulose by lignin; cellulose sheathing by hemicellulose; and fiber strength. The removal of lignin enhances biomass digestibility up to the point where the effect of the presence of lignin no longer limits it. Highly crystalline cellulose is less accessible to cellulase attack than amorphous cellulose, and the cellulose accessibility to cellulase is one of the most important factors in enzymatic hydrolysis when the effect of lignin is minimized (Ávila et al., 2018, 2020; Bhatia et al., 2020). Ultrafine grinding leads to smaller particle sizes, changing polymerization and porosity, which enhances enzymatic hydrolysis. The bio-based research efforts targeting woody biomass should be focused on upstream activity such as size reduction and physicochemical pretreatment to improve direct microbial utilization of polysaccharides and sugar yields following enzymatic saccharification (W. Zhu et al., 2010).

### I.3. Pretreatment and fractionation of lignocellulosic materials

The pretreatment of lignocellulosic materials is a crucial step, which aims to disorganization of the recalcitrant structure of the lignocellulosic cell wall, enabling, release of fermentable sugars and greater accessibility of enzymes to the substrate during enzymatic hydrolysis (**Figure 12-1**) (Shill et al., 2011). Therefore, pretreatment is essential for the removal of lignin, reduction of cellulose crystallinity and increased porosity of the material. The ideal biomass pretreatment method should have the following characteristics: no need for biomass size reduction, preservation of the cellulosic and hemicellulose part, no toxic inhibitors generated, lower energy demands, low cost, and recycling of chemicals (Singh et al., 2014).

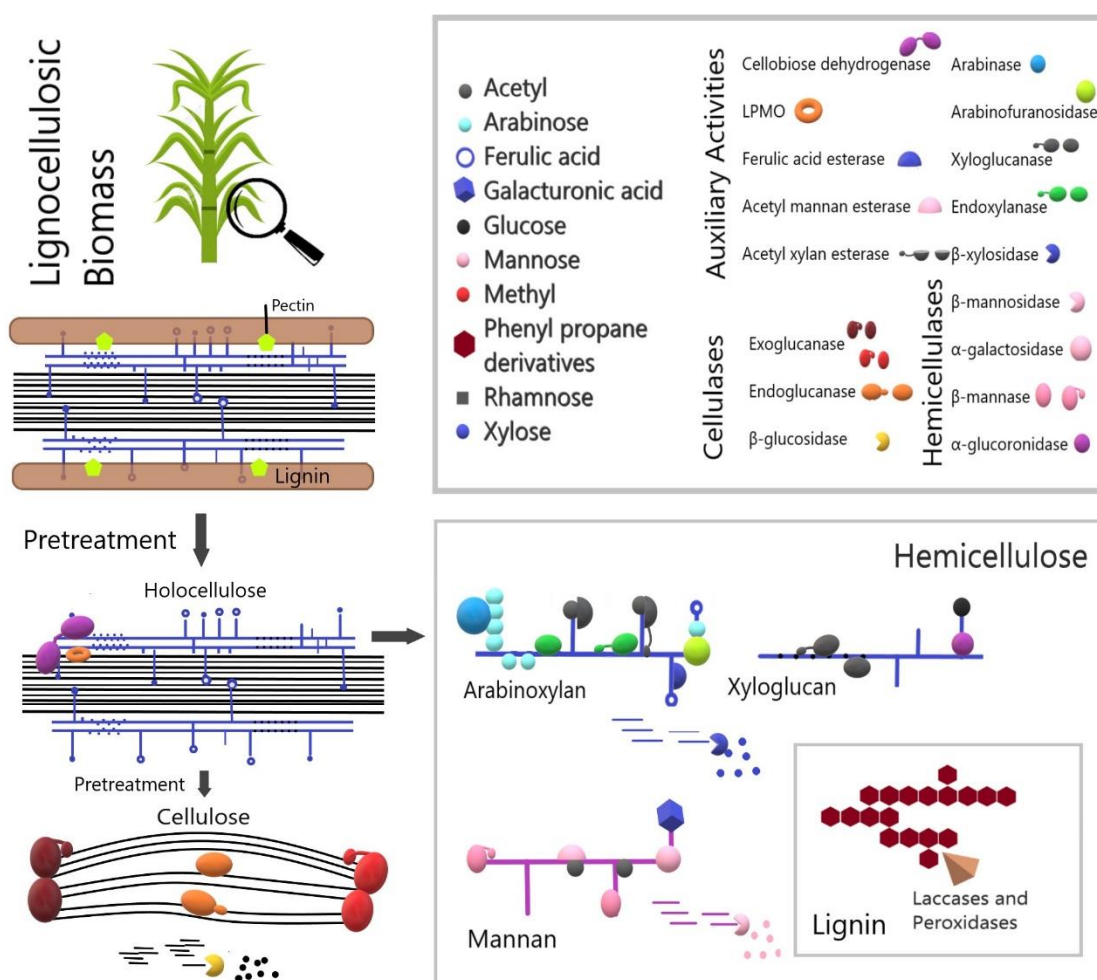


**Figure 12-1** Lignocellulose biomass structure, pretreatment and enzymes enrolled in the deconstruction of the lignocellulosic biomass.

Different lignocellulosic biomass requires different pretreatments based on their composition to convert these into free sugars. A generalized classification of pretreatment

methods groups them into; physical, chemical, biological and multiple or combinatorial pretreatment. In combinatorial pretreatment methods, physical parameters such as temperature, pressure or a biological step are combined with chemical treatments and are termed physicochemical or biochemical pretreatment methods (Agbor et al., 2011). Combinatorial pretreatment strategies are generally more effective in enhancing biomass digestibility, and often employed in designing leading pretreatment technologies (Brandt et al., 2013). This section covers concepts and advances of chemical and physicochemical pretreatments methods (item 3.1 and 3.2) to overcome lignocellulosic biomass intrinsic recalcitrant nature prior to enzymatic hydrolysis treatment for bioethanol production, which will be discussed in the next section as well as treatments for fractionating lignocellulosic biomass (item 3.3) to produce other high-value added bio base products.

### **I.3.1. Chemical pretreatments**

#### ***I.3.1.1. Alkaline pretreatment***

Pretreatment with alkali such as NaOH, KOH,  $\text{Ca(OH)}_2$ , hydrazine and anhydrous ammonia cause swelling of biomass, which increases the internal surface area of the biomass, and decreases both the degree of polymerization, and cellulose crystallinity (Rocha et al., 2009). Alkaline pretreatment disrupts the lignin structure and breaks the linkage between lignin and the other carbohydrate fractions in lignocellulosic biomass, increasing the accessibility to the carbohydrates in the lignocellulosic matrix structure (Agbor et al., 2011). Acetyl and other uronic acid substitutions on hemicellulose that lessen the accessibility of enzymes to cellulose surface are also removed by alkali pretreatments. Alkali pretreatment is most effective with low lignin content biomass like agricultural residues but becomes less effective as lignin content of the biomass increases (Ali et al., 2020). A number of studies have combined alkaline pretreatment with other pretreatment methods, such as wet oxidation, steam explosion, ammonia fiber explosion (AFEX), and ammonia recycled percolation (ARP), which are discussed in later sections.

#### ***I.3.1.2. Acidic pretreatment***

Acidic pretreatment involves the use of concentrated and diluted acids to break the rigid structure of the lignocellulosic material. The most commonly used acid is dilute sulphuric acid ( $\text{H}_2\text{SO}_4$ ), which has been commercially used to pretreat a wide variety of biomass types switchgrass (Sun et al., 2015), corn stover (Y. Zhu et al., 2004), sugarcane biomass (Goldbeck et al., 2014; Ávila, Forte and Goldbeck, 2018). Acid pretreatment enhances the hydrolysis of hemicelluloses and portion of amorphous cellulose and thus, increases the recovery of

hemicelluloses as monomers in liquid fraction and digestible cellulose in solid fraction. However, concentrated acids are toxic, corrosive and hazardous in nature (Singh et al., 2014).

Diluted acid pretreatment (0.5–1.5%, temperature above 160°C) is more favorable for industrial applications since a high sugar yield is obtained from hemicelluloses (Singh et al., 2014). A key advantage of acid pretreatment is that a subsequent enzymatic hydrolysis step is sometimes not required, as the acid itself hydrolyses the biomass to yield fermentable sugars. Hemicellulose and lignin are solubilized with minimal degradation, and the hemicellulose is converted to sugars. However, extensive washing and/or a detoxification step is required to remove the acid before a fermentation step. Due to the corrosive nature and toxicity of most acids, adequate material for the reactor is required in order to withstand the required experimental conditions and corrosiveness of the acids. Another drawback is the production of fermentation inhibitors like furfural and HMF (hydroxymethyl furfural) that reduces the effectiveness of the pretreatment method and further processes (Agbor et al., 2011; Bhatia et al., 2020; Brodeur et al., 2011).

#### ***1.3.1.3. Ionic liquids***

A new approach on chemical pretreatment of biomass is the use of Ionic liquids (ILs). ILs are a new class of solvents with low melting points (below 100 °C), high polarities, high thermal stabilities, negligible vapor pressure, consisting entirely of ions (cations and anions). Most ILs used in biomass fractionation are imidazonium salts. Studies show that 1-allyl-3-methylimidazonium chloride and 1-butyl-3-methylimidazonium chloride can be used effectively as a solvent for the dissolution of cellulose at temperatures below 100 °C (Brandt et al., 2013). A possible dissolution mechanism suggests that these ionic liquids compete with lignocellulosic components for hydrogen bonding, thus disrupting its three dimensions network. Through this dissolution mechanism, ILs are employed in the dissolution of cellulose presenting good solubilities (5–20%) (Arora et al., 2010). ILs can remove the lignin from lignocellulosic biomass in the range of 15–92% and partial hemicellulose, while the cellulose part remains intact (Brandt et al., 2013).

The cost, recovery and reuse of ILs are important considerations for the industrial application. Almost a full recovery of ILs was achieved by adding glycerol to carboxylate ILs (Clough et al., 2016). The high cost of imidazolium cations is a major obstacle limiting the industrial application of ILs for biomass pretreatment. Therefore, novel ionic liquids based on cations have been developed. Ferrari et al. (2019) have studied strategies to obtaining the low-cost and easily synthesizable ethanol ammonium-based ILs to be applied as a pretreatment step of sugar cane

straw biomass. The IL mixtures improved enzymatic digestibility at higher solids loading and water addition in the reaction medium under mild temperature conditions (90 °C), resulting in 98% of cellulose conversion after enzymatic hydrolysis under nonoptimized conditions.

ILs can be also synthesized using vanillin, p-anisaldehyde and furfural derived from lignin and hemicellulose as starting material. The techno economic analysis suggested the raw materials cost to 13.71\$/kg for the biomass-derived ILs. However, the modification of the reductive amination step in the synthesis of ILs could significantly lower the raw material cost to 4 \$ / kg (Socha et al., 2014).

#### ***1.3.1.4. Wet oxidation***

Wet oxidation (WO) utilizes oxygen dissolved in water. There are two reactions that occur during this process. One is low temperature hydrolysis and the other is a high temperature oxidation (Brodeur et al., 2011). Typically, the procedure for wet oxidation consists of drying and milling lignocellulosic biomass to obtain particles that are 2 mm in length, to which water is added at a ratio of 1 L to 6 g biomass. This method of pretreatment is performed at 195°C for a range of 10 to 20 minutes (Pedersen & Meyer, 2009). WO has been shown to be effective in pretreating a variety of biomass such as wheat straw, corn stover, sugarcane bagasse, cassava, peanuts, rye, canola, faba beans, and reed to obtain glucose and xylose after enzymatic hydrolysis (Martín & Thomsen, 2007; Pedersen & Meyer, 2009). During WO, lignin is decomposed to carbon dioxide, water, and carboxylic acids. The amount of lignin removed after pretreatment ranges from 50% to 70% depending on the type of biomass pretreated and the conditions used. For bagasse with a pretreatment time of 15 minutes 50% of lignin was removed which resulted in 57.4% conversion of cellulose compared to only 35% lignin removal and 48.9% cellulose conversion for steam explosion, explained later, under the same conditions (Martín and Thomsen, 2007).

### **1.3.2. Physicochemical pretreatment**

#### ***1.3.2.1. Steam-Explosion***

Steam-Explosion pretreatment is one of the most commonly used pretreatment options, as it uses both chemical and physical techniques in order to break the structure of the lignocellulosic material. This method subjects the material to high pressures and temperatures for a short duration of time after which it rapidly depressurizes the system, disrupting the structure of the fibrils (Brodeur et al., 2011).

A two-step steam-explosion pretreatment option has also been looked at in order to solubilize the structure in stages creating an optimum cellulose fraction during hydrolysis.

The first step involved temperatures of 180° C in order to solubilize and remove the hemicellulose fraction. The second stage used a high-temperature pressurized pretreatment with temperatures up to 210°C, not exceeding 240°C, in which the cellulose fraction was subject to breaking down of its carbohydrate linkage (Brodeur et al., 2011; Agbor et al., 2011). This two-step process increased the downstream ethanol yield by increasing accessibility to cellulose structure because of the reduction of the hemicellulose fraction. Operation costs also decreased as less enzyme dosage was required due to increased accessibility of the cellulose fraction (Bathia et al., 2020). However, increased costs of equipment needed for processing and the additional energy usage of a second steam-explosion process is required (Brodeur et al., 2011).

#### ***1.3.2.2. Liquid hot water***

Liquid hot water pretreatment uses water at elevated temperatures and high pressures to maintain its liquid form in order to promote disintegration and separation of the lignocellulosic matrix (Singh et al., 2014). Temperatures can range from 160°C to 240°C and over length soft immersing from a few minutes up to an hour with temperatures dominating the types of sugar formation and time dominating the amount of sugar formation (Agbor et al., 2011). Xylose recovery is high (88–98%) and no acid or chemical catalysis is needed in this process, which makes it economically interesting and environmentally attractive (Mosier et al., 2005). However, it requires higher energy due to high pressures and a large amount of water. Ammonia fibre/freeze explosion (AFEX), Ammonia recycle percolation (ARP) and Soaking aqueous ammonia (SAA).

AFEX, ARP and SAA are alkaline pretreatment methods that use liquid ammonia to pretreat biomass. As a physicochemical process, AFEX is similar to steam explosion to operating at high pressure, but it is conducted at ambient (<90°C) temperatures, while ARP is carried out at higher temperatures. SAA is a modified version of AFEX utilizing aqueous ammonia to treat biomass in a batch reactor at moderate temperatures (25–60 °C) to reduce the liquid through put during pretreatment (Tae & Lee, 2005). At ambient temperatures the duration could be up to 10–60 days while at higher temperatures (150–190 °C) the effect of ammonia is rapid and the duration of pretreatment is reduced to minutes (Alizadeh et al., 2005).

In both mentioned processes the lignocellulosic material is exposed to ammonia at a given temperature and high pressure, which causes swelling and phase change in cellulose crystallinity in addition to the alteration and removal of lignin. This increases the reactivity of the remaining carbohydrates after pretreatment. The pretreated biomass is easily hydrolysable with close to theoretical yields after enzymatic hydrolysis at low enzyme loadings compared to

pretreated biomass from other pretreatments methods (Agbor et al., 2011; Tae & Lee, 2005). In an attempt to optimize the process and establish a continuous process, by dropping the liquid ammonia through put, Kim et al. (2006), reported a low liquid ARP (LLARP) using one reactor void volume with 15 wt.% ammonia. The digestibility of the LLARP pretreated biomass under optimum condition was at least 86% of the theoretical maximum even at the lowest enzyme loading of 7.5 FPU/g glucan.

#### ***1.3.2.3. Supercritical fluid***

A supercritical fluid (SFC) is a material which can be either liquid or gas, used in a state above the critical temperature and critical pressure where gases and liquids can coexist (Agbor et al., 2011). SCF has the ability to penetrate the crystalline structure of lignocellulosic biomass overcoming the mass transfer limitations encountered in other pretreatments (King & Srinivas, 2009).

Supercritical carbon dioxide (CO<sub>2</sub>) with a critical temperature (T<sub>c</sub>) of 31° C and a critical pressure (P<sub>c</sub>) of 7.4 MPa, typically, is also used as an extraction solvent (Schacht, Zetzl, and Brunner, 2008). Currently it has gained importance as a solvent for pretreatment of different varieties of biomass. When used in combination with water, it forms carbonic acid which favors polymer hydrolysis (Brodeur et al., 2011). Once the biomass is pretreated, explosive release of CO<sub>2</sub> disrupts the cellulose and hemicellulose structure, thus increasing the access for enzyme hydrolysis. The lower temperatures used in the process aids in the stability of the sugars and prevents degradation observed in other pretreatments (Agbor et al., 2011). Even though SFC is being investigated by a considerable number of researchers for pretreatment, the whole process has not proven to be economically viable with the high pressures involved being a deterrent. Improvements need to be made to implement the process on a large scale (King & Srinivas, 2009).

#### **1.3.3. Fractionating treatments of lignocellulosic compounds**

Another approach to overcoming the cost barrier is the production of high value-added co-products from lignocellulose. Prior to producing these co-products, separation of each component in lignocellulosic biomass is required. Each component can be utilized by itself or converted into the intermediates of various products (Zheng et al., 2009). Fractionation process can contribute to reducing the cost in the downstream process, because a significant amount of energy, chemical and water are consumed in the downstream process (Z. Zhang et al., 2014).

The alkaline treatment also releases other polysaccharides, such as lignin, thus decreasing the arabinoxylan purity. Alkaline peroxide is an effective reagent for delignification

and improving the whiteness of hemicelluloses from cereals. In alkaline media, hydrogen peroxide forms the highly active hydroperoxide anion ( $\text{HOO}^-$ ), which produces the oxidation of lignin structures by reacting with the hydrophilic (carboxyl) groups, breaking some interunit bonds, resulting in the separation of lignin and hemicelluloses. A process that is gaining prominence is the organosolv, which consists of the fractionation of lignocellulosic biomass, using a mixture of organic solvents and water at high temperatures to separate cellulose, hemicellulose and lignin. There are a variety of solvents, but the most used are: acetone, ethanol, methanol and organic acids (acetic acid and formic acid). The choice of the solvent is defined according to the studied process, the costs and the ease of acquisition of the solvent (Z. Zhang et al., 2014). The organosolv process is generally carried out without the addition of catalysts, however it can be used to accelerate the reactions. The commonly used catalysts are acetic acid,  $\text{H}_2\text{SO}_4$ ,  $\text{HCl}$ ,  $\text{NaOH}$ ,  $\text{CaCl}_2$ ,  $\text{NH}_3$ ,  $\text{AlCl}_3$ ,  $\text{Fe}_2(\text{SO}_4)_3$ ,  $\text{Al}_2(\text{SO}_4)_3$ , anthraquinone, oxalic acid and sulfonic acid. The main disadvantages of using catalysts are the possibility of equipment corrosion, additional catalyst recovery and recycling steps, contamination of the extracted lignin and the consequent increase in the cost of the process (Skrebets et al., 2004). Direct extraction of lignin in biomass, without prior removal of cellulose and hemicellulose, it implies that more than 60% of mass added to the extraction reactor are polysaccharides (cellulose and hemicellulose), which can suffer degradation during the extraction process, reducing the degree of purity of the extracted lignin (Yamamoto et al., 2014). Generally, during the lignin extraction process, the hemicellulose is also extracted in large quantities (> 70%) (Esteves et al., 2008).

#### **I.4. Enzymatic Hydrolysis of Lignocellulosic Biomass**

Although chemical and physical catalysis are able to hydrolyze lignocellulosic biomass to products of interest, enzymatic reactions have some advantages since they occur at moderate temperatures, have stereospecificity and eliminate the use of chemicals and toxic reagents, that hinder purification and are difficult to recycle, which is particularly beneficial for the synthesis of biomolecules for food or pharmaceutical use. The use of enzymes is ecologically friendly and well established industrially, applied in the manufacture of biofuels, resins, processed meats, cheeses, juices, oils, alcoholic beverages etc. Currently, purification techniques and genetic engineering have expanded the performance of enzymatic processes (Lopes et al., 2018). Due to the variety and complexity of the structures of lignocellulose, it requires a coordinated action of an enzymatic complex for its complete degradation. Information about the characteristics and classifications of lignocellulosic enzymes (Figure 12-1) is found in the Carbohydrate-Active Enzyme (CAZy) database (<http://www.cazy.org>). The heterogeneity of



biomass results in a multiplicity of enzymes that differ in their structural and physical-chemical properties, mode of action and specificity for the substrate (Obeng et al., 2017).

#### **I.4.1. Cellulases**

Key enzymes required for hydrolysis of microcrystalline cellulose, the main polysaccharide in the cell wall, are the glycoside hydrolases endoglucanases, exoglucanases and  $\beta$ -glucosidases, working cooperatively to enforce hydrolysis of the complex microfibrils of cellulose. These enzymes commonly have a catalytic domain that cleaves the glycosidic bond, and a carbohydrate binding module that targets the catalytic domain to the substrate (Obeng et al., 2017).

Endo-1,4- $\beta$ -D-glucanases (EC 3.2.1.4) are short loops that randomly hydrolyze the internal bonds of cellulose fibers present in the amorphous part of cellulose, releasing cello-oligosaccharides and cellobiose, which is hydrolyzed to glucose by the action of glucosidase or cellobiases (EC 3.2.1.21) (Polizeli et al., 2017; Wyman et al., 2005).

B- glucosidases possess a rigid structure with the active site inside a large cavity, that is a site pocket formed by four hydrophilic loops with different conformations to enhance carbohydrate binding, which favors the entry of cellobiose. Also, they are essential to relieve the feedback inhibition from the products of glucanases. However,  $\beta$ -glucosidases also face inhibition from their products and, thus, the search for glucose-tolerant  $\beta$ -glucosidases is developing (Obeng et al., 2017).

Exo-1,4- $\beta$ -D-glucanases have long loops and affinity for the crystalline sites along external cellulose chains. The loops form a tunnel around the catalytic site to where the substrate is directed. They include the 1,4- $\beta$ -D-glucan glucohydrolases (EC 3.2.1.74), which liberate glucose from 1,4- $\beta$ -D-glucans and slowly hydrolyze cellobiose, and 1,4- $\beta$ -D-glucan cellobiohydrolases (CBH), which liberate cellobiose from 1,4- $\beta$ -glucans from the reducing- (CBH I) or nonreducing ends (CBHII). Moreover, CBH can hydrolyze highly crystalline cellulose (Obeng et al., 2017; Polizeli et al., 2017; Wyman et al., 2005).

Cellobiose dehydrogenase (EC 1.1.99.18), capable of oxidizing cellobiose, belongs to the auxiliary activities (AA) family and has a synergistic auxiliary activity, activating hydrolytic cellulases by relieving enzymatic inhibition by cellobiose product, similarly to  $\beta$ -glucosidases (Moreira et al., 2011; Obeng et al., 2017).

Hydrolytic enzymes also from the auxiliary activities family that helps in cellulose degradation are LPMOs (lytic polysaccharide monooxygenases) (Moreira et al., 2011; Obeng et al., 2017). These copper-dependent enzymes have received attention because of their synergic

effect on cellulase activity and their activity toward a range of substrates besides cellulose: chitin, starch, xyloglucan, glucomannan (Filho et al., 2020). The active sites of LPMOs are allocated in the center of a flat face. This flat structure promotes efficient interaction with oligomers. Unlike the other cellulases which require the presence of a loose substrate, the strong mechanism of LPMO seems to allow oxidation at the surface of a cellulose crystalline microfibril (Levasseur et al., 2013). The catalysis occurs from the binding of active oxygen molecules from the atmosphere to the monomeric  $\text{Cu}^{2+}$ , which culminates in the interaction of the active site with the polysaccharide chains (Obeng et al., 2017). It is believed that LPMOs constitute the first attack on lignocellulose polymer, cleaving bonds by oxidizing C1 and C4, improving the degradation of this recalcitrant polymer, making the material more accessible to other enzymes, although the exact mechanism of action is still unclear (Johansen, 2016).

#### **I.4.2. Ligninolytic Enzymes**

Lignin is the most abundant aromatic polymer consisting of phenolic and non-phenolic compounds. There are two families of ligninolytic enzymes which play an important role in enzymatic degradation: phenol oxidase (laccase) and peroxidases (lignin peroxidase, versatile peroxidase and manganese peroxidase), both from the category auxiliary activities (Baruah et al., 2018). The delignification of the phenylpropane polymers involve laccases (EC 1.10.3.2), also called benzenediol oxygen oxidoreductase, a multicopper enzyme which oxidize the phenolic compounds and aromatic amines, boosting cellulase activity by liberating cellulose from binding sites on lignin (Obeng et al., 2017); lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) are enzymes of low specificity capable of oxidizing the lignin (Crestini et al., 2011). Versatile peroxidase (EC 1.11.1.16) also oxidizes phenolic and non-phenolic aromatic compounds. These enzymes have the catalytic activities of both manganese and lignin peroxidase and are able to oxidize  $\text{Mn}^{2+}$  as well as high-redox potential non-phenolic compounds (Baruah et al., 2018). Vanillyl alcohol oxidase (EC 1.1.3.38) catalyzes conversion of phenolic compounds released during breakdown of lignin, and 1,4-benzoquinone reductase (EC 1.6.5.6) degrades aromatic compounds (Levasseur et al., 2013).

##### ***I.4.2.1. Pectic Enzymes***

While abundant in fruits, very little pectin is present in traditional biomass, such as wood, sorghum or sugarcane bagasse. Pectic substances are deconstructed by endo and exopoligalacturonases (EC 3.2.1.15 and 3.2.1.67), cleaving the chain formed by polygalacturonic acid, releasing oligo and monogalacturonates, respectively. The Carbohydrate Esterase CE8 pectinesterases (EC 3.1.1.11) deesterify the ester bonds of the methyl group, releasing methanol

(Jayani et al., 2005). Additional rhamnogalacturonases (EC 3.2.1.-) and rhamnogalacturonan lyases (EC 4.2.2.-) are responsible for degradation of the “hairy regions” in the pectin and CE16 pectin acetyl esterases (EC 3.1.1.6) remove acetyl groups from acetylated homogalacturonan and rhamnogalacturonan (Van Dyk & Pletschke, 2012).

#### ***1.4.2.2. Mannases***

Mannans, hemicellulosic polysaccharides, are degraded by the synergistic action of  $\beta$ -mannanase (EC 3.2.1.78), which acts on the main mannan chain,  $\beta$ -mannosidase (EC 3.2.1.25), which releases mannose from the non-reducing ends, and  $\beta$ -glucosidase (EC 3.2.1.21), which cleaves  $\beta$ -D-1,4-glucopyranose in the final non-reducing region of the oligosaccharides released from glucomannan and galactoglucomannan by  $\beta$ -mannanase, releasing monosaccharides. Acetyl mannan esterase (EC 3.1.1.6) and  $\beta$ -galactosidase (EC 3.2.1.22) cleave the bonds of the acetyl and galacturonic acid groups respectively (Lopes et al., 2018; Moreira et al., 2011).

#### ***1.4.2.3. Xylanases***

Xylan, due to its complex and heterogeneous nature, has more complicated enzymatic hydrolysis than other plant polysaccharides. Different enzymes are required depending on the specific type of xylan to be hydrolyzed and the product to be obtained. The enzymes involved in the xylan depolymerization process can be categorized into three groups: (1) enzymes that hydrolyze backbones; (2) enzymes that remove side chains; (3) accessory enzymes that remove substituted residues, such as acetyl groups. Due to the insolubility and recalcitrance of plant cell wall, many hemicellulases are modular proteins, pursuing besides their catalytic domain other functional modules such as carbohydrate binding module. A detailed explanation about these enzymes is described hereafter (Wyman et al., 2005).

#### **Backbone enzymes**

The core for xylan degradation is the endo-1,4- $\beta$ -xylanases (EC 3.2.1.8), that cleave glycosidic bonds between xylose units in the main xylan chain, releasing oligomers of lesser degree of polymerization and xylo-oligosaccharides. Only two families belong to this group of enzymes: GH 10 and GH 11. While the GH 10 family, exhibiting strand-loop-helix-turn ( $\beta/\alpha$ )<sub>8</sub> folding with a deep active site groove at the loops  $\beta \rightarrow \alpha$ , is able to hydrolyze xylan chains closer to the branching points, releasing shorter chains of xylo-oligosaccharides, the GH 11 family, with a  $\beta$ -jelly-roll folding, is able to cleave in more distant regions of the side chains. Thus, branching residue distribution interferes in the action of these enzymes on the xylan backbone.  $\beta$ -xylosidases (EC 3.2.1.37) hydrolyze small xylo-oligosaccharides and xylobiose from their

nonreducing ends to xylose. They are usually not able to hydrolyze xylan but can cleave artificial substrates such as p-nitrophenyl- $\beta$ -D-xylopyranoside (Polizeli et al., 2005).

#### **Side chains enzymes**

$\alpha$ -glucuronidase (EC 3.2.1.131) hydrolyzes  $\alpha$ -1,2 bonds between glucuronic acid and  $\beta$ -D-xylopyranosyl residues found in glucoxylans; arabinose (EC 3.2.1.99) removes residues of L-arabinose from positions 2 and / or 3 in residues of  $\beta$ -D-xylopyranosyl; arabinofuranosidases (EC 3.2.1.55) hydrolyze  $\alpha$ -1,2-,  $\alpha$ -1,3-, and  $\alpha$ -1,5-L-arabinofuranosidic bonds in L-arabinose-containing polysaccharides, removing residues substituted at positions 2 and 3. They are active on the synthetic substrate p-nitrophenyl  $\alpha$ -L-arabinofuranoside; xyloglucanases (EC 3.2.1.151) hydrolyze xyloglucan backbone (glucose  $\beta$ -1,4 linkages) in glucose residues (Polizeli et al., 2005; Segato et al., 2014).

#### **Accessory enzymes**

Other enzymes needed for complete depolymerization of xylan: acetyl xylan esterase (EC 3.1.1.72) removes the O-acetyl groups from positions 2 and/or 3 in the residues  $\beta$ -D-xylopyranosyl, facilitating the action of endoxylanases; ferulic acid esterase (EC 3.1.1.73) and p-coumaric acid esterase (EC 3.1.1.-) cleave ester bonds from the xylan side chains and also pectin (Segato et al., 2014). Additional auxiliary enzymes, glyoxal oxidase (EC 1.1.3.-) converts aldehydes to carboxylic acids, and galactose oxidase (EC 1.1.3.9) oxidizes carbohydrates and primary alcohols (Levasseur et al., 2013).

#### **I.4.2.4. Enzyme Synergism**

Lignocellulosic biomass is an exceedingly complex substrate, requiring several enzymes to degrade. It is therefore anticipated that there is synergism among these enzymes in nature, producing much higher yields when acting in a cooperative manner. In addition, more effective cooperation reduces enzymatic loadings, and consequently the production costs. Great efforts have been devoted to the improvement of enzyme cocktails for specific substrates (Lopes et al., 2018; Van Dyk & Pletschke, 2012).

Cellulosomes are a natural means of harnessing the synergistic nature of certain hydrolytic enzymes to degrade lignocellulose. A cellulosome is an extracellular enzymatic complex found on cell surface of extracellular medium growth of some anaerobic fungi (e.g., *Neocallimastix frontalis*) and bacteria (e.g., *Clostridium thermocellum*) where several saccharolytic enzymes are anchored to scaffolding proteins (Schuster & Chinn, 2013; Wyman et al., 2005). Since its discovery, many investigators have used a variety of preparations to demonstrate the synergy among various lignocellulosic enzymes: more recently, Goldbeck et al. (2014)

investigated enzymatic mixtures do enhance hemicellulose depolymerization, and Ávila et al. (2020) reported the synergy between endoxylanase and arabinofuranosidase, used to optimize xylo-oligosaccharides production (Martins et al., 2020). In another approach, Furtado et al. (2013) designed a bifunctional enzyme showing synergism between  $\beta$ -glucanase (EC 3.2.1.73) and the copper-dependent oxidase laccase (EC 1.10.3.2) that catalyzes the oxidation of aromatic compounds, enhancing reducing sugars production. Inspired by nature, artificial chimeric proteins, that are domains of two or more enzymes expressed in a single peptide sequence, have been designed to reduce the production costs and improve the enzymatic performance (Saadat, 2017).

### **I.3. Biorefinery – Biobased Chemicals Platform**

#### **I.3.1. Contextualization - bioeconomic and biorefinery**

World development over the years has undergone several transformations to meet global dynamics (Francois et al., 2020). Oil exploration, in the mid-20th century, generated a low-cost energy source that triggered global industrialization. Since then, oil has been applied not only as a fuel source, but as a base for most chemicals currently available (Hess et al., 2016). Burning fossil fuels releases more than 21.3 billion tons of carbon dioxide (CO<sub>2</sub>) per year (Shinde et al., 2020), which is associated with the greenhouse effect, bringing poses serious risks to human health and the environment. Thus, current economic development requires the preservation and management of the various resources available, in order to promote a profitable and bio-based productive system. Therefore, innovative materials, methods and technologies have been the main challenge for researchers to provide alternative and viable subsidies for energy and chemicals to the economy. Bioeconomic is the term given to the economic sector for the production of value-added products through the sustainable conversion of bio-based raw materials. More recently, the term circular bioeconomy was defined, since it was realized that circularity and efficiency are not automatically present in bioeconomic mechanisms (D'Adamo et al., 2020). In practice, the main motivation of the circular bioeconomy refers to the transformation of conventional industrialists through the implementation of efficient biorefineries, in order to enable economic growth unrelated to environmental degradation (Escobar & Laibach, 2021; Mohan et al., 2020). The American National Renewable Energy Laboratory (NREL) defines biorefineries as an installation of integrated biomass conversion processes to produce fuels, energy and chemicals (Kamm et al., 2006).

The chemical market based on biological platform has an expected annual growth of 12.6%, with an expectation of reaching US \$ 22.75 billion in 2025 (López-Porfiri et al.,

2020). Biofuels represent an important part of that market. Bioethanol, for example, has an extensive production volume, contributing to energy sufficiency. However, the economic viability of biorefineries is linked to the ability to manage multiple processes. Analogous to an oil refinery, a biorefinery can take advantage of the different fractions of biomass to produce various compounds and maximize their gains (Bozell & Petersen, 2010). More than 200 value-added chemicals can be produced from refining lignocellulosic biomass. Chemical products and platform intermediates, also called building blocks, represent the key to the growth of bioeconomics (Isikgor & Becer, 2015). Advances in bioethanol production and the main biologically based chemicals will be covered in sections 5.2 and 5.3, respectively.

In general, biomass has a higher amount of oxygen and lower fractions of hydrogen and carbon compared to oil. Due to this differentiated composition, a wider range of products can be obtained. However, a greater number of processes and technologies must be developed. To produce biofuels, biomass needs to be deoxygenated. In general, the presence of oxygen increases the polarity of the biofuel, making it difficult to mix with fossil fuels, in addition to decreasing energy capacity (Cherubini & Strömman, 2010). Oxygen is rejected in the typical form of  $\text{CO}_2$  or  $\text{H}_2\text{O}$ . Both forms have disadvantages. Whereas in the first case, half a mole of carbon is lost per mole of oxygen (loss of energy); in the second, two moles of hydrogen are unwantedly removed per mole of oxygen. The removal of oxygen can be favored by the elimination of undesirable elements, especially sulfur and nitrogen ( $\text{SO}_2$  and  $\text{NO}_2$ , respectively) (Lange, 2007). Other chemicals may require much less deoxygenation, because the presence of oxygen generally provides greater diversification of products with interesting physical and chemical properties (Wang & Liu, 2020).

### **I.3.2. Bioethanol**

Bioethanol is produced from the fermentation of sugars obtained from biomass. Bioethanol feedstock must contain either sucrose (e.g. sugarcane, sugar beet) or starch (e.g. corn, wheat) or a lignocellulosic material (e.g. sugarcane bagasse, wood and straw). Sugarcane is the feedstock used in Brazil and, so far, the most efficient raw material for bioethanol production. The CAZymes could be naturally expressed by a high number of microorganisms in the medium such as aerobic and anaerobic prokaryotes and eukaryotes (Champreda et al., 2019; Himmel et al., 2010) or could be heterologous expressed in microbial cell factories (Claes et al., 2020). The cost of these enzymes could constitute up to 30% of the bioethanol production from lignocellulosic biomass (Valdivia et al., 2016), thus the reduction of costs is paramount.

Typically, during the sugarcane processing, large amounts of bagasse are produced (~240 kg per ton of sugarcane), which are burnt in boilers for steam and electricity generation. But, nowadays, better technologies for cogeneration and optimization of bioethanol production allow it be used as a raw material for producing second-generation bioethanol (E2G) and other biobased products. The E2G could be produced from the plant biomass by use of microorganisms in four different bioprocesses: the SHF (Separate hydrolysis and fermentation), where the enzymatic hydrolysis and the fermentation occurs in separate vessels, and the SSF (Simultaneous saccharification and fermentation), SSCF (Simultaneous saccharification and co-fermentation) and CBP (Consolidated bioprocessing), where the enzymatic hydrolysis is associated to the fermentation in the same compartment (Olson et al., 2012; Salehi Jouzani & Taherzadeh, 2015). The combination of hydrolysis and fermentation at the same compartment could reduce costs associated to a reduced number of compartments and energy (Olofsson et al., 2008; Salehi Jouzani & Taherzadeh, 2015). The SHF, SSF and SSCF processes use exogenous enzymes (Figure 12-2), obtained from specialized companies or produced on-site at the same refinery, with are an additional cost. However, the consolidated bioprocessing (CBP) could remove this additional cost in the bioethanol production by allowing the microorganism to perform the production of CAZymes and the fermentation simultaneously, also allowing a lower contamination and inhibition of the cellulases (Olson et al., 2012; Salehi Jouzani & Taherzadeh, 2015).

In order to develop an ideal microorganism for bioethanol production in a CBP system, several authors performed modifications in *S. cerevisiae* strains, such as expression of heterologous cellulases from other microorganisms (Claes et al., 2020) or modification of genes associated to improved phenotypes (Caspeta et al., 2014).

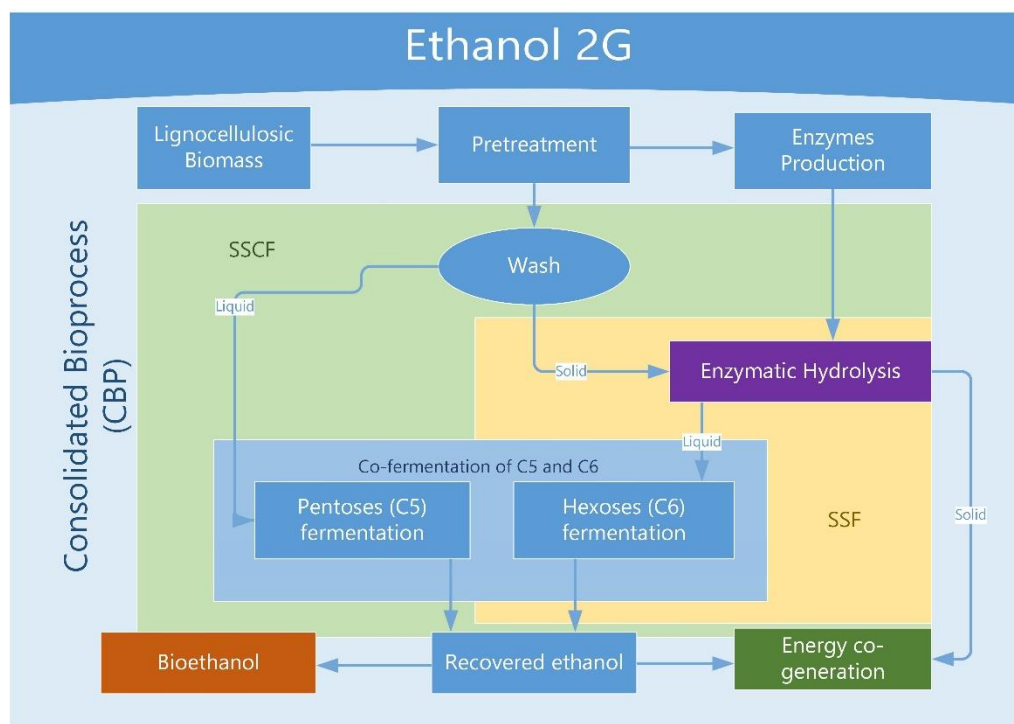


**Figure 12-2** Strategies used for hydrolysis of the pre-treated biomass and fermentation at the same compartment using the SSF (Simultaneous saccharification and fermentation), SSCF (Simultaneous saccharification and co-fermentation) in a CBP (Consolidated bioprocess)

### I.3.3. Other Value-added Bioproducts Obtained from Lignocellulosic Biomass

In the context of biorefinery, one of the main challenges is the application of the most diverse biotechniques to optimize bioprocesses in order to obtain the most diversified compounds with high added value from agro-industrial residues, aiming at the application in other industrial sectors (Silva et al., 2020). From each fraction of lignocellulosic biomass, through different strategies, the most diverse bioproducts can be obtained. **Figure 12-3** shows a scheme of the main compounds obtained from each fraction.

In the face of advances in nanotechnology, researchers around the world have been looking for the development of nanomaterials with properties of interest for multiple applications (Pires et al., 2019). In this respect, nanocellulose has stood out, in view of its multiple properties and applications. It is defined as a cellulosic material with at least one of its dimensions (length, width, or diameter) within the nanometric scale, and can be classified into three: crystalline nanocellulose (NCC), fibrillated nanocellulose (NFC) and bacterial nanocellulose (BNC) (Thomas et al., 2020). In this chapter we will focus on the first two types since they can be obtained from lignocellulosic biomass.



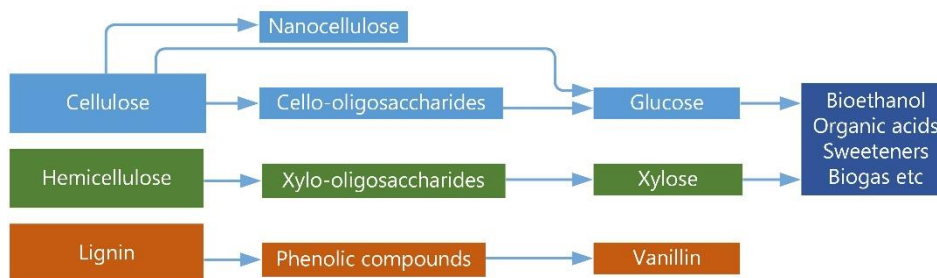


**Figure 12-3 Main Bioproducts Obtained from the Different Fractions of Lignocellulosic Biomass.**

### ***1.3.3.1. Nanocellulose***

NCC, also known as cellulose nanocrystals or nanocrystalline cellulose, are terms used to denote nanostructures of a crystalline nature up to 500 nm in length and up to 70 nm in width (Moon et al., 2011). They stand out for their chemical purity, rigid structure, resistance, can disperse easily and are usually amphiphilic (Thomas et al., 2020). Currently, NCC is commonly obtained from acid hydrolysis of lignocellulosic biomass or cellulosic isolate. Sulfuric acid is the main reagent used in this process, usually in high concentrations, mainly guaranteeing good colloidal stability of the crystals. Other acids such as phosphoric, hydrochloric, citric, among others are used as an alternative approach. In order to promote a more sustainable and ecologically correct commercial production of NCC, researchers have been developing recovery techniques for reusing the acids used in the treatment, in addition to studying applications of other less abrasive and more ecological techniques such as enzymatic hydrolysis, application of ultrasound among others. Crystalline nanocellulose has multiple industrial applications (Cui et al., 2016). In the food industry it has been used mainly in coatings, films and food packaging, in view of promoting improvements in the mechanical properties of polymers, in addition to being able to be used as a functional ingredient (dietary fiber), emulsifying agent and stabilizers. In the medical field, NNC has been applied in the development of materials with great potential in therapies that involve the repair, replacement and regeneration of tissues and organs. In the materials industry, cellulose nanocrystals have an important application in the development of high resistance polymers, aiming to minimize the excessive dependence on plastics and other synthetic polymers (Thomas et al., 2020).

NFC, also known as cellulose nanofibers or nanocellulose fibers, are terms used to designate individual cellulosic fibers in nano dimensions, usually with a diameter less than 100 nm in width and a few micrometers in length (Moon et al., 2011). Cellulose nanofibers have interesting properties, such as low thermal expansion, high aspect ratio, strengthening effect, good mechanical, and optical properties. Due to these specific characteristics, nanocellulose

fibers have been used in composites, food packaging, coating additives, air gel, membranes, as a gas barrier material, fillers, flocculants, emulsifiers, food thickeners and reinforcement material (Czaikoski et al., 2020). Mechanical breakdown or defibrillation is one of the main methods used to produce NFC, from the amorphous regions of cellulose present in lignocellulosic biomass. Other methods have also been used together to optimize the production of possible nanofibers, such as, for example, electrostatic induction, enzymatic and oxidative treatments (Thomas et al., 2020).

### ***1.3.3.2. Prebiotics***

Currently, in the face of changes in the population's eating habits, both the consumption and the development of functional foods have gained considerable prominence. Among the main functional ingredients, prebiotics are selective substrate of microorganisms naturally present in the organism, such as bifidobacteria and lactobacillus, thus promoting several health benefits (Hurtado-Romero et al., 2020). In the context of biorefinery, involving the use of lignocellulosic biomass, there is the possibility of obtaining two important classes of prebiotics: the cello-oligosaccharides (COS) and the xylooligosaccharides (XOS).

Cello-oligosaccharides or cello-oligomers are defined as oligomers that consist of 2 to 6 glucose units linked by  $\beta$ -1,4 type bonds. COS can be obtained from the cellulosic fraction of biomass through different treatments, such as acid hydrolysis, hydrolysis on carbon catalysts, hydrothermal process and controlled enzymatic hydrolysis. The latter is considered the most selective method in comparison to the others, in view of the greater control regarding the generation of monomers, thus providing higher yields of cello-oligosaccharides. COS are functional molecules of great interest to the food, feed and pharmaceutical industry, since they have a prebiotic effect, favoring the growth of probiotic strains in the large intestine, which inhibit the development of pathogenic bacteria and also help to combat intestinal constipation, maintaining intestinal health. In addition, cello-oligosaccharides can be used to produce bioethanol (Barbosa et al., 2020).

Xylooligosaccharides or xylooligomers are defined as oligomers consisting of xylose units, interconnected through  $\beta$ -1,4 bonds (Samanta et al., 2015). The XOS can be obtained from the xylan found in the hemicellulosic fraction of the biomasses by different treatments and enzymatic procedures, in a similar way to the COS. In addition to their prebiotic properties, encouraging the development of probiotic microorganisms, as well as COS, XOS present another important biological activity which is its antioxidant activity, helping to capture free radicals, inhibiting the development of cancer cells, especially, in the small intestine, colon and

liver tissues; promote the reduction of blood glucose, triglycerides and cholesterol levels, helping to fight diabetes mellitus and obesity, and can also act as immunomodulating agents, helping to strengthen the immune system, especially in the fight against infectious diseases (H. Zhang et al., 2017). In the pharmaceutical industry, these prebiotics are mainly used in the production of drugs related to the fight against obesity and in the treatment of gastrointestinal diseases. In the food industry, there are several possibilities for applications, in addition to acting as prebiotics, xylooligosaccharides can be used as sugar and/or fat substitutes, acting as preserving, stabilizing and gelling agents (de Freitas et al., 2019).

#### ***1.3.3.3. Organic Acids***

Organic acids are one of the main products in the chemical platform of biorefineries. The biotechnological production of these compounds is preferable to traditional methods, in view of the high selectivity and purity, cost-benefit, working in the optimized conditions, and because it is more ecological. Organic acids have a wide industrial application, standing out in the sectors of food and beverages, pharmaceutical products, textile, detergents, solvents, dyes, adhesives, rubbers etc. Many organic acids, such as levulinic acid, acetic acid, lactic acid, succinic acid etc., can be produced from renewable raw materials, such as lignocellulosic biomasses, which provide simple sugars, derived from cellulosic and hemicellulosic fractions, which serve as a source of carbon for the most diverse microorganisms to which through different metabolic routes it is possible to obtain these organic molecules (Rajendra Singh et al., 2017).

Levulinic acid is a chain fatty acid linked with a ketone carbonyl and a carboxylic acid group. This organic molecule is recognized as one of the chemicals with the highest added value because it is a promising precursor to other valuable chemicals such as  $\gamma$ -valerolactone (GVL), methyltetrahydrofuran (MTHF), hydroxymethylfurfural (HMF), ethyl levulinate, succinic acid, 2-pentanol, 2-butanol etc (A. Kumar et al., 2019). Levulinic acid can be produced from glucose, fructose, sucrose, and xylose, which can be found in the biomass of agricultural residues, for example, rice straw, rice husk, wheat straw, corn straw, bagasse, grass etc. On an industrial scale, levulinic acid is produced through various routes, such as the synthesis route, biological route, and extraction route (Eda et al., 2018). The biological route through fermentation is preferred by researchers, mainly because it avoids the use of large amounts of chemical conductors. This organic acid compound is fundamental for several industries, such as the oil industry, the pharmaceutical industry due to its applications in biofuels, capsules, tablets and

injections; in the food industry, as a flavor regulator, and in the agricultural industry in plant cultivation (A. Kumar et al., 2019).

Lactic acid is an important bioproduct used in a wide range of applications in the food, pharmaceutical, cosmetic, and leather industries. It has great potential to produce biodegradable and biocompatible polylactic acid polymer, which has vital applications in the pharmaceutical industry for the construction of prosthetic devices, sutures, and internal dosage of medicines. The obtaining of lactic acid can be carried out by chemical method or by fermentation. Fermentative production is more advantageous because in addition to producing a racemic mixture of lactic acid, this type of production uses sugars from low-cost renewable substrates and minimizes energy consumption. The lactic acid bacteria most used for commercial production of lactic acid, belongs to the genus *Lactobacillus*, such as *L. rhamnosus*, *L. casei*, *L. plantarum*, *L. pentosus*, *L. delbrueckii*. These bacteria consist of one of the most industrially important groups, since most of them are considered GRAS (Generally Considered Safe) and are used to efficiently convert sugars derived from various raw materials into lactic acid (Karnaouri et al., 2020).

Succinic acid is defined as a dicarboxylic acid composed of four carbons. Today, this important molecule is being recognized as the foundation for many other important chemicals applied in the food, chemical and pharmaceutical industries. It has great potential in the application in the manufacture of polybutylene succinate, surfactants, detergents, flavors and fragrances, herbicides, fungicides, and food additives. Currently, most of this acid is produced from fossil fuels. However, recent studies have demonstrated an efficient production of this important acid from renewable raw materials, making the process more economical and environmentally friendly (R. Kumar et al., 2020). Naturally, bio-succinic acid is formed by plants, animals, and microorganisms as a fermentation product. However, the greatest production is achieved by anaerobic microbial fermentation. Several microorganisms can be used to produce this acid, among them the bacterium *Actinomyces* *succinogenes* is recognized as one of the most promising microorganisms for industrial production, for producing relatively large quantities and for being able to use a wide range of carbon sources including lactose, xylose, arabinose, cellobiose and other reduced sugars, to produce succinic acid as the main end product. In addition, several renewable and inexpensive resources have been used by *A. succinogenes* to produce bio-succinic acid, especially fruit peels and husks, sugarcane bagasse, corn straw and fiber, rapeseed flour, wheat bran, bread residues, corn stalk, cotton stalk, cane molasses, fresh cassava root lentils, cheese whey, among others (Putri et al., 2020).

#### ***1.3.3.4. Sweeteners***

Currently, a significant portion of the population is seeking healthier lifestyle habits. As a result, researchers are looking for new ingredients that can be applied to food products to meet these requirements (Hong et al., 2019). In this scenario, the production of sugar substitutes, such as xylitol and arabitol, end up becoming the target of research by scientists from around the world.

Xylitol is defined as a type of alcohol pentahydrate (polyol) that is characterized by being a white, crystalline, odorless, non-toxic powder with a sweet taste. Its main application is as a sweetener, replacing or reducing the amount of sucrose in formulations, but it can also be used as a preservative and stabilizing agent in foods. Another aspect of great relevance of this substance is its high anticariogenic action, in addition to helping to combat various physiological dysfunctions of the human organism. Due to all these characteristics, xylitol has a high added value, presenting great interest in food and pharmaceuticals (Dasgupta et al., 2017). Biotechnological production of xylitol on a large scale usually occurs through fermentative processes, using microorganisms, such as bacteria, yeasts, and filamentous fungi capable of assimilating pentoses, as a substrate. Among these microorganisms, yeasts of the genus *Candida* sp. present great potential to be used in these bioprocesses. This large-scale production is influenced by operational parameters such as pH, temperature, initial xylose concentration and oxygen transfer rate (Tamburini et al., 2015).

Arabitol is another important polyol, widely used in the food, chemical and pharmaceutical industries. It has a sweetness like sucrose, low in calories (0.2 kcal/g) and can be absorbed slowly by the human digestive tract. Like xylitol, it also has an important anticariogenic action for oral health, in addition to being able to compose products and medicines for diabetics. In addition, D-arabitol has been used as a promising raw material for the biosynthesis of some value-added products, including xylitol, arabinoic acid, xylonic acid, propylene, ethylene glycol, enantiopure compounds, immunosuppressive glycolipids, herbicide formulations, etc. Since the last decades, arabitol has been biosynthesized from different substrates or hydrolysates derived from lignocellulosic biomasses, composed of glucose, xylose, crude glycerol, arabinose, fructose, xylose, and galactose. A wide variety of microorganisms, mainly osmophilic yeasts, have been reported to produce arabitol from different substrates, including *Candida*, *Pichia*, *Debaryomyces* and *Zygosaccharomyces* species (Guo et al., 2019).

#### ***1.3.3.5. Biogas***

Biogas is produced by anaerobic digestion of organic raw material through a process mediated by microorganisms, which occurs in four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. It is mainly composed of methane (40-65% v/v) and CO<sub>2</sub> (35-55% v/v) with small amounts of hydrogen sulfide (H<sub>2</sub>S) (0.1-3% v/v), moisture, and other traces of contaminants depending on the raw material (Kapoor et al., 2020). Several lignocellulosic biomasses can be used as organic material for this anaerobic digestion process, such as rice straw, corn straw, sunflower residues, wheat straw etc., requiring some pretreatment, such as diluted sulfuric acid, sodium hydroxide, ionic liquids, in order to reduce the recalcitrance of biomass, removing lignin and consequently increasing access to cellulosic and hemicellulosic fractions (Mirmohamadsadeghi et al., 2021). Choosing the inoculum as the right microbiological community is also important to reduce the process (15 to 40 days) and increase biogas production. It is also important to have a balance between the abundance of lignocellulolytic and methanogenic bacteria in the inoculum to obtain greater productivity (Vu et al., 2020).

#### ***1.3.3.6. Vanillin***

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the main component of the vanilla aroma, presenting a sweet and creamy odor. Its flavor and fragrance properties have made this compound important worldwide, especially for the food and pharmaceutical industries. Vanillin can be obtained from vanilla orchid grains by means of extraction. However, vanillin yield is considerably low because it accumulates at low levels in the plant. The biotechnological production of vanillin using microorganisms has attracted a lot of attention in recent years, as it provides higher yields, in the optimized conditions of the process, can be obtained from lignocellulosic biomasses (Furuya et al., 2017). The biochemical route for obtaining vanillin has as a precursor ferulic acid (4-hydroxy-3-methoxycinnamate), which is a phenolic compound that is linked to the cell wall of plant matrices, through ester bonds and can be released through enzymatic reactions. Some strains of microorganisms, such as the bacterium *Bacillus subtilis* and the yeast *Streptomyces sannanensis*, as well as agro-industrial residues, such as wheat bran for example, have shown success in the production of vanillin from ferulic acid esters in bioreactors (de Albuquerque et al., 2019).

## I.4. References

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## **Appendix II: Enzymatic bioconversion trends for lignocellulosic biorefineries**

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### **Abstract**

Biorefinery platforms emerge as a promising and sustainable approach to replace technologies based on chemical synthesis. Several challenges for the future are in line with the development of biorefineries, such as seasonal availability, land use, logistics and yields. Among all, from a technical point of view, enzymatic hydrolysis is one of the biggest critical points, with several bottlenecks to be overcome. However, it is a unique process capable of converting lignocellulose with the production of non-conventional bioproducts with greater purity, such as prebiotic oligosaccharides, fermentable sugars, and generating opportunities for the development of emerging products, such as bacterial nanocellulose. Thus, this chapter emphatically describes the biotransformation of lignocellulose from different sources, addressing enzymatic technology and processes, to produce emerging bioproducts, highlighting future challenges and opportunities for the research and industrial fields.

**Keywords:** Bioeconomy, cello-oligosaccharides, enzymatic hydrolysis, cellulose nanocrystals, fibrillar nanocellulose, xylo-oligosaccharides

## **II.1. Introduction**

A biorefinery integrates several unit processes such as pre-treatments and enzymatic conversion to produce a variety of products, enhancing yields and reducing time and costs. The value chain of a biorefinery is influenced by the feedstock according to the number of products that can be obtained, and their use in multiple applications before it ends up as waste. The first-generation feedstock for biorefineries (1G biorefineries) are food and oil-based crops such as corn, sugarcane, soybeans, and sunflower. The second-generation (2G) feedstock comprises biomass residues, agricultural wastes such as stems and stalks, and industrial waste such as husks, peels, and other wastes. The valorization of lignocellulose from these wastes contributes to waste management and a circular economy approach, which emphasizes the retrieval and restoration of products and energy after each product's life cycle. The 2030 agenda agreement for sustainable development claims the sustainable reduction of waste generation through prevention, reduction, recycling, and reuse (Nagarajan et al., 2022; Pires & Martinho, 2019; Velvizhi et al., 2022).

The efficient renewable feedstuffs through environmentally friendly bioconversion routes accrues from hydrolytic enzymes, whose costs are still a bottleneck in this process, along with the obtainment of an efficient pre-treatment of recalcitrant plant cell wall polymers, land usage, and biomass transportation costs (Lopes et al., 2021). Enzymatic hydrolysis is considered one of the most efficient and eco-friendly methods for depolymerization of lignocellulosic biomass fractions, which allows the formation of most diverse bioproducts of industrial interest. The main players include the types of enzymes, enzyme mixtures, hydrolysis conditions and the characteristics and composition of the lignocellulosic material employed (Goldbeck et al., 2014).

This chapter presents the main strategies and mechanisms for the enzymatic deconstruction of lignocellulosic biomass to obtain bioproducts involved in the integrated biorefinery, the challenges faced and future perspectives for research and industrial investments.

## **II.2. Lignocellulose characteristics and sources**

Cellulose is the core component of lignocellulosic biomass and is considered the most important candidate for replacement of fossil derived products as it represents the most abundant material of photosynthetically fixed carbon. Cellulose has been considerably valorized since its supply from the forest industry products and agriculture waste is abundant and inexpensive. Additionally, cellulose presents excellent characteristics such as hydrophilicity,

biocompatibility, reactive hydroxyl groups and stereo regularity, thus becoming a resource for a wide range of biofuels, platform chemicals and biomaterials.

Cellulose is the most abundant structural polymer in nature, and it can be obtained from agricultural residues, such as sugarcane bagasse and straw, rice straw, wheat and corn straw, fruit husks and bamboo culm, among others. It is classified as a straight-chain homopolymer composed of glucose units joined by  $\beta$ -1,4-type glycosidic bonds. The degree of polymerization is approximately 4000–6000 units in woody biomass, varying for each type of plant as well as for each stage of its development. The polymers are interconnected through hydrogen bonds and Van der Waals-type bonds, forming a microfibril structure with crystalline and amorphous regions, covered by hemicellulose and lignin. The crystalline form is highly ordered and stable, and less accessible than the amorphous form for physical, physical-chemical, and biochemical treatments in terms of breaking the structure and releasing bioproducts of interest, such as prebiotic oligosaccharides, nanocellulose, and fermentable sugars (Kamel et al., 2020). Crystalline cellulose fiber parts joined together by non-covalent hydrogen bonds, provide 3–30 times less degradability than the amorphous part. Cellulases, for example, are easily capable of hydrolyzing the more accessible amorphous cellulose, but they are not as effective in degrading the less accessible crystalline portion, thus requiring specific pre-treatments aimed at reducing the crystallinity and recalcitrance of lignocellulosic biomasses (Kamel et al., 2020).

Hemicellulose is the second-largest polysaccharide in lignocellulosic biomass. It supports cellulose microfibrils by hydrogen bonds and binds to lignin by covalent bonds. Unlike cellulose, hemicellulose is classified as a heteropolysaccharide, composed of different carbohydrates, presenting an amorphous structure with a polymerization degree of 50 to 300 units (Monteiro et al., 2021). This polysaccharide is composed of pentose units (xylose and arabinose), hexoses (glucose, galactose, and mannose), and sugar acids such as D-glucuronic acid, 4-O-methyl-D-glucuronic acid, and D-galacturonic acid. The composition and structure of hemicellulose vary depending on the plant species. Furthermore, there are considerable differences in the composition of hemicellulose between the stem, branches, roots, and bark. There is evidence that, on average, the number of different polysaccharides characterized in hemicellulose in plant species does not exceed four (Saha, 2003).

Lignin is a complex amorphous polyphenolic polymer composed of o-methoxylated p-hydroxyphenyl propanoic units, variable depending on the plant source. These monomeric units give rise to p-hydroxyphenyl, guaiacyl and syringyl subunits, which when incorporated into each other form this phenolic polymer (Bhatia et al., 2020). Lignin ends up acting as a connector, aggregating, and enveloping cellulose and hemicellulose, providing greater

integrity to the biomass. It makes the biomass recalcitrant by restricting the accessibility of enzymes, in addition to promoting mechanical and microbiological resistance to the treatments the lignocellulosic biomasses are subjected to. This highlights an obstacle that researchers have bypassed by separating it from other parts of interest, that is, cellulose and hemicellulose (Bichot et al., 2018). The great challenge today is to find conditions and pre-treatments that are efficient in reducing recalcitrance, can remove lignin, and do not degrade cellulose and hemicellulose into furfural and hydroxymethylfurfural, in addition to being economically viable with low environmental impact (da Silva et al., 2020).

### **II.3. Enzymes hydrolyzing lignocellulose**

Enzymatic lignocellulose hydrolysis requires synergistic action of several different enzyme complexes. Cellulases and hemicellulases are the major enzymes of this process; however, it is known that pectinases, ligninases and oxidative enzymes are important accessory enzymes that improve sugar yields. Understanding the individual and synergistic mechanism of each enzyme and designing mixtures in appropriate proportions is the key to a successful hydrolysis, with higher efficiencies and lower cost (M. Martins, Dinamarco, et al., 2020).

Cellulases are the main enzymes responsible for cellulose hydrolysis. Endo-1,4- $\beta$ -glucanases (EC 3.2.1.4) are enzymes that randomly break the internal glycosidic bonds of the amorphous region of cellulose, releasing free ends of the cellulose chain. Cellobiohydrolase (CBH, exo-1,4- $\beta$ -glucanases, EC 3.2.1.91) hydrolyzes the outer part, releasing short-chain oligosaccharides, such as cellobiose. The  $\beta$ -glucosidase (EC 3.2.1.21) breaks down cellobiose into two glucose molecules, completing the synergistic hydrolysis process (Du et al., 2018). It is important to highlight that the limitation of  $\beta$ -glucosidase is reported as one of the main reasons for inhibition problems, since the accumulation of cellobiose inhibits the other enzymes by feedback. Another relevant point is the performance of cellobiohydrolase (CBH) (EC 3.2.1.176 and EC 3.2.1.91), which can hydrolyze the terminal region of the cellulose, with specificity towards the reducing (CBHI) and non-reducing (CBHII) part, respectively (Rytioja et al., 2014).

Xylanolytic and ligninolytic enzymes act in synergism with cellulases for reducing sugars production. Unlike cellulose, hemicellulose, because it is a heteropolymer, releases, as already mentioned, different fermentable sugars. The most important hemicellulose degradation enzymes are endo- $\beta$ -1,4-xylanase (EC 3.2.1.8) and  $\beta$ -D -xylosidases (EC 3.2.1.37) that degrade xylan to xylose (Terra & Ferreira, 1994). L-arabinan is hydrolyzed by arabinanases (EC

3.2.1.99) and arabinofuranosidases (EC 3.2.1.55), releasing arabinose or arabino oligomers (Lim et al., 2011). Galactoglucomannan (most abundant hemicellulose in softwood) consists of a backbone of mannose and glucose units, decorated with galactose and acetyl moieties. The mannan backbone is broken into shorter oligosaccharides by the action of endo-1,4- $\beta$ -mannanases (EC 3.2.1.78), while  $\beta$ -mannosidases (EC 3.2.1.25),  $\beta$ ,  $\beta$ -glucosidases (EC 3.2.1.21),  $\alpha$ -galactosidases (EC 3.2.1.22), and acetyl esterases (EC 3.1.1.72) release mannose, glucose, galactose, and acetyl groups, respectively (Domingues et al., 2018). Glucuronic acid can be obtained by degradation of glucuronan groups by  $\beta$ -glucuronidases (EC 3.2.1.31) and xylan  $\alpha$ -1,2-glucuronidases (EC 3.2.1.131) (Nascimento et al., 2014). Accessory enzymes of the ligninolytic complex are also important in the process of hydrolysis, for example, lytic polysaccharide monooxygenase (LMPO, EC 1.14.99.54 and EC 1.14.99.56), pectin lyase (EC 4.2.2.10), and feruloyl esterase (EC 3.1.1.73), the latter enzyme acts by cleaving the ester bonds between the side chains of arabinose, releasing ferulic acid, which is of great economic interest due to its antioxidant properties (Nascimento et al., 2014).

## **II.4. Bioproducts**

### **II.4.1. Fermentable Sugars**

Fermentable sugars are defined as simple chain saccharides that can be easily metabolized by microorganisms, serving as a carbon source and which can be used to produce various bioproducts (Jelonek et al., 2022). One of the most common examples in which fermentable sugars are used is bioethanol production where these sugars are fermented into biofuel. The challenge lies in producing high amounts of fermentable sugars with high conversion from lignocellulosic biomass, which will help in the development of economically viable processes (Zhang et al., 2017).

From the cellulosic fraction, glucose can be obtained, which is the main fermentable sugar, it belongs to the class of hexoses and is widely used in fermentation processes to produce ethanol, bacterial nanocellulose and other bioproducts from fermentation (Gao et al., 2021). From the hemicellulose fraction, xylan can be obtained, which, when degraded, releases both xylo-oligosaccharides and xylose, which is another fermentable sugar, belonging to the class of pentoses (R. P. Martins et al., 2021). Also, this fraction can be used to obtain other sugars such as galactose, arabinose, mannose, glucuronic acid. Biotechnological production of xylitol and, more recently, arabitol, which are non-cariogenic sweeteners of low caloric value,



has increased the demand of fermentable sugars, xylose, and arabinose, respectively (Farias et al., 2022).

#### II.4.2. Cello-oligosaccharides

##### *Definition and characteristics*

Cello-oligosaccharides (COS) are short-chain D-glucose units linked by  $\beta$ -1,4 bonds derived from cellulose partial hydrolysis. Due to their chemical structure, COS constitute a class of non-digestible oligosaccharides (NDOs). Unlike other groups of NDOs, such as fructo-oligosaccharides (FOS), xylo-oligosaccharides (XOS), and galacto-oligosaccharides (GOS), which already have extensive literature on their properties and applications, COS still have plenty to be discovered. COS are classified according to their degree of polymerization (DP): cellobiose (2 glucose units), cellotriose (3 units), cellotetraose (4 units), cellopentaose (5 units), and cellohexaose (6 units) (**Figure 12-4**). Those COS with up to 6 glucose units are water soluble under normal conditions, facilitating the separation from non-hydrolyzed cellulose, which is an insoluble material (Ávila et al., 2021a). COS may still have glycosidic bonds different from  $\beta$ -1,4-type bonds, especially under water deficient operating conditions, where glycosylation can create  $\alpha$ -1,4,  $\alpha/\beta$ -1,3,  $\alpha/\beta$ -1,6, and  $\alpha/\beta$ -1,2 bonds. These bonds can induce the formation of branches in the structure of the COS, which even favors solubility. However, these same bonds can modify the bioactivity of the COS. This fact highlights the importance of carefully characterizing the glycosidic bonds in the final product (Shrotri et al., 2013).

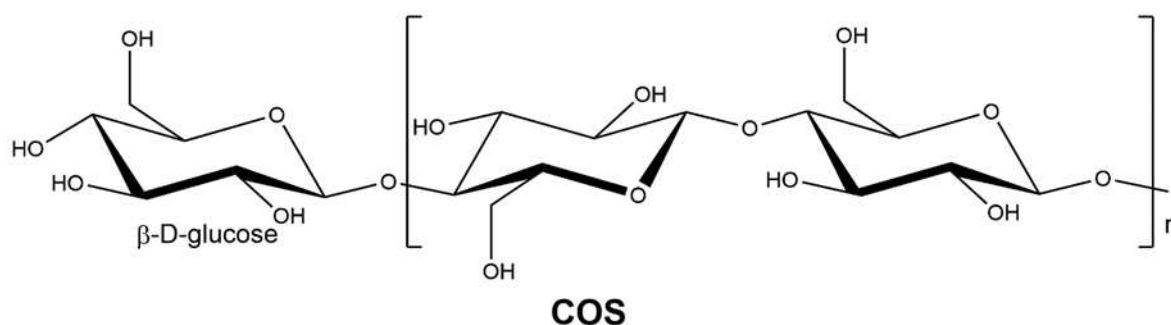


**Figure 12-4** Cello-oligosaccharides (COS) chemical structure, where "n" represents the number of glucose units.

The availability of COS in the market is still rare, even for research. However, the development of COS synthesis and purification processes has been driven by their potential application, mainly due to the beneficial action on the human body, either as a prebiotic for the intestinal microbiota or as an antibiotic for pathogenic bacteria (Chen et al., 2021).

### ***Production***

The production and isolation of COS with economic viability are of great interest. The large-scale production of COS is favored by the fact that cellulose is the most abundant polymer in nature, as it is the main constituent of the plants cell wall. However, the scale up of COS production comes up against the difficulty of controlling the DP of the product and recovering it, along with the recalcitrant nature of cellulose (Chen et al., 2021). Despite the vast literature on the complete depolymerization of cellulose, there are still few reports on its partial depolymerization to obtain COS. However, with the discoveries regarding the prebiotic potential of COS, the development of partial depolymerization processes of cellulose is on the rise. Physicochemical and enzymatic methods of partial hydrolysis of cellulose aiming at the production of COS are briefly described below.

Physicochemical treatments are very effective in the partial hydrolysis of cellulose, producing COS and glucose monomers. However, adequate operating conditions of temperature, residence time, acid load, and biomass loading are crucial for good yields. Controlling these conditions is essential, as the monomers and oligomers that are released can dehydrated to 5-hydroxymethyl furfural, followed by the production of levulinic acid. The treatment with supercritical water is successful in favoring the dissolution of hydrolyzed cellulose such as COS and short-chain polymers, rather than monomers (Ávila et al., 2021b). The DP of the produced COS depends on the treatment temperature. Through this method, Tolonen et al. (Tolonen et al., 2015) obtained a reaction liquid content of 6.1% glucose and 42.0% COS. This was attributed to the rate of dissolution of cellulose which increases faster than its rate of hydrolysis, allowing a high recovery of the dissolved compounds.

Chemical hydrolysis in an acid medium is commonly used to produce glucose, xylose, furan derivatives, levulinic acid, among others. As cellulose has a rigid crystalline structure, the acid has difficulty in penetrating its dense network, being necessary to boost the hydrolysis with concentrated or supported catalyst acids, such as sulfuric acid and hydrochloric acid. Despite the few reports of COS production by concentrated acid hydrolysis, it has already been demonstrated that it is possible to obtain a COS with an DP lower than 20 (Ávila et al., 2021b). However, processes with these strong acids tend to be difficult to control and can generate products with reduced nutritional quality and form toxic substances (Chen et al., 2021).

The hydrolysis of cellulose using carbon support catalysts is interesting since they are easily separated from the liquid medium with the product dissolved after the reaction (Chen et al., 2021). These catalysts must have oxygenated functional groups to present a high density of accessible and stable acid sites under the milling and hydrothermal conditions necessary for

the hydrolysis of cellulose. These catalysts have large surface areas and cellulose adsorption capacity, essential characteristics for contact between cellulose and catalyst because both are water insoluble. Thus, during the reaction, the cellulose adsorbs on the catalyst surface via interaction with the carbons present there, and then the glycosidic bonds are hydrolyzed by the acid functional group presented on the catalyst surface (Ávila et al., 2021b). The carbon catalysts in a continuously operating system favor the synthesis of COS by rapidly removing them from the reaction medium, preventing their further hydrolysis. The combination of the benign nature of the carbon catalyst with the easy separation of the COS results in a product practically ready for biological applications (Chen et al., 2021).

A recent approach to transforming cellulose into water-soluble products is mechanical catalytic depolymerization, based on the synergistic effect between strong acids and mechanical friction. Depolymerization takes place during the milling of cellulose impregnated with a strong acid in the absence of a solvent. The obtained COS showed a non-linear configuration, which contributes to the higher water solubility. These ramifications may have been formed by acid-catalyzed repolymerization of glucose and other small molecules during the milling process (Chen et al., 2021). Despite the advantages of mechano-catalysis over more conventional processes, the production of branched COS is currently undesired based on current knowledge.

Cellulose hydrolysis by cellulolytic enzymes is more interesting than acid hydrolysis as it is conducted under mild operating conditions and does not generate undesirable by-products, thus requiring fewer purification steps. In addition, the enzyme specificity facilitates hydrolysis control. Thus, the enzymatic synthesis of COS is adapted from processes of total cellulose depolymerization to obtain glucose. For this, it is necessary to modify the enzyme preparation to limit the hydrolysis of the produced soluble oligosaccharides (Chen et al., 2021). This preparation is composed by three types of cellulases: endoglucanases, which randomly hydrolyze internal  $\beta$ -1,4 glycosidic bonds of the amorphous region of cellulose, exoglucanases (cellobiohydrolases), which hydrolyze  $\beta$ -1,4 glycosidic bonds of the reducing or non-reducing chain ends to release cellobiose, and  $\beta$ -glucosidases, which hydrolyze  $\beta$ -1,4 glycosidic bonds of the soluble cellobiose and other COS releasing glucose (Ávila et al., 2021b). The GH7 Cellobiohydrolase (CBHI) and the GH5 endoglucanase (EGI) were considered enzymes of fundamental importance for COS synthesis. As hydrolysis progresses, enzymes that act specifically on the amorphous regions of the cellulose are needed. The synergistic action of cellobiohydrolases and endoglucanases radically increases the COS production yields (Ávila et al., 2021b; Karnaouri et al., 2019).

Therefore, a strategy to preserve the COS produced by endoglucanases and exoglucanases is to remove  $\beta$ -glucosidases from the enzyme preparation or at least inhibit their activity (Chen et al., 2021). One of the methods for this is to promote the adsorption of endoglucanases and exoglucanases to the cellulosic substrate since  $\beta$ -glucosidases have a low affinity for cellulose. In this way, the separation takes place by filtration easily. Instead of removing  $\beta$ -glucosidases, another strategy is to add adsorbent material for oligosaccharides, such as activated carbon. Thus, the produced COS are protected from the action of  $\beta$ -glucosidases. The accumulation of glucose and other products during the process inhibits the activity of endoglucanases. To overcome this problem, a multi-stage system can be used to remove the main products and add new enzymes to the reaction medium (Chu et al., 2014). Another interesting strategy is to convert glucose into high value-added products, such as glucose mono-decanoate, a known biodegradable and non-ionic surfactant. The simultaneous production of COS and this high value-added product increases the degree of purity of the obtained COS (Zhou et al., 2020).

To increase the contact surface of the cellulose to facilitate the access of the enzymes, the cellulose must undergo a pre-treatment that can be carried out by mechanical grinding, acid or alkali treatment, steam, ammonia explosion, and ionic liquids (Chen et al., 2021). Recently, it has been verified that the use of auxiliary enzymes, such as lytic polysaccharide monooxygenases (LPMOs), can increase the chain breaks promoting the defibrillation of crystalline cellulose, increasing the contact surface, thus facilitating the activity of endoglucanases. The synergistic action of LPMOs and endoglucanases releases neutral and oxidized sugars (Barbosa, Kendrick, et al., 2020).

Despite increasing the COS production yields, the advance in the enzymatic hydrolysis application for cellulose depolymerization process comes up against the high cost of enzymes and the purification processes. However, as the commercial demand for COS increases, the enzymatic process becomes more economically viable (Chen et al., 2021). Even the discovery of new enzymes that further optimize the biochemical process, such as LPMOs, highlights the promising future of this process.

### ***Properties and applications***

COS have great potential application in food, pharmaceutical products, cosmetics, animal feed, bioenergy, chemical industries in general, among other products. This is due to their physical, rheological, and functional properties, such as excellent oil retention capacity, good compressive strength and malleability, and considerable heat and acid resistance (Kluge et al., 2019).

As it is a new material, there are still few reports of its isolated application, also there is no description of its specific characteristics, thus configuring an excellent research field to be explored. One of the most important properties of COS is their prebiotic activity, given the increasing demand for functional foods. A prebiotic substance promotes the growth of probiotic bacteria of the intestinal microbiota, such as *Lactobacillus* sp. and *Bifidobacterium* sp. stimulating them to produce biologically important compounds, such as short-chain fatty acids (Karnaouri et al., 2019).

As COS are not digested by humans, they resist the stomach digestion process and are not absorbed by the small intestine, thus arriving practically intact in the large intestine where they will have their prebiotic action. Probiotic bacteria produce short-chain fatty acids such as acetate, propionate, and butyrate, which reduce luminal pH, thus inhibiting the development of entero-pathogens, and are substrates for the growth of colonic epithelium, inducing the production of immunomodulatory cytokines (Ávila et al., 2021b). In this context, prebiotics intake is directly associated with several health benefits, such as stimulation of the immune system, regulation of gastrointestinal function, anti-obesity effect, and prevention of carcinogenesis (Chen et al., 2021). Like other prebiotic substances, COS can be consumed directly in the form of capsules or incorporated into foods such as cereal bars, baked goods, sweets, ice cream, juices, sauces, among others.

The prebiotic effect and other beneficial properties of COS have already been identified in several livestock animals, such as calves and pigs, favoring the functioning of their gastrointestinal tract (Chen et al., 2021). In addition to the prebiotic potential, COS have shown excellent inhibitory action against important human pathogenic bacteria, such as *Helicobacter pylori*, a bacterium that resists stomach pH and can cause severe gastroenteritis, (Ávila et al., 2021b; Karnaouri et al., 2019).

COS can also improve the efficiency of cosmetics. They have a significant moisturizing effect, in addition to improving the skin barrier, mainly inhibiting bacteria that cause dermatitis, such as *Staphylococcus* spp. and *Pseudomonas* spp. (Ávila et al., 2021b). Another potential application of COS is as a substrate for ethanol production by fermentation. The advantage over the conventional process, which uses glucose as a substrate, is to avoid inhibition by the high concentration of glucose. Yeasts must be modified to be able to digest these oligosaccharides, which consequently ends up increasing the speed of the process and reducing the risk of contamination (Barbosa, Kendrick, et al., 2020).

### II.4.3. Nanocellulose

#### *Definitions and classification*

Given the advances in nanotechnology in recent years, researchers around the world have been seeking the development of nanomaterials with properties of interest for multiple applications. In this aspect, nanocellulose has stood out, considering its multiple properties and possibility of applications. Nanocellulose is defined as a cellulosic material with at least one of its dimensions (length, width, or diameter) within the nanometric scale (**Figure 12-5**). Nanocellulose can be classified into three categories: crystalline nanocellulose (NCC), fibrillated nanocellulose (NFC) and bacterial nanocellulose (BNC) (Thomas et al., 2020). In this chapter we will focus on the first two types since they can be obtained directly from the cellulosic fraction from lignocellulosic biomasses. Table 12-1 summarizes the properties and potential applications of NCC and NFC.

**Table 12-1 Main properties and potential applications of NCC and NFC**

|                                        | <b>Production</b>                                                                                                          | <b>Average Dimensions</b>                | <b>Properties</b>                                                                                                                                                                                                   | <b>Applications</b>                                                                                                                                                                                                                                                                                                            |
|----------------------------------------|----------------------------------------------------------------------------------------------------------------------------|------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Crystalline nanocellulose (NCC)</b> | Acid hydrolysis of crystalline cellulose                                                                                   | Diameter 3-50 nm<br>Length: 30 to 300 nm | High strength, electromagnetic response, large surface area, stiffness, thermostability                                                                                                                             | Bandage for wound healing, packaging, coating, films, food fiber, emulsifying and stabilizing agent                                                                                                                                                                                                                            |
| <b>Fibrillated nanocellulose (NFC)</b> | High pressure homogenization, Microfluidization, Grinding, Cryocrushing, Ultrasound, Steam explosion, Enzymatic hydrolysis | Diameter: 5-60 nm<br>Length: microns     | Low thermal expansion, high aspect ratio, strengthening effect, light weight, dimensional stability, thermal stability, high optical transparency, high thermal conductivity, low oxygen permeability, non-toxicity | Support/carrier for nanoparticles, textile coatings, films, papermaking, automobile parts. Smaller volume applications include cosmetics, pharmaceuticals, paints additives, sensors, construction, aerospace materials, organic LEDs, flexible electronics, photovoltaics, 3D-printing photonic films, recyclable electronics |

NCC, also known as cellulose nanocrystals or nanocrystalline cellulose are terms used to designate nanostructures of a crystalline nature with lengths of up to 500 nm and width of up to 70 nm, obtained from the hydrolysis of the crystalline region of cellulose (Moon et al., 2011). They stand out for their chemical purity, rigid structure, resistance, easy dispersion and are normally amphiphilic (Thomas et al., 2020).

On the other hand, NFC, also known as cellulose nanofibers or nanocellulose fibers, are terms used to designate individual cellulosic fibers in nanodimensions, normally with

a diameter of less than 100 nm and a length of a few micrometers, obtained from the hydrolysis of the amorphous region of cellulose. Cellulose nanofibers have interesting properties, such as low thermal expansion, high aspect ratio, strengthening effect, good mechanical, and optical properties (Moon et al., 2011).

### ***Nanocellulose production and its challenges***

Cellulose nanocrystals are obtained from the hydrolysis of the crystalline fraction of cellulose. In the current scenario, the industrial scale production of NCCs commonly occurs from the acid hydrolysis of lignocellulosic biomass or cellulosic isolates. Concentrated sulfuric acid is the main reagent used in this process, usually in high concentrations, mainly ensuring good colloidal stability of the crystals. Other acids such as phosphoric, hydrochloric, citric, among others, are used as an alternative approach. To promote a more sustainable and ecologically correct commercial production of NCC, researchers have been developing recovery techniques to reuse the acids used in the treatment, in addition to studying applications of other less abrasive and more ecological techniques, such as enzymatic hydrolysis, which can be applied together with other technologies such as ultrasound, for example (Durairaj et al., 2022).

Mechanical breakdown or defibrillation is one of the main methods used to produce NFC, from the amorphous regions of cellulose present in lignocellulosic biomass. Other methods have also been used together to optimize the production of possible nanofibers, such as electrostatic induction, enzymatic treatments, ultrasound, and oxidative methods (Thomas et al., 2020).

To avoid the significant consumption of acids, as well as to save energy, avoiding mechanical methods, researchers have been facing challenges in producing nanocellulose by more sustainable, simple, and promising routes. Therefore, the application of enzymatic hydrolysis ends up being an advantageous method, of high efficiency, without pollution, in addition to low energy consumption. In addition, other treatments have also been used together, such as the ultrasonic treatment, for example, which provides an increase in the contact area between the enzyme and the substrate, accelerating the cellulose hydrolysis reaction and thus favoring higher concentrations of nanocrystals and nanofibrils of cellulose (Li et al., 2016).

Among the main enzymes that can act on the cellulosic fraction, there are the endoglucanases, which act on the internal chains of the amorphous region of cellulose and that, under conditions of partial hydrolysis (usually up to 12 h, at 50 °C and pH 5), can release the cellulose nanofibrils. On the other hand, cellobiohydrolases I and II enzymes can act in the crystalline region, at the reducing and non-reducing ends, respectively, and can fragment the

crystalline region of cellulose through partial hydrolysis conditions, releasing nanoparticles of cellulosic crystalline nature, in addition to cellobiose (cello-oligosaccharide with a lower degree of polymerization) (Ávila et al., 2021b; Barbosa, Kendrick, et al., 2020).

The application of oxidative enzymes, such as LMPOs, can also help in the fragmentation of the crystalline region of cellulose, in view of its high resistance to hydrolysis since it is in a high degree of organization and structural compaction. Treatments such as ultrasound can also be viable to be applied in these enzymatic hydrolysis processes, to promote more cavitation, more turbulence and more interactions between enzymes and substrate, allowing higher yields of the bioprocess (Barbosa, Kendrick, et al., 2020; Barbosa, Martins, et al., 2020).

### *Applications of Nanocellulose*

Crystalline nanocellulose has multiple industrial applications. In the food industry it has been used mainly in coatings, films, and food packaging, considering that they promote improvements in the mechanical properties of polymers, in addition to being able to be used as a functional ingredient (food fiber), emulsifying and stabilizing agent. In the medical field, NCC has been applied in the development of materials with great potential in therapies that involve the repair, replacement and regeneration of tissues and organs. In the materials industry, cellulose nanocrystals have an important application in the development of high strength polymers, aiming to minimize the excessive dependence on plastics and other synthetic polymers (Thomas et al., 2020).

Due to their interesting rheological, functionals and structural properties, nanocellulose fibers have been used in food, chemical, pharmaceutical and environmental processes. They are usually applied in composites, biodegradable food packaging, coating additives, aerogels, membranes, as a gas barrier material, fillers, flocculants, emulsifiers, food thickeners and reinforcement material (Czaikoski et al., 2020).



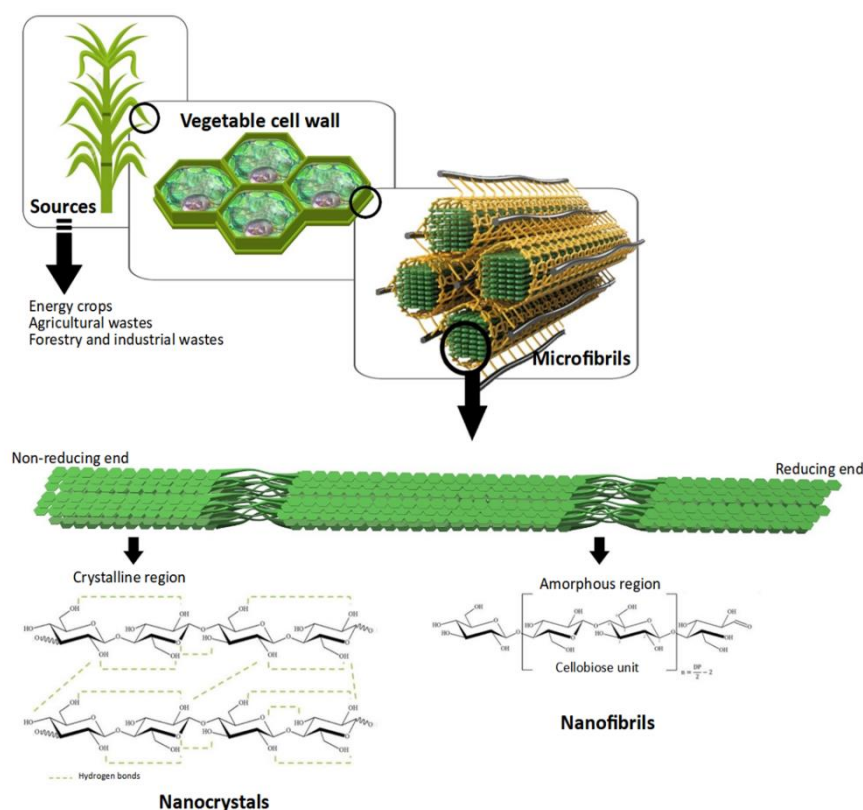


Figure 12-5 Illustration of cellulose nanofibrils' and nanocrystals' structure.

### *Definitions and classification*

Xylo-oligosaccharides (XOS) or xylo-oligomers consist of a class of non-digestible oligosaccharides (NDOs) and have been the subject of intense research in recent years to seek optimized and economically viable conditions using lignocellulosic biomass, specifically the hemicellulose (xylan) fraction, which is the (second polymer most abundant polymer in nature and is of great importance from the point of view of an integrated lignocellulosic biorefinery. They XOS have technological potential due to their functional properties and potential applications in the food, pharmaceutical and chemical industries (Farias et al., 2022).

Chemically, XOS are defined as oligomers formed by xylose units, linked through  $\beta$ -1,4 bonds (**Figure 12-6**). XOS can be differentiated by the degree of polymerization and can be classified into xylobiose (two monomers), xylotriose (three monomers), xylotetraose (four monomers), xylopentose (five monomers) and xylohexaose (six monomers). XOS with up to four monomeric xylose units are classified as having a low degree of polymerization, while the others have a high degree of polymerization, which ends up directly influencing the functional properties, mainly the prebiotic effect, as well as the technological properties, such as a stabilizing effect (de Freitas et al., 2019) .

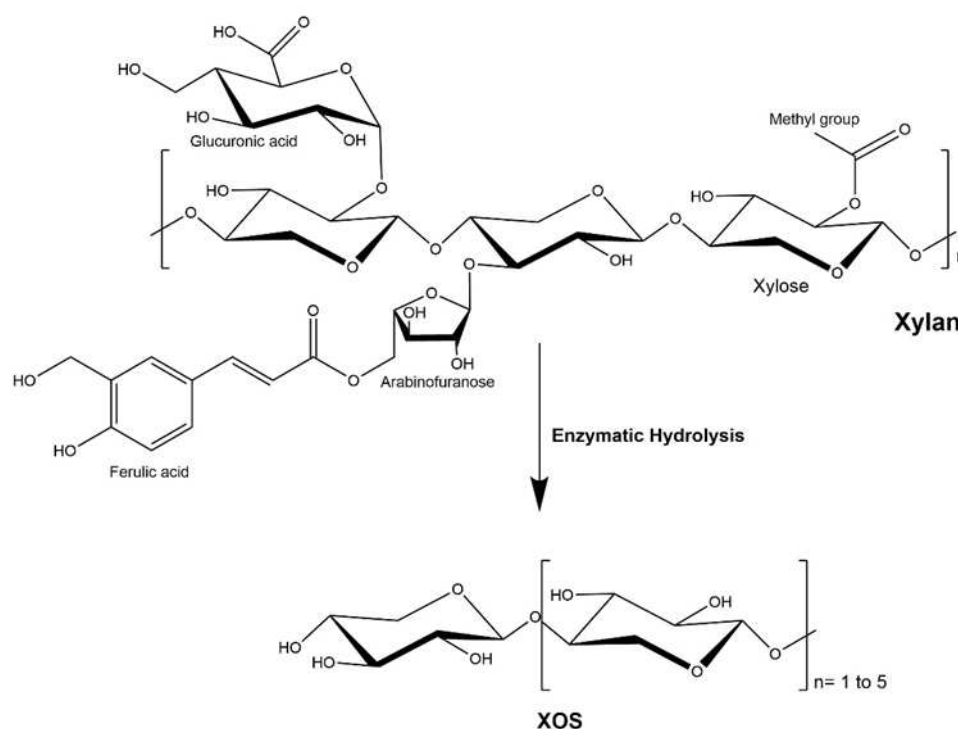


Figure 12-6 Xylo-oligosaccharides from enzymatic hydrolysis of Xylan, where "n" represents the number of xylose units from 1 to 5 (polymerization degree from 2 to 6)

### *Production of xylo-oligosaccharides*

Xylo-oligosaccharides (XOS) are naturally present in fruits, vegetables, milk, and honey, but in low concentrations. The large-scale production accrues from the hemicellulosic fraction of lignocellulosic biomass through chemical or biotechnological processes (da Silva et al., 2020). The recalcitrance of lignocellulosic biomass must be overcome by pre-treatments to deconstruct the three-dimensional structure of lignocellulose, avoiding carbohydrate degradation and, mainly, preserving the usefulness of the hemicellulose fraction. Among the main pre-treatments aimed at the production of XOS, the dilute acid treatment (0.5% sulfuric acid) is the most applied technology allowing the breaking of the glycosidic bonds in the chains. There is also the application of alkaline reagents, such as sodium or potassium hydroxide, in which the hydrogen bonds between cellulose and hemicellulose are cleaved by means of hydroxide ions. Another proposed methodology is the solubilization of hemicellulose material using organic solvents such as ethanol, which can separate the hemicellulose and precipitating the polysaccharides from the biomass, isolating them from the lignin (de Freitas et al., 2019).

After pretreatment, the hemicellulose-rich hydrolysate proceeds to the production of XOS itself. Large-scale production of XOS occurs through the cleavage of chemical bonds in the xylan chain (the main polymer of the hemicellulose fraction formed by monomeric xylose

units). Xylan hydrolysis can occur through physical-chemical treatments, such as acid, alkaline or hydrothermal processes, or, preferably, also through biotechnological processes such as enzymatic hydrolysis (**Figure 12-6**), which is the most sustainable and environmentally -friendly way (Farias et al., 2022).

The chemical production of XOS normally occurs through the action of dilution acids, such as acetic, sulfuric, and hydrochloric acids. These acids act randomly cleaving the  $\beta$ -1,4 glycosidic bonds of xylan, decreasing the degree of polymerization and promoting the release of xylo-oligomers and xylose. Acid concentrations, as well as treatment time, are important process variables that must be controlled, because if excessive they can lead to the total depolymerization of xylan to xylose. Furthermore, there is also the possibility of intensifying the degradation of xylose to furfural, which is an undesirable compound responsible for the inhibition of the bioprocess. Commonly, acid concentrations vary from 0.5 to 5%, with a hydrolysis time of up to 2 hours, which may or may not be combined with a thermal process (da Silva et al., 2020).

To obtain XOS through a hydrothermal process, also known as auto-hydrolysis, lignocellulosic biomass is treated only with water, usually in autoclaves or specific reactors. In these, high pressures and temperatures are applied, which promote the release of acetic acid from the acetyl groups of the xylan chain, acidifying the medium and promoting the depolymerization of the xylan. The hydrolysis conditions proposed by this methodology must be moderate to avoid the degradation of xylo-oligomers to xylose, in addition to the formation of inhibitors such as furfural and hydroxymethylfurfural, which must be removed from formulations containing XOS, due to their toxic effects. Normally, in this process, temperatures in the range of 150-250 °C are applied, at pressures of 1 – 15 MPa, for a maximum of 1 hour of process (Farias et al., 2022).

The enzymatic hydrolysis of the hemicellulose fraction of the biomass is considered one of the most efficient and interesting methods to produce XOS. In addition, it is considered a very selective process, allowing high yields of these biocompounds, it can be easily controlled, as it requires mild pH and temperature conditions, minimizing the generation of undesirable compounds in the subsequent steps, such as furfural and hydroxymethylfurfural, which end up being generated in the other processes (da Silva et al., 2020). Normally, the production of XOS by enzymatic hydrolysis occurs in a range of temperatures around 40-65 °C, although many studies indicate an optimal temperature of 50-55 °C. As for pH, sodium acetate solution is normally used at 0.05 to 0.1 mol/L in pH ranges between 4.5 and 6. All these parameters

depend on the optimal biochemical characteristics of the xylanolytic enzymes that act in the bioprocess (M. Martins, Ávila, et al., 2020).

The endoxylanases of the GH 10 and GH 11 families are the main families of enzymes that act on the hydrolysis of xylan to produce XOS. These are mainly secreted by filamentous fungi of the genus *Trichoderma* spp. and *Aspergillus* spp. The endoxylanases of the GH 10 family produce xylo-oligomers with a low degree of polymerization, while the GH 11 family produces XOS with a high degree of polymerization, since polymerization since they act more internally and in the middle of the hemicellulose chains (M. Martins, Ávila, et al., 2020).

These enzymes can hydrolyze the  $\beta$ -D-1,4-type internal bonds between the xylose units that make up the xylan, releasing the xylo-oligomers as a result. In addition to the action of endoxylanases, other enzymes, classified as accessory, are very important and interesting to be applied, as they act synergistically providing higher concentrations of xylo-oligosaccharides. Accessory enzymes  $\alpha$ -L-arabinofuranosidases and feruloyl esterases can improve the accessibility of the main ones, aiding in their catalytic activities and promoting higher yields from the bioprocess (M. Martins, Ávila, et al., 2020). These enzymes act by removing the arabinose and ferulic acid groups that may be present in the hemicellulosic fraction, depending on the type of lignocellulosic biomass used. Researchers found a higher production of XOS from a mixture of enzymes composed of endoxylanases,  $\alpha$ -L-arabinofuranosidase, feruloyl esterases from xylans extracted from coffee husks and sugarcane straw, obtaining 10.28 and 8.29 g/L of total XOS (Farias et al., 2022).

Furthermore, with advances in molecular biology and genetic engineering, the development and application of recombinant enzymes obtained by heterologous expression to produce XOS with greater prebiotic effects have been reported. Researchers produced a recombinant endoxylanase expressed in *Pichia pastoris* and applied it together with  $\alpha$ -L-arabinofuranosidase on the xylan extracted from sugarcane straw, thus obtaining a maximum release of XOS of 4.07 g/L of total XOS composed mainly of xylobiose and xylotriose (M. Martins, Ávila, et al., 2020).

### ***Properties and Applications of Xylo-oligosaccharides***

Among their main physicochemical characteristics, XOS are solid substances, of crystalline nature, with variable color depending on the xylan source in the lignocellulosic biomass used. XOS has a sweetening power of 30 to 50% of that of sucrose, has a sensory odor accepted by consumers, is non-cariogenic and low in calories. They also have higher

hygroscopicity when compared to xylose, in addition to being stable over a wide range of temperatures (up to 100 °C) and pH (2.5-8.0) (Amorim et al., 2019).

XOS are well known for their prebiotic functional properties, promoting the growth of probiotic cultures in the final gastrointestinal tract, such as those of the genus *Lactobacillus* sp. and *Bifidobacterium* sp., which mainly help to combat constipation, in addition to inhibiting the development of potentially pathogenic bacteria, such as *Clostridium perfringens*, *Escherichia coli* and *Staphylococcus aureus*. These prebiotics also have antioxidant and anticancer activity, inhibiting the development of cancer cells, mainly in the tissues of the small intestine, colon, and liver. XOS also promotes the reduction of blood glucose, triglycerides, and cholesterol levels, helping to combat diabetes mellitus and obesity. In addition, they can also act as immunomodulatory agents, helping to strengthen the immune system, especially in the fight against infectious diseases (Farias et al., 2022).

Because of their benefits in promoting human health, there is a growing interest in using XOS in various products, expanding the range of industrial applications. In the pharmaceutical industry, these prebiotics are mainly used in the production of drugs related to the fight against obesity and in the treatment of gastrointestinal diseases. In the food industry, in addition to acting as a prebiotic, XOS can be used as a substitute for sugar and/or fat, acting as a preservative, stabilizer or gelling agent, and can be applied in formulations for ice cream, dairy desserts, cookies, bakery products, bars of cereals, jellies, juices, nectars, confectionery, sauces, in symbiotic foods, among others (de Freitas et al., 2019). Lately, several patents have been granted in a wide range of applications where XOS is used, for example weight loss food formulations, nutritional formulations for cancer patients and constipation treatment.

## **II.5.Economic outlook**

There is a great economic opportunity for the development of lignocellulose-derived biochemicals, since the value of these bioproducts is comparable to the fuel and energy industries but requires a much smaller fraction of feedstock. This facilitates the development of small-scale biorefineries near the biomass generating environment. Furthermore, although the integrated biorefinery for both bio-based materials and energy production is a superior approach for the sustainable valorization of lignocellulosic biomass, the production of chemicals and biomaterials are the key to the economic viability of this platform (FitzPatrick et al., 2010).

New markets have started the demand for lignocellulosic based products due to the productive expansion or substitution of analogues of chemical synthesis or that require arable land. The polysaccharides market, for example, is expected to reach a value of US\$ 22 billion

by 2030, of which it is estimated that US\$ 140 million will come from XOS (Polysaccharides and Oligosaccharides Market, 2022). Market studies show that among the main prebiotics commonly produced industrially, XOS are those with the highest added value, costing up to 30 US\$/kg (Amorim et al., 2019). The global XOS market was valued at USD 102.1 million in 2020 and is expected to reach USD 135.7 million by the end of 2026, growing at an annual rate of 4.1% during 2021-2026 (The Market Reports, 2020). Among the main players in this market are Cargill Incorporated (USA), Bright Dairy & Food Co., LTD. (China), Abbott Laboratories (USA), Cosucra Groupe Warcoing Sa (Germany), Parmalat S.P.A (Italy) and Beneo-Orafti (Germany) (Amorim et al., 2019). In comparative terms, Galacto-oligosaccharides (GOS), which have a more developed techno-productive chain, should reach the mark of US\$ 1,394 million in the same period. In this sense, the XOS represent a still small portion in view of the possibilities of the market. The same statement can be said for cello-oligosaccharides, although less information is available. In both cases, demand is emerging, especially in the prebiotic ingredients market. However, the main limitations are technical, to achieve higher yields, and bureaucratic, due to the need to release the use and consumption of products from unestablished raw materials (Amorim et al., 2019; Precup et al., 2022).

Fermentable sugars are one of the most versatile building blocks, and can be applied in different markets, which makes it difficult to measure economics value without equivocation, however their availability is one of the primary economic factors in deciding the installation of a biotechnology platform. It is still worth mentioning the high economic value of NC, since the world market for NC is expected to reach US\$ 2.712 billion by 2025, with an annual growth of 18.80% from 2018 to 2025. All these aspects explain and highlight the high added value of oligosaccharides, encouraging new research for the development and optimization of bioprocesses, based on lignocellulosic biomass (Thomas et al., 2020).

## **II.6. Future perspectives**

Current research efforts point to the lignocellulosic biorefinery as an effective and sustainable platform for the generation of value-added materials, overcoming the growing problems associated with petroleum derivatives and the global reduction of greenhouse gas emissions (Ubando et al., 2020). However, degradation of lignocellulosic biomass is a major challenge as it is necessary to integrate pretreatments and catalytic conversion to increase yields, reduce reaction time and cut costs (Barbosa, Kendrick, et al., 2020). However, the abundant availability and diversity of types of lignocellulosic raw materials allow the effective use of a wide range of bioproducts in an economical way. The main problems for this consolidation are

fluctuations in oil prices, political conditions, technological advances, and public attitudes towards renewable energies (FitzPatrick et al., 2010). As a result, the development of technologies for lignocellulosic biorefineries must be implemented more seriously (globally) from 2030 onwards, through more research, process quality certifications, government investments, tax cuts, subsidies, and socio-political revisions to avoid the competition between biorefineries and the food market. The isolation of new wild microorganisms and the tools of genetic engineering, metabolic and synthetic biotechnology approaches will design more resistant and efficient microorganisms for enzymatic hydrolysis and fermentation as the main strategies for optimizing processes to produce bioproducts from lignocellulosic substances (Amorim et al., 2019; Farias et al., 2022; Precup et al., 2022).

#### CONSENT FOR PUBLICATION

Not applicable.

#### CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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**Appendix III: Polysaccharide degradation for oligosaccharide production  
with nutraceutical potential for the food industry**

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### III.1. Introduction

Oligosaccharides (OS) are considered functional fibers or prebiotics containing mainly carbohydrates in their composition. These molecules stimulate the growth of beneficial bacteria in the gut microbiota showing a beneficial effect on body weight control, improving blood glucose and lipid levels (J. Wang et al., 2011), and reducing the symptoms of colon cancer (Aachary et al., 2015). In fact, prebiotics are substrates for probiotic microorganisms that produce important metabolites, recently called postbiotics, which are associated with most health-promoting effects (Żółkiewicz et al., 2020). Prebiotics are also cited among the "Top Ten Functional Food Trends", and foods containing these compounds will continue to find a welcome market (Sloan, 2020).

As life expectancy rises, population aging demands health-promoting factors to enhance wellness and prevent diseases through lifestyle changes, practice of physical exercises, and adoption of a healthy diet. This philosophy, powered by research efforts to identify properties and potential applications of natural substances, accrues from public consciousness in health-related information and the growing demand for products containing fewer chemicals and synthetic additives in the food and pharmaceutical market. Prevention is the most desired feature, a health guarantee path from nutraceuticals and functional foods consumption, with minimal side effects that, if consumed daily, increase the health promotion (Keservani et al., 2010; Moldes et al., 2017).

The neologism "Nutraceutical" (a contraction of nutrition and pharmaceutical) is simply a scientific definition to describe any product derived from natural sources that benefits health. In contrast to pharmaceutical products, nutraceutical substances usually do not have patent protection. It does not mean that nutraceuticals can be compared to medicines. But instead, it generates a new field of research on the mechanisms of action and benefits that can be derived from consuming evidence-based dietary patterns, discerning the contribution of specific compounds. Proof-of-concept studies, based on clinical trials and population-based prospective, establish safe and efficacious quantities of the bioactive compound without unbalancing the overall macronutrient and calorie intake. Regarding the potential applications, genetic engineering and biotechnology often present novel technologies for feasible large-scale production of any new bioactive compound, which can come even from agro-industrial residues such as peels, husks, and seeds (Ansari et al., 2013; Keservani et al., 2010; Minuz et al., 2017; Moldes et al., 2017).

According to the Grand View Research market report, the nutraceutical global market size was valued at USD 412.7 billion in 2020. It's expected to have a Compound Annual Growth Rate (CAGR) of 8.3% until 2027. Increasing application for the treatment of cardiovascular disorders, obesity, diabetes, malnutrition, and the rising healthcare costs and aging stimulate the growth of this market. The categories of the nutraceutical market are dietary supplements, functional beverages, and functional foods. The beverages segment led the overall commerce for nutraceuticals in 2019, followed by functional foods and supplements (Research, 2019, 2020).

Nutraceuticals can be classified based on their natural source (plant, animal, and microbial), chemical structure (amino acid-based, fatty acids, carbohydrates derivatives, minerals, phenolic compounds, and isoprenoids), or mechanism of action (antioxidant, antibacterial, antihypertensive, anti-hypercholesterolemic, anti-inflammatory, anticarcinogenic, osteoprotective, and so on). The food sources used as nutraceutical can be categorized as dietary fiber, probiotic, prebiotic, fatty acids, antioxidants, and polyphenols (**Figure 12-7**) (Ansari et al., 2013; Keservani et al., 2010).

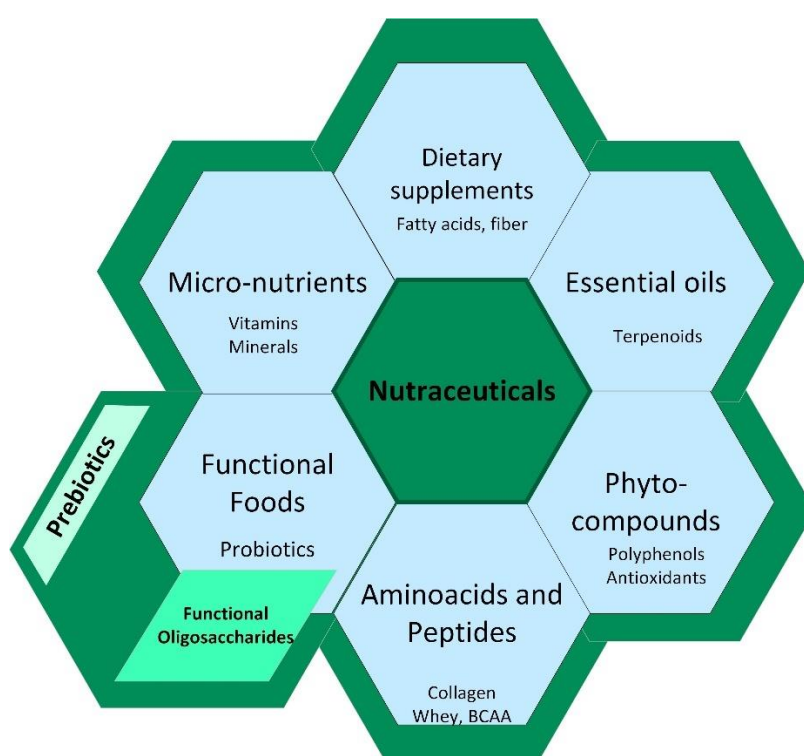


**Figure 12-7** Categories and examples of nutraceutical compounds

Some sources have clear reasons to be established as a nutraceutical, such as vitamins or minerals. However, some non-obvious sources have gained outstanding popularity for

their benefits. Dietary carbohydrates are shown to be more than just an energy basis for calorie intake balance. They also consist of edible plants remains, polysaccharides, lignin, and associated substances resistant to digestion by endogenous humans enzymes. Carbohydrate fibers include cellulose, hemicelluloses, lignin, gums, mucilage, oligosaccharides, pectin, and other associated minor substances. More than that, several insoluble and non-fermentable fibers can be hydrolyzed by enzymatic or physicochemical process to become soluble, fermentable and functional. Oligosaccharides (OS), especially the non-digestible, obtained from bacteria, algae, fungi, and higher plants, have been used as dietary fiber, sweetener, and humectant. Notably, these functional OS have also been effective in gastrointestinal normal flora proliferation and pathogen suppression, enhancement of immunity, mineral absorption facilitation, antioxidant and antibiotic activities (O. Ibrahim, 2018; Patel & Goyal, 2011).

In addition to providing modifications to food and applications of flavor and physicochemical characteristics, many of these sugars possess beneficial properties for the health of the consumer. Thus, this chapter discusses the types of food-grade OS studied on a laboratory scale or already commercially available, their properties, applications, recent developments, and manufacturing trends.

## **III.2. Functional Oligosaccharides**

### **III.2.1. Properties and Food Industrial Application**

Functional oligosaccharides (OS) are non-digestible carbohydrates containing up to 10 monosaccharides joined by glycosidic bonds. These oligosaccharides are found in small quantities in milk, honey and structural plant fibers, such as garlic, onions, asparagus, bananas, and wheat bran. Their large-scale production is via hydrolysis of polysaccharides, such as xylan, chitosan, pectin, and inulin, and transglycosylation mechanisms when sucrose and lactose are the substrates. Fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are currently the most utilized functional OS in the industry. However, agro-residue-derived OS gained attention due to their relatively low production costs (Babbar et al., 2016; Moreno et al., 2017; Samanta et al., 2015).

As nutraceuticals, oligosaccharides are used in beverages (fruit drinks, coffee, cocoa, tea, soda, soya milk, soft drinks, health drinks and alcoholic beverages) and milk products (fermented milk, instant powders, powdered milk, yoghurt and ice cream). Also, the functional oligosaccharides are amended in desserts (puddings and sherbets), table spreads (jellies, jams, marmalades, honey products), confectionary (candy, cookies, biscuits, chocolate, sweets), bakery products (breads and pastries), breakfast cereals, meat products (fish paste and tofu) and

food for infants, elders and diabetics (Wu et al., 2005). Their trade began in the 1980s as low-calorie bulking agents. Currently, they arouse a lot of interest in the food and pharmaceutical sector due to the consumer preference for healthier food (O. Ibrahim, 2018). Japan first introduced the concept of functional food, where several oligosaccharides received the classification of foods for specific health use (FOSHU) (Patel & Goyal, 2011).

The field of interest in functional OS study is growing in the food and pharmaceutical industries mainly due to their activity as prebiotics. Their prebiotic definition comes from the selective growth stimulation of microbiota, impairing the growth of pathogenic organisms. Besides, enhance minerals absorption, improve the ratio of HDL/LDL, decrease serum lipids and blood cholesterol, improve blood pressure, decrease fecal pH, remove toxins from the body, and decrease the glycemic index by slowing down the carbohydrates absorption rate. Their metabolization produces short-chain fatty acids related to beneficial effects on the colon against several diseases such as cardiovascular disease, cancer, obesity, and type-2 diabetes (Bali et al., n.d.; Gómez et al., 2016; José Sánchez-Martínez et al., n.d.; Singh et al., 2015; Zhao et al., 2017).

A recent study reported the anti-microbial, anti-adhesive, and anti-inflammatory properties of functional oligosaccharides such as alginate oligosaccharides (AOS) and chitosan oligosaccharides (CHOS). These oligosaccharides might decrease antibiotic concentration against pathogenic bacteria. On the other hand, several studies have published the association of increased consumption of OS with the resurgence of symptoms of irritable bowel syndrome (Asadpoor et al., 2021; Charoenwongpaiboon et al., 2019; Huazano-García et al., n.d.). Hence, quality control of foods containing OS is necessary for ensuring food safety and quality (Catenza & Donkor, 2021).

Functional oligosaccharides have about 0.3-0.6 times the sweetness of sucrose and a low caloric density. The  $\beta$ -glycosidic bonds are generally stronger than  $\alpha$ -linkages, and hexoses stronger linked than pentoses (Patel & Goyal, 2011). Technological properties of OS, such as gel-forming ability and water holding characteristic, can improve the sensory and physico-chemical characteristics of food products, leading to the increased OS application in the food industry (Balthazar et al., 2015, 2017; Farias et al., 2019). They also present good stability over a broad range of temperature and pH, proving their suitability for food incorporation (Patel & Goyal, 2011).

### III.2.2. Polysaccharides Sources and Production Process

Plants and algae are the richest sources of functional oligosaccharides (Patel & Goyal, 2011; Zhao et al., 2017) (**Table 12-2**), which can be obtained via extraction from a natural source. However, they generally are produced through the enzymatic hydrolysis of polysaccharides and other substrates at the industrial level (Catenza & Donkor, 2021). Commercial FOS are either obtained from enzymatic hydrolysis of oligofructose (DP 10 – 12) from chicory roots (Chikkerur et al., 2020), yacon root (*Smallanthus sonchifolius*) (Wagner et al., 2019), and Jerusalem artichoke (C. M. Martínez et al., 2019) or enzymatic transfructosylation of sucrose by fructosyltransferase enzymes (José Sánchez-Martínez et al., n.d.; Ojwach et al., 2020). Similarly, commercial GOS are produced from transglycosylation reactions by  $\beta$ -galactosidase enzymes using lactose as substrate (Gao et al., 2019; Rico-Rodríguez et al., 2020). However, these processes account for high production costs due to the high concentration requirement and procurement costs of starting materials. As a result, recent methodologies have sought low-cost alternatives to produce functional oligosaccharides, such as agricultural wastes (Catenza & Donkor, 2021; O. Ibrahim, 2018).

Lignocellulose sources are considered attractive raw materials to produce non-starch oligosaccharides. Three main classes of polymers constitute the lignocellulosic biomass: cellulose, hemicellulose, and lignin, which can be cleaved by enzymatic hydrolysis or autocatalysis, releasing a wide range of compounds with different properties, such as glucose, xylose, furfural, xylo-oligosaccharides (XOS), and cello-oligosaccharides (COS) (Bhatia et al., 2019). The lignocellulose hydrolysate composition depends on the type of raw material, pretreatment, and hydrolysis conditions. Depolymerization of suitable raw materials or partial enzymatic hydrolysis of purified pectin produces pectic oligosaccharides (POS) (Gullón et al., 2013). Pectin depolymerization by acid treatment is scarce due to the autohydrolysis and enzymatic hydrolysis advantages. Depending on the nature and concentration of the acid used, a variety of POS can be obtained, as pectin is composed of neutral and acidic sugars linked in different ways (Cano et al., 2020).

Generally, enzymatic processes are preferred due to mild operational conditions and low release of monosaccharides and inhibitory compounds (e.g., furfural and hydroxymethylfurfural). Moreover, it is eco-friendly and facilitates the downstream process. The enzymatic reaction stops by changes in pH or an increase in temperature, and the downstream process feasibility is dependent on the final product. Several microorganisms can produce specific enzymes to fit industrial needs, but usually, recombinant enzymes are the most applied due to safety and productivity. Genetically modified GRAS microorganisms, such as

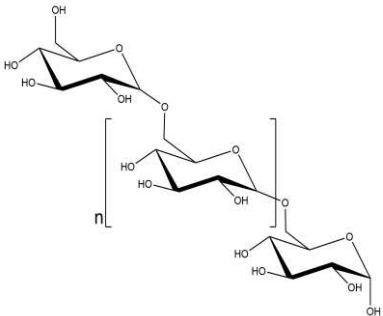
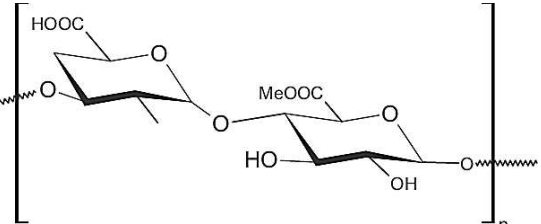


*Saccharomyces cerevisiae*, *Pichia pastoris*, and *Aspergillus niger*, can express enzymes with enhanced thermotolerance and catalytic activity (A. M. M. Lopes et al., 2021).

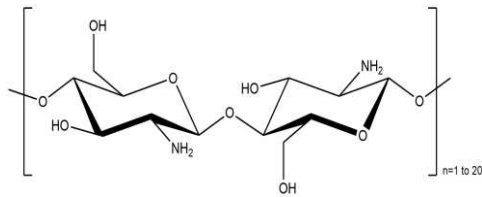
The large-scale purification process of oligosaccharides is usually ion-exchange chromatographic, nanofiltration, precipitation with ethanol, or activated charcoal. The cation-exchange resins are the most used due to the highest affinity for monosaccharides, thus oligosaccharides are the first to elute from the column, and activated charcoal presents a higher affinity for oligo than mono and disaccharides, increasing the advantage at the industrial level with low substrate losses (Torres et al., 2010; Vera et al., 2016).

Due to the growing interest in these functional oligosaccharides, researchers have developed enzymatic processes that contribute to reducing production costs (up and downstream), facilitating purification and increasing yields, replacing the costly natural extraction in plant tissue (Varzakas et al., 2016). This chapter presents details from each functional oligosaccharide class obtained from polysaccharides, classified in 1. Sucrose-related oligosaccharides; 2. Lactose-related oligosaccharides; 3. Starch-related oligosaccharides; 4. Non-starch oligosaccharides; 5. Algae-oligosaccharides.

**Table 12-2 Oligosaccharides obtained from natural sources by enzymatic processes based on work of Patel and Goyal (2011) and Ibrahim (2018).**

| Oligosaccharide and molecular structure                                                                              | Polysaccharide source and production mechanism                                                                                                                                                                             |
|----------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>Isomalto-oligosaccharides</p>  | <p>Starch/maltose: wheat, barley, potato, rice, cassava, honey.</p> <p>Hydrolysis by <math>\alpha</math>-amylase, pullanase and <math>\beta</math>-amylase and transglycosylation by <math>\alpha</math> D-glucosidase</p> |
| <p>Pectin-oligosaccharides</p>    | <p>Pectin: Apple pulp, sugar beet, onion skin, potato pulp, soy hull, olive pomace, citrus waste.</p> <p>Hydrolysis by polygalacturonase, pectin lyase and pectin esterase.</p>                                            |

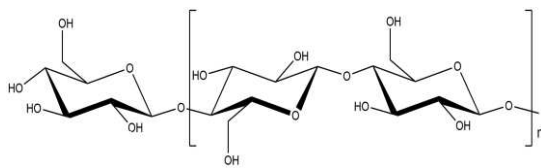
### Chitosan-oligosaccharides



Chitin or chitosan: crab and shrimp shell wastes.

Hydrolysis by chitosanase

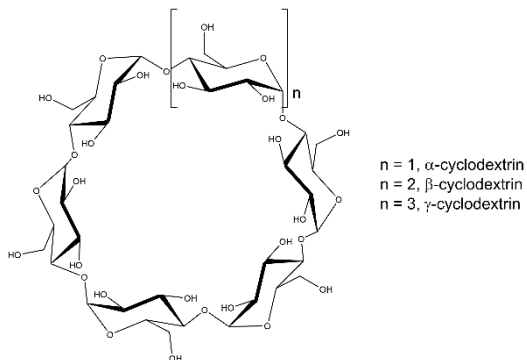
### Cello-oligosaccharides



Cellulose: sugarcane straw, birch, spruce, wheat straw, maize bran, corncob.

Hydrolysis by cellulases: (cellobiohydrolase, endoglucanases); lytic polysaccharide monooxygenases

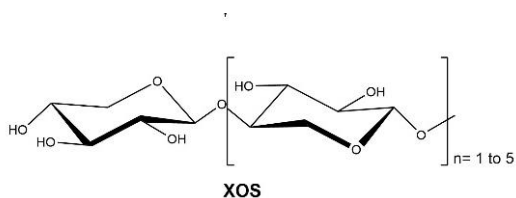
### Cyclodextrins oligosaccharides



Starch: transformation of starch by certain bacteria such as *Bacillus macerans*.

Transglycosylation: Cyclodextrin glucanotransferase and partly α-amylases.

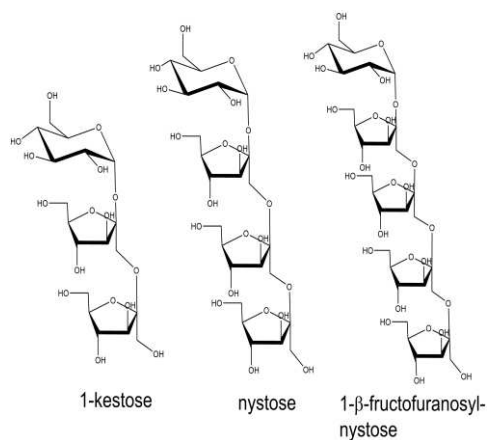
### Xylo-oligosaccharides



Xylan: hardwood, corncob, wheat straw, rice hull, barley straw, sugarcane biomass.

Hydrolysis by 1,4-β-endoxylanases and arabinofuranosidases

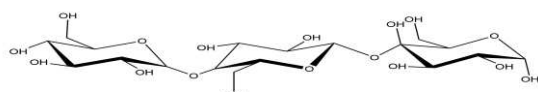
### Fructo-oligosaccharides



Sucrose/inulin: artichoke, garlic, onion, asparagus, chicory.

Hydrolysis by Fructosyl-transferase/ β-fructofuranosidase

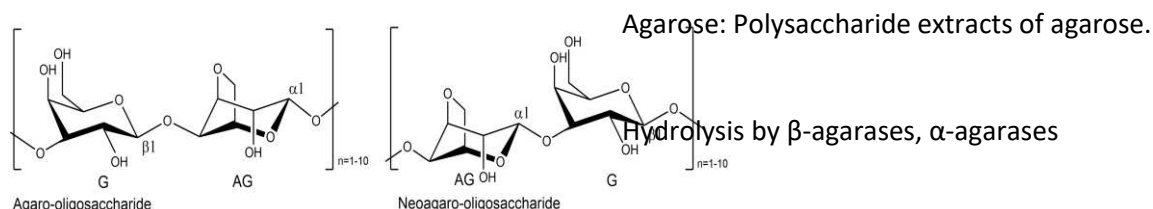
### Galacto-oligosaccharides



Lactose: Milk

Galactosylation by β-galactosidase

### Algal-oligosaccharides



## III.3. Sucrose-related Oligosaccharides

### III.3.1. Fructooligosaccharides

FOS are water-soluble, thermostable, stable in pH from 4.0 to 7.0, and 0.3 to 0.6 times the sweetness of sucrose, depending on the ratio of the three chemical structures that constitute FOS, which are 1-kestose, nystose, and 1-β-fructofuranosyl nystose (**Table 12-2**). These three FOS chemical structures have higher molecular weight than sucrose, thus higher viscosity at the same concentration as sucrose. Additionally, FOS are recognized as a low calorie sweetener ranging from 1.5 to 2.0 kcal/g, about half the caloric values of sucrose (Mabel

et al., 2008). The configuration of the glycosidic bond resists hydrolysis by saliva and intestinal enzymes reducing the consumer's blood sugar, making them useful for dietetic formulations (Shah et al., 2021). These characteristics are acceptable for applications in foods, beverages, pharmaceutical drugs, and cosmetics products as bulk agents and humectants, in addition to sweetener or prebiotic fiber for constipation and to enhance the growth of probiotic microorganisms. Besides, FOS demonstrated to improve mineral absorption, decrease the levels of cholesterol and triglycerides in blood (Costa et al., 2015), and treat the symptoms of irritable bowel syndrome (Currò, 2021) and functional abdominal pain (Gholizadeh et al., 2021).

The daily intake of FOS that is acceptable to avoid side effects is up to 20 g/day for adults and not more than 4.2 g/day for children. Exceeding these limits may cause bloating, stomach cramps, and diarrhea (Kumar et al., 2012). In addition, excess FOS intake could negatively affect patients suffering from irritable bowel syndrome and small intestinal bacterial overgrowth syndrome and might also lead to other chronic health conditions (Catenza & Donkor, 2021; Charoenwongpaiboon et al., 2019; Huazano-García et al., n.d.).

FOS emerged in the 1980s, and since then its demand has continued rising. In 2015, the global market reached \$ 1.2 billion, with an annual growth rate of 10.4%. FOS market may reach \$ 3.9 billion by the year 2027. Major key players in global FOS manufacturing and marketing include Cargill Incorporation (USA), Ingredion Incorporated (USA), Beneo-Orafti SA (Belgium), Cosucra-Groupe Warcoing SA (Belgium), and Beghin Meiji (Japan) (O. Ibrahim, 2021).

The FOS structure is fructose units linked by  $\beta$ -(2 $\rightarrow$ 1) glycosidic bonds with terminal glucose, joined to fructose by  $\alpha$ -(1 $\rightarrow$ 2) glycosidic bonds. These oligosaccharides present higher concentrations in chicory roots and Jerusalem artichoke and lower concentrations in grains like wheat and barley. For commercial purposes, their manufacturing utilizes disaccharide sucrose or the polysaccharide inulin as substrates (O. Ibrahim, 2021).

#### ***Sucrose as substrate***

The enzymes that convert sucrose into FOS, on a large scale, are fructosyl-transferase and  $\beta$ -fructofuranosidase. The reaction also releases free glucose units, which are enzyme inhibitors, decreasing the bioconversion efficiency and lowering the yield of FOS. There are two approaches to circumvent this limitation: 1) the incorporation of the enzyme glucose oxidase (EC 1.1.3.4), which converts  $\beta$ -D-glucose into D-glucono-1,5-lactone and then hydrolyzes it to gluconid acid. Glucose oxidase requires flavin adenine dinucleotide (FAD), a common

cofactor for biological oxidation-reduction; 2) cross-flow ultrafiltration membrane to remove the glucose units (Duan et al., 1994; O. Ibrahim, 2021).

Fructosyl-transferase (EC 2.4.1.9) occurs in higher plants, such as asparagus, chicory roots, and onions, and in microorganisms such as *Aspergillus niger* and *Saccharomyces cerevisiae*. This hydrolase catalyzes the transfer of fructosyl group (fructose) from the disaccharide sucrose as a donor to the second disaccharide or a short-chain FOS as an acceptor (**Figure 12-8**). The fructose released from the sucrose plays a dual role as donor and receptor. The reaction equilibrium shifts from the hydrolysis of sucrose towards the synthesis of fructo-oligosaccharides (FOS).  $\beta$ -fructofuranosidase (EC 3.2.1.26) found in similar plants and microorganisms as fructosyl-transferase, is an invertase that hydrolyzes sucrose into glucose and fructose (Madlová et al., 2000).

In short, sucrose is hydrolyzed into glucose and fructose by  $\beta$ -fructofuranosidase. Glucose is released, and fructose is bound to the enzyme fructosyl-transferase. The fructose bound to this enzyme is released and becomes a donor to another sucrose molecule to form 1-kestose, a shorter chain of FOS; the continuous enzymatic reaction produces longer chains of FOS molecules.

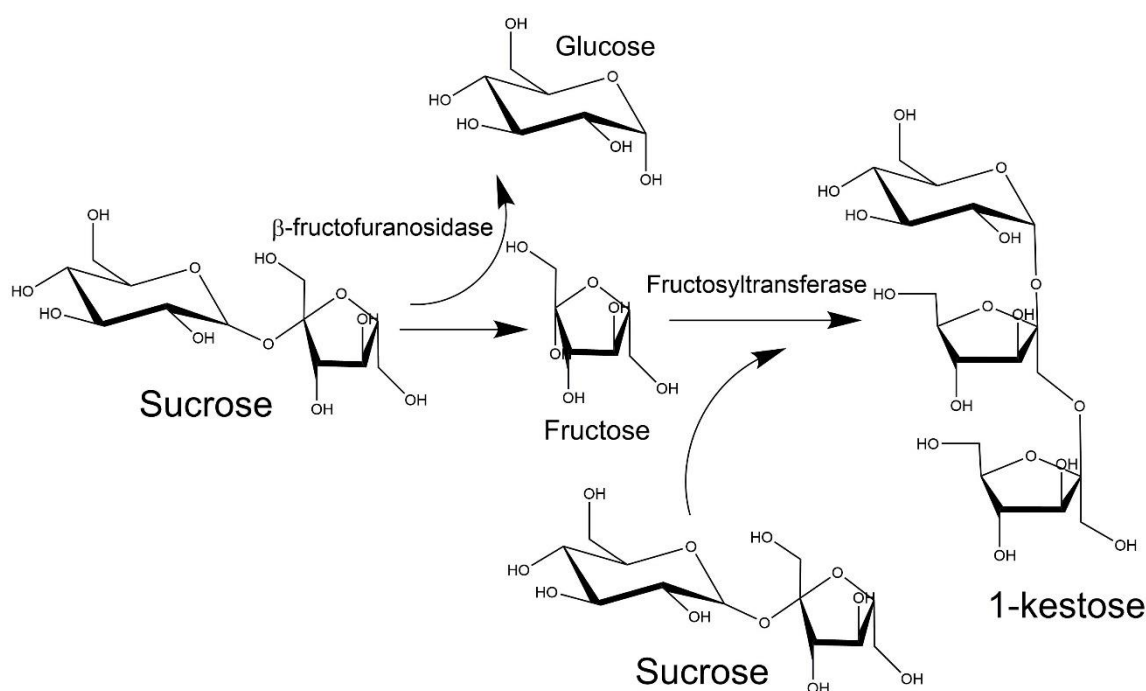


**Figure 12-8** Fructo-oligosaccharides synthesis

#### *Inulin as substrate*

High yields of FOS from inulin as substrate derive from the use of the microbial enzyme endo-inulinase ( $\beta$ -fructan-fructanohydrolase, EC 3.2.1.7). This enzyme randomly hydrolyzes long chains of fructose polymers (inulin) into short chains FOS by cleaving  $\beta$ -(2 $\rightarrow$ 1)

glycosidic bonds yielding a mixture of FOS of 2 to 9 fructose units joint with  $\alpha$ -(1→2) glycosidic bonds, and the terminal fructose is linked to a glucose unit by  $\beta$ -(1→2) glycosidic bond (O. Ibrahim, 2021). Higher plants, such as chicory roots (Chikkerur et al., 2020) and industrial wastes, such as *Stevia rebaudiana* stems (S. M. S. Lopes et al., 2017), are sources rich in inulin, extracted by hot water and precipitated with ethanol or ion exchange methodologies. The refined inulin is concentrated by evaporation and dried using a spray dryer or lyophilization to produce a powder form acceptable as a substrate for FOS production (S. M. S. Lopes et al., 2017; O. Ibrahim, 2021) using endonucleases in batch or immobilized process (Chikkerur et al., 2020).

### III.4. Lactose-related Oligosaccharides

#### III.4.1. Galactooligosaccharides, lactulose and lactosucrose

The general mechanism for lactose enzymatic hydrolysis involves sequential reactions with other disaccharides and higher saccharides, named galactooligosaccharides (GOS), as intermediate products. GOS are a mixture of lactose derivatives containing 2 to 8 saccharide units, with one of these units being terminal glucose and the remaining units being galactose (**Table 12-2**) (Torres et al., 2010). GOS are currently produced in a reaction of lactose transgalactosylation catalyzed by  $\beta$ -galactosidases (E.C. 3.2.1.23) from different microbial strains (Vera et al., 2016).

Among all types of functional oligosaccharides, it is the only non-synthetic oligosaccharide derived from animal milk (Fara et al., 2020). The use of GOS emerged in Japan in the 1970s as a substitute for human milk-oligosaccharides for infant formulas to promote a healthy gut microflora of not breast-fed newborns (Vera et al., 2016). In 2013, GOS with different degrees of purity and formats (concentrated syrup or solid powder) were supplied by four major companies: Friesland Foods Domo, Yakult Honsha, Nisin Sugar Manufacturing Co, and Ingredion. In 2020, the global GOS market reached 175.7 kilotons worth USD 1.01 billion, driven by the increasing attention on the unique properties of GOS among the prebiotics on food nutrition.

The most recent studies demonstrated the potential of GOS to improve colonic healing and surgical recovery (Hajjar et al., 2021), inhibit the attachment of pathogenic bacteria to the colonic epithelium, reduce serum cholesterol and blood pressure, prevent colon cancer and enhance immunity (Fara et al., 2020; Gibson et al., 2017). As food ingredients/sweeteners, GOS present a low-glycemic index, low-calorie (1 to 2 kcal/g), and resistance to acidic pH values and high temperatures (pasteurization and baking) (López-Sanz et al., 2015), besides the

well-known prebiotic effect, preserving probiotic strains during freeze-drying processes (Fara et al., 2020). Most of the GOS prophylactic health effects arise from their selective consumption by bifidobacteria and lactobacilli. Supplemented infant formulas usually contain 6.0 to 7.2 g/L GOS mixed with 0.6 to 0.8 g/L FOS. To avoid side effects, like diarrhea, the daily dose should not exceed 0.3 to 0.4 g/kg body weight, or 20 g per person (Torres et al., 2010).

GOS production involves the kinetically controlled two-step mechanism: first, a molecule of lactose binds to the  $\beta$ -galactosidase active site to form the galactosyl-enzyme complex while liberating one glucose; second, the transition intermediate reacts with another lactose molecule forming a gal-gal-glu trisaccharide (GOS-3), which in turn acts as an acceptor of the galactosyl-enzyme complex to generate a gal-gal-gal-glu tetrasaccharide (GOS-4) and so on to produce longer chain GOS (**Figure 12-9**). In this way, lactose acts both as donor and acceptor of galactose moiety. Water is nucleophile and can also act as an acceptor of the galactosyl-enzyme complex, releasing galactose, resulting in the hydrolysis of lactose into its monosaccharide components glucose and galactose (Vera et al., 2016).  $\beta$ -(1 $\rightarrow$ 2),  $\beta$ -(1 $\rightarrow$ 3),  $\beta$ -(1 $\rightarrow$ 4),  $\beta$ -(1 $\rightarrow$ 6) GOS vary in oligosaccharide content depending on  $\beta$ -galactosidase specificities for building glycosidic linkages (Intanon et al., 2014). Other hydroxyl-containing compounds, like alcohols, can act as acceptors of the galactosyl group as well, producing lactulose, lactosucrose, and lactitol (**Figure 12-10**) (Guerrero et al., 2011; Vera et al., 2016).

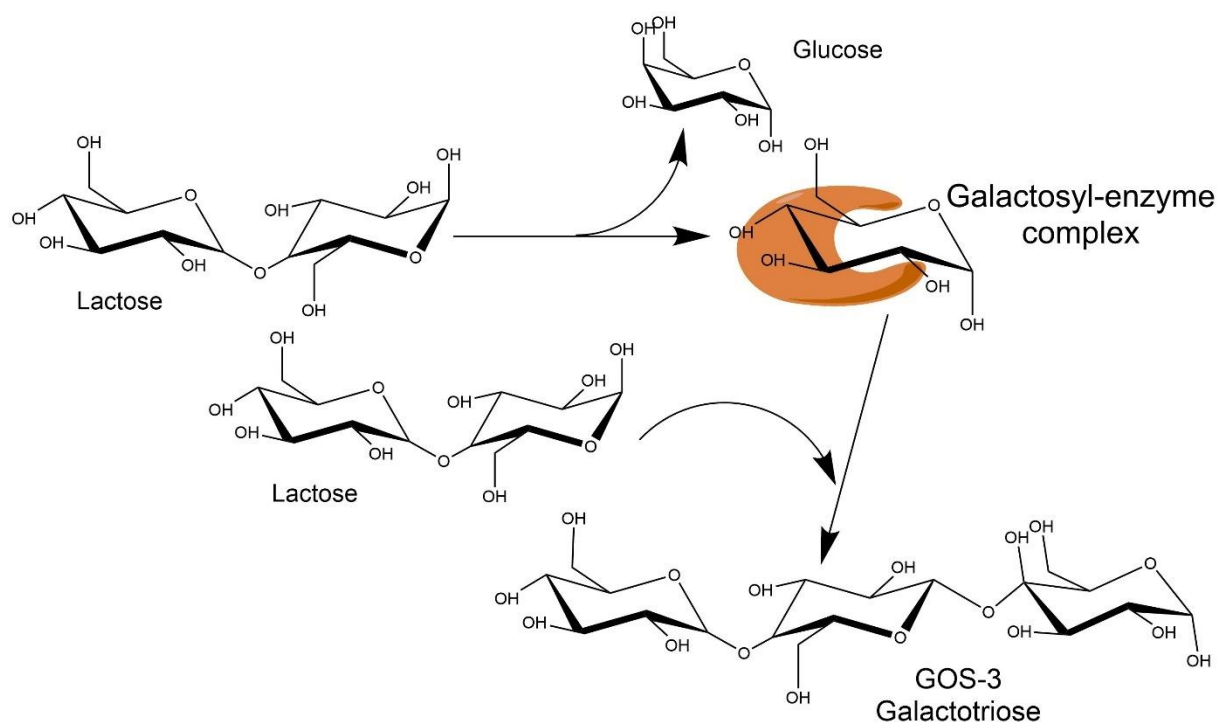


**Figure 12-9** Galacto-oligosaccharides structure and synthesis.

The synthetic disaccharide lactulose is a well-established prebiotic produced by transgalactosylation of fructose with lactose, and it has been demonstrated that the reaction can be driven to the synthesis of GOS or lactulose by controlling the proportions of lactose and fructose in the medium (Guerrero et al., 2011, 2015); the mixture of GOS and lactulose is often interesting for prebiotic supplements (Vera et al., 2016). Lactosucrose is a potential prebiotic that can also be produced with GOS by transgalactosylation of sucrose but is mostly produced via lactose transfructosylation with  $\beta$ -fructofuranosidase. Lactitol is a polyhydroxyalcohol with prebiotic potential obtained by catalytic hydrogenation of the glucose moiety in lactose (Vera et al., 2016).

At the onset of reaction, the high concentration of lactose favors transgalactosylation, making GOS reach maximum concentration when the hydrolysis and transgalactosylation rates become equal, and then the reaction must stop; if not, hydrolysis will prevail and reduce GOS concentration (Vera et al., 2016). Generally, the synthesis yield of GOS from lactose can increase using a high starting concentration of lactose, decreasing water thermodynamic activity (e.g., micro-aqueous environment), removing the final products or inhibitors from the medium, and modifying the enzyme (Torres et al., 2010). Although  $\beta$ -galactosidase can originate from various microorganisms, *Aspergillus oryzae* is the most promising source for commercial application in terms of food safety and expenditure (G. Wang et al., 2021), fundamentally because of its ability to catalyze the transgalactosylation and its affinity for the GOS production (Torres et al., 2010).

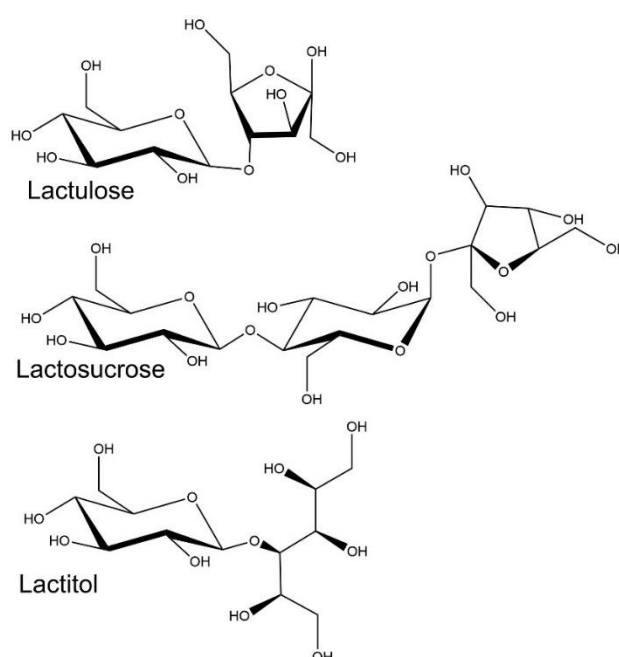


**Figure 12-10** Molecular structure of lactulose, lactosucrose, and lactitol



### III.5. Starch-related Oligosaccharides

#### III.5.1. Malto-oligosaccharides

Malto-oligosaccharides (MOS) consist of 3 to 10  $\alpha$ -D-glucose units linked by  $\alpha$ -1,4 glycosidic linkages and are mainly applied in food products, as syrup or powder to improve hygroscopicity, viscosity, permeability, stability and gelling. These characteristics make them suitable for sugar and fat substitution, artificial preservatives, and encapsulation material. Regarding their health effects, MOS is shown to fight fatigue, improve intestinal function, regulate blood sugar and cholesterol levels, and prevent constipation. The large-scale production of MOS comes from starch hydrolysis by the action of amylases (EC 3.2.1.1). A second enzyme, pullulanase (EC 3.2.1.9), added to break down  $\alpha$ -(1 $\rightarrow$ 6) glycosidic bonds in the branched amylopectin, improves production yield (Jaafar et al., 2021; O. Ibrahim, 2018).

#### III.5.2. Trehalose

Trehalose is a non-reducing disaccharide composed of two glucose residues linked by  $\alpha$ -(1 $\rightarrow$ 1) glycosidic bond. It is naturally present in mushrooms and commercially produced from yeast fermented dough for baked goods and also from MOS enzymatic hydrolysis. Two mutases are involved in this process: MOS synthase (EC 5.4.99.15) that shift the  $\alpha$ -(1 $\rightarrow$ 4) glycosidic bond from reducing end of the two glucose units into  $\alpha$ -(1 $\rightarrow$ 1) glycosidic bond; and malto-oligosyltrehalose trehalohydrolase (EC 3.2.1.141) that cleave the  $\alpha$  (1 $\rightarrow$ 4) glycosidic bond next to the  $\alpha$ -(1 $\rightarrow$ 1) glycosidic bond in the terminal disaccharide of malto-oligosyltrehalose to produce the end product of trehalose and a shorter chain maltooligosaccharide (Elbein, 2003).

As opposed to the other oligosaccharides, trehalose can be digested by the intestinal enzyme trehalase (EC 3.2.1.28) into two glucose molecules, providing 4 kcal/g, similar to calorie generated from sucrose. It has 45% sweetness of sucrose and heat stability, that helps to cryo-preserve cell structure, biological materials, and foods after heating or freezing (O. Ibrahim, 2018). Trehalose triggers a small increase of blood insulin levels, which may improve glucose tolerance and reduce the progress of insulin resistance. Some researchers suggested that trehalose can potentially reduce the development of metabolic syndrome and reduce other associated lifestyle-related diseases such as type 2 diabetes (Mizote et al., 2016).

#### III.5.3. Isomalto-oligosaccharides

Isomalto-oligosaccharides (ISMO) are composed of 3 to 6 glucose molecules linked by  $\alpha$ -(1 $\rightarrow$ 6) glycosidic bonds (**Table 12-2**). They are present naturally at low concentrations in honey, and some fermented foods such as, in soy sauce, and are manufactured on large

scale by enzymatic hydrolysis of starch. This process involves first the enzymes amylase (EC 3.2.1.1 and 3.2.1.2), endodextranase (E.C. 3.2.1.11),  $\alpha$ -glucosidases (E.C. 3.2.1.20), pullulanase (E.C. 3.2.1.41), and maltogenic  $\alpha$ -amylase (E.C. 3.2.1.133) for starch liquefaction and saccharification, followed by transglucosidase (EC 3.2.1.20) to convert the glucose glycosidic bonds  $\alpha$ -(1 $\rightarrow$ 4) into indigestible glycosidic bonds of  $\alpha$ -(1 $\rightarrow$ 6) in isomalto-oligosaccharides. This process generates some impurities of glucose, maltose and maltotriose that can be removed by yeast fermentation. The final products are isomaltose, panose, isomaltotriose, and other higher oligosaccharides. Simultaneous saccharification, engineered recombinant enzymes, fusion of enzymes, and enzyme immobilization are new technologies developed to enhance ISMO yields (O. Ibrahim, 2018; Sorndech et al., 2018).

Isomalto-oligosaccharides are low caloric and 60% as sweet as sucrose, applied in food as bulking agent, dietary fibers, and to mask unpleasant taste of artificial sweeteners such as saccharine, aspartame, stevia, sucralose, and acesulfame-k. ISMO are partially digested by isomaltase in the jejunum and residual oligosaccharides are fermented by gut microbes. Hence, they are considered slowly digestible prebiotic oligosaccharides (Goffin et al., 2011). ISMO have high stability, high-moisture-retaining properties, lower water activity and are not digested by yeasts. Their health benefits include prebiotic effect, improving colonic microbial profile, intestinal function and blood cholesterol, and help mineral absorption. Worldwide regulatory agencies approve ISMO as safe (GRAS) with maximum daily intake of 30 g/day (Sorndech et al., 2018; Yen et al., 2011).

#### III.5.4. Cyclodextrins

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of glucose units creating a cone shape of  $\alpha$ -cyclodextrin containing six glucose units,  $\beta$ -cyclodextrin containing seven glucose units, and  $\gamma$ -cyclodextrin containing eight glucose units in a ring-forming shape of glucopyranose molecules (**Table 12-2**). They are a result of intramolecular transglycosylation reaction from the degradation of starch by cyclodextrin glucanotransferase (CGTase) enzyme (EC 2.4.1.19) (O. Ibrahim, 2018).

Unlike  $\gamma$ -cyclodextrin, both  $\alpha$ - and  $\beta$ -cyclodextrin cannot be hydrolyzed by human saliva and pancreatic amylases (O. Ibrahim, 2018; Reineccius et al., 2002). They are described as non-digestible prebiotic carbohydrates, which support the beneficial intestinal microflora in the colon. Overall, the metabolic fate of  $\alpha$ -cyclodextrin ingested is similar to that of other non-digestible but fermentable carbohydrates such as resistant starch or inulin. The carbohydrate which is slowly but still fully digested in the small intestine could have important applications

in the dietary management of diabetes (avoidance of night-time hypoglycemia), sports nutrition (sustained release of glucose for endurance events), and management of glycogen storage disorders (reduction in fasting hypoglycemia) (Fenyvesi et al., 2016). In addition, they are reported as useful for controlling the body weight and blood lipid profile (Del Valle, 2004; Fenyvesi et al., 2016).

Cyclodextrins,  $\alpha$ ,  $\beta$ , and  $\gamma$  are generally recognized as safe by FDA in the USA and approved by other worldwide organizations for the application in both food and pharmaceutical formulations. In the pharmaceutical industry are used as complexing agents to increase the aqueous solubility of poorly water-soluble drugs, increase both drugs bioavailability and stability, reduce or prevent gastrointestinal irritation, reduce or eliminate unpleasant smells or tastes, prevent drugs interactions, and convert oils and liquid drugs into microcrystalline or amorphous powders (Matencio et al., 2020; O. Ibrahim, 2018). In the food industry, CDs are used for stabilization of flavors, flavor delivery, elimination of undesired tastes and microbiological contaminations, and developing browning reactions in foods (O. Ibrahim, 2018). In Japan, CDs have been approved as ‘modified starch’ for food applications for more than two decades, serving to mask odors in fresh food and stabilize fish oils. One or two European countries, for example, Hungary, have approved  $\gamma$ -cyclodextrin for use in particular applications due to its low toxicity (Fenyvesi et al., 2016). The complexation of CDs with sweetening agents such as aspartame stabilizes and improves the taste, and eliminates the bitter aftertaste of other sweeteners such as stevioside, glycyrrhizin, and rubusoside. Enhancement of flavor has been also claimed for alcoholic beverages such as whisky and beer (Del Valle, 2004). The bitterness of citrus fruit juices is a dominant problem in the industry caused by limonoids (essentially limonin) and flavonoids (mainly naringin). Cross-linked cyclodextrin polymers are useful to remove these bitter components by inclusion complexes (Matencio et al., 2020).

In the cosmetic industry, cyclodextrins control the release of fragrances in products such as detergents, perfumes, and room fresheners (Del Valle, 2004). In the agriculture industry, cyclodextrins are used in manufacturing complex formulas of pesticides, herbicides, and insect repellents for safe and environmentally friendly products. In general, the three types of CDs have a wide range of applications, and  $\beta$ -cyclodextrin seems to be the most used especially in the pharmaceutical industry due to its efficacy, and its lower production cost (Figueiras et al., n.d.; O. Ibrahim, 2018).

### III.6.Non-starch Oligosaccharides

#### III.6.1. Xylo-oligosaccharides

The amorphous polymer xylan is the most abundant component in hemicellulose, composed of a backbone of xylose units linked via  $\beta$ -(1 $\rightarrow$ 4) glycosidic bonds. The depolymerization of xylan by xylanolytic enzymes (mainly endoxylanases) releases short chain xylo-oligosaccharides (XOS) (**Table 12-2**). The structure of XOS can vary greatly in terms of side groups (acetyl, arabinofuranosyl residues, 4-O-methyl, and uronic acids) and degree of polymerization (2 to 10), as well as the conditions for their production (pre-treatment of lignocellulosic biomass and enzymatic cocktails) (Freitas et al., 2019; Samanta et al., 2015).

XOS present higher bifidogenic activity compared to other prebiotics, and many studies reported their potential to inhibit carcinogenesis (Le et al., 2020), anti-inflammatory and antioxidant properties (Yu et al., 2015). The antioxidant activity of XOS is associated to the presence of acetyl and uronyl groups and also ester-linked hydroxycinnamic acid derivatives as caffeic, ferulic, syringic and coumaric acid residues and methyl glucuronic acid from xylan side chains (Ávila et al., 2020; Poletto et al., 2020). As a food ingredient, XOS are stable over a wide range of temperatures (up to 100 °C) and pH (from 2.5 to 8), have acceptable odor and flavor, and relative sweetness. They are commonly used as a gelling agent to modify viscosity and stabilize foam (Catenza & Donkor, 2021).

Although a disaccharide is not an oligosaccharide, the disaccharide xylobiose is considered a XOS for food purposes (Vázquez et al., 2000). Xylobiose has 30% of the sweetness of sucrose and low energy-content (1.5 kcal/g). The possibility of having a sweetener with antioxidant and prebiotic activity and low calorie implies several advantages for food enrichment. The recommended daily dose is 8-12 g per day for healthy adults (Pinales-Márquez et al., 2021; Samanta et al., 2015). Recent research applied XOS as a fat replacer in dairy products, improving elasticity and firmness of low-fat cream cheese (Ferrão et al., 2018), and increasing the storage stability in dulce de leche (Ledomado et al., 2021) and cookies (Ayyappan et al., 2016). XOS also showed potential application in microencapsulation systems to protect probiotic microorganisms (Martins et al., 2021).

#### III.6.2. Cello-oligosaccharides

Cello-oligosaccharides (COS) is a new class of non-digestible oligosaccharides and have been studied by researchers in recent years, seeking optimal conditions to obtaining them from lignocellulosic biomass with properties for industrial applications. From a biochemical point of view, COS are defined as important biomolecules constituted by glucose units

interconnected by  $\beta$ -1,4-type bonds (Ávila et al., 2021). They are differentiated according to their degree of polymerization and can be classified and/or characterized as: cellobiose (2 glucose monomers), cellotriose (3 glucose monomers), cellotetraose (4 glucose monomers), cellopentose (5 glucose monomers) and cellohexose (6 glucose monomers) (**Table 12-2**).

COS are intermediates from the hydrolysis of cellulose to glucose, using two main strategies: acid-based and enzyme-based cellulose hydrolysis. The latter has been considered more attractive due to milder reaction conditions and lower production of monomers. Cellobiohydrolases (CBHs) and some endoglucanases (EGs) have been reported as fundamental enzymes for COS production, especially cellobiose. Both belong to a group called processive enzymes, whose main characteristic is the release of soluble products from the terminal of the cellulose chains, carrying out several hydrolysis cycles moving in the same chain (Karnaouri et al., 2018). Lately, new approaches for optimizing COS production have been using regular EGs, processive EGs and auxiliary enzymes such as lytic polysaccharide monooxygenases (LPMOs) (Barbosa, Kendrick, et al., 2020; Karnaouri et al., 2018, 2019). Lignocellulosic biomass such as forest residues pretreated with organosolv (birch and spruce) have been tested in the presence of processive EGs of bacterial and fungal origin, belonging to the GH9, GH6 and GH48 families and LPMOs for their ability to release COS (Karnaouri et al., 2019). Barbosa et al. (Barbosa, Kendrick, et al., 2020; Barbosa, Martins, et al., 2020) also have optimized COS production, using different pretreated sugarcane straw biomass using a combination of heterologous expressed processive EGs of bacterial and fungal origin, belonging to GH9 and GH45, LPMOs and cellobiose dehydrogenase enzyme. However, there is a lack of information regarding the large-scale production, which could be associated with the recalcitrant nature of cellulose compared to other polysaccharides, which hinders the hydrolysis of its dense network (Ávila et al., 2021).

Regarding the physical-chemical, rheological and functional properties, COS became important compounds with high potential application in the most diverse segments of the food and pharmaceutical industry. One of the most important properties currently studied is their prebiotic functional activity. They may be able to resist the initial stages of the digestion process, serving as a substrate for beneficial microorganisms present in the intestinal microbiota (Cezar et al., n.d.). Furthermore, they play an important role in inhibiting pathogenic microorganisms, in addition to other properties that make them biomolecules of great interest and target of research in the medical, pharmaceutical and cosmetic fields. By oral ingestion, COS are also responsible for lowering total cholesterol and neutral fat concentration in the liver, preventing or helping to cure diseases and, consequently, promoting a better lifestyle (Kluge et al., 2019).

All these mechanisms triggered by their prebiotic effect favor cello-oligosaccharides, considering that, like other prebiotic substances, COS have considerable potential to be incorporated into food formulations, such as cereal bars, bakery products, sweets, ice creams, dairy desserts, juices, nectars, sauces, symbiotic foods, etc. They can also be incorporated into dietary supplements or even into medicines that assist in the replacement of the intestinal microbiota (de Freitas et al., 2019). In the field of cosmetics, COS has shown potential to improve the effectiveness of moisturizing creams. When combined with fatty acids, they can still be used as new biobased amphiphilic compounds (Billès et al., 2016). Hydroxyl groups in COS can be derived by a variety of methods, adjusting their properties and opening other fields of application (Kluge et al., 2019).

### III.6.3. Pectin-oligosaccharides

Pectin comprises a family of acidic polymers, with several neutral sugars/polymers such as arabinans, galactans and arabinogalactans (attached as side chains) (Strasser & Amado, 2001). Four main pectic components have been identified, namely, homogalacturonan (HG), rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II), apiogalacturonan (AG), and xylogalacturonan (XG) (**Figure 12-11**). All these pectic components are connected by either covalent or ionic cross links (Gullón et al., 2013). The extraction of these neutral and acidic polymers in the form of pectic oligosaccharide (POS) is a promising step towards the manufacture of prebiotics from agricultural by-products such as apple pulp, sugar beet, onion skin, potato pulp, soy hull, olive pomace and citrus waste, which contain significant amounts of pectin (Babbar et al., 2016). The most common and well-known POS are arabinogalacto-oligosaccharides, arabinoxyl-oligosaccharides, arabino-oligosaccharides, galacto-oligosaccharides, oligogalactouronides and rhamnogalacturonan-oligosaccharides (Babbar et al., 2016; Concha Olmos & Zúñiga Hansen, 2012; M. Martínez et al., n.d.). The advantage of POS over pectin (a fermentable fiber too), is the higher prebiotic potential associated with the reduction in the size of the molecule chain, a less complex structure and a low steric hindrance. These characteristics favor their fermentation yielding larger amounts of short-chain fatty acids (Tingirikari, 2018).

POS have been obtained by either enzymatic or chemical-physical methods. Various enzymes have been widely used for the production of POS because of their specificity and selectivity. In addition, the use of enzymes over other pre-treatment methods is regarded as safe due to minimum adverse chemical modifications of products

The fermentation properties of these oligosaccharides have been investigated using pH-controlled fecal batch cultures (Olano-Martin et al., n.d.). POS were more selective for

bifidobacteria and lactobacilli than pectin, and also more selective for bifidobacteria than FOS (Manderson et al., 2005). The colonic fermentation of prebiotic POS results in the generation of short-chain fatty acids (SCFA), which exerts several health effects like inhibition of pathogenic bacteria, relief of constipation, reduction in blood glucose levels, improvement in mineral absorption, decreased incidence of colonic cancer and modulation of the immune system (Gulón et al., 2013). The literature also suggests that POS can act as phytoalexin elicitor, flowering inducer and antibacterial agent in plants (Iwasaki et al., 2014). However, to further substantiate the claim of the prebiotic efficacy and other health benefits of POS, more rigorous *in vitro* investigations are required, and *in vivo* studies will validate the claim (Babbar et al., 2016).

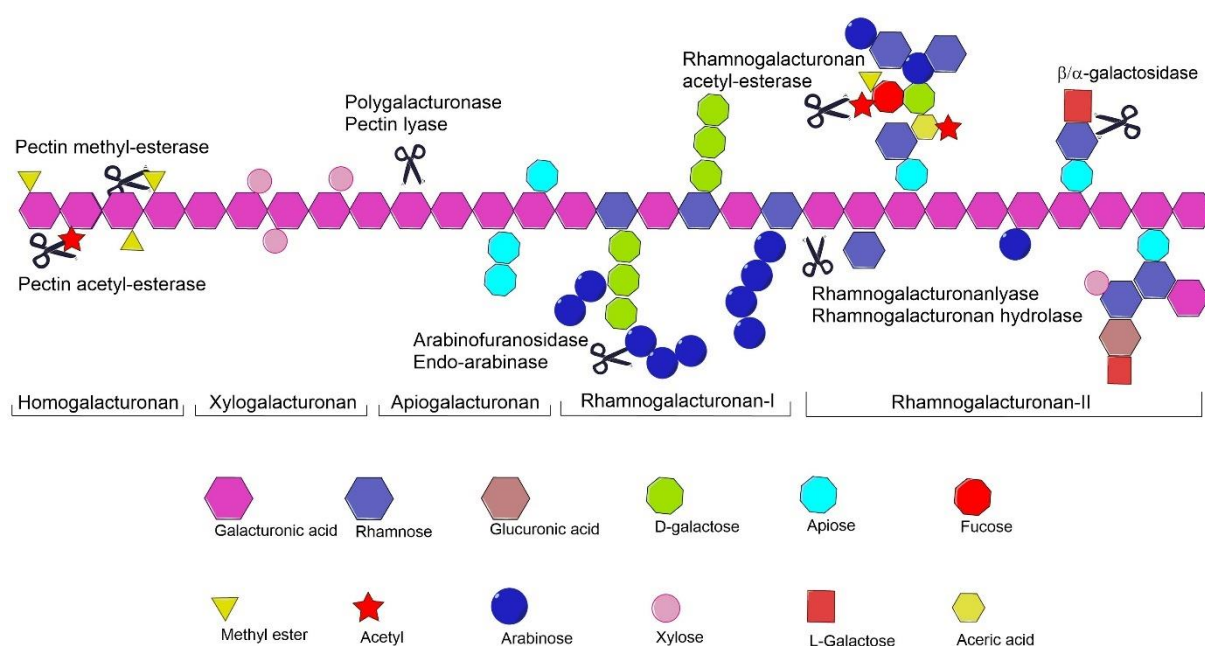


Figure 12-11 Schematic structure of pectin and the main enzymes responsible for its hydrolysis

#### III.6.4. Soy-oligosaccharides

Soy-oligosaccharides refer to oligosaccharides found in soybeans, which account for approximately 40–50 g/kg of dry weight of soybean meal (Lan et al., 2007). The principal oligosaccharides in mature soybeans are stachyose (14-41 g/kg), raffinose (1-9 g/kg), and verbascose (2-3 g/kg). Raffinose is a trisaccharide containing galactose linked by  $\alpha$ -(1→6) bond to the glucose unit of sucrose. Stachyose is a tetrasaccharide containing extra galactose unit linked to raffinose by  $\alpha$ -(1→6) bond. Verbascose is pentasaccharide containing extra galactose unit linked to stachyose by  $\alpha$ -(1→6) bond. Ajugose is a hexasaccharide, that is, verbascose that has an additional unit of  $\alpha$ -D-galactopyranose attached by  $\alpha$ -(1→6) bond (**Figure 12-12**) (Lan et al., 2007; O. Ibrahim, 2018).

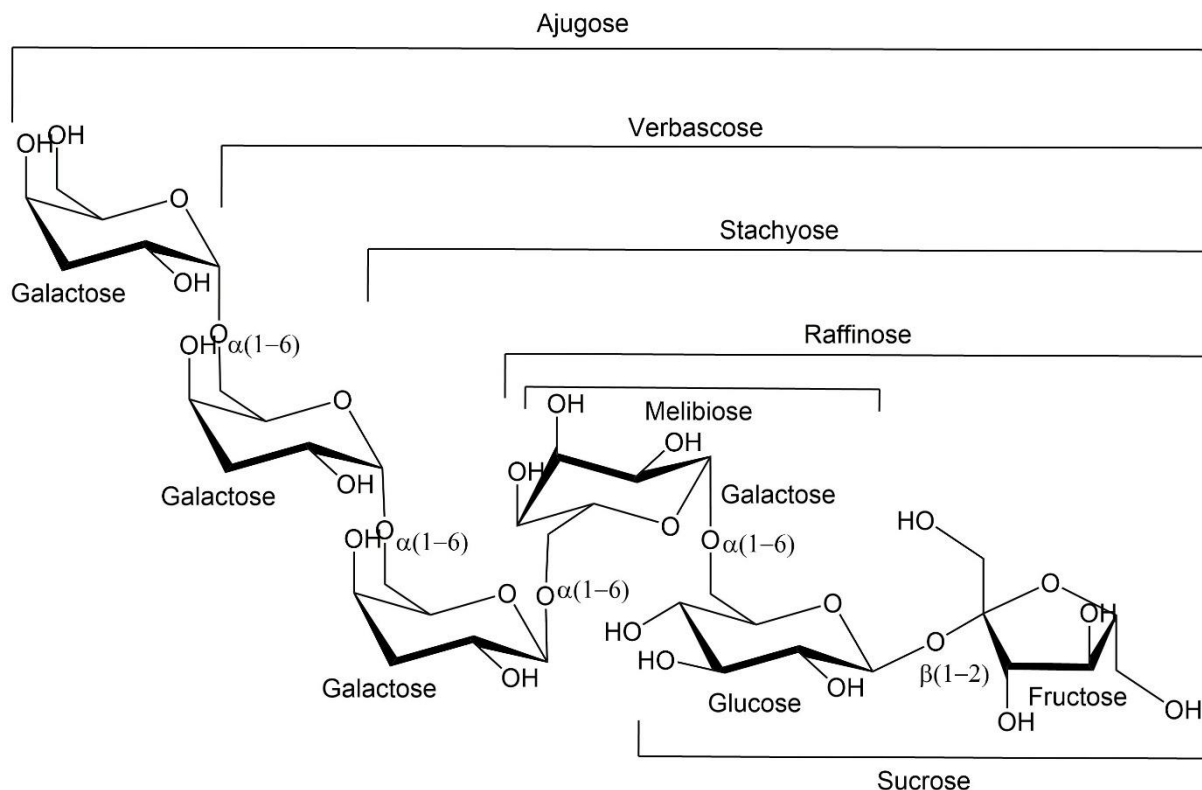


Figure 12-12 Soy-oligosaccharides structures

Soy-oligosaccharides are low calorie sweeteners and soluble fiber that can make the stools softer. The recommended daily intake of these compounds is 4 g/day to improve absorption of calcium and other minerals, increase microbial metabolites of short-chain fatty acid that help reducing the risk of colon cancer, and minimize toxic metabolites production that effect liver. However, it is important to highlight that over consumption of soy-oligosaccharides may trigger abdominal bloating, excessive gas, and diarrhea (O. Ibrahim, 2018; Rastall, 2010).

The characteristic of  $\alpha(1\rightarrow6)$  linked bonds in soy-oligosaccharides chemical structures is unbreakable due to the lack of  $\alpha$ -galactosidase enzyme in the human digestive system. The undigested soy-oligosaccharides reached the colon intact, where is hydrolyzed by  $\alpha$ -galactosidase of intestinal microflora into fermentable sugars that enhance the growth of enteric bacteria in the colon (O. Ibrahim, 2018). However, some scientists did not consider soy-oligosaccharides as prebiotic due to conflict in published research reports. Some researchers have observed a significant increase only in beneficial bacteria of *Bifidobacterium* species (Bouhnik et al., 2004), while others, observed significant increase in both *Bifidobacterium* species and the pathogenic *Clostridium* species (Dong et al., 2014). These disputed research reports on the prebiotic property of soy-oligosaccharides still need further investigation.



### III.6.5. Chitosan-oligosaccharides

Chitosan-oligosaccharides (CHOS) are the degraded products of chitin/chitosan by acid hydrolysis, enzymatic degradation or both (**Table 12-2**) (F. F. Wang et al., 2011). They are characterized by a degree of deacetylation (DD) >90% (Kweon Park et al., 2011; Naveed et al., 2019), a degree of polymerization (DP) <20, and average molecular weight (Mw) <3900 Da (Liang et al., 2016; Xia et al., 2011). Although, the definition of CHOS is not restricted to these specifications. These compounds have captured the attention of numerous researchers in various fields such as food, cosmetics, biomedicine, and agriculture (Zhao et al., 2017).

In the last decade, studies have focused on the health benefits of CHOS, decreasing blood cholesterol, controlling high blood pressure, protecting from infections, improving the antitumor properties, antidiabetic, anti-obesity, anti-HIV-1, anti-Alzheimer's disease, calcium absorption, and hemostatic effects. Furthermore, CHOS have showed higher cellular transduction and completely absorbability via intestinal epithelium due to the cationic sphere exposed on the more exposed shorter N-glucosamine (N-Glc) units (Naveed et al., 2019; Zou et al., 2015). Researches also have examined the relationship between CHOS biological activities and physicochemical properties. They revealed that the multiple biological effects are directly related to the Mw, DD, DP, and pattern of charge distribution on the N-Glc unit (Xia et al., 2011). With close structure examination, CHOS possess significant reactive functional groups that are integrally responsible to their biological activities, as well as being the site for further suitable chemical modifications, which include primary and secondary hydroxyl groups at C3 and C6 respectively, the amine/acetamide group and glycosidic link between the N-Glc units applications (Tanaka et al., 2003). Thus, several of these modifications and their chemical and biological effects are worth consideration concerning the mechanism of actions and their proposed applications (Naveed et al., 2019).

### III.7. Algal-oligosaccharides

Algal-oligosaccharide (AOS) lysates are soluble and low molecular weight carbohydrates (2 to 10 monomer units) derived from algal polysaccharides (**Table 12-2**). The water solubility makes AOS biocompatible and provides a wide range of bioactivity, with potential application in pharmaceutical and nutraceutical industries as dietary fiber, hydrocolloid, anti-cancer, anticoagulant, drug delivery, and tissue engineering (Tanna & Mishra, 2019). Algal-oligosaccharide-lysates have antioxidant properties owing to their high polyphenol content (Wu et al., 2005). AOS have been commercialized since the 1980s as low-calorie bulking agents,

but they received much interest in food industry after the findings that non-digestible oligosaccharides can be included as functional food and credited as prebiotics (Gurpilhares et al., 2019).

The complex nature of the algal polysaccharides requires specific enzymes for the production of each AOS: agaro, alginate, carrageenan, fucoidan, laminarin, porphyrin, oligosaccharides. Studies on the bioactivity of enzymatically produced ulvan oligosaccharides were not found. AOS are produced by the action of the enzymes agarase, carrageenase, porphyranase, fucoidanase, and laminarinase. Polysaccharide lyase (PL) is the second group of enzymes which break down  $\beta$ -(1  $\rightarrow$  4) glycosidic bond between anionic sugars of the algal polysaccharides. Carrageenase, agarase and alginate lyases are the most studied enzymes for the AOS production. Several microbes (bacteria and fungus) associated with the marine macroalgae, molluscs, seaweed sludge, and decaying forms produce extracellular enzymes that break down the complex algal polysaccharides to AOS (Jagtap & Manohar, 2021).

#### **III.7.1. Agaro and neoagaro-oligosaccharides**

Agar is a linear polysaccharide composed by alternating units of 3-O- $\beta$ -D-galactose (G) and 4-O-3, 6-anhydro- $\alpha$ -L galactose (AG) and is hydrolyzed by GH 96  $\alpha$ -agarases (EC 3.2.1.158) and, mainly,  $\beta$ -agarases (EC 3.2.1.81, GH 16, 50, 86, and 118) to produce agaro and neoagaro-oligosaccharides, respectively (Hu et al., 2006). Identified  $\beta$ -agarases are more abundant than  $\alpha$ -agarases in both the CAZy database ([http://www.cazy.org/fam/acc\\_GH.html](http://www.cazy.org/fam/acc_GH.html)) and published studies (Fu & Kim, 2010; Jagtap & Manohar, 2021). Therefore, the greater recombinant expression of  $\beta$ -agarases leads to a higher content of neoagaro-oligosaccharides functional application studies, showing antioxidant, antiglycemic, anti-inflammatory and prebiotic activities (Hu et al., 2006; Jagtap & Manohar, 2021; S.-Y. Xu et al., 2018). NAOS also alleviated hepatocyte swelling and recovered pancreatic  $\beta$ -cells involved in insulin secretion (Lin et al., 2019).

#### **III.7.2. Alginate-oligosaccharides**

Alginate is a complex polymer of  $\alpha$ -L-glucuronic acid (GlcA) and its C5 epimer  $\beta$ -D-mannuronic acid (MnA) hydrolyzed by mannuronate lyase (EC 4.2.2.3) and glucuronate lyase (EC 4.2.2.11) that cleavage the  $\beta$ -(1  $\rightarrow$  4) linkage to produce alginate oligosaccharides (**Figure 12-13**). Several alginate lyases are reported from a wide range of marine, soil, and human gut microorganisms. The enzymatically produced alginate-oligosaccharides present prebiotic, antioxidant, immunoregulatory, anti-inflammatory, and anti-glycemic activities (Han et al., 2019; Jagtap & Manohar, 2021; Xing et al., 2020). They also induced nitric oxide production, nitric oxide synthase, and stimulated tumor necrosis factor- $\alpha$  production (X. Xu et al., 2014).

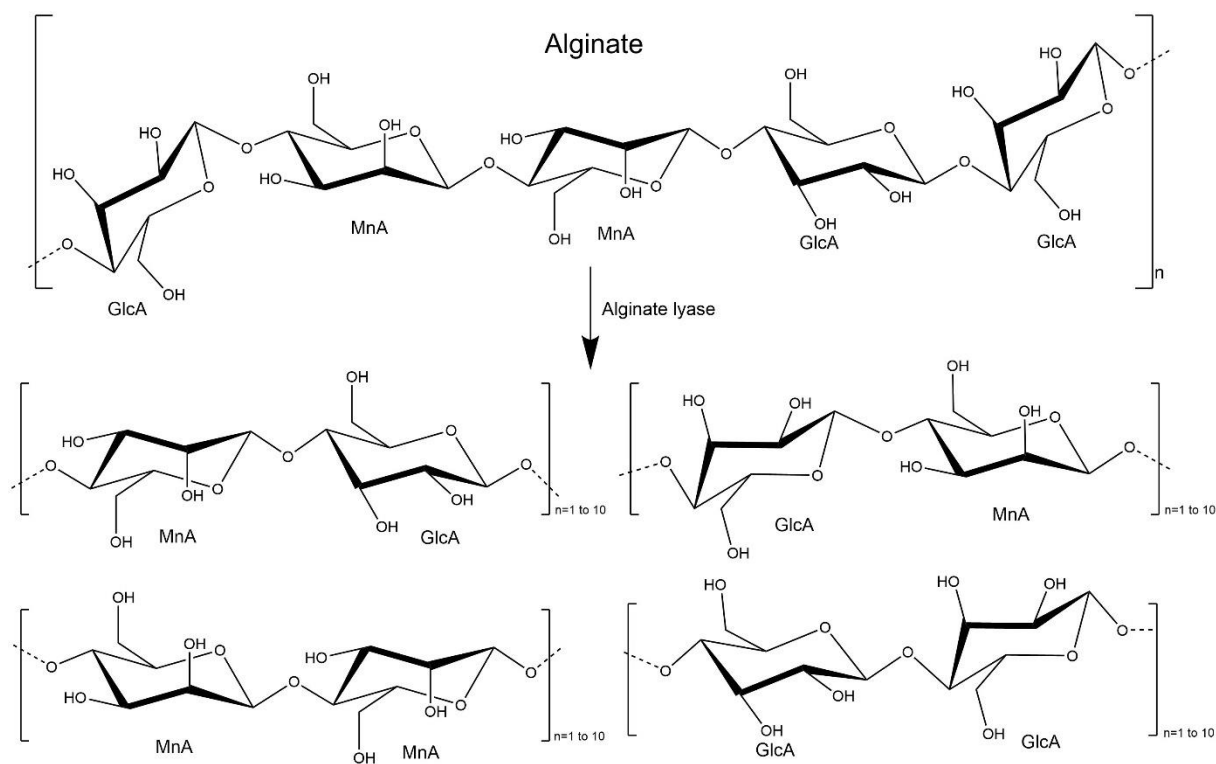


**Figure 12-13 Alginate-oligosaccharides structure**

### III.7.3 Carrageenan-oligosaccharides

Carrageenans are linear sulfated polysaccharides mainly composed of repeating disaccharide units of 3-O-linked- $\beta$ -D-galactose (G) and 4-O-linked-3, 6-anhydro- $\alpha$ -L galactose (AG), categorized as  $\kappa$ ,  $\iota$ , and  $\lambda$  based on the number of sulfate groups:  $\kappa$ -carrageenan has one sulfate group,  $\iota$ -carrageenan has two, and  $\lambda$ -carrageenan has three sulfate groups per disaccharide unit (**Figure 12-14**). Each carrageenan is hydrolyzed by its specific endohydrolase  $\kappa$ -carrageenase (GH 16, EC 3.2.1.83),  $\iota$ -carrageenase (GH 82, EC 3.2.1.157), and  $\lambda$ -carrageenases (GH 150, EC 3.2.1.162), which cleavage the  $\beta$ -(1  $\rightarrow$  4) linkages. The enzymatically produced oligosaccharides showed potential antioxidant, immunoregulatory, and antitumor activity than carrageenan polysaccharide (Jagtap & Manohar, 2021).  $\kappa$ -carrageenan-oligosaccharides are the most studied recently, showing antioxidant (Sun et al., 2015), anti-angiogenic (Yao et al., 2014), antitumor (Yuan et al., 2011), and antibacterial activities (F. F. Wang et al., 2011).

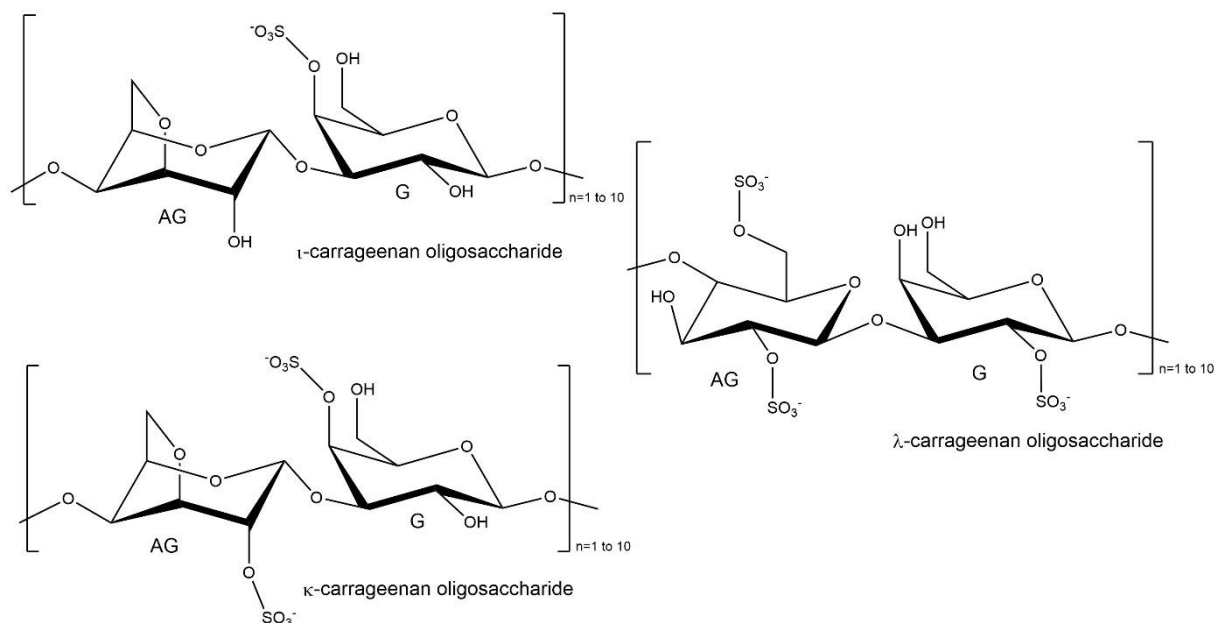


Figure 12-14 Carrageenan-oligosaccharides structure

### III.7.4. Fucoidan-oligosaccharides

Fucoidan is a highly sulfated polysaccharide composed of α-L-fucoses linked by (1 → 4) and (1 → 3) glycosidic bond with sulfation at C2, C3, or C4 depending on the specie (**Figure 12-15**). Fucoidanase (GH 107, EC 3.2.1.44) catalyze the hydrolysis of α-L-fucoside linkage without removal of substitute groups and produce low molecular weight fucoidan oligosaccharides (Jagtap & Manohar, 2021) that, recently, showed anticoagulating and anticancer activities (Anastyuk et al., 2012; Cui et al., 2018; W. J. Kim et al., 2010).

### III.7.5. Laminarin-oligosaccharides

Laminarin is a water-soluble glucan found in brown macroalgae consisting of glucose units linked through β-(1 → 3) and some β-(1 → 6) glycosidic bonds. The exo-β-(1 → 3)-glucanase (EC 3.2.1.58) hydrolyze laminarin at the reducing end and formed monomer units. The endo-β-(1 → 3)-glucanase (EC 3.2.1.6 and EC 3.2.1.39) hydrolyze the β (1 → 3) linkage randomly within the chain of laminarin into β-(1 → 6) linked laminarin oligosaccharides (**Figure 12-15**) (K.-H. Kim et al., 2006). The side chain in laminarin limits the activity of the endoglucanase due to steric hindrance. Endo-β-(1 → 3)-glucanase belongs to different GH families including GH16, GH17, GH55, GH64, and GH81 (Jagtap & Manohar, 2021).

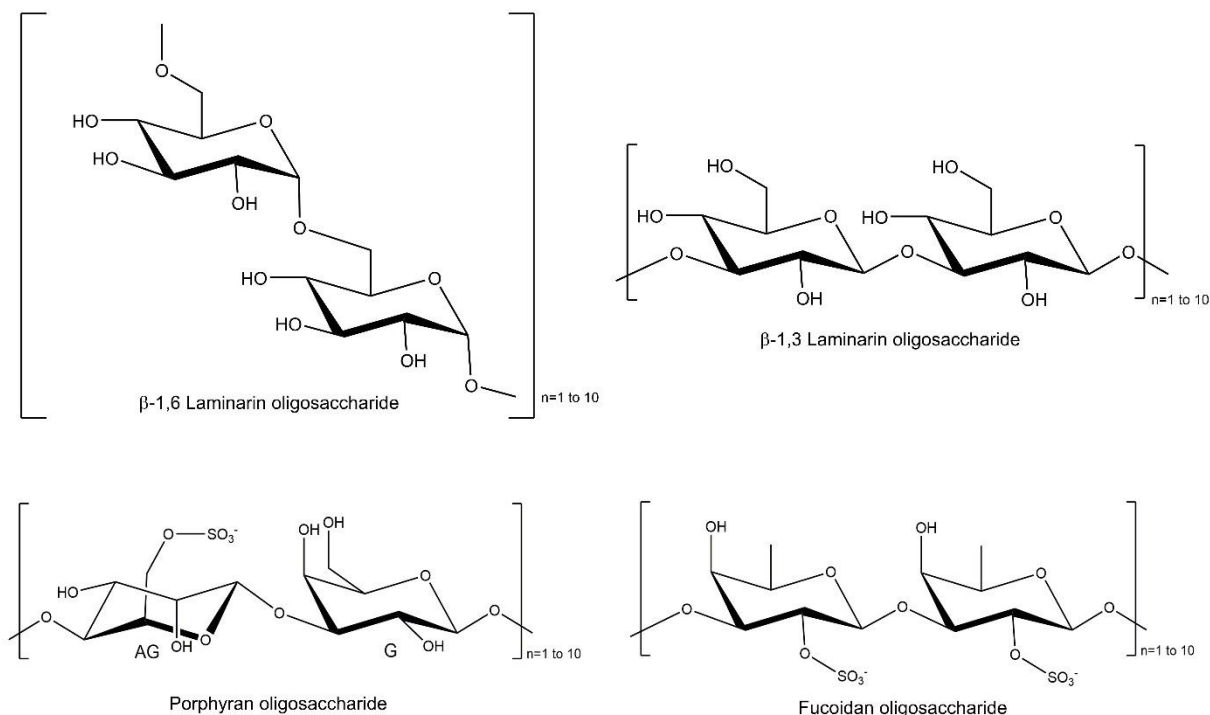


**Figure 12-15** Fucoidan, laminarin, and porphyran oligosaccharides structure

### III.7.6. Porphyran-oligosaccharides

Porphyran is a sulfated polysaccharide mainly composed of a linear backbone of alternating 3-linked methylated  $\beta$ -D-galactose (G) and sulfated 3,6-anhydro- $\alpha$ -L-galactose (AG) units, whose  $\beta$ -(1  $\rightarrow$  4) linkages are hydrolyzed by  $\beta$ -porphyranases (GH 16 and 86, EC 3.2.1.178) (**Figure 12-15**). The low molecular weight porphyran oligosaccharides alleviate dysbiosis and showed antitumor and antiobesity activities (Jagtap & Manohar, 2021; Osumi et al., 2002; X. Wang et al., 2021; Zhang et al., 2019).

### III.8. Concluding remarks

The interest in functional oligosaccharides is driven by their unique properties, health benefits, diverse industrial applications, and the growing popularity of natural products. As nutraceuticals, functional oligosaccharides have been targeted of intensive research during the last decade, and their applications in the food and pharmaceutical industries open space for a new market of enzymatically manufactured oligosaccharides and the prospection of new raw material, especially those from renewable and abundant sources such as agro-industrial wastes.

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## III.9. References

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**Appendix IV – Patent of Invention**

**Processo de produção de pectina e xilooligossacarídeos a partir de resíduo industrial de suco de laranja**



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**República Federativa do Brasil**

Ministério do Desenvolvimento, Indústria, comércio e Serviços

Instituto Nacional da Propriedade Industrial

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24/04/2024**(54) Título:** PROCESSO DE PRODUÇÃO DE PECTINA E XILO- OLIGOSSACARÍDEOS A PARTIR DE RESÍDUO INDUSTRIAL DE SUCO DE LARANJA E XILO- OLIGOSSACARÍDEOS**(51) Int. Cl.:** C12P 19/02; C08B 37/06; C13K 13/00.**(71) Depositante(es):** UNIVERSIDADE ESTADUAL DE CAMPINAS.**(72) Inventor(es):** ROSANA GOLDBECK; MANOELA MARTINS.

**(57) Resumo:** PROCESSO DE PRODUÇÃO DE PECTINA E XILO-OLIGOSSACARÍDEOS A PARTIR DE RESÍDUO INDUSTRIAL DE SUCO DE LARANJA E XILOOLIGOSSACARÍDEOS. A presente invenção refere-se a um processo integrado para produção de pectina e xilo-oligossacarídeos a partir de resíduo de casca de laranja. O processo otimizado de extração com ácido cítrico para a produção de pectina foi adaptado para o resíduo de casca de laranja. Esta otimização rendeu um resíduo sólido mais rico em xilana e passível de ser utilizado para produção enzimática de xilo-oligossacarídeos. Além disso, durante o processo de extração de pectina, também foram produzidas quantidades consideráveis de xilooligossacarídeos, que podem ser recuperados e purificados da fração líquida da extração de pectina. A hidrólise enzimática da xilana foi otimizada para a produção de xilooligossacarídeos, avaliando a concentração de substrato e enzima utilizadas no processo. A presente invenção, além de produzir pectina com mais de 80% de pureza e 0,7 g/L de xilooligossacarídeos no processo de extração de pectina, também rendeu mais de 9g/L de xilo-oligossacarídeos após 48h de hidrólise de xilana a 50° C e 2% de sólidos, valor que representa mais de 70% de conversão da xilana em xilooligossacarídeos. Adicionalmente, os xilos oligossacarídeos produzidos se destacam pela sua (...).

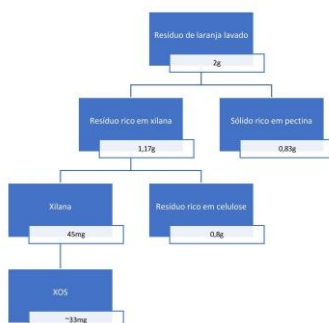


FIGURA 3

**PROCESSO DE PRODUÇÃO DE PECTINA E XILO-OLIGOSSACARÍDEOS A  
PARTIR DE RESÍDUO INDUSTRIAL DE SUCO DE LARANJA E XILO-OLI-  
GOSSACARÍDEOS**

**CAMPO DA INVENÇÃO**

[1] A presente invenção refere-se a um processo integrado para produção de pectina e xilo-oligossacarídeos a partir de resíduo de casca de laranja. Os xilooligossacarídeos produzidos se destacam pela sua capacidade pré-biótica e antioxidante e, ainda, são passíveis de serem aplicados na indústria de alimentos e/ou farmacêutica.

[2] A presente invenção se insere no campo da engenharia de alimentos, mais precisamente na área de biotecnologia.

**FUNDAMENTOS DA INVENÇÃO**

[3] Cascas de frutas cítricas representam um dos resíduos mais subaproveitados do setor industrial. Após a extração do suco das frutas, o resíduo de casca pode atingir mais de 50% do peso úmido da fruta. Cascas de laranja são constituídas por 20% de matéria seca (açúcar, celulose, hemicelulose, pectina e D-limoneno) e 80% de água. O reaproveitamento deste resíduo tem sido focado principalmente na extração de D-limoneno e, em menor proporção, pectina. Tradicionalmente, estas cascas são secas e os compostos de interesse são extraídos com ácidos, e uma pequena porção de casca seca é aproveitada na alimentação animal. Entretanto, a maior parte deste material é descartada de forma inadequada no solo devido aos custos de secagem e uso de ácidos, gerando séria poluição orgânica por conta da elevada demanda de oxigênio biológico para sua decomposição (Balu et al., 2012; Ortiz-Sanchez et al., 2021).

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[4] Uma alternativa para se contornar o problema é o reaproveitamento das frações de pectina e lignocelulose. A pectina pode compor até 50% do peso seco deste resíduo, e em torno de 5% de xilana. Este teor mais baixo de xilana presente na casca de laranja pode ser compensado pelo alto volume de resíduo gerado todos os anos nas indústrias de sucos. Além disso, ao se extrair a fração de pectina, o resíduo sólido remanescente pode conter até 50% do peso seco de xilana, tornando este resíduo sólido da extração de pectina uma fonte abundante para produção enzimática de xilooligossacarídeos (XOS).

[5] Xilo-oligossacarídeos são fibras alimentares parcialmente digeridas por humanos. As porções não digeridas servem de alimento para as bactérias intestinais, atuando como prebióticos, estimulando o crescimento e a atividade de bactérias benéficas do intestino, aumentando a produção de ácidos graxos voláteis antiulcerogênicos, inibindo o crescimento de bactérias nocivas e retardando a absorção de carboidratos no trato gastrointestinal, sendo relatado um efeito benéfico no diabetes mellitus tipo 2. Os benefícios proporcionados aos sistemas digestivo e imunológico levam a potencial aplicação em alimentos funcionais. Uma vantagem do XOS é a possibilidade de produção a partir de resíduos agroindustriais por hidrólise de xilana.

[6] A produção industrial de pectina é custosa devido a grande quantidade de água, etanol e energia (elétrica, vapor e refrigeração). A produção industrial de XOS também é um desafio devido aos custos relacionados com o uso de enzimas e álcalis (KOH ou NaOH) durante a etapa de pré-tratamento. Apesar disso, a indústria de alimentos



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demanda uma alta produção de pectina, devido ao seu uso como agente espessante em diversos produtos como geleias, iogurtes e manufatura de bioembalagens, e de XOS para a produção de alimentos funcionais (Amorim et al., 2019; Tsouko et al., 2020). A literatura reporta o aproveitamento de cascas de laranja para a produção de óleo essencial, pectina, biogás e XOS (Ortiz-Sanchez et al., 2021; Praveen et al., 2017; Teigiserova et al., 2022; Tsouko et al., 2020).

[7] Entretanto, informações relacionadas às combinações economicamente viáveis que possibilitem o aproveitamento simultâneo de várias frações deste resíduo são escassas, especialmente relacionadas à produção de XOS. O desenvolvimento de um processo integrado dentro do conceito de uma biorefinaria deve acessar a eficiência energética e de custos por meio da otimização das etapas sequenciais do processo, visando reduzir tempo e consumo de água e reagentes, mantendo a integridade do material residual de uma etapa para ser utilizado na etapa seguinte.

[8] Desta forma, devido à otimização dos métodos utilizados para a extração de pectina, extração de xilana e hidrólise enzimática de xilana, o principal diferencial da presente invenção é o rendimento em xilo-oligossacarídeos e a pureza de pectina obtida.

[9] A presente invenção otimizou o método de extração com ácido cítrico já aplicado na indústria para a produção de pectina, adaptando-o para o resíduo de casca de laranja. Esta otimização também rendeu um resíduo sólido mais rico em xilana passível de ser utilizado para produção enzimática de xilo-

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oligossacarídeos. Além disso, durante o processo de extração de pectina, também foram produzidas quantidades consideráveis de xilo-oligossacarídeos, que podem ser recuperados e purificados da fração líquida da extração de pectina.

[10] Alguns documentos do estado da técnica descrevem processos para produção de pectina e xilo-oligossacarídeos, adicionalmente, alguns dos processos ocorrem a partir de resíduos de casca de laranja, tais como:

[11] O documento científico intitulado "VALUE ADDITION OF ORANGE FRUIT WASTES IN THE ENZYMATIC PRODUCTION OF XYLOOLIGOSACCHARIDES" refere-se ao uso de biomassa de laranja no processo de preparo de xilo-oligossacarídeos (XOS). Adicionalmente, são reveladas as mesmas condições de processo e o uso de enzimas endo-xilanases comerciais.

[12] Todavia, a presente proposta aproveita a fração de pectina para a produção de uma xilana mais pura, enquanto o documento utiliza o resíduo integral de casca de laranja para a extração de xilana e produção de XOS. Além disso, o referido documento analisa a produção de XOS para um curto tempo (6h), o que não representa um rendimento industrial. O presente processo otimiza as condições coerentes com uma aplicação industrial, a maior pureza da xilana extraída rende mais de 9g/L XOS. Além disso, XOS foram liberados durante o processo de extração de pectina, no total, produziu-se aproximadamente 10g/L de XOS. Foi utilizada uma menor concentração de etanol e álcali, 2,5% hidróxido de potássio (KOH), apresentando menor impacto ambiental e garante uma maior recuperação da xilana extraída de casca de laranja, por outro lado o referido documento utiliza hidróxido de sódio (NaOH), ao invés de KOH, que não é o melhor álcali para extrair xilana de casca e bagaço de laranja (uma vez

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que cada biomassa requer um tratamento diferente).

[13] O referido documento científico não deixa claro como foi realizado o planejamento experimental, não foi construída uma superfície resposta estatisticamente significativa para ser considerada uma otimização do processo. A melhor condição apresentada pelo documento produziu 0,630g/L de XOS. Ainda, não foi apresentada a caracterização química do material utilizado e a pureza obtida para a xilana extraída, a fim de se calcular o balanço de massa e eficiência do processo. É apresentada uma curva de calibração de xilose, mas o documento não deixa claro qual a concentração de enzima (massa de proteína) e a concentração de substrato (xilana extraída) foi utilizada para os ensaios de hidrólise.

[14] O documento científico intitulado "PRODUÇÃO DE XILO-OLIGOSSACARÍDEOS POR HIDRÓLISE ENZIMÁTICA DE XILANAS" refere-se o uso de enzimas comerciais, como a *Shearzyme*, para avaliar a atividade de endo- $\beta$ -xilanasases. Ainda, o referido documento revela que endo- $\beta$ -1,4-xilanasases foram utilizadas na hidrólise da xilana em xilo-oligossacarídeos. O estudo aponta que endo- $\beta$ -xilanasases da *Shearzyme* tem um baixo valor de  $K_m$  e é capaz de hidrolisar de forma mais eficiente a xilana.

[15] Contudo, o referido documento analisa a cinética de produção de XOS a partir de substratos comerciais, variando-se pH e temperatura. A caracterização e os parâmetros cinéticos das enzimas comerciais foram determinados utilizando xilana de faia (substrato comercial, não um resíduo agroindustrial), não sendo utilizada a casca de laranja. O melhor rendimento em XOS foi obtido com xilana

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extraída de casca de arroz, 5,67 g/L XOS.

[16] No referido documento, o método 2 para extração de xilana utiliza maior concentração de álcali e maiores tempos (12h a 60°C + 3h a 100°C). Para a acidificação, foi utilizado ácido clorídrico (HCl), que reage com NaOH e precipita grande quantidade de cloreto de sódio (NaCl), o que pode inibir a atividade enzimática, exige etapas adicionais de lavagem de xilana para a remoção do excesso de sal, além de ser mais caro (\$0,1/kg). Já o método 1 não apresenta potencial para aplicação industrial. Não fica clara a pureza da xilana extraída. Não fica claro o volume de enzima adicionado às reações.

[17] Ressalta-se que a presente proposta utiliza um resíduo agroindustrial abundante e de custo baixo. A quantidade de enzima usada é a menor concentração necessária para atingir a saturação enzimática. O método de extração de xilana requer menores tempo e concentração de álcali, e utiliza ácido acético na acidificação, apresentando baixa toxicidade e menor impacto ambiental, não forma NaCl e é mais barato (\$0,045/kg). Na presente invenção apresenta ainda quantidades de XOS obtidas são superiores ao do documento, além de haver o duplo aproveitamento do resíduo, para produção de pectina e XOS.

[18] O documento científico intitulado "PRODUÇÃO DE XILOOLIGOSSACARÍDEOS A PARTIR DE RESÍDUOS LIGNOCELULÓSICOS E FUNGOS FILAMENTOSOS" refere-se ao uso de resíduos de materiais lignocelulósicos para obtenção de xilooligossacarídeos como ingredientes prebióticos. Ainda, descreve sobre a hidrólise enzimática e o uso de  $\beta$ -1,4endoxilanase no processo.

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[19] No referido documento é aplicada fermentação em estado sólido utilizando casca de arroz como substrato na primeira etapa e posteriormente é aplicada a enzima produzida em uma etapa de hidrólise. Não há extração da xilana, o substrato é usado sem pré-tratamento. A maior concentração de XOS atingida foi 75 mg/g de substrato.

[20] Em relação ao referido documento, quando se trata da produção de oligossacarídeos, um dos pontos mais custosos é o *downstream* para purificação. Na fermentação em estado sólido, o substrato é adicionado ao meio de crescimento do fungo, que secreta diversas enzimas e pigmentos. Ao final do processo, não apenas XOS, mas diversos outros açúcares estarão presentes no meio, além de proteínas do fungo. Em relação à etapa de hidrólise realizada com a enzima produzida por *A. brasiliensis*, pela análise da composição de XOS ao longo do tempo de hidrólise, a maior produção de XOS aconteceu nas primeiras 3h. A partir deste ponto, houve intensa queda na quantidade de oligossacarídeos e produção de xilose, indicativo de atividade de  $\beta$ xilosidases.

[21] Por outro lado, a presente invenção realiza a etapa de hidrólise enzimática separadamente ao processo de produção da enzima e utilizando um substrato pré-tratado, o que resulta em uma hidrólise mais eficiente, com pouca ou nenhuma presença de contaminantes, sem pigmentos e com alta concentração de XOS. Não há necessidade de uma etapa de purificação, uma vez que o hidrolisado, por ser apenas concentrado por evaporação gera um produto com mais de 80% de pureza em oligossacarídeos prebióticos. Ainda, a etapa de pré-tratamento reduz a recalcitrância do material e aumenta

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a eficiência de hidrólise e facilita a etapa *downstream*. A maior concentração de XOS atingida por 255 mg/g de substrato. [22] O documento científico intitulado "OBTAINING XYLO-OLIGOSACCHARIDES FROM SUGARCANE STRAW USING RECOMBINANT ENDOXYLANASE" refere-se, particularmente, aos xilooligossacarídeos como uma das classes de prebióticos que pode ser obtida a partir da biomassa lignocelulósica, pela reação de hidrólise dos resíduos agroindustriais.

[23] No referido documento a xilana de cana é extraída com borohidreto de sódio e 24% KOH, ocorre a aplicação de endoxilase recombinante produzida por *Pichia pastoris* e a hidrólise é realizada a 1% de sólidos, 65°C. O uso de borohidreto de sódio eleva custos de produção e de tratamento dos resíduos. É um produto de uso controlado e tóxico e 24% KOH é uma concentração de álcali elevada que pode inviabilizar economicamente o processo. O documento possui as condições de hidrólise ainda não otimizadas e baixa variedade dos XOS produzidos.

[24] Em contrapartida, na presente invenção foi eliminado o uso de borohidreto de sódio, pois o material é pobre em lignina, e reduziu-se o tempo de reação e concentração de álcali para a extração da xilana. As condições de hidrólise foram otimizadas e o melhor resultado foi utilizando a concentração de substrato 2%. O material lignocelulósico é mais bem aproveitado, para produção de açúcar fermentescível, pectina e XOS.

[25] O documento científico intitulado "ESTUDO CINÉTICO ENZIMÁTICO DA HIDRÓLISE DE BAGAÇO DE LARANJA" refere-se a hidrólise enzimática (com enzima comercial xilanase da Novozymes) do bagaço de laranja para obtenção de

bioprodutos e destaca O resíduo do processamento de frutos cítricos possui alto valor agregado devido à sua composição. Esse subproduto é de grande interesse industrial apresentando grande potencial para ser utilizado na obtenção de uma gama produtos através da hidrólise enzimática.

[26] O foco do referido documento é a produção de açúcares fermentescíveis a partir da hidrólise total de resíduo cítrico, empregando concentrados enzimáticos com diversas atividades (arabinase, pectidase, xilanase, celulase e glucanase). Foram gerados 7,34 g/L de açúcares (concentração medida indireta de absorbância). A falta de pré-tratamento da biomassa afeta a eficiência da hidrólise devido à recalcitrância e presença de lignina, diminuindo a quantidade de material acessível às enzimas. Não há discriminação do tipo de açúcar fermentescível produzido (pentose ou hexose), o que impacta a potencial aplicação industrial. O produto obtido é de baixo valor agregado no mercado. Ainda, reitera-se que determinar a concentração de açúcar redutor por DNS pode gerar erros de superestimativa pois oligômeros (também presentes no hidrolisado, uma vez que a degradação não foi completa) apresentam maior intensidade de absorbância do que monômeros, causando uma medição acima da real.

[27] A presente proposta aproveita o resíduo cítrico para gerar dois produtos com alto valor agregado nas indústrias de alimentos: pectina e XOS, e como subproduto gera-se açúcar fermentescível naturalmente presente no material. As etapas de lavagem da casca, extração de pectina com ácido cítrico e extração de xilana com KOH permitem melhor aproveitamento das frações e aumentam a eficiência de

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hidrólise. Gerou-se 9 g/L XOS (quantificados por HPLC), além do aproveitamento da fração de pectina com alto teor de pureza e da produção de açúcar fermentescível durante a etapa de lavagem da casca de laranja (24g por 100g de casca).

[28] O documento científico intitulado "STRUCTURAL CONSIDERATIONS ON THE USE OF ENDO-XYLANASES FOR THE PRODUCTION OF PREBIOTIC XYLOOLIGOSACCHARIDES FROM BIOMASS" refere-se ao interesse em xilo-oligossacarídeos como prebióticos. Adicionalmente, é discutido que xilooligossacarídeos podem ser obtidos a partir de grandes frações de biomassa, incluindo subprodutos agrícolas. O documento conclui que endoxilanases são enzimas chave e de alto valor para a produção de xilo-oligossacarídeos a partir de xilana.

[29] O referido documento é um artigo de revisão que não apresenta resultados de experimentos próprios. O documento analisa a estrutura e a função de endoxilanases para a produção de XOS. Não há dados de aplicação, teor de pureza de xilana, concentração de sólidos, balanço de massa e carga enzimática, além de dados de concentração de XOS obtidos após a hidrólise dos resíduos agroindustriais mencionados. Não há análise das condições de hidrólise e extração de xilana.

[30] Por outro lado, a presente proposta otimizou o processo de extração de xilana e etapa de hidrólise enzimática especificamente para o aproveitamento da fração hemicelulósica de resíduo de casca de laranja, além da fração de pectina e açúcar fermentescível. Foram apresentados os devidos dados e balanços de massa para permitir o desenvolvimento de uma análise técnico-econômica do



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processo.

[31] O documento científico intitulado "SYNERGIC RECOMBINANT ENZYME ASSOCIATION TO OPTIMIZE XYLO-OLIGOSACCHARIDES PRODUCTION FROM AGRICULTURAL WASTE" refere-se a hidrólise enzimática da xilana da palha da cana-deaçúcar utilizando endoxilanase para produção de XOS com alta atividade prebiótica como uma possível forma sustentável de reduzir custos enzimáticos e de matéria-prima.

[32] O documento descreve uma análise de sinergia de duas enzimas, endoxilanase recombinante e arabinofuranosidase comercial, para otimizar a produção de XOS a partir de hemicelulose de palha de cana. A xilana é extraída com 24% KOH e borohidreto de sódio devido à elevada quantidade de lignina no material (20%).

[33] A presente invenção, por sua vez, descreve que apenas a enzima endoxilanase é necessária para a produção de XOS a partir de casca de laranja uma vez que a fração hemicelulósica deste resíduo não é constituída por arabinoxilana, ao contrário da palha de cana, e sim xilana.

[34] A extração de xilana foi otimizada para reduzir a quantidade de KOH utilizado, e não houve o uso de borohidreto de sódio, visto que o teor de lignina da biomassa é baixo (~1%), não havendo necessidade de potencializar a extração da lignina no processo. Além disso, há a valorização de duas frações do resíduo agroindustrial: pectina e XOS.

[35] O documento científico intitulado "ENDOXYLANASES AS TOOLS FOR PRODUCTION OF SUBSTITUTED XYLOOLIGOSACCHARIDES WITH PREBIOTIC PROPERTIES" refere-se às endoxilanases. É descrito que as enzimas são capazes de clivar a cadeia de xilana em locais de clivagem determinados

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pelo padrão de substituição e, assim, produzem diferentes padrões de produtos de oligossacarídeos. Ainda, o referido documento apresenta que a capacidade dos xilo-oligossacarídeos da dieta de funcionar como prebióticos em humanos é governada por seus padrões de substituição. As endoxilanases são, portanto, excelentes ferramentas para adaptar os oligossacarídeos prebióticos para estimular vários tipos de bactérias intestinais e causar fermentação em diferentes partes do trato gastrointestinal.

[36] O referido documento é um artigo de revisão de que não apresenta resultados de experimentos próprios. O documento analisa a estrutura e a função de endoxilanases para a produção de XOS. Não há dados de rendimento, teor de pureza de xilana, concentração de sólidos e carga enzimática, além de dados de concentração de XOS obtidos após a hidrólise de hemicelulose. A presente proposta, por outro lado, otimiza o processo de extração de xilana e etapa de hidrólise enzimática especificamente para o aproveitamento da fração hemicelulósica de resíduo de casca de laranja, além de também haver o aproveitamento da fração de pectina. Foram apresentados os devidos dados e balanços de massa para permitir o desenvolvimento de uma análise técnico-econômica do processo

[37] O documento científico intitulado "METHOD FOR SEPARATING LIGNIN AND PRODUCING XYLOOLIGOSACCHARIDE FROM LIGNOCELLULOSE ALKALINE OXIDATION PRETREATMENT LIQUID" refere-se um método que utiliza lignocelulose para obtenção de xilo-oligossacarídeos com hidrólise de xilanases.

[38] O referido documento solubiliza a fração hemicelulósica com peróxido de hidrogênio e conta com duas

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etapas de neutralização e hidrólise para produzir XOS. A concentração de XOS obtida é em torno de ~35%. Peróxido de hidrogênio pode não ser a melhor escolha de álcali dependendo da biomassa aproveitada. Neste caso, a xilana de casca de laranja é mais bem extraída com KOH ao invés de H<sub>2</sub>O<sub>2</sub>. São necessárias duas etapas de neutralização (acidificação) e hidrólise enzimática para a produção de XOS.

[39] Na presente invenção, é descrito que a biomassa utilizada é pobre em lignina, não havendo necessidade para sua remoção. Pode-se aproveitar a fração de pectina e xilana para gerar dois produtos de alto valor agregado. A eficiência de conversão de xilana em XOS foi maior que 70%. Além disso, há o potencial aproveitamento da fração de celulose remanescente da extração de xilana e dos açúcares fermentescíveis separados na etapa de lavagem das cascas de laranja.

[40] Os documentos científicos, em conjunto, podem revelar um processo para produção de xilo-oligossacarídeos a partir de resíduo de casca de laranja e que utiliza a enzima endoxilanase em reação de hidrólise enzimática, porém diferem da presente proposta em relação às frações aproveitadas e pré-tratamento. A presente invenção descreve um processo com condições viáveis para aplicação industrial, considerando o uso de reagentes (principalmente KOH, etanol e enzima). Assim, o presente processo caracterizou as frações de pectina extraída e resíduo sólido para alcançar a condição de melhor recuperação da xilana e aumentar o rendimento. Isso permitiu elevar a pureza da extração de pectina e a conservação da xilana presente no resíduo sólido. É o processo integrado da presente proposta que rende a elevada

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produção de XOS, a pureza de pectina, além dos açúcares fermentescíveis. O uso da enzima endoxilanase para a hidrólise enzimática foi deduzido após a caracterização do resíduo sólido da extração de pectina, que indicou a presença de aproximadamente 30% de hemicelulose, o dobro do teor inicialmente presente na biomassa.

[41] Alguns dos documentos embasam o uso de resíduos agroindustriais para obtenção de xilo-oligossacarídeos a

partir da hidrólise enzimática empregando  $\beta$ -1,4-endoxilanase para obter XOS, mas diferem da presente proposta pelo prétratamento utilizado para a biomassa. A casca de laranja apresenta baixo teor de lignina, não sendo necessária grande quantidade de álcali (KOH ou NaOH) e dispensa o uso de borohidreto de sódio para a quebra das ligações éster e remoção da lignina. O uso de KOH se mostrou a melhor opção para a recuperação da xilana de casca de laranja e foi possível reduzir a sua concentração para 2,5%, reduzindo também o volume de ácido para a neutralização (menor custos e descarte de substâncias químicas). O uso de HCl não é uma alternativa devido à grande quantidade de sal precipitado em conjunto com a xilana extraída, além de ser mais caro que o ácido acético. O sal gerado eleva os custos com as lavagens do material (desperdício de água e perda de sólidos) e pode resultar em redução da atividade enzimática na etapa de hidrólise.

[42] Além disso, o volume de etanol para a precipitação da xilana foi avaliado no presente processo para evitar a precipitação de outras frações de hemicelulose que reduziriam a pureza da xilana extraída, como arabinana e manana. Vale ressaltar que a hidrólise enzimática

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utilizando endoxilanase não é o único caminho para a produção de XOS. Existem outras metodologias como tratamentos hidrotérmicos e explosão a vapor para a produção de XOS, a depender da biomassa e da estratégia de trabalho. Por isso a caracterização da biomassa e de cada fração do processo é importante para a avaliação da viabilidade. A presente proposta aproveita todas as correntes do processo, desde a lavagem para a remoção de açúcares fermentescíveis, até a extração de pectina e xilana com alto grau de pureza e produção de XOS com baixo teor de xilose.

[43] Os documentos concluem que endoxilanasas são enzimas chave e de alto valor para a produção de xilo oligossacarídeos a partir de xilana, mas essa afirmação depende do tipo de hemicelulose trabalhada. Hemicelulose de grãos é constituída por arabinoxilana, por exemplo, e apenas a endoxilanase não garante bom rendimento em XOS. No caso da casca de laranja, a fração hemicelulósica é composta por xilana, assim a endoxilanase é suficiente para degradar o material em XOS. Mas esta conclusão advém da caracterização química do material e do ensaio de saturação e cinética enzimática para se determinar a menor concentração de enzima necessária para este processo em específico.

[44] Além disso, a produção de XOS a partir de casca de laranja só se mostrou viável após a eficiente extração da pectina, uma vez que a concentração de hemicelulose na casca de laranja in natura é baixa. A grande quantidade de pectina atrapalha a extração da xilana e deve ser previamente extraída com ácido cítrico para aumentar a disponibilidade de xilana. O eficiente pré-tratamento da xilana, com redução do uso de KOH e etanol, resulta em um hidrolisado mais limpo

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e rico em XOS, reduzindo custos na etapa *downstream* de purificação.

[45] Sendo assim, percebe-se que a produção de XOS a partir de casca de laranja já foi documentada no estado da técnica, contudo nenhum dos documentos é capaz de avaliar a extração dos açúcares fermentescíveis e a pectina para aumentar a disponibilidade da fração hemicelulósica e garantir melhor rendimento na hidrólise enzimática. A concentração de xilana no material in natura é baixa e seria inviável a produção de XOS a partir deste resíduo em escala industrial sem estas prévias etapas de remoção de açúcar e pectina. Devido a isso, a presente proposta alcançou mais de 9g/L de XOS após a hidrólise de xilana, valor significativamente maior que os demais documentos apontados. As otimizações das etapas prévias garantem um hidrolisado limpo e livre de compostos inibidores, contribuindo para a redução dos custos com as etapas de purificação dos produtos.

[46] O uso de endoxilanase para a produção de XOS já é de domínio do estado da técnica, mas os volumes e as misturas sinérgicas dependem do material utilizado. É necessária a caracterização da fração hemicelulósica e a avaliação das condições de precipitação para garantir um substrato enzimático com maior pureza e menos contaminantes. A presente proposta reduziu a concentração de KOH para a extração de xilana devido à baixa concentração de lignina no material e facilidade de solubilização de hemicelulose presente no resíduo sólido. O volume de etanol utilizado para precipitar a xilana também foi reduzido para elevar a pureza do material e garantir melhor eficiência de conversão. A presente proposta, além de apresentar alto rendimento em

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XOS, reduziu o consumo de KOH, etanol e enzima.

[47] Nenhum dos documentos apontados caracterizaram ou aproveitaram todas as frações que a casca de laranja apresenta para avaliar a viabilidade do processo. A presente proposta busca a redução do uso de reagentes e a otimização das etapas de extração e hidrólise para aproveitar a biomassa de casca de laranja como um todo.

[48] Apontadas as diferenças e a relevância da presente proposta em relação ao estado da técnica, percebe-se que a presente proposta é um processo otimizado para o aproveitamento integral do resíduo de casca de laranja, o qual pode se tornar poluição orgânica caso não seja devidamente processado. As etapas de processamento eficientemente geram três produtos principais: açúcar fermentescível e celulose, com potencial aplicação na indústria energética; pectina para aplicação em indústrias de alimentos; e XOS, prebiótico com aplicações tanto nas indústrias de alimentos quanto farmacêuticas.

#### **BREVE DESCRIÇÃO DA INVENÇÃO**

[49] A presente invenção refere-se a um processo integrado para produção de pectina e xilo-oligossacarídeos a partir de resíduo de casca de laranja. Os xilos oligossacarídeos produzidos se destacam pela sua capacidade prebiótica e antioxidante e, ainda, são passíveis de serem aplicados na indústria de alimentos e/ou farmacêutica.

[50] A presente invenção otimizou o método de extração com ácido cítrico já aplicado na indústria para a produção de pectina, adaptando-o para o resíduo de casca de laranja. Esta otimização também rende um resíduo sólido mais rico em xilana passível de ser utilizada para produção

enzimática de xilo-oligossacarídeos. Além disso, durante o processo de extração de pectina, também foram produzidas quantidades consideráveis de xilo-oligossacarídeos, que podem ser recuperados e purificados da fração líquida da extração de pectina. O resíduo sólido desta extração foi então aproveitado para a extração da xilana. As condições de extração alcalina para possibilitar uma xilana mais pura para a hidrólise enzimática também foram otimizadas, com significativa redução na concentração de KOH e etanol. A hidrólise enzimática da xilana foi otimizada para a produção de xilo-oligossacarídeos, avaliando a concentração de substrato e enzima utilizadas no processo.

[51] A presente invenção, além de produzir pectina com mais de 80% de pureza e 0,7 g/L de xilo-oligossacarídeos no processo de extração de pectina, também rendeu mais de 9g/L de xilo-oligossacarídeos após 48h de hidrólise de xilana a 50°C e 2% de sólidos, valor que representa mais de 70% de conversão da xilana em xilo-oligossacarídeos.

#### **BREVE DESCRIÇÃO DAS FIGURAS**

[52] Para obter uma total e completa visualização do objetivo desta invenção, são apresentadas as Figuras as quais se faz referências, conforme segue.

[53] A Figura 1 ilustra graficamente o rendimento de extração (barra cinza escura) e pureza da pectina extraída (barra cinza clara) utilizando as proporções 1:20, 1:40 e 1:50 m/v.

[54] A Figura 2 ilustra graficamente a superfície resposta de massa de pectina extraída (A) e recuperação da pectina presente originalmente na biomassa (B),  $R^2 > 90\%$ .

[55] A Figura 3 apresenta um fluxograma ilustrando o



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balanço de massa do processo de extração de pectina e produção de XOS a partir de resíduo de casca de laranja.

[56] A Figura 4 ilustra graficamente a superfície resposta das condições ótimas determinadas para o coquetel *Shearzyme*.

[57] A Figura 5 ilustra graficamente a curva de saturação enzimática para o coquetel *Shearzyme* diluído 1:50.

[58] A Figura 6 ilustra, através de um fluxograma, as etapas apresentadas pelo processo integrado para produção de pectina e xilo-oligossacarídeos.

#### **DESCRIÇÃO DETALHADA DA INVENÇÃO**

[59] A presente invenção refere-se a um processo integrado para produção de pectina e xilo-oligossacarídeos a partir de resíduo de casca de laranja. Os xilos oligossacarídeos produzidos se destacam pela sua capacidade prebiótica e antioxidante e, ainda, são passíveis de serem aplicados na indústria de alimentos e/ou farmacêutica.

[60] O processo integrado, compreende as seguintes etapas conforme apresentadas pelo fluxograma da Figura 6: I. Caracterização química;

II. Extração de pectina;

III. Extração de xilana;

IV. Hidrólise enzimática; e,

V. Quantificação de xilo-oligossacarídeos.

[61] Sendo assim, para demonstrar o potencial do referido processo, a presente invenção será mais detalhadamente descrita sob aspecto das etapas realizadas e os seus respectivos parâmetros.

#### **Caracterização química**

[62] Na primeira etapa O material foi lavado duas

vezes a 40°C durante 1h com agitação para remoção de açúcares livres remanescentes da polpa. Após as lavagens, o material foi seco em estufa a 50°C e triturado a partículas 60Mesh ou menores.

[63] A caracterização química analisou o teor de celulose, hemicelulose, lignina e cinzas: 300 mg foram hidrolisados com ácido sulfúrico 72 % (0,3mL) a 30 °C por 1 h, diluídas até 4% (p/v) adicionando 84mL de água destilada e autoclavada a 121 °C por 1 h, arrefecida e filtrada em cadinho de fundo poroso (4-5µm). A lignina insolúvel foi quantificada como quantidade de sólido insolúvel, obtido a partir da massa de cinzas insolúveis. A lignina solúvel foi medida em meio alcalino a 280nm. O teor de cinzas foi determinado pela queima de 1 g de amostra durante 6 h a 600 °C até peso constante.

[64] A caracterização de pectina seguiu o método colorimétrico utilizando o reagente m-hidroxidifenil e padrão de ácido galacturônico mono hidratado. 1 ml de amostra (ou 1 mg em 1ml de água) foi adicionado em béquer de 20ml contendo 5mL de solução 0.0125M tetraborato de sódio em ácido sulfúrico. A solução foi aquecida a 80°C por 6 minutos e esfriada em gelo até temperatura ambiente. 0.1mL de solução 0,15% m-hidroxidifenil em 0,5% NaOH foi adicionado, homogeneizado em vórtex. A leitura foi feita a 520nm, aguardando o maior valor de absorbância.

#### **Extração de pectina**

[65] Na segunda etapa do processo, 2g de casca lavada e triturada foram adicionados a 80ml de solução de ácido cítrico 2% (pH 2.2). Um delineamento central composto rotacional (DCCR) avaliou as condições de temperatura e tempo

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de extração, de 70 a 130°C e 1 a 70 minutos. Após extração, as amostras foram centrifugadas a 9000g e 4°C por 10 minutos e filtradas a vácuo. Ao filtrado, adicionou-se 80mL de etanol, e as amostras foram mantidas a 4°C por 12 horas para precipitação da pectina. Após este período, a pectina foi recuperada por centrifugação e seca em estufa. Os sobrenadantes das extrações foram reservados para análise da concentração de xilo-oligossacarídeos obtidos. O resíduo sólido da extração de pectina de cada ponto do DCCR foi caracterizado quanto ao teor de celulose e hemicelulose.

#### **Extração de xilana**

[66] Na terceira etapa do processo, o resíduo sólido da extração de pectina com maior teor de hemicelulose foi lavado com água destilada para remoção do excesso de ácido cítrico e 2g adicionados a solução de KOH com concentrações variando de 2,5% a 24%. As extrações foram avaliadas variando também a temperatura de 30°C a 120°C, e o tempo de 30 minutos a 12 horas. Após extração, o pH foi ajustado com ácido acético até atingir o ponto isoelétrico da xilana extraída (pH ~5,5). Etanol gelado foi adicionado na proporção de 10x o volume de ácido acético adicionado. As amostras foram mantidas a 4°C por 12 horas, centrifugadas, e os sólidos recuperados foram lavados com solução 50% etanol, centrifugados novamente e secos em estufa.

#### **Hidrólise enzimática**

[67] Na quarta etapa do processo, as condições ótimas de Endo- $\beta$ -1,4-xilanase comercial (Shearzyme, NS50030 Novozymes) foram previamente determinadas por DCCR, avaliando temperatura e pH. A curva de saturação enzimática foi realizada nas condições ótimas determinadas de 50°C e pH

5. A concentração de saturação da enzima foi utilizada para as hidrólises enzimáticas da xilana extraída, variando a concentração de sólidos de 1 a 5%. Após 48h, as amostras foram aquecidas a 98°C para desativação da enzima, centrifugadas para remoção dos sólidos não hidrolisados e reservadas para quantificação de xilo-oligossacarídeos. A Figura 4 apresenta as condições enzimáticas ótimas e a Figura 5 a curva de saturação enzimática previamente determinada para 50°C e pH 5.

[68] Desta forma, foram utilizados 1% (v/v) de enzima *Shearzyme* diluída 1:50 (concentração final de proteína 0,165mg/mL). Hidrólise conduzida a 50°C, pH 5, 48h.

#### **Quantificação de xilo-oligossacarídeos**

[69] A quinta etapa descreve que a quantificação dos sobrenadantes resultantes da hidrólise foi feita por cromatografia de troca iônica com detecção amperométrica pulsada (HPLC-PAD), utilizando o cromatógrafo Dionex® (Sunnyvale, CA, Estados Unidos) equipado com coluna Carbopac PA-1 (4 x 250 mm) e coluna de proteção Carbopac PA-1 (4x50 mm), bomba GP50, detector eletroquímico ED40 e detector de índice de refração gradiente A (NaOH 100mM) e B (NaOAc 300 mM, NaOH 100mM), software PEAKNET, Áreas de pico integradas serão ajustadas com padrões Megazyme® xilobiose (X2), xilotriose (X3), xilotetraose (X4), xilopentaose (X5), xilohexaose (X6).

[70] A seguir a presente invenção descreve os resultados obtidos

#### **Resultados obtidos pela presente invenção**

[71] Como resultados obtidos pelo processo empregado na presente invenção tem-se por etapa:

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**i.** Extração de pectina:

- 58,5% de resíduo sólido rico em xilana; e,
- 81,2% de pureza da pectina extraída (100°C 70min); e 0,7 g/L XOS.

**ii.** Extração de xilana:

- 69% de pureza (oligômeros de xilose) (2,5% KOH, 120°C, 30min, 1 volume etanol).

**iii.** Hidrólise enzimática da xilana extraída: - 9g/L XOS; (2% de sólidos, 48h, 50°C); e, - 6,7g/L Xilotetraose.

[72] Para demonstrar o seu potencial a presente invenção também será mais detalhadamente descrita sob aspecto dos exemplos concretizados. Cabe ressaltar que a descrição a seguir tem apenas a finalidade de elucidar o entendimento da invenção proposta e revelar, de forma mais detalhada, a concretização da invenção sem limitá-la ao mesmo. Dessa forma, variáveis similares ao exemplo também estão dentro do escopo da invenção.

**Exemplos de Concretização***Lavagem e caracterização química*

[73] O resíduo de casca de laranja oriundo de indústria de suco de laranja foi fornecido por Vita Citrus (Ribeirão Preto, SP). A primeira etapa deste processo consistiu na lavagem na casca de laranja oriunda de indústria de suco de laranja com água morna para remoção de açúcares da polpa. O material foi lavado duas vezes a 40°C durante 1h com agitação para remoção de açúcares livres remanescentes da polpa, utilizando uma concentração de sólidos de 50 g/L. Após as lavagens, o material foi seco em estufa a 50°C e triturado a partículas 60Mesh ou menores.

[74] A Tabela 1 apresenta a composição centesimal do resíduo de casca de laranja utilizado neste trabalho, assim como dos subsequentes resíduos e produtos de cada etapa.

**Tabela 1:** Composição centesimal de resíduo de casca de laranja e sólidos das extrações de pectina e xilana, valores com erro padrão de 5%

| %                                                                                         | Casca lavada | Pectina extraída* | Resíduo sólido | Xilana extraída | Resíduo xilana |
|-------------------------------------------------------------------------------------------|--------------|-------------------|----------------|-----------------|----------------|
| Pectina                                                                                   | 40,5         | 81,2              | 11,2           | 10,0            | 15,4           |
| Hemicelulose                                                                              | 14,8         | 4,9               | 30,1           | 69,0            | 8,0            |
| Celulose + amido                                                                          | 39,0         | 14,3              | 56,7           | 21,0            | 76,6           |
| Cinzas                                                                                    | 1,0          | -                 | -              | -               | -              |
| Lignina                                                                                   | 1,2          | -                 | -              | -               | -              |
| Proteína                                                                                  | 3,2          | -                 | -              | -               | -              |
| Óleos                                                                                     | 2,3          | -                 | -              | -               | -              |
| Total                                                                                     | 102,5        | 100,4             | 97,1           | 101,0           | 101,0          |
| * Resultados das condições otimizadas e adotadas para a produção de xilo-oligossacarídeos |              |                   |                |                 |                |

*Extração de pectina*

[75] O material lavado e seco foi utilizado para extração da pectina. Os pontos principais desta etapa são a pureza da pectina extraída e a integridade do resíduo sólido remanescente, utilizado na hidrólise enzimática para produção dos xilo-oligossacarídeos. Além disso, produtos de degradação (furfural, HMF e ácido fórmico), monossacarídeos e xilo-oligossacarídeos também foram quantificados e avaliados. A extração de pectina pode ser realizada com diversos ácidos como sulfúrico, clorídrico, nítrico e tartárico. No entanto, a extração de pectina com ácido cítrico melhora a economia do processo e apresenta baixo

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impacto ambiental. Para as extrações, empregou-se solução de ácido cítrico 2% (pH 2,2).

[76] Inicialmente foram avaliados os rendimentos e as purezas da pectina extraída utilizando diferentes proporções de sólido e líquido para a extração, 1:20, 1:40 e 1:50 m/v (Figura 1) a 100°C durante 30 minutos. Embora a proporção 1:50 tenha apresentado maior rendimento de extração devido à menor viscosidade da solução, e não tenha havido diferença estatisticamente significativa em relação à pureza do material extraído, utilizar proporções mais altas de solventes pode levar a custos de processamento mais altos e auto-hidrólise da pectina devido à diluição excessiva.

[77] Desta forma, empregou-se a proporção 1:40 m/v no delineamento central composto rotacional (DCCR) para otimização das condições de extração de pectina e recuperação do resíduo sólido: 2g de biomassa foram adicionados a 80 mL de solução de ácido cítrico 2% (pH 2,2). O DCCR avaliou as condições de temperatura e tempo de extração, de 70 a 130°C e 1 a 70 minutos. Após extração, as amostras foram centrifugadas a 9000g e 4°C por 10 minutos e filtradas a vácuo. Ao filtrado, adicionou-se 80mL de etanol, e as amostras foram mantidas a 4°C por 12 horas para precipitação da pectina. Após este período, a pectina foi recuperada por centrifugação e seca em estufa. Os sobrenadantes das extrações foram reservados para análise da concentração de xilooligossacarídeos obtidos. O resíduo sólido da extração de pectina de cada ponto do DCCR foi caracterizado quanto ao teor de celulose e hemicelulose.

**Tabela 2:** Resultados do DCCR de extração de pectina

|    | °C  | M i<br>n | pH final | Extrato (g) | Rend<br>imen to | %<br>Purez a<br>de<br>pecti na | Massa pec-<br>tin a<br>extraí da (g) | %<br>Recupe ração<br>de<br>pectin a | Sólid os<br>resid uais<br>(g) | %<br>Pect ina<br>no sólido<br>resi<br>dual | % Hemice<br>lulose no<br>sólido re-<br>sidu al |
|----|-----|----------|----------|-------------|-----------------|--------------------------------|--------------------------------------|-------------------------------------|-------------------------------|--------------------------------------------|------------------------------------------------|
| 1  | 80  | 1<br>0   | 2.42     | 0.12        | 6%              | 40%                            | 0.048                                | 4.2%                                | 1.880                         | 21%                                        | 10.1%                                          |
| 2  | 120 | 1<br>0   | 2.36     | 0.24        | 12%             | 58%                            | 0.139                                | 12.1%                               | 1.760                         | 23%                                        | 11.90%                                         |
| 3  | 80  | 6<br>0   | 2.42     | 0.26        | 13%             | 40%                            | 0.104                                | 9.0%                                | 1.740                         | 22%                                        | 10.7%                                          |
| 4  | 120 | 6<br>0   | 2.35     | 0.84        | 42%             | 58%                            | 0.487                                | 42.3%                               | 1.160                         | 9%                                         | 7.6%                                           |
| 5  | 70  | 3<br>5   | 2.4      | 0.14        | 7%              | 35%                            | 0.049                                | 4.2%                                | 1.860                         | 25%                                        | 8.6%                                           |
| 6  | 130 | 3<br>5   | 2.33     | 0.77        | 39%             | 57%                            | 0.439                                | 38.1%                               | 1.230                         | 11%                                        | 12.5%                                          |
| 7  | 100 | 1        | 2.4      | 0.23        | 12%             | 20%                            | 0.046                                | 4.0%                                | 1.770                         | 24%                                        | 16.7%                                          |
| 8  | 100 | 7<br>0   | 2.43     | 0.83        | 42%             | 81%                            | 0.678                                | 58.5%                               | 1.170                         | 13%                                        | 15.9%                                          |
| 9  | 100 | 3<br>5   | 2.4      | 0.64        | 32%             | 61%                            | 0.390                                | 33.9%                               | 1.360                         | 16%                                        | 13.7%                                          |
| 10 | 100 | 3<br>5   | 2.4      | 0.67        | 34%             | 60%                            | 0.402                                | 34.9%                               | 1.330                         | 16%                                        | 14.1%                                          |
| 11 | 100 | 3        | 2.4      | 0.66        | 33%             | 59%                            | 0.389                                | 33.8%                               | 1.340                         | 16%                                        | 14.0%                                          |



|  |  |   |  |  |  |  |  |  |  |  |
|--|--|---|--|--|--|--|--|--|--|--|
|  |  | 5 |  |  |  |  |  |  |  |  |
|--|--|---|--|--|--|--|--|--|--|--|

**Tabela 3:** Resultados do DCCR de extração de pectina: oligossacarídeos, monossacarídeos e produtos de degradação (continuação da Tabela 2).

|    | °C  | Min | XOS<br>g/L | Monossacarídeos<br>mg/L | HMF   | Furfural | Ácido<br>acético | Ácido<br>fórmico | Arabinose |
|----|-----|-----|------------|-------------------------|-------|----------|------------------|------------------|-----------|
| 1  | 80  | 10  | 0.660      | 0.348                   | 0     | 0        | 0.030            | 0.014            | 0         |
| 2  | 120 | 10  | 0.541      | 0.481                   | 0.007 | 0        | 0.034            | 0.021            | 0         |
| 3  | 80  | 60  | 0.683      | 0.351                   | 0     | 0        | 0.034            | 0.020            | 0.025     |
| 4  | 120 | 60  | 1.301      | 0.478                   | 0.010 | 0        | 0.030            | 0.029            | 0.342     |
| 5  | 70  | 35  | 0.845      | 0                       | 0     | 0        | 0.043            | 0.300            | 0         |
| 6  | 130 | 35  | 0.889      | 0.402                   | 0.009 | 0        | 0.030            | 0.022            | 0.107     |
| 7  | 100 | 1   | 0          | 0                       | 0     | 0        | 0                | 0                | 0         |
| 8  | 100 | 70  | 0.695      | 0.400                   | 0.007 | 0        | 0.033            | 0.016            | 0.122     |
| 9  | 100 | 35  | 0.681      | 0.376                   | 0     | 0        | 0                | 0                | 0.015     |
| 10 | 100 | 35  | 0.679      | 0.365                   | 0     | 0        | 0                | 0                | 0.015     |
| 11 | 100 | 35  | 0.680      | 372.0                   | 0     | 0        | 0                | 0                | 0.015     |

[78] A condição para extração de pectina que proveu melhor grau de pureza e menor degradação do material foi obtida a 100°C e 70 minutos (ensaio 8). A pureza da pectina nesta condição foi de 81%. A caracterização detalhada desta pectina está apresentada na Tabela 1. Também foram produzidos ~0,7 mg/mL de xilo-oligosacarídeos (xilobiose e xilotriose). A segunda melhor condição de extração de pectina foi obtida a 120°C durante 10 e 60 minutos (ensaios 2 e 4). O material extraído apresentou 58% de pectina e foram produzidos 0,54 mg/mL e 1300 mg/mL de xilo-oligossacarídeos, respectivamente. O alto rendimento em xilo-oligossacarídeos do ensaio 4 se deve à auto hidrólise ácida da hemicelulose do material, uma conversão de 70,35% da xilana presente na biomassa em XOS. Entretanto, esta produção acompanha inibidores, além de um menor rendimento de extração e pureza de pectina. A Figura 2 apresenta a superfície resposta do DCCR para extração de pectina ( $p < 0.10$ ). Não foi possível obter uma superfície resposta com adequado valor de  $R^2$  para a pureza da pectina.

**Tabela 4:** Composição dos xilo-oligossacarídeos quantificados nos sobrenadantes da extração de pectina em cada ponto do DCCR.

|                 | 1     | 2     | 3     | 4     | 5     | 6     | 7 | 8     | 9     | 10    | 11    |
|-----------------|-------|-------|-------|-------|-------|-------|---|-------|-------|-------|-------|
| Xilobiose (x2)  | 0,327 | 0,275 | 0,339 | 0,446 | 0,431 | 0,453 | - | 0,347 | 0,341 | 0,341 | 0,344 |
| Xilotriose (x3) | 0,334 | 0,266 | 0,343 | 0,398 | 0,414 | 0,436 | - | 0,349 | 0,339 | 0,340 | 0,339 |
| Xilotetraose    | -     | -     | -     | 0,200 | -     | -     | - | -     | -     | -     | -     |

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|                      |       |       |       |       |       |       |            |       |       |       |       |
|----------------------|-------|-------|-------|-------|-------|-------|------------|-------|-------|-------|-------|
| (x4)                 |       |       |       |       |       |       |            |       |       |       |       |
| Xilopentaose<br>(x5) | -     | -     | -     | 0,133 | -     | -     | -          | -     | -     | -     | -     |
| Xilohexaose<br>(x6)  | -     | -     | -     | 0,125 | -     | -     | -          | -     | -     | -     | -     |
| XOS Total<br>(g/L)   | 0,660 | 0,541 | 0,683 | 1,301 | 0,845 | 0,889 | 0,00<br>00 | 0,695 | 0,680 | 0,681 | 0,681 |

### *Extração de xilana*

[79] A xilana foi extraída do resíduo sólido remanescente da extração de pectina (ensaio 8). As porcentagens de hemicelulose presente nos resíduos sólidos da extração de pectina estão apresentadas na Tabela 2. O ensaio 8 rendeu a maior pureza de pectina e o resíduo sólido com maior concentração de hemicelulose. A xilana foi extraída deste material com 2,5%, 5%, 15% e 24% de KOH, a 120°C por 30 min e proporção de sólidos 1:10. Foram analisados diferentes volumes de etanol adicionado para precipitação da xilana extraída.

[80] Seguindo a metodologia, que recomenda a adição de 10 volumes de etanol para cada volume de ácido acético adicionado para neutralizar o KOH, quanto maior a concentração de KOH, maior a quantidade de etanol adicionada, o que aumenta custos de reagentes. Por exemplo, 10mL 2,5% KOH necessita 1mL de ácido acético, já 10mL 24% KOH necessita 5mL de ácido para neutralizar o pH, o que aumenta o volume de etanol de 10mL para 50mL. Percebeu-se que maiores volumes de etanol reduziram a pureza da xilana extraída de 69% (com 2.5% KOH) para 15% (com 24% KOH). Desta forma fixou-se o

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volume de etanol para 1 volume reacional. Ou seja, para cada 10mL de solução de KOH e biomassa, seriam adicionados ácido acético em quantidade suficiente para neutralizar a solução e 10mL (mais o volume de ácido) de etanol gelado. Desta forma não foram apresentadas diferenças nas massas extraídas com diferentes concentrações de KOH.

[81] Constatou-se que 2,5% de KOH e 1 volume de etanol rendeu a xilana mais pura, de 69%, levando-se em conta apenas a composição de oligômeros de xilose (os demais 30% são referentes a outros tipos de hemicelulose também extraídas no processo, principalmente arabinanas, além de pequena quantidade de celulose). Menor concentração de KOH e menor quantidade de etanol para precipitação da xilana resultaram em maior pureza e rendimento de hidrólise. A eficiência de extração deste processo também foi alta, uma vez que o resíduo da extração de xilana apresentou menos de 8% de hemicelulose e 76% de celulose.

#### *Hidrólise enzimática da Xilana extraída*

[82] A hidrólise enzimática para produção de XOS empregou a xilana extraída da etapa 3. O resíduo sólido da extração de pectina da etapa 2 também foi utilizado para comparação dos rendimentos.

[83] O resíduo sólido, rico em hemicelulose (Tabela 2, ensaio 8), rendeu 0.46mg de XOS por mg de xilana (pura) presente no material, utilizando uma concentração de sólidos de 3%. Maiores concentrações de sólido resultaram em inibição enzimática e menos de 0.2mg de XOS por mg de xilana (Tabela 5).

**Tabela 5:** Rendimentos da hidrólise de resíduo sólido de pectina (ensaio 8)

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| % Resíduo sólido | XOS (g/L) | XOS (mg/g xilana) |
|------------------|-----------|-------------------|
| 3                | 2,226     | 0,463             |
| 4                | 1,665     | 0,231             |
| 5                | 1,618     | 0,202             |
| 9                | 1,824     | 0,127             |
| 12               | 1,772     | 0,092             |

[84] Já a hidrólise de xilana extraída na melhor condição apresentada na etapa 3 (69% de pureza) rendeu 9,16 g/L de XOS, o que representa uma conversão de 72,68% de xilana em XOS, utilizando concentração de sólidos de 2% (Tabela 6), comprovando a necessidade da extração de xilana para aumentar a eficiência de conversão e produção de XOS. Maiores concentrações de sólido inibiram a enzima. O produto de hidrólise apresentou 0,32g/L de monossacarídeos. O principal oligossacarídeo obtido foi xilote-traose (x4), o que corresponde a aproximadamente 74% dos XOS produzidos (Tabela 7).

[85] Considerável fração de compostos fenólicos é extraída com a fração de xilana (Tabela 2). Pode-se afirmar que o hidrolisado é rico em XOS e compostos fenólicos antioxidantes, e que boa parte da atividade antioxidante advém dos xilo-oligossacarídeos, uma vez que o produto de hidrólise que apresentou maior concentração de XOS também foi o que apresentou maior atividade antioxidante, não proporcional à quantidade de fenóis totais.

[86] A Figura 3 apresenta o balanço de massa dos processos de extração e produção de XOS. A composição química de cada fração está apresentada em suma na Tabela 1.

**Tabela 6:** Hidrólise enzimática de xilana extraída de resíduo sólido de pectina, quantificação de fenóis totais e atividade

antioxidante total (CAT).

| Substrato de hidrólise | XOS (g/L) | % conversão | µg eq ác. gálico | µM eq Trolox |
|------------------------|-----------|-------------|------------------|--------------|
| 1% xilana              | 5,802     | 84,10%      | 52,33            | 3803         |
| 2% xilana              | 9,157     | 72,68%      | 104,62           | 20279        |
| 3% xilana              | 4,391     | 21,23%      | 154,32           | 11587        |
| 4% xilana              | 2,955     | 10,72%      | 193,80           | 15279        |

**Tabela 7:** Composição dos XOS produzidos

|                   | 1%    | 2%    | 3%    | 4%    |
|-------------------|-------|-------|-------|-------|
| Xilose            | 0,343 | 0,349 | 0,348 | 0,352 |
| Xilobiose (x2)    | 0,319 | 0,334 | 0,329 | 0,339 |
| Xilotriose (x3)   | 0,320 | 0,326 | 0,309 | 0,341 |
| Xilotetraose (x4) | 3,522 | 6,690 | 1,820 | 1,976 |
| Xilopentaose (x5) | 0,117 | -     | -     | -     |
| Xilohexaose (x6)  | 1,524 | 1,807 | 0,497 | 1,736 |
| XOS Total (g/L)   | 5,802 | 9,157 | 2,955 | 4,391 |
| XOS (mg/g xilana) | 0,840 | 0,663 | 0,107 | 0,212 |

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<https://doi.org/10.1016/j.renene.2020.05.108>.

### REIVINDICAÇÕES

1. Processo de produção de pectina e xilo-oligossacarídeos a partir de resíduo industrial de suco de laranja, caracterizado pelo fato de que compreende as etapas de:

- I. Caracterização química;
- II. Extração de pectina;
- III. Extração de xilana;
- IV. Hidrólise enzimática; e,
- V. Quantificação de xilo-oligossacarídeos.

2. Processo, de acordo com a reivindicação 1 caracterizado pelo fato de que na etapa (I) de caracterização química o material é lavado duas vezes a 40°C durante 1h com agitação para remoção de açúcares livres remanescentes da polpa, após as lavagens, o material é seco em estufa a 50°C e triturado a partículas 60Mesh ou menores.

3. Processo, de acordo com a reivindicação 2 caracterizado pelo fato de que na caracterização química são analisados os teores selecionados dentre: celulose, hemicelulose, lignina e cinzas, em que 300 mg foram hidrolisados com 0,3mL ácido sulfúrico 72% a 30°C por 1h, diluídas até 4% (p/v) adicionando 84mL de água destilada e autoclavada a 121°C por 1h, arrefecida e filtrada em cadinho de fundo poroso 4-5µm.

4. Processo, de acordo com a reivindicação 2 ou 3 caracterizado pelo fato de que a lignina insolúvel é quantificada como quantidade de sólido insolúvel, obtido a partir da massa de cinzas insolúveis e a lignina solúvel é medida em meio alcalino a 280nm; em que o teor de cinzas é determinado pela queima de 1g



de amostra durante 6h a 600°C até peso constante.

5. Processo, de acordo com a reivindicação 2 caracterizado pelo fato de que a caracterização da pectina ocorre pelo método colorimétrico utilizando o reagente mhidroxidifenil e padrão de ácido galacturônico mono hidratado, em que 1ml de amostra, ou 1mg em 1ml de água, é adicionado em béquer de 20ml contendo 5mL de solução 0.0125M tetraborato de sódio em ácido sulfúrico, a solução posteriormente é aquecida a 80°C por 6 minutos e esfriada em gelo até temperatura ambiente, 0,1mL de solução 0,15% mhidroxidifenil em 0,5% NaOH é adicionado e homogeneizado em vórtex; em seguida é feita leitura a 520nm.

6. Processo, de acordo com a reivindicação 1 caracterizado pelo fato de que na etapa (II) de extração de pectina 2g de casca lavada e triturada são adicionados a 80ml de solução de ácido cítrico 2% com pH 2.2, o delineamento central composto rotacional (DCCR) avaliou as condições de temperatura e tempo de extração na faixa de 70 a 130°C e 1 a 70 minutos, em que após a extração as amostras são centrifugadas a 9000g e 4°C por 10 minutos e filtradas a vácuo, ao filtrado, adiciona-se 80mL de etanol, e mantem-se as amostras a 4°C por 12 horas para precipitação da pectina, em seguida a pectina é recuperada por centrifugação e seca em estufa.

7. Processo, de acordo com a reivindicação 6 caracterizado pelo fato de que é utilizada a proporção 1:40 m/v no DCCR para otimização das condições de extração de pectina e recuperação do resíduo sólido.

8. Processo, de acordo com a reivindicação 6 caracterizado pelo fato de que a etapa (II) de extração da

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pectina obtém à 100°C durante 70min:

- 58,5% de resíduo sólido rico em xilana;
- 81,2% de pureza da pectina extraída; e -

0,7g/L XOS.

9. Processo, de acordo com a reivindicação 1 caracterizado pelo fato de que na etapa (III) de extração de xilana o resíduo sólido da extração de pectina com maior teor de hemicelulose é lavado com água destilada para remoção do excesso de ácido cítrico e 2g adicionados a solução de KOH com concentrações variando de 2,5% a 24%, em que as extrações são avaliadas variando a temperatura na faixa de 30°C a 120°C e o tempo de 30 minutos a 12 horas; após a extração, o pH é ajustado com ácido acético até atingir o ponto isoelétrico da xilana extraída pH 5,5, etanol gelado é adicionado na proporção de 10 vezes o volume de ácido acético adicionado, as amostras são mantidas a 4°C por 12 horas, centrifugadas, e os sólidos recuperados são lavados com solução 50% etanol, centrifugados novamente e secos em estufa.

10. Processo, de acordo com a reivindicação 9 caracterizado pelo fato de que a etapa (III) de extração da xilana obtém em 2,5% de KOH, 120°C, 30min e 1 volume etanol: - 69% de pureza de oligômeros de xilose.

11. Processo, de acordo com a reivindicação 1 caracterizado pelo fato de que na etapa (IV) de hidrólise enzimática as condições ótimas de Endo- $\beta$ -1,4-xilanase são determinadas por DCCR e a curva de saturação enzimática é realizada nas condições ótimas de 50°C e pH 5, em que a concentração de saturação da Endo- $\beta$ -1,4-xilanase é utilizada para as hidrólises enzimáticas da xilana extraída, variando

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a concentração de sólidos de 1 a 5%.

12. Processo, de acordo com a reivindicação 11 caracterizado pelo fato de que após 48h, as amostras são aquecidas a 98°C para desativação da enzima, centrifugadas para remoção dos sólidos não hidrolisados e reservadas para quantificação de xiloligossacarídeos.

13. Processo, de acordo com a reivindicação 11 ou 12 caracterizado pelo fato de que são utilizados 1% (v/v) de enzima diluída 1:50, com concentração final de proteína 0,165mg/mL e a hidrólise é conduzida a 50°C, pH 5, 48h.

14. Processo, de acordo com qualquer uma das reivindicações 11 a 13 caracterizado pelo fato de que a enzima Endo-β-1,4-xilanase comercial preferencial é a *Shearzyme* NS50030.

15. Processo, de acordo com qualquer uma das reivindicações 11 a 14 caracterizado pelo fato de que a etapa (IV) de hidrólise enzimática da xilana extraída obtém: - 9,16g/L XOS; (2% de sólidos, 48h, 50°C); e, - 6,7g/L Xilotetraose.

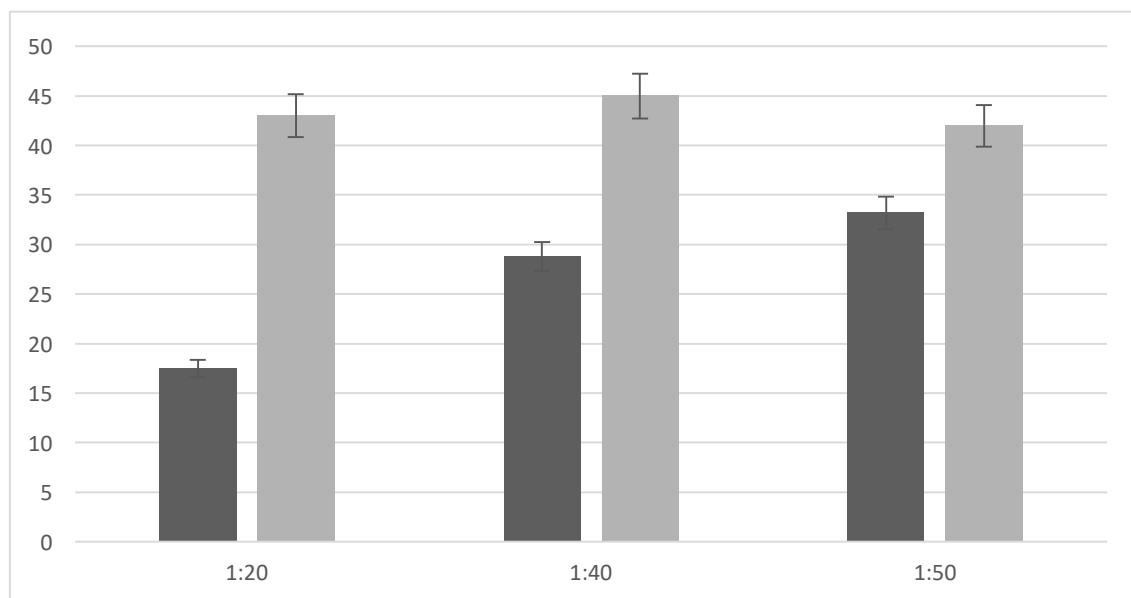
16. Processo, de acordo com qualquer uma das reivindicações 11 a 15 caracterizado pelo fato de que na etapa (IV) de hidrólise enzimática 72,68% de xilana são convertidos em XOS utilizando concentração de sólidos de 2%.

17. Processo, de acordo com qualquer uma das reivindicações 11 a 16 caracterizado pelo fato de que O produto de hidrólise apresentou 0,32g/L de monossacarídeos.

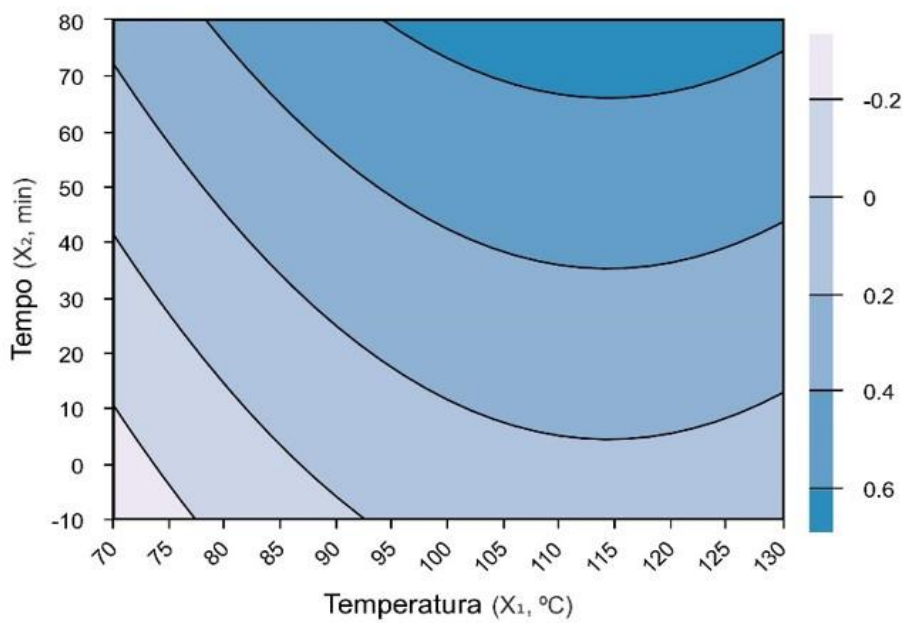
18. Processo, de acordo com a reivindicação 17 caracterizado pelo fato de que o principal oligossacarídeo obtido é xilotetraose, correspondendo a 74% dos XOS produzidos.

19. Xilo-oligossacarídeos, produzido de acordo com a reivindicação 1, caracterizado por ser pré-biótico e antioxidante.

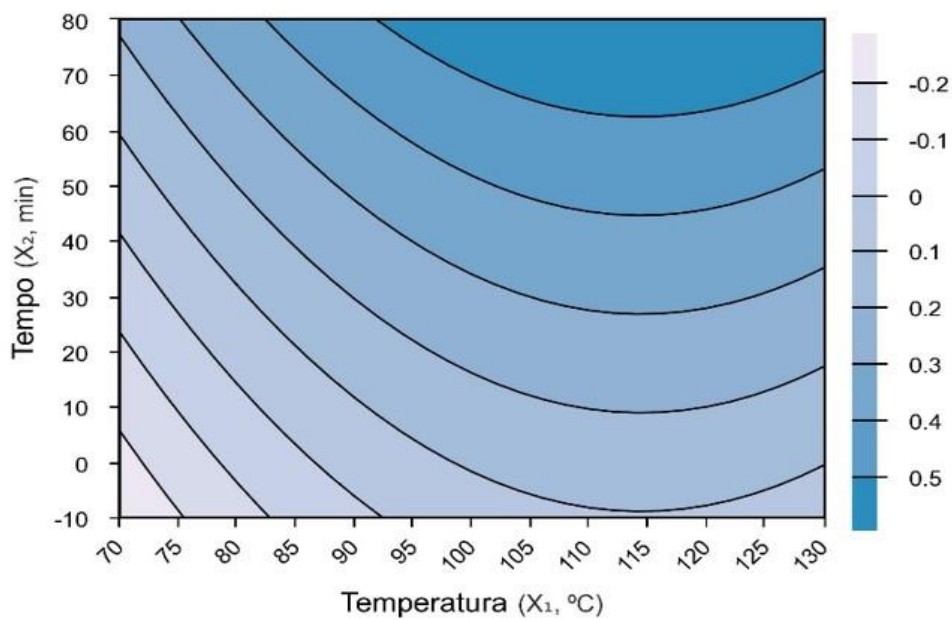
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**FIGURA 1**

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A



B

FIGURA 2

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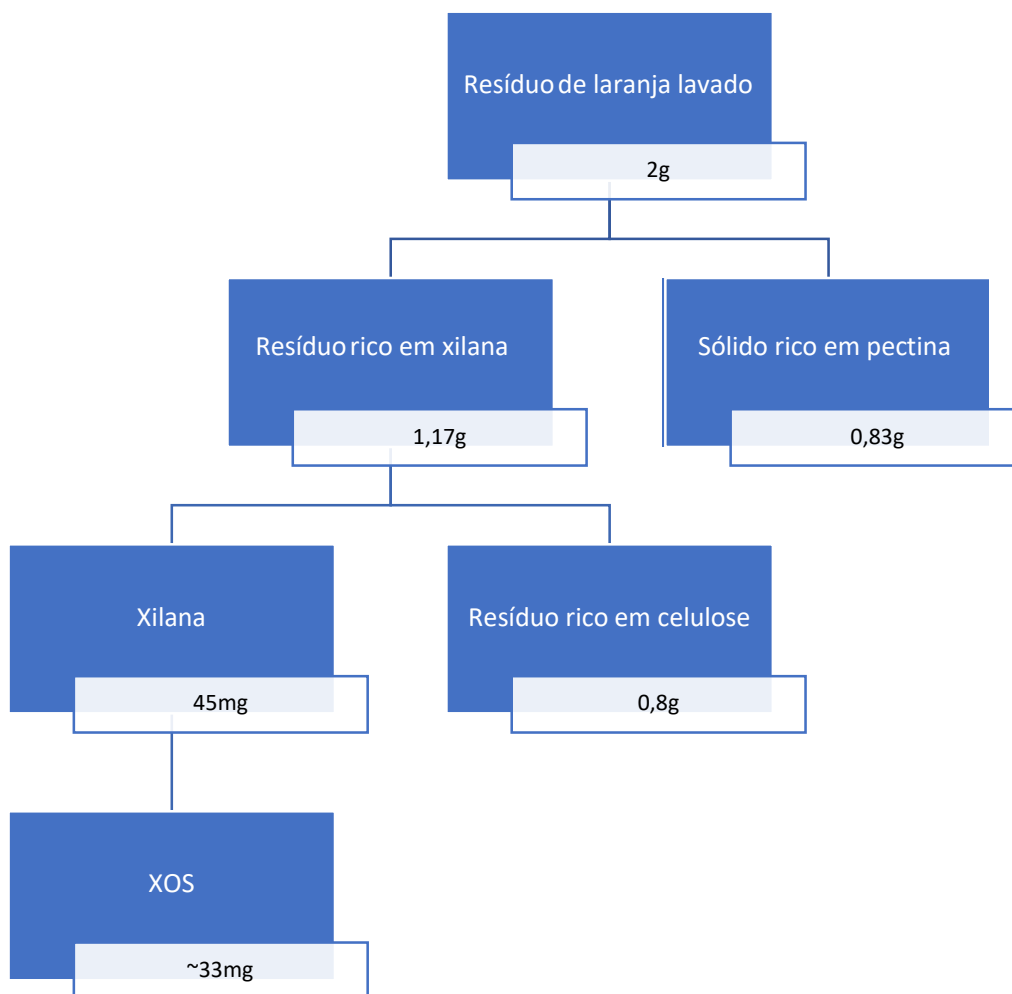


FIGURA 3

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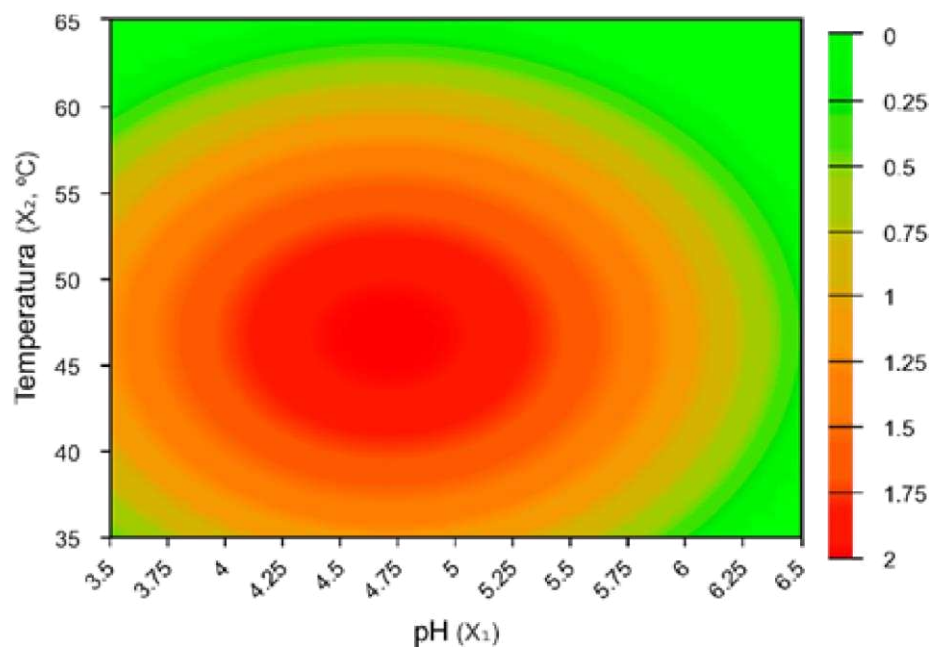


FIGURA 4

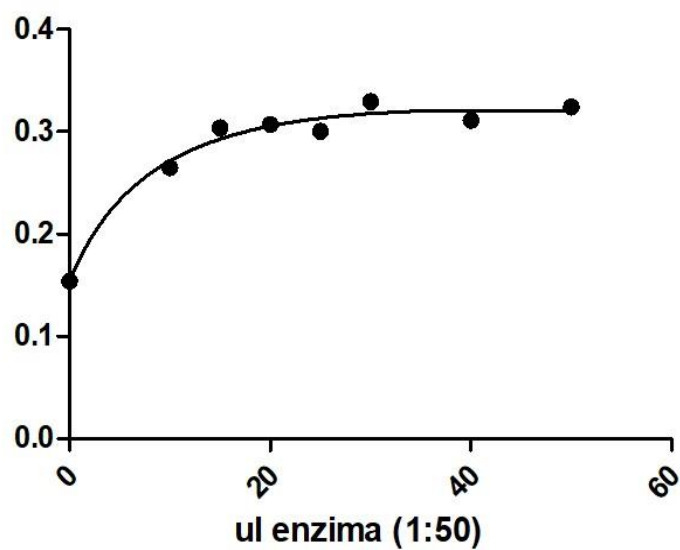


FIGURA 5



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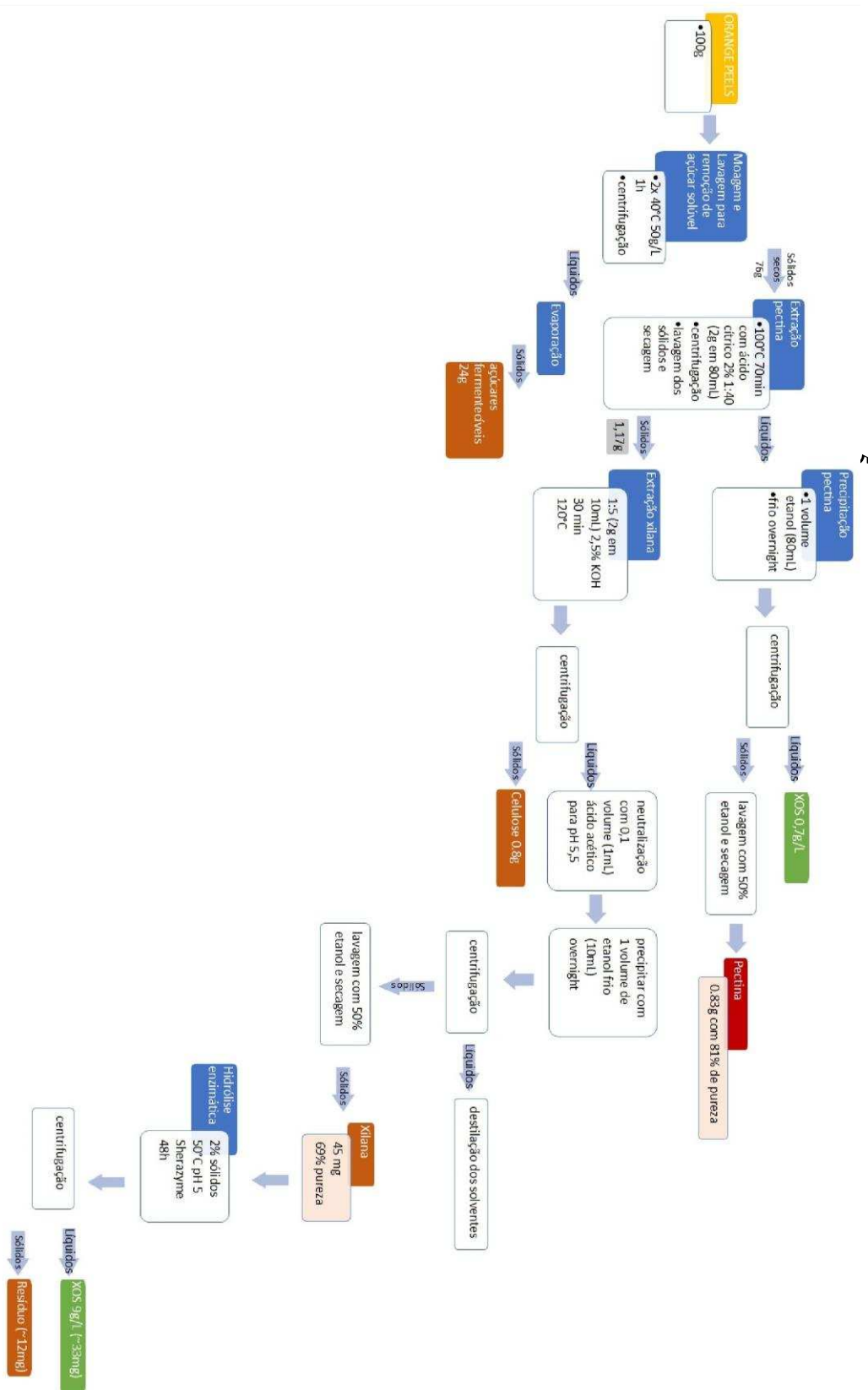


FIGURA 6

RESUMO**PROCESSO DE PRODUÇÃO DE PECTINA E XILO-OLIGOSSACARÍDEOS A PARTIR DE RESÍDUO INDUSTRIAL DE SUCO DE LARANJA E XILO-OLIGOSSACARÍDEOS**

A presente invenção refere-se a um processo integrado para produção de pectina e xilo-oligossacarídeos a partir de resíduo de casca de laranja. O processo otimizado de extração com ácido cítrico para a produção de pectina foi adaptado para o resíduo de casca de laranja. Esta otimização rendeu um resíduo sólido mais rico em xilana e passível de ser utilizado para produção enzimática de xilo-oligossacarídeos. Além disso, durante o processo de extração de pectina, também foram produzidas quantidades consideráveis de xilooligossacarídeos, que podem ser recuperados e purificados da fração líquida da extração de pectina. A hidrólise enzimática da xilana foi otimizada para a produção de xilooligossacarídeos, avaliando a concentração de substrato e enzima utilizadas no processo. A presente invenção, além de produzir pectina com mais de 80% de pureza e 0,7 g/L de xilooligossacarídeos no processo de extração de pectina, também rendeu mais de 9g/L de xilo-oligossacarídeos após 48h de hidrólise de xilana a 50°C e 2% de sólidos, valor que representa mais de 70% de conversão da xilana em xilooligossacarídeos. Adicionalmente, os xilos oligossacarídeos produzidos se destacam pela sua capacidade pré-biótica e antioxidante, passíveis de serem aplicados na indústria de alimentos e/ou farmacêutica.

## Appendix V - Publishers' authorization

### *Lignocellulose Bioconversion through Chemical Methods, Platform Chemicals, and New Chemicals*



**Book:** Lignocellulose Bioconversion Through White Biotechnology  
**Chapter:** Lignocellulose Bioconversion through Chemical Methods, Platform Chemicals, and New Chemicals  
**Author:** Manoela Martins, Patrícia F. Ávila, Marcos Felipe Silva, et al  
**Publisher:** John Wiley and Sons  
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**Enzymatic Bioconversion Trends for Lignocellulosic Biorefineries**  
**Author:** Manoela Martins, Marcos Felipe da Silva, Allan Henrique Félix de Mélo et al  
**Publication:** Springer eBook  
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***Polysaccharide degradation for oligosaccharide production with nutraceutical potential for the food industry***



Chapter: Chapter 13 Polysaccharide degradation for oligosaccharide production with nutraceutical potential for the food industry  
 Book: Polysaccharide-Degrading Biocatalysts  
 Author: Manoela Martins, Patrícia F. Ávila, Patrícia Poletto, Rosana Goldbeck  
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***Recent advances in xylo-oligosaccharides production and applications: A comprehensive review and bibliometric analysis***



**Recent advances in xylo-oligosaccharides production and applications: A comprehensive review and bibliometric analysis**

Author: Manoela Martins, William Gustavo Sganzerla, Tânia Forster-Carneiro, Rosana Goldbeck  
 Publication: Biocatalysis and Agricultural Biotechnology  
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***Recombinant chimeric enzymes for lignocellulosic biomass hydrolysis***



**Recombinant chimeric enzymes for lignocellulosic biomass hydrolysis**

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***Synergism for xylooligosaccharides, p-coumaric acid, ferulic acid, and thermostability modulation of GH 62  $\alpha$ -L-arabinofuranosidase***



**Synergism for xylo-oligosaccharides, p-coumaric and ferulic acid production, and thermostability modulation of GH 62  $\alpha$ -L-arabinofuranosidase**

**Author:** Manoela Martins, Robson Tramontina, Fábio Márcio Squina, Taísa Magnani Dinamarco, Rosana Goldbeck

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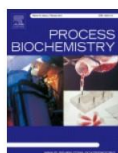
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***Novel bi-functional thermostable chimeric enzyme for feasible xylo-oligosaccharides production from agro-industrial wastes***



**Novel bi-functional thermostable chimeric enzyme for feasible xylo-oligosaccharides production from agro-industrial wastes**

**Author:** Manoela Martins, Marcos Fellipe Silva, Taísa Magnani Dinamarco, Rosana Goldbeck

**Publication:** Process Biochemistry

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Thermostability Enhancement of GH 62  $\alpha$ -L-Arabinofuranosidase by Directed Evolution and Rational Design

Author: Manoela Martins, Alberto M. dos Santos, Clauber H. S. da Costa, et al

Publication: Journal of Agricultural and Food Chemistry

Publisher: American Chemical Society

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***Integrated biorefinery for xylooligosaccharides, pectin, and bioenergy production from orange wastes***



Integrated biorefinery for xylooligosaccharides, pectin, and bioenergy production from orange waste

Author: Rosana Goldbeck, Manoela Martins

Publication: Biofuels, Bioproducts and Biorefining

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## Recent advances in xylo-oligosaccharides production and applications: A comprehensive review and bibliometric analysis

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### ARTICLE INFO

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Lignocellulosic biomass  
Prebiotic  
Xylan

### ABSTRACT

This comprehensive review elucidates the state of the art of xylo-oligosaccharides (XOS) production and applications. Emerging trends, gaps, and opportunities for future research were evaluated by bibliometric analysis, covering XOS production by chemical and enzymatic methods, purification, health benefits, and new products development. In total, 1256 publications (1217 articles and 39 reviews) were recorded from 2000 to 2021, the selected timespan for the bibliometric analysis. The network analysis of keywords revealed that XOS research had been divided into five main knowledge areas: prebiotics, autohydrolysis, enzymatic hydrolysis, xylan pretreatment, and xylanase. The prebiotic activity of XOS is a deeply investigated field, and this hot topic still demands future research. Autohydrolysis was shown to be a base niche, while enzymatic hydrolysis is gaining more interest. Most recent research is focused on the development of new technologies for XOS production toward a biorefinery concept, especially with the valorization of agro-industrial wastes in a circular bioeconomy. In conclusion, XOS is a value-added biological macromolecule with scientific and industrial interest that can be obtained from lignocellulose biorefineries and applied to develop functional foods.

### 1. Introduction

The high occurrence of public health problems, especially obesity, hypertension, and diabetes, raises people's awareness and increases the demand for natural foods with reduced fat, salt, and sugar, as well as functional foods containing prebiotics and probiotics, as they are considered potential therapeutics with health benefits (Ozen et al., 2012). Non-digestible oligosaccharides (NDOs) are prebiotic carbohydrates made up of low molecular weight compounds that stimulate the growth of beneficial intestinal microbiota. Among the different classes of NDOs, xylo-oligosaccharides (XOS) are an emergent food ingredient produced from inexpensive and renewable sources such as agro-industrial by-products. The multidimensional influence of XOS on human health makes it a potential agent against several gastrointestinal disorders, insulin resistance, and even cancer (Vázquez et al., 2000). There is a growing awareness of the role of human gut microflora in maintaining host health. Several studies have revealed the beneficial effects of XOS for its bifidogenic activity, reduction in blood cholesterol and glucose levels, and increase in mineral absorption, immunomodulation, antioxidant, and anti-inflammatory activities (Ávila et al., 2020; Li et al., 2022; Valls et al., 2018; Yu et al., 2015).

At present, more investigation is necessary to fully understand the molecular mechanisms underlying the beneficial effects of XOS on health. However, the scientific community and industries have made efforts to prove that it is possible to produce XOS from several types of agro-industrial wastes composed of cellulose, hemicellulose, and lignin (Ávila et al., 2020; Gullón et al., 2011), including

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forest and pulping waste from hardwood and softwood. Xylan, the hemicellulosic main chain, is highly important for XOS production via autohydrolysis or enzymatic hydrolysis. Many treatments have been investigated to optimize the recovery of xylan from these wastes and its conversion to short-chain xylose oligomers (i.e., XOS). Additionally, from the lignocellulosic material, several bioproducts can be produced and applied in the food, energy, and chemical industries in a biorefinery concept advocating circular bioeconomy (Ferreira et al., 2021; Sganzerla et al., 2021). The production of biocompounds and biofuels from lignocellulosic biomass is increasing due to the new policies of replacement of fossil-based carbon, sustainability, waste management, and land competition for food production (Álvarez et al., 2017; Gullón et al., 2011; Pereira et al., 2021; Pinales-Márquez et al., 2021; Praveen et al., 2017).

Recent reviews presented the production of XOS from lignocellulosic biomass, purification technologies, applications, and health benefits (Anarado et al., 2022; Pinales-Márquez et al., 2021; Santibáñez et al., 2021). However, a bibliometric analysis of XOS was not reported in the literature. The use of bibliometric tools can be a promising approach to perform a complete analysis of scientific publications related to all research areas, addressing gaps, identifying trends, and supporting the establishment of future research and perspectives (Aria and Cuccurullo, 2017; van Eck and Waltman, 2010). Due to the increasing interest in XOS production from sustainable sources and their application in functional food, this study aimed to address the status of XOS research regarding the technological routes of production, the health benefits investigation, and advances toward the development of an integrated biorefinery for the valorization of agro-industrial wastes as well as the application as a functional food ingredient.

## 2. Methodology for literature search and bibliometric analysis

The bibliometric study used scientific output data from the core collection of Science Citation Index Expanded (SCI-E) Clarivate Analytics' ISI Web of Science®. The type of search applied was advanced search applying the following logic operation: TS = ( xylo-oligosaccharides OR xylooligosaccharides OR xyloligosaccharides OR xylooligosaccharide OR xylooligosaccharide OR xylooligosaccharide ) NOT KP = ( xylo-oligosaccharides OR xylooligosaccharides OR xyloligosaccharides OR xylooligosaccharide OR xylooligosaccharide OR xyloligosaccharide ).

This search query was used to obtain reliable and accurate details based on selected words included in the title, abstract, author's keywords and not in the keywords-plus of each document on the topic. The types of documents were filtered to article and review. The systematic search was conducted from 2000 to 2021, aiming to observe the publication's evolution over the years and the most frequent research areas. This timespan was selected based on guidelines established in previous studies (Gabriel da Rosa et al., 2022; Sganzerla and da Silva, 2022; Sillero et al., 2022).

In the selected timespan, 1256 publications were recorded (1217 articles and 39 reviews). The data from the documents obtained were exported and analyzed using bibliometric software. The Bibliometrix package (R language) was used to obtain a three-field plot and the top authors' production over the timing diagram (Aria and Cuccurullo, 2017). After that, the dataset was investigated on VOSviewer® software to construct the authors, sources and country's networks, and the authors' keyword clusters (van Eck and Waltman, 2010). All the data used in the bibliometric analysis were obtained from the Web of Science®. The most cited documents were evaluated to understand the importance and repercussions of different aspects of XOS.

### 2.1. Bibliometric analysis of xylo-oligosaccharides

The bibliometric analysis of XOS production and application are presented in the following sections, providing insightful data on research progress, updates, and trends.

### 2.2. Publication evolution and research areas

Fig. 1 reveals a total of 1256 publications (1217 articles and 39 reviews) related to XOS over the selected timespan (2000–2021), with significant increases from 2020 to 2021, jumping from 105 to 174 publications. The constant increase in the number of publications represents the active interest of researchers in the subject and market trend. *Biotechnology Applied Microbiology* is the predominant area (22.7%), followed by *Food Science Technology* (16.2%), which can be associated with the increased demand for functional foods and is considered a hot topic in the field. Notwithstanding, the market of XOS presents an annual growth rate of approximately 4.1% and is projected to reach US\$ 130 million by 2025 (Brenelli et al., 2022). The application of XOS in food mainly includes the development of symbiotic foods (containing both prebiotic and probiotic microorganisms) such as drinks composed of probiotic bacteria, XOS, tea extract, and minerals (Amorim et al., 2019).

### 2.3. Study of the main keywords

Co-occurrence keyword analysis has been widely used to identify hot research topics and gaps (Tables 1 and 2). From this search over the previous 21 years, there are 2717 keywords. The 20 most used keywords are displayed in Table 3. In addition, the bibliometric coupling of the most used keywords is presented in Fig. 2, where the 75 most used keywords were divided into 5 clusters. Each cluster was associated with a specific research field, and the keywords were considered given a minimum of 7 occurrences. The keyword xylo-oligosaccharides was the keyword with the highest link strength and occurrence. As previously elucidated, there are different keyword combinations to express the XOS, but with less occurrence. The most frequent keywords used in the ranked documents according to the strongest link were xylan and xylanase, which reveals the importance of the enzymatic hydrolysis technology of xylan bioconversion into XOS. Interestingly, xylanase appears in a different cluster than enzymatic hydrolysis, suggesting the greater importance of xylanase in this field for XOS production compared to other xylanolytic enzymes. In fact, the hemicellulose fraction of the lignocellulosic biomass utilized for XOS production is mainly composed of xylose units, followed by branched chains of

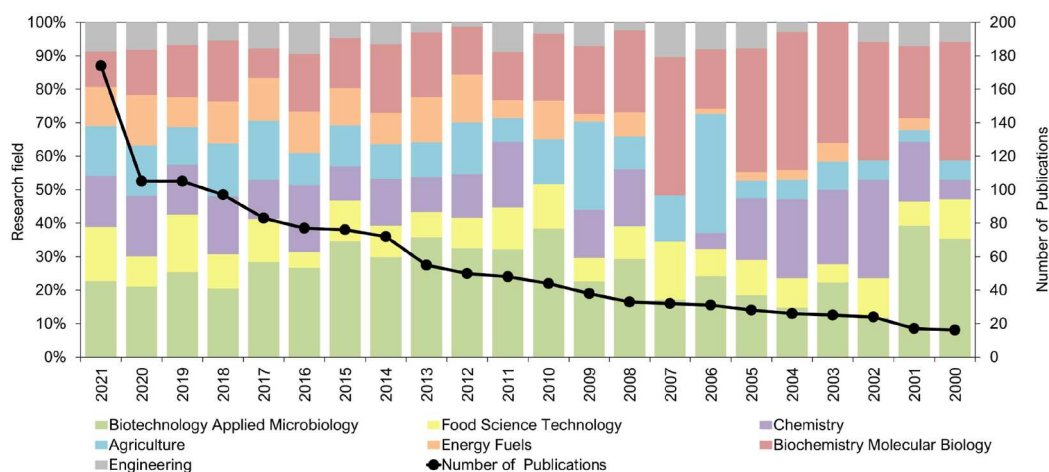


Fig. 1. Evolution of the number of publications over the years (2000–2021) in the field of xylo-oligosaccharides.

Table 1

Clusters of the main keywords obtained on VOSviewer software.

| Clusters | Number of items | Keywords on the VOSviewer network                                                                                                                                                                                                                                                                                                                                                                                             |
|----------|-----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1        | 21              | Alpha-L-arabinofuranosidase, <i>Aspergillus niger</i> , $\beta$ -D-xylosidase, $\beta$ -xylosidase, carbohydrate-binding, characterization, crystal structure, endo-xylanase, glycoside hydrolase, heterologous expression, hydrolysis, immobilization, <i>Pichia pastoris</i> , purification, steam Explosion, thermostability, thermostable xylanase, transglycosylation, xylan hydrolysis, xylanase, xylo-oligosaccharide. |
| 2        | 15              | Arabinoxylan, <i>Bacillus subtilis</i> , bifidobacteria, fermentation, growth performance, gut microbiota, microbiota, oligosaccharides, prebiotic, prebiotics, probiotic, probiotics, synbiotic, XOs, xylooligosaccharide.                                                                                                                                                                                                   |
| 3        | 12              | Antioxidant activity, arabinofuranosidase, corncob, glucoronoxylan, glycoside hydrolase Family, NMR, substrate specificity, wheat straw, wheat bran, xylo-oligosaccharides, xylobiose, xylooligosaccharides (XOs).                                                                                                                                                                                                            |
| 4        | 12              | Bioethanol, biomass, cellulase, cellulose, enzymatic hydrolysis, hemicellulose, hydrothermal pretreatment, lignin, lignocellulose, poplar, <i>Trichoderma reesei</i> , xylooligosaccharides.                                                                                                                                                                                                                                  |
| 5        | 8               | Autohydrolysis, biorefinery, furfural, hemicelluloses, oligosaccharide, response surface methodology, sugarcane bagasse, xylose.                                                                                                                                                                                                                                                                                              |
| 6        | 7               | Corn stover, endoxyanase, lignocellulosic biomass, pretreatment, xylan, xylanases, xylosidase.                                                                                                                                                                                                                                                                                                                                |

Table 2

Clusters of the main keywords obtained on Bibliometrix software.

| Clusters | Main keyword          | Number of items | Keywords on Bibliometrix network                                                                                                                             |
|----------|-----------------------|-----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1        | B-xylosidase          | 10              | B-xylosidase, xylose, xylo-oligosaccharide, glycoside hydrolase, substrate specificity, xylobiose, crystal structure, wheat bran, cellulase, oligosaccharide |
| 2        | Enzymatic hydrolysis  | 10              | Enzymatic hydrolysis, autohydrolysis, biorefinery, bioethanol, corncob, lignin, hemicellulose, hydrothermal pretreatment, biomass, pretreatment              |
| 3        | Xylooligosaccharides  | 10              | Xylooligosaccharides, xylanase, xylan, xylooligosaccharide, hemicellulose, purification, sugarcane bagasse, xylanases, hydrolysis, characterization          |
| 4        | Xylo-oligosaccharides | 10              | Xylo-oligosaccharides, oligosaccharides, prebiotic, XOs, fermentation, gut microbiota, arabinoxylan, synbiotic, growth performance, probiotic                |
| 5        | Prebiotic             | 4               | Prebiotic, probiotics, synbiotics, <i>Bifidobacterium</i>                                                                                                    |

other types of sugars, such as arabinose and glucose. Moreover, it was observed that the keyword prebiotic was presented in a separate cluster, which indicates a distinct research field with a strong connection between the other clusters.

The network analysis of keywords can be plotted over the publication years (Fig. 2b). The colors ranging from blue to yellow illustrate the trend of XOS over the years, considering the average terms used. The research fields related to prebiotic, microbiota, and enzymatic hydrolysis were most expressive between 2016 and 2018, while the fields related to autohydrolysis were expressive in approximately 2012 but not in most recent years. Hence, there is a trend in studying XOS production by enzymatic methods rather than autohydrolysis, as well as the bioactivities of XOS as prebiotic and their effects on microbiota.

Fig. 3 shows a thematic map grouping the keywords according to the relevance and development degree of the research field. This map is an intuitive research theme plot divided into four quadrants: i) niche (very specialized) themes; ii) motor themes; iii) emerging or declining themes, and iv) basic themes (de Oliveira et al., 2022). In this bibliometric study, the keywords xylooligosaccharides, xylan, and xylanase are considered basic themes, which means that they are important for the research field but are not fully developed, that is, these keywords present low density and high centrality and more research and analysis on this matter is required, and when the research around these keywords will be further substantiated, they may become motor themes. The re-

**Table 3**

The top 20 keywords in the field (rank based on the occurrences).

| Ranking | Keyword               | Occurrences | Total link strength |
|---------|-----------------------|-------------|---------------------|
| 1       | Xylooligosaccharides  | 243         | 429                 |
| 2       | Xylanase              | 172         | 324                 |
| 3       | Xylan                 | 103         | 233                 |
| 4       | Xylooligosaccharide   | 75          | 124                 |
| 5       | Enzymatic hydrolysis  | 72          | 144                 |
| 6       | Xylo-oligosaccharides | 72          | 110                 |
| 7       | Prebiotics            | 61          | 97                  |
| 8       | B-xylosidase          | 54          | 109                 |
| 9       | Oligosaccharides      | 47          | 62                  |
| 10      | Prebiotic             | 44          | 82                  |
| 11      | Autohydrolysis        | 43          | 93                  |
| 12      | Hemicellulose         | 40          | 90                  |
| 13      | Xylose                | 38          | 92                  |
| 14      | Purification          | 29          | 66                  |
| 15      | Biorefinery           | 26          | 59                  |
| 16      | Xylo-oligosaccharide  | 26          | 27                  |
| 17      | Sugarcane bagasse     | 24          | 45                  |
| 18      | Glycoside hydrolase   | 23          | 57                  |
| 19      | Xylanases             | 22          | 37                  |
| 20      | Bioethanol            | 20          | 54                  |

sults suggest that the technology around xylan pretreatment and xylanase production still requires improvements to increase XOS yield and feasibility (Pinales-Márquez et al., 2021). Further studies could be conducted to elucidate biomass pretreatment technologies, the choice of xylan sources, and enzyme engineering to develop thermostable and highly specific xylanases.

Otherwise, xylose and  $\beta$ -xylosidase are classified as motor themes. However, these keywords are not related to XOS production since  $\beta$ -xylosidase is responsible for the cleavage of xylobiose and xylose release. The enzymatic cocktail for XOS production must be poor in  $\beta$ -xylosidase activity to improve yields and avoid an excessive production of xylose, which is considered an impurity of the production and prejudice the downstream purification process (Carvalho et al., 2013). B-Xylosidases are often studied to improve the efficiency of saccharification and fermentation of XOS. Bioethanol production from xylose (product of XOS depolymerization) is essential for reducing enzymatic inhibition and taking full advantage of lignocellulose biomass (Niu et al., 2019).

At the center of relevance and occupying the niche and motor themes, enzymatic hydrolysis, autohydrolysis, and biorefinery are important keywords found in every research paper related to XOS production. There is constant investigation toward the development of an integrated biorefinery of XOS using sustainable biomass from lignocellulosic wastes (straws, bagasse, peels, and husks from crops) by autohydrolysis or enzymatic hydrolysis. Both are efficient technologies, but each presents specific bottlenecks that must be assessed (Kumar et al., 2021).

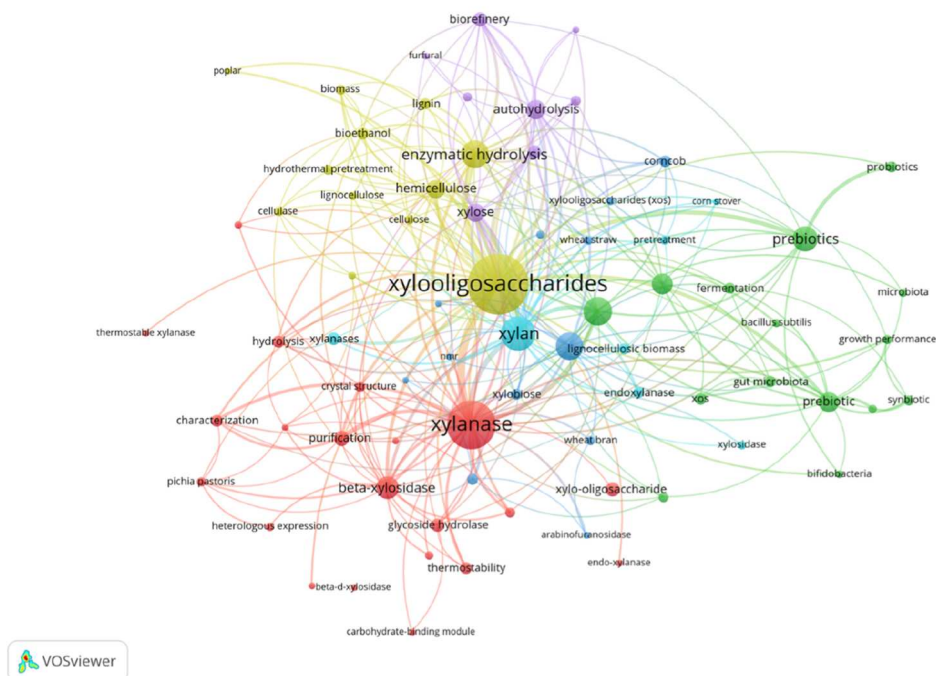
The keywords prebiotic, probiotic, and synbiotic are located at the center of development degree (research density) between niche themes and emerging or declining themes. Two conclusions may be obtained from this chart: i) the prebiotic potential of XOS was the main reason for the growing interest in XOS production; and ii) the development of functional food with XOS could be of industrial interest, especially if obtained from sustainable sources, to replace artificial sweeteners, emulsifiers, and fat while providing several health benefits, such as antioxidant and prebiotic activities. Therefore, the bioactivities of XOS should be deeply investigated to consolidate XOS as an emerging prebiotic to raise industrial interest. At the same time, new investigations are being made to explore further the potential application of XOS in the field of gut microbiota. Therefore, the research field of XOS bioactivities is very specialized and represents a recent research trend.

#### 2.4. The most cited publications

A good indicator to identify the most advanced and influential topics in a research field is the most cited publications. Table 4 presents the ten most cited research articles and review papers in the XOS field over the last 20 years. The first four papers and the 7th are related to the prebiotic potential, mainly *in vitro* and one in mice. These papers did not exclusively report XOS effects, but XOS presented the most promising results compared to the other evaluated prebiotics. The attention given to these articles shows the strong interest in establishing XOS as a functional ingredient in the food industry. The 3rd paper was the most recent, from 2014, while the 1st is the oldest, published in 2001. The following most cited articles reported the production of XOS by autohydrolysis of agro-industrial by-products. Only the 9th paper of this list reported enzymatic hydrolysis using xylanase.

In contrast, the most cited review presented a comprehensive overview of manufacturing and application of XOS. The 1st and 2nd papers are quite similar and present the production from lignocellulosic biomasses, application in food, and market perspectives. The reviews occupying the 3rd, 7th, 9th, and 10th positions compile the health-related effects, but they are not focused solely on the XOS, discussing other classes of prebiotic and cereal-derived oligosaccharides. The 4th paper discusses the utilization of xylan to obtain bioproducts, including XOS. Although not focused on XOS itself, this review establishes xylan from agro-industrial wastes as the most important source for XOS production. The 5th paper was wrongly submitted as a review paper and is a research article about the xylan autohydrolysis kinetic model for XOS production. The 6th paper presents structural features and mechanisms of xylanases. It

(a)



(b)

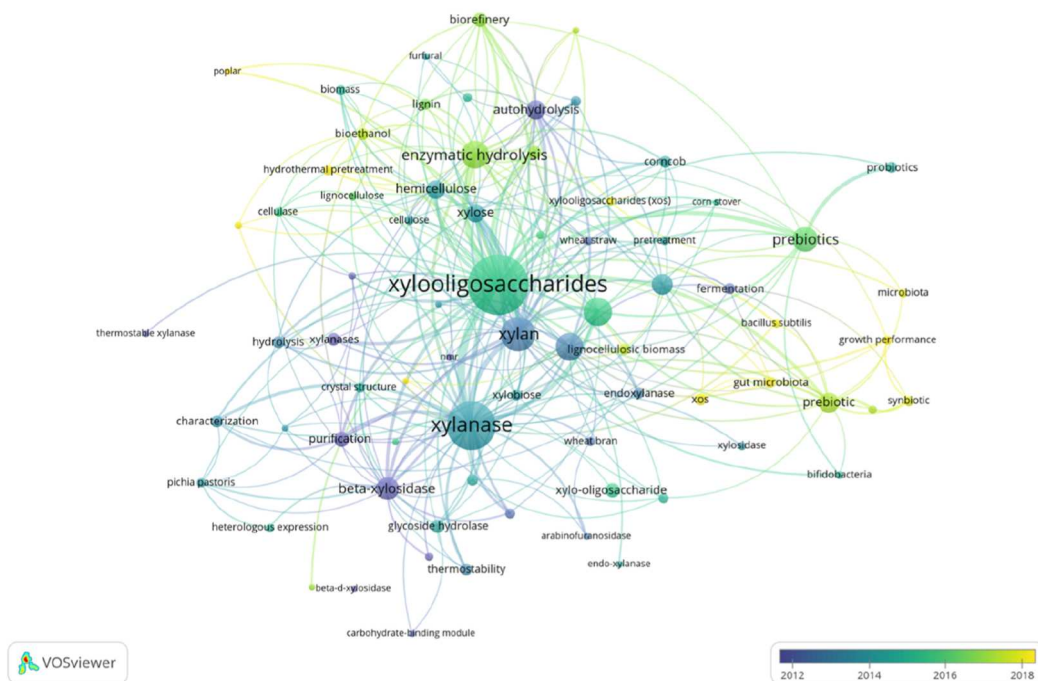


Fig. 2. Clustering of the 75 most frequent authors' keywords. (a) Term map based on different clusters; (b) Term average year map; (c) Density visualization map.

should be analyzed along with the 4th paper to determine the best strategy for the enzymatic hydrolysis of xylan depending on the source of lignocellulosic material, and the 8th, which highlights the possibility of discovering and designing new xylanolytic enzymes for XOS production.

Based on the most cited documents, the prebiotic potential is well established and still under constant investigation; autohydrolysis is commonly associated with its production; however, enzymatic hydrolysis from agro-industrial wastes and the development of new enzymes seems to be a trend in the XOS research field, following Fig. 3.

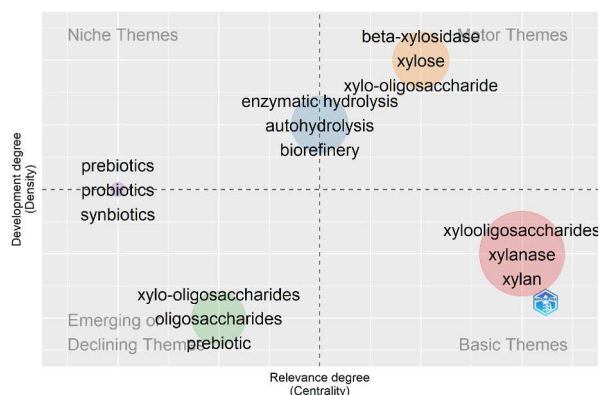


Fig. 3. Thematic map of the clusters from the 100 most frequent authors' keywords.

### 2.5. Bibliometric study of authors, journals, institutions, and countries

The most productive researchers in the field can be identified after the evaluation of the most cited papers. Fig. 4 presents the analysis of the top 20 authors' productivity over the last 20 years. The size of the circles is defined by the number of articles, and the marker color represents the citations per year, where the more transparent the marker is, the lower the number of total citations. Additionally, the authors shown in Fig. 4 are not necessarily the first authors of the paper. Of the listed authors, only Parajo JC, Garrote G., and Biely P. are among the most cited authors. Biely P. was the most productive researchers in the field, accounting for 24 publications. In 2016, the number of citations registered for this author reached the maximum number of 20 citations. Xu was the author with the most publications (10) and citations (20) in 2021.

The main journal dedicated to publishing XOS research was *Bioresource Technology*, with 92 publications and representing 7.3% of total publications, followed by *Applied Biochemistry and Biotechnology* and *Industrial Crops and Products*, both with 34 publications (2.7%). The scope of the listed journals is similar and related to biomass, waste management and feedstock utilization, biotransformation, bioprocesses, and bioproducts. The 5th and 9th most cited research articles were published in *Bioresource Technology*. In addition, *Carbohydrate Polymers* is the 5th journal listed, with 33 publications, including the 8th and 10th most cited research articles (Table 5).

The countries and institutions related to XOS research were ranked according to the number of publications (Table 5). Globally, 68 countries are engaged in research in this field. Fig. 5 shows the distribution of publications among the countries. China is the most productive country, accounting for 345 publications (27.46%), followed by the USA (139 publications, 11.06%), Japan (125 publications, 9.95%), Brazil (95 publications, 7.56%), and Spain (90 publications, 7.16%). The massive contribution of China to this field is notable. In addition to China, this topic is well spread throughout the globe, with assistance from all continents.

Regarding the most prestigious institutions, *Nanjing Forestry University* and the *Chinese Academy of Science*, both from China, stand out, followed by the *United States Department of Agriculture* (USA) and the *University of Virgo* (Spain). The *University of Campinas* (Brazil) is the 6th most prestigious institution in XOS research. Fig. 5b illustrates the collaboration network between the countries. The number of citations per country defines the size of the circles, and the lines among the authors represent the number of co-authorships, with the distance between them determined by the strength of the relation. In general, they are assembled into 4 clusters, and the shorter the distance between the circles is, the more similar the research fields are.

Fig. 6 presents a three-field plot relating the top 15 authors, keywords, and journals to illustrate how they are connected through a Sankey diagram. This plot is widely used to depict a flow from one item to another, where the heavier the links are, the stronger the connection between the items. Xu and Yong presented the heaviest link to enzymatic hydrolysis; Garrote and Parajo, to autohydrolysis. These two keywords share the weighty link to *Bioresource Technology Journal*, suggesting that this journal covered most publications with these keywords. Garrote, Parajo, Xu, and Dominguez presented the heaviest link to the keyword xylo-oligosaccharides, which also showed the strongest link to *Bioresource Technology Journal*.

## 3. Advances and trends of xylo-oligosaccharides

### 3.1. General properties and production methods

The linear XOS known for their prebiotic potential and health benefits are xylobiose (degree of polymerization (DP) = 2), xylotriose (DP = 3), xylotetraose (DP = 4), xylopentose (DP = 5) and xylohexose (DP = 6) (Fig. 7). They are low-calorie sweetener (1.5 kcal/g), solid, and colorless, although this is dependent on the xylan source and purification process. The recommended daily intake is 1.4–2.8 g/day for healthy adults (Finegold et al., 2014); it is suitable for diabetic patients and has antioxidant and cytoprotective activity. XOs are generally recognized as safe (GRAS); however, excess intake could result in distension of the intestine, nausea, flatulence, and diarrhea (Samanta et al., 2015).

In the depolymerization process (chemical or enzymatic), xylan is converted into high molecular weight xylooligomers, branched or linear, or is degraded to furfural, depending on the severity of the treatment and biomass nature. Xylan is a large hemicellulosic fraction found in different cheap raw materials, especially lignocellulosic biomass of agricultural wastes. From the xylan fraction in



**Table 4**

The top 10 most cited articles and reviews.

| Ranking  | Title                                                                                                                                        | Issue                                                                                                                                                                                                                                                                                                               | Journal                                        | Citations | Year | Reference                                     |
|----------|----------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------|-----------|------|-----------------------------------------------|
| Articles |                                                                                                                                              |                                                                                                                                                                                                                                                                                                                     |                                                |           |      |                                               |
| 1st      | A comparative <i>in vitro</i> evaluation of the fermentation properties of prebiotic oligosaccharides                                        | Comparison of <i>in vitro</i> fermentation properties of commercial prebiotic oligosaccharides.                                                                                                                                                                                                                     | Journal of Applied Microbiology                | 467       | 2001 | <a href="#">Rycroft et al. (2001)</a>         |
| 2nd      | The Impact of a Consortium of Fermented Milk Strains on the Gut Microbiome of Gnotobiotic Mice and Monozygotic Twins                         | Characterization of the fecal microbiomes and metatranscriptomes of mice after consumption of fermented milk containing probiotics. <i>B. lactis</i> was the dominant member of the consortium due to the catabolism of xylooligosaccharides.                                                                       | Science Translational Medicine                 | 384       | 2011 | <a href="#">McNulty et al. (2011)</a>         |
| 3rd      | Prebiotic stimulation of human colonic butyrate-producing bacteria and <i>bifidobacteria</i> , <i>in vitro</i>                               | Reports carbohydrate utilization patterns for representative butyrate-producing anaerobes on different substrates. XOS was the most selective growth substrate.                                                                                                                                                     | Fems Microbiology Ecology                      | 235       | 2014 | <a href="#">Scott et al. (2014)</a>           |
| 4th      | <i>In vitro</i> fermentation of cereal dietary fiber carbohydrates by probiotic and intestinal bacteria                                      | A range of probiotics and other intestinal bacteria were examined for their ability to ferment the dietary fiber. XOS was efficiently fermented by <i>Bacteroides</i> isolates, <i>Bifidobacterium</i> , and <i>Lactobacillus</i> but not by <i>E. coli</i> , enterococci, and <i>Clostridium</i> species examined. | Journal of the Science of Food and Agriculture | 225       | 2002 | <a href="#">Crittenden et al. (2002)</a>      |
| 5th      | Production of oligosaccharides by autohydrolysis of brewery's spent grain                                                                    | Various temperatures and reaction times were investigated for the hemicellulose treatment of brewery's spent grain. The maximal XOS yield (61% of the feedstock xylan) was obtained at 190 °C after 5 min of reaction.                                                                                              | Bioresource Technology                         | 214       | 2004 | <a href="#">Carvalho et al. (2004)</a>        |
| 6th      | Autohydrolysis of corn cob: the study of nonisothermal operation for xylooligosaccharide production                                          | Up to 94% of xylan was removed, producing XOS and other byproducts (including xylose and furfural). Series reactions modeled the autohydrolysis.                                                                                                                                                                    | Journal of Food Engineering                    | 208       | 2002 | <a href="#">Garrote et al. (2002)</a>         |
| 7th      | Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats         | This study reports that XOS is superior to FOS in microbiota growth, decreasing the cecal pH and serum triglyceride concentration and increasing the total cecal weight and <i>bifidobacteria</i> population.                                                                                                       | Journal of Nutrition                           | 200       | 2004 | <a href="#">Hsu et al. (2004)</a>             |
| 8th      | Autohydrolysis of agricultural byproducts for the production of xylo-oligosaccharides                                                        | Six agricultural residues were tested for XOS production. The yield of XOs depended on the content of xylan and its accessibility and was proportional to the acetyl content of the raw materials.                                                                                                                  | Carbohydrate Polymers                          | 185       | 2007 | <a href="#">Nabarlatz et al. (2007)</a>       |
| 9th      | Production of xylooligosaccharides from corn cob xylan by fungal xylanase and their utilization by probiotics                                | Corn cob xylan was extracted using the alkaline treatment and hydrolyzed by fungal xylanase. XOS purification was done using activated charcoal. Prebiotic effects were also evaluated <i>in vitro</i> .                                                                                                            | Bioresource Technology                         | 176       | 2012 | <a href="#">Chapla et al. (2012)</a>          |
| 10th     | Hydrothermally treated xylan-rich byproducts yield different classes of xylo-oligosaccharides                                                | Branched XOS was obtained by autohydrolysis of 4 xylan-rich agricultural residues                                                                                                                                                                                                                                   | Carbohydrate Polymers                          | 176       | 2002 | <a href="#">Kabel (2002)</a>                  |
| Review   |                                                                                                                                              |                                                                                                                                                                                                                                                                                                                     |                                                |           |      |                                               |
| 1st      | Xylooligosaccharides: manufacture and applications                                                                                           | A comprehensive review on xylooligosaccharides production from lignocellulosic materials by chemical or enzymatic methods, purification, and application as food ingredients                                                                                                                                        | Trends In Food Science & Technology            | 459       | 2000 | <a href="#">Vázquez et al. (2000)</a>         |
| 2nd      | Advances in the manufacture, purification, and applications of xylo-oligosaccharides as food additives and nutraceuticals                    | A comprehensive review on XOS production from lignocellulosic materials by chemical or enzymatic methods, utilization in synbiotic preparations, technological properties, and market perspectives.                                                                                                                 | Process Biochemistry                           | 383       | 2006 | <a href="#">Moure et al. (2006)</a>           |
| 3rd      | Prebiotic and Other Health-Related Effects of Cereal-Derived Arabinoxylans, Arabinoxylan-Oligosaccharides, and Xylooligosaccharides          | This review focuses on the AXOS and XOS prebiotic effects in the colon of humans and animals. <i>In vitro</i> experiments and <i>in vivo</i> intervention studies on animals or humans are discussed that have investigated potential health-related effects resulting from the dietary intake of AX, AX            | Critical Reviews in Food Science and Nutrition | 349       | 2011 | <a href="#">Broekaert et al. (2011)</a>       |
| 4th      | From plant biomass to biobased chemicals: Latest developments in xylan research                                                              | This review is focused on xylan and xylan-derived products (ethanol, xylitol, xylo-oligosaccharides). It discusses extraction and purification methodologies, chemical modification, and analytical methods.                                                                                                        | Biotechnology Advances                         | 187       | 2012 | <a href="#">Deutschmann and Dekker (2012)</a> |
| 5th      | Production of xylooligosaccharides by autohydrolysis of lignocellulosic materials <sup>a</sup> research article classified as a review paper | This study determined the composition of xylan-containing raw materials and produced nonlinear XOS by autohydrolysis under optimal conditions deduced from reported kinetic models.                                                                                                                                 | Trends In Food Science & Technology            | 166       | 2004 | <a href="#">Parajó et al. (2004)</a>          |

(continued on next page)

Table 4 (continued)

| Ranking | Title                                                                                                           | Issue                                                                                                                                                                                                        | Journal                             | Citations | Year | Reference                                  |
|---------|-----------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|-----------|------|--------------------------------------------|
| 6th     | Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families | This study analyzes the 3D structures of xylanases to provide insight into the mechanisms and strategies by which xylanases bind and hydrolyze structurally different heteroxylans and XOS.                  | Critical Reviews In Biotechnology   | 164       | 2010 | <a href="#">Pollet et al. (2010)</a>       |
| 7th     | Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine              | This review focused on the prebiotic potential of AXOS and the intestinal microbiota involved in their transformation processes                                                                              | Trends In Food Science & Technology | 164       | 2007 | <a href="#">Grootaert et al. (2007)</a>    |
| 8th     | Toward enzymatic breakdown of complex plant xylan structures: State of the art                                  | This review highlights the possibility of the discovery of novel xylanolytic enzyme construction of multifunctional chimeric enzymes and xylanosomes in parallel with increasing knowledge of XOS structure. | Biotechnology Advances              | 145       | 2016 | <a href="#">Biely et al. (2016)</a>        |
| 9th     | Prebiotics and gut microbiota in chickens                                                                       | This review presents the current knowledge of the chicken gastrointestinal microbiota and the most recent publications related to prebiotics in the modulation of the gut microbiota and immune functions.   | Fems Microbiology Letters           | 143       | 2015 | <a href="#">Pourabedin and Zhao (2015)</a> |
| 10th    | The functionality of prebiotics as immunostimulant: Evidence from trials on terrestrial and aquatic animals     | This review covers the scientific outcomes of the direct and indirect mechanisms through which prebiotics can affect the intestinal immunity of animals.                                                     | Fish & Shellfish Immunology         | 108       | 2018 | <a href="#">Nawaz et al. (2018)</a>        |

<sup>a</sup> The number of citations was recorded until 2020.

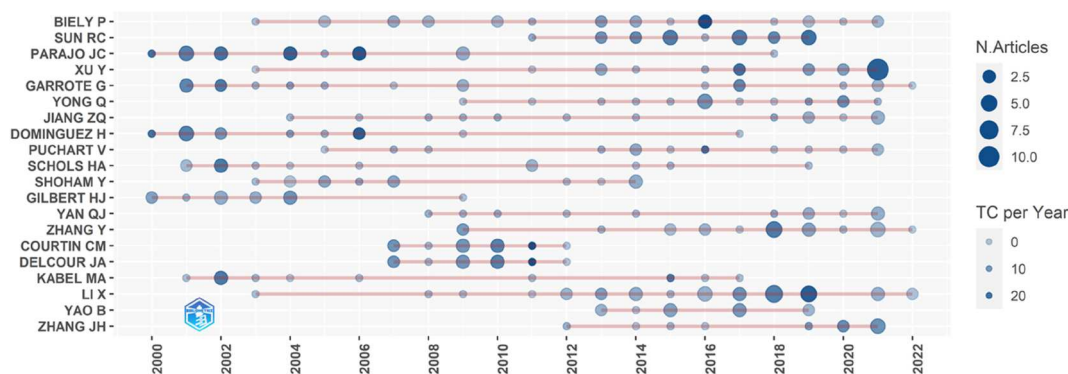


Fig. 4. Bibliometric analysis of the top 20 authors over time.

the biorefinery context, XOS and several high value-added products (ethanol, xylitol, lactic acid, furfural, xylose, arabinose) can be obtained for the food, bioenergy, and pharmaceutical industries. XOS are prebiotic compounds with 2–6 xylopyranosyl residues linked by  $\beta$ -1,4 xylosidic bonds. It is important to mention that these compounds stand out for their overall thermal and pH stability since XOS are stable at temperatures above 100 °C and are capable of withstanding pH variations between 2.5 and 8 ([Gullón et al., 2011](#)). The fundamentals of XOS production start with drying and grinding biomass to reduce the crystallinity and enhance the contact surface. The next stage generally involves a treatment to remove lignin and reduce biomass recalcitrance. These treatments can be physical (ultrasound and microwave), hydrothermal (steam explosion or liquid hot water) ([Ruiz et al., 2023](#)), biological (using microorganisms), or chemical (acid, alkali, ionic, or oxidative). These treatments solubilize xylan and can produce a wide range of oligosaccharides and several degradation products ([Poletto et al., 2020](#)).

Enzymatic treatment for XOS production has gained relevance as it is a controlled process that prevents the formation of undesirable compounds such as furfural and phenolic acids. The hemicellulosic enzymes used in these processes are mainly composed of  $\beta$ -1,4-endoxylanases (E.C.3.2.1.8), which divide the  $\beta$ -1,4-xylose bonds inside the heteroxylan chain. The accessory enzymes (mainly esterases and arabinofuranosidases) act on the side chains of xylan. To improve the enzymatic production of XOS, the activity of  $\beta$ -xylosidase must be low to release the lower content of monomers possible. The ability of endoxylanases to release short XOS and thermostability are some of the crucial selection parameters ([Lopes et al., 2021](#); [Pinales-Márquez et al., 2021](#); [Polizeli et al., 2005](#)).

XOS can be produced from xylan depolymerization by chemical or enzymatic methods; however, both processing routes have advantages and disadvantages compared to each other. Chemicals are cheaper than enzymes; nevertheless, improper treatments can lead to the degradation of monosaccharides and the production of toxic compounds ([Fig. 8](#)). On the other hand, the use of enzymes means much more specific, environmentally friendly, and controlled processes carried out under mild temperature and pH conditions ([Poletto et al., 2020](#)). The combination of chemical and enzymatic methods can be established to modulate the proportion of various DP. The hydrothermal treatment and subsequent endo-xylanase hydrolysis resulted in a XOS with conversion yield of 44.6% containing 78.7% xylobiose and xylotriose starting from the initial xylan in raw poplar ([Su et al., 2021](#)). In another study, the acidic condition of subcritical carbon dioxide and inorganic salts in seawater significantly promoted the degradation of xylan and increased the

**Table 5**

Ranking 10 top publication areas, affiliations, countries, journals, and authors.

| Ranking | Research areas                                                             | Number | % <sup>a</sup> |
|---------|----------------------------------------------------------------------------|--------|----------------|
| 1st     | Biotechnology Applied Microbiology                                         | 448    | 35.67          |
| 2nd     | Biochemistry Molecular Biology                                             | 310    | 24.68          |
| 3rd     | Chemistry                                                                  | 265    | 21.09          |
| 4th     | Agriculture                                                                | 195    | 15.52          |
| 5th     | Food Science Technology                                                    | 190    | 15.12          |
| 6th     | Energy Fuels                                                               | 163    | 12.97          |
| 7th     | Microbiology                                                               | 124    | 9.87           |
| 8th     | Engineering                                                                | 109    | 8.67           |
| 9th     | Polymer Science                                                            | 70     | 5.57           |
| 10th    | Science Technology Other Topics                                            | 58     | 4.61           |
| Ranking | Affiliations (Country)                                                     | Number | % <sup>a</sup> |
| 1st     | Nanjing Forestry University (China)                                        | 46     | 3.66           |
| 2nd     | Chinese Academy of Sciences (China)                                        | 39     | 3.10           |
| 3rd     | United States Department of Agriculture (USA)                              | 35     | 2.78           |
| 4th     | University of Vigo (Spain)                                                 | 32     | 2.54           |
| 5th     | National Center for Scientific Research (France)                           | 31     | 2.46           |
| 6th     | University of Campinas (Brazil)                                            | 30     | 2.38           |
| 7th     | South China University of Technology (China)                               | 30     | 2.38           |
| 8th     | Slovak Academy of Sciences (Slovakia)                                      | 29     | 2.30           |
| 9th     | Beijing Forestry University (China)                                        | 28     | 2.22           |
| 10th    | National Research Institute for Agriculture, Food and Environment (France) | 28     | 2.22           |
| Ranking | Countries                                                                  | Number | % <sup>a</sup> |
| 1st     | China                                                                      | 345    | 27.46          |
| 2nd     | United States of America                                                   | 139    | 11.06          |
| 3rd     | Japan                                                                      | 125    | 9.95           |
| 4th     | Brazil                                                                     | 95     | 7.56           |
| 5th     | Spain                                                                      | 90     | 7.16           |
| 6th     | India                                                                      | 87     | 6.92           |
| 7th     | England                                                                    | 51     | 4.06           |
| 8th     | Portugal                                                                   | 51     | 4.06           |
| 9th     | France                                                                     | 47     | 3.74           |
| 10th    | Thailand                                                                   | 41     | 3.26           |
| Ranking | Journals                                                                   | Number | % <sup>a</sup> |
| 1st     | Bioresource Technology                                                     | 92     | 7.32           |
| 2nd     | Applied Biochemistry and Biotechnology                                     | 34     | 2.70           |
| 3rd     | Industrial Crops and Products                                              | 34     | 2.70           |
| 4th     | Applied Microbiology and Biotechnology                                     | 33     | 2.62           |
| 5th     | Carbohydrate Polymers                                                      | 33     | 2.62           |
| 6th     | Biotechnology for Biofuels                                                 | 29     | 2.30           |
| 7th     | Applied and Environmental Microbiology                                     | 28     | 2.22           |
| 8th     | Carbohydrate Research                                                      | 28     | 2.22           |
| 9th     | Process Biochemistry                                                       | 26     | 2.07           |
| 10th    | International Journal of Biological Macromolecules                         | 24     | 1.91           |
| Ranking | Authors                                                                    | Number | % <sup>a</sup> |
| 1st     | Biely P                                                                    | 24     | 1.91           |
| 2nd     | Sun RC                                                                     | 20     | 1.59           |
| 3rd     | Parajo JC                                                                  | 19     | 1.51           |
| 4th     | Xu Y                                                                       | 19     | 1.51           |
| 5th     | Garrote G                                                                  | 18     | 1.43           |
| 6th     | Yong Q                                                                     | 15     | 1.19           |
| 7th     | Jiang ZQ                                                                   | 14     | 1.11           |
| 8th     | Dominguez H                                                                | 13     | 1.03           |
| 9th     | Puchart V                                                                  | 13     | 1.03           |
| 10th    | Schols HA                                                                  | 13     | 1.03           |

<sup>a</sup> Percentage of 1256 documents (automatically calculated in WoS).

contents of low-DP XOS, enhancing XOS yields (51.44%) and increasing the pore area and porosity of the pretreated biomass, further enhancing enzymatic hydrolysis (Zhang et al., 2022). Also, chemical methods may present synergistic effects and can be combined for a better exploitation of biomass, achieving the coproduction of XOS, lignin fractionation, and cellulose-rich substrates, for example hydrothermal and deep eutectic solvent pretreatments (Shen et al., 2021).

### 3.2. Development of biorefineries for XOS production

Most recent publications in the field of XOS production focus on the development of integrated processes in a biorefinery concept. A biorefinery is an industrial complex using organic raw materials, especially wastes, as feedstock to produce biomaterials and bioen-



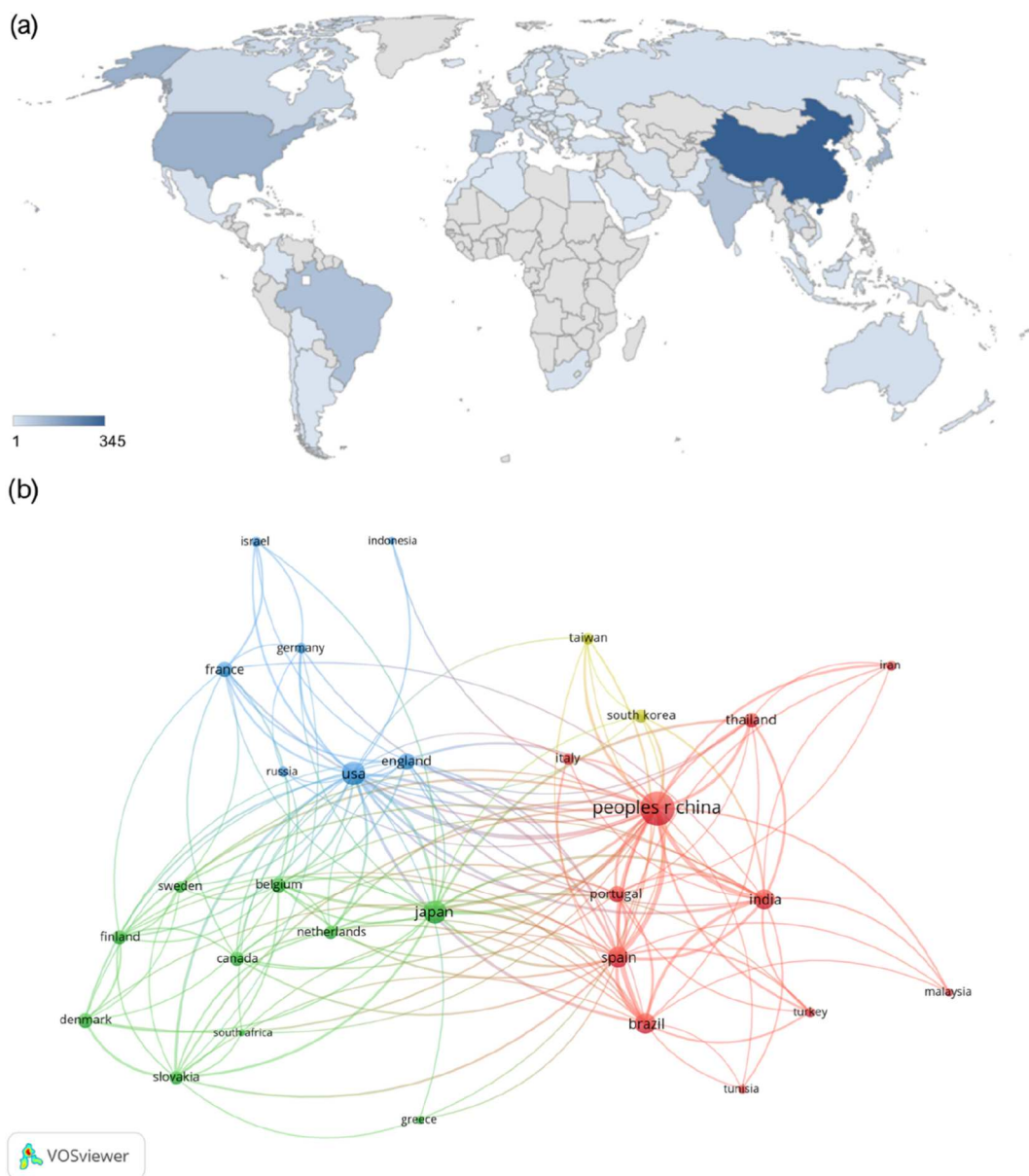


Fig. 5. The most expressive countries. (a) Map representation of the number of publications per country. (b) Collaboration network strength between the countries according to co-authorship.

ergy (Pinales-Márquez et al., 2021). Processing wastes through the biorefinery to obtain multiple value-added products leads to cost reduction and creates a competitive advantage, given the increased interest in the food and cosmetics industries due to their biological and physicochemical properties. Different processing stages can be designed to couple byproducts from cellulose, hemicellulose, and lignin, promoting better use of the feedstock (Tsouko et al., 2020). Agro-industrial wastes represent an advantageous raw material to produce XOS and other bioproducts due to their quantity, availability, and elevated xylan content, ranging from 20 to 50% depending on the biomass (Table 6) (Maity, 2015). Important environmental benefits emerge with this strategy, as it helps manage millions of tons of waste generated annually (Amorim et al., 2019).

The industrial application of XOS has been greatly limited due to product quality and impurities. The expensive purification methods have become the key to successful industrial application. Additionally, the autohydrolysis method has high energy consumption and production cost, and the corrosive acid used in the process generates many undesirable compounds (Yan et al., 2022). Therefore, XOS separation and purification steps still need further study to improve the purity and lower the production cost. The bibliometric analysis showed that bioconversion of xylan by enzymatic hydrolysis had gained relevance within the scientific community as it is a controlled process and prevents the formation of undesirable compounds (such as furfural, 5-hydroxymethylfurfural, and formic

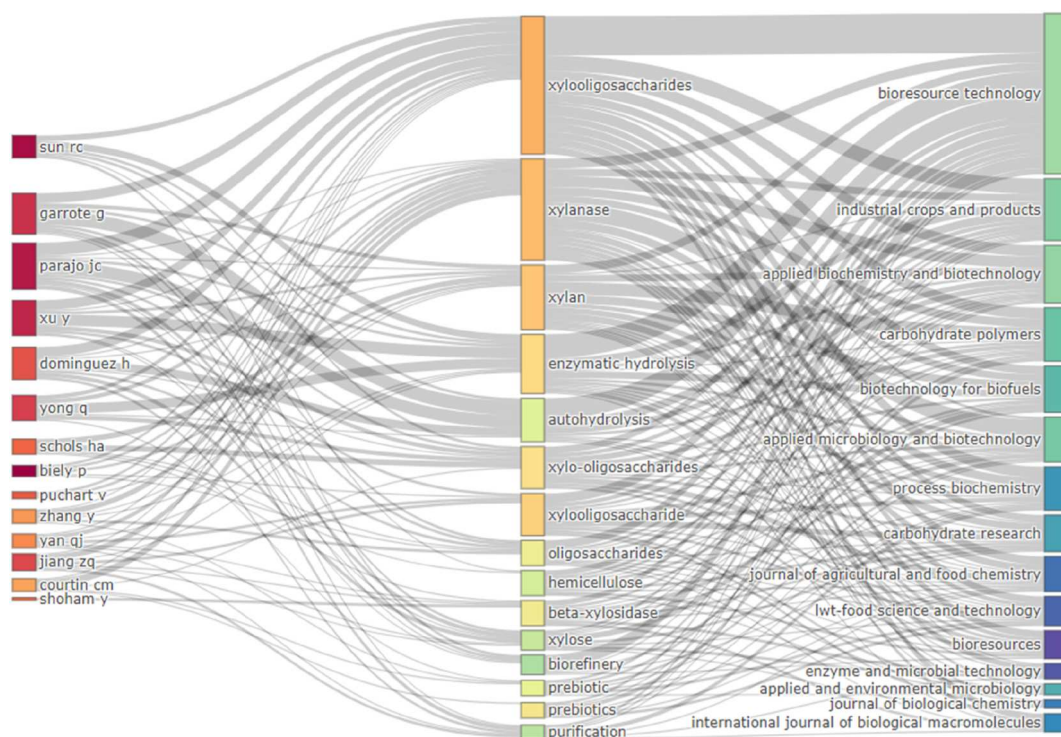


Fig. 6. The three fields plot correlating the top 15 authors, keywords, and journals.

acid), reducing costs related to downstream processing. However, the costs of enzyme production and long reaction times raise the value of the process (Pinales-Márquez et al., 2021).

There are several technological opportunities for increasing the performance of pretreatments and enzymes toward greener polymerization (Pinales-Márquez et al., 2021). Physical or chemical pretreatment is still required for enzymatic hydrolysis because of the biomass recalcitrance to dissolve the hemicellulose. Among the methods, the steam explosion was shown to be an effective green pretreatment with low energy consumption and short reaction time, reducing the chemicals usage (Yan et al., 2022). For example, Shandong Longlive, one of the most important XOS producers, patented a preparation method for pure XOS production by which the first step is a continuously steam explosion pretreatment with diluted acid (0.1–1.5%) (SHANDONG LONGLIVE BIO-TECHNOLOGY Co Ltd, 2014).

The search for sustainable energy and food resources accrues from the population increase, raising the need to develop technologies and policies to regulate the use of soils to avoid the conflict between energy and food production. Additionally, the 1.3 billion tons of lignocellulosic biomass generated annually does not compete with the land for food cultivation. It takes advantage of the development of second-generation biorefineries, becoming a sustainable economic model (Nanda et al., 2015). The global demand for bioproducts in the biorefinery concept and circular bioeconomy sets the investment at 2.6 to 5.8 trillion dollars from 2025 to 2030. In 2023, XOS will have an estimated value of 130 million dollars in the prebiotic market, which shows an excellent opportunity for the economic development of compounds produced from xylan (Poletto et al., 2020). According to the global prebiotic ingredient market, the annual growth rate for 2019–2024 is forecasted to be 12.2% and valued at 3.85 billion dollars (Pinales-Márquez et al., 2021).

### 3.3. XOS as a functional food ingredient

Longlive (Shandong, China), YuanLong Chem (Tianjin, China), Henan Shengtai Biotechnology Co. (Zhengzhou, China), Henan Kangwei (Xinjiang, China), HFsugar (Xinjiang, China), and Yibin Yatai (Sichuan, China) are the most important XOS industries worldwide. XOS can be purchased in bulk at US\$ 20 to 52, depending on the powder or syrup purity. Several researchers have made efforts the investigation the use of XOS as a novel food ingredient for its bioactive properties, flavor, and physicochemical properties. Although XOS is a potential sugar and fat replacement and emulsifier in beverages, dairy products, chocolates, and bakery products, the studies are directed toward the development of cost-effective production routes rather than their application fields. Here, the most recent studies regarding the application of XOS in food are described.

A pioneering study elucidated the cryoprotectant effect of a mixture of xylobiose and xylotriose on porcine myofibrillar proteins during frozen storage (Chou and Lin, 2010). The gel strength increased with increasing XOS concentration up to 6%, and protein solubility improved with increasing XOS concentration up to 14%. XOS provided the lowest syneresis compared to other oligosaccharides.

In another study, different combinations of XOS and sucrose were incorporated into a meatball recipe. The 4% XOS treatment provided the lowest cooking loss and a similar value of sucrose, and no difference in overall acceptability was found. There was no significant difference in the water holding capacity or pH after cooking or frozen storage. Incorporating XOS into meatballs resulted in a

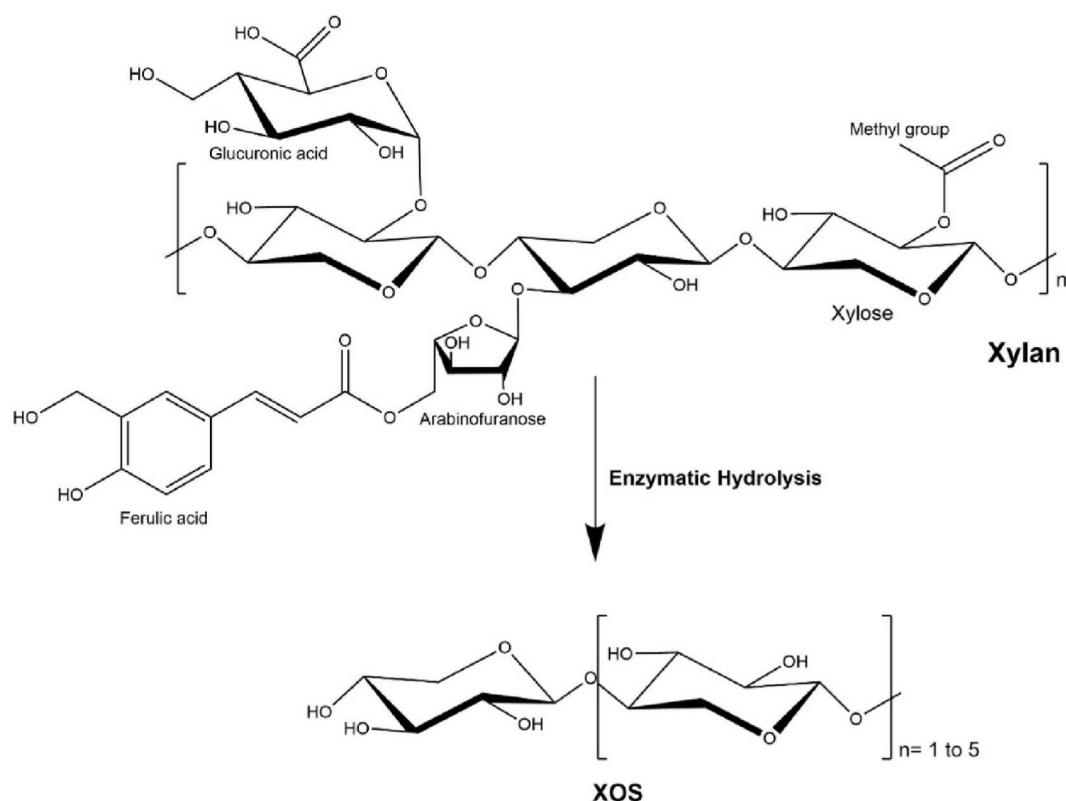


Fig. 7. General structure of arabinoxylan and obtainment of XOS by enzymatic hydrolysis. (where  $n = 1$  is xylobiose;  $n = 2$  is xylotriose;  $n = 3$  is xylotetraose;  $n = 4$  is xylopentose; and  $n = 5$  is xylohexaose).

product comparable to the traditional sucrose-containing recipe in terms of sensory and physicochemical properties (Wu and Lin, 2011).

XOS was also utilized as a cryoprotectant to increase the emulsion stability of the meat batters compared to sucrose, trehalose, and sorbitol. Non-significant difference was observed in the water holding capacities and freeze-thaw stabilities during the frozen storage of any samples. However, these values increased with increasing cryoprotectant concentration. The water-soluble protein remained higher for the XOS samples during frozen storage. The relative surface hydrophobicity indicated that the XOS containing batters exhibited less change in protein structure during frozen storage (Wu and Lin, 2014).

The functionality of plant proteins may also be improved by conjugation with XOS. The application of plant proteins is limited due to their low solubility. The combination of XOS and pea protein increased thermal stability, antioxidant activity, and solubility from pH 3 to 10. These results can be the foundation for the development of novel plant-based foods (Zhao et al., 2022a, 2022b). Additionally, whey beverages enriched with XOS presented better functional characteristics, such as greater antioxidant activity, angiotensin-converting enzyme (ACE) inhibitory activity,  $\alpha$ -amylase, and  $\alpha$ -glucosidase inhibition. The inhibition of ACE activity leads to a decrease in the angiotensin II concentration and an increase in the bradykinin level, and consequently in the reduction of hypertension, one of the major causes of chronic diseases and a high-risk factor of cardiovascular diseases worldwide (Paiva et al., 2017), while the  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibition contributes to blood sugar modulation as it decreases the digestion of carbohydrates into glucose, promoting anti-diabetic effect (Jeong et al., 2012). The beverages also presented higher viscosity, volatile profile, and sensory acceptance, in addition to providing adequate concentrations of the prebiotic (1.25 g/mL) (Souza et al., 2019).

XOS may also substitute sugar in food. For instance, snack hawthorn leathers were formulated with 25% XOS and 75% fructooligosaccharides (FOS) and exhibited comparable morphology and sensorial properties to the traditional one containing sucrose, showed anti-obesity activity with suppressed body weight gain, blood glucose, and oxidative stress, improved the histology of liver and subcutaneous fat tissues, and decreased inflammatory responses in obese mice (Li et al., 2022); cookies enriched with 5% XOS were found to be the most acceptable and were stable for 21 days at room temperature ( $25 \pm 2^\circ\text{C}$ ) (Ayyappan et al., 2016).

The incorporation of XOS in vegan products can already be found commercially, such as the sparkling fermented tea and kombucha from Wonder Drink, and many others are being developed. For example, berry juice containing XOS improved skin brightness, moisture, elasticity, spots, UV spots, and brown spots of subjects, indicating that prebiotic juice may provide an alternative route to daily skincare. However, the underlying mechanism of XOS for skin improvement is still unclear (Chang et al., 2020). In another study, the addition of XOS to rice cakes effectively reduced cooking loss and promoted the regeneration of starch, decreasing the digestibility rate (Yang et al., 2021).

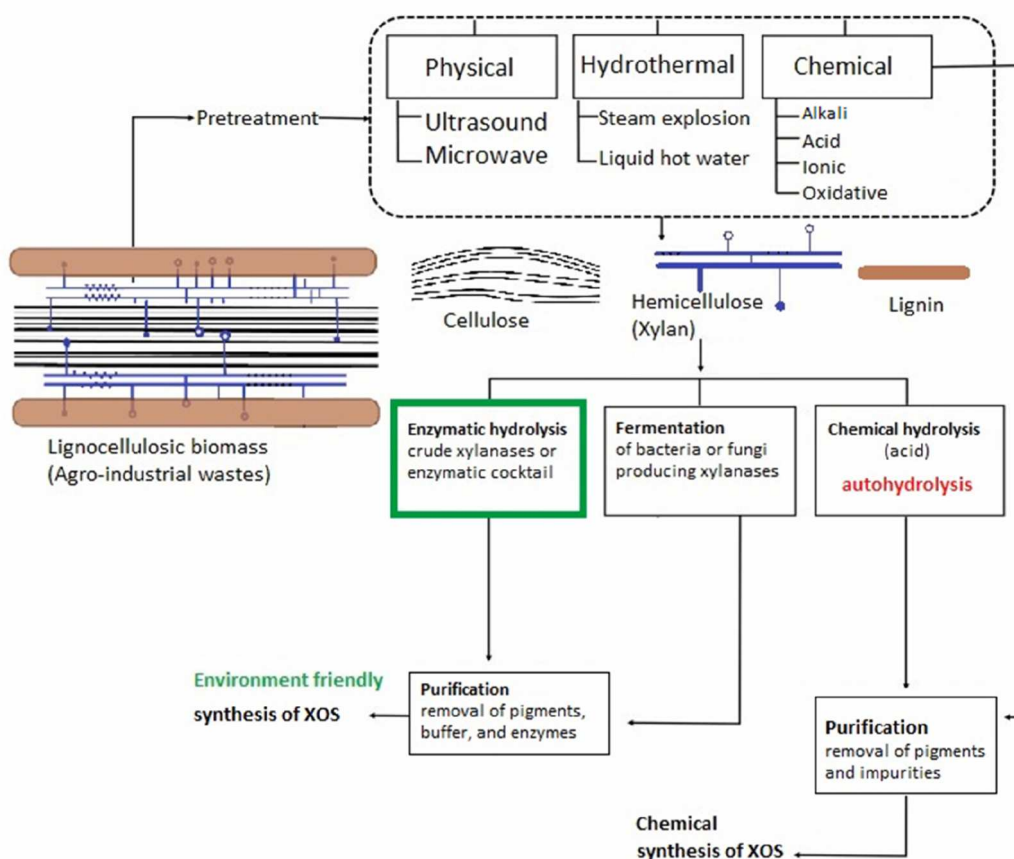


Fig. 8. Steps for XOS production from lignocellulosic biomass.

Table 6

Agro-industrial wastes as raw materials to produce XOS with different pretreatments.

| Feedstock            | Method                                            | XOS yield (xylan conversion) | Other value-added bioproducts                            | Ref.                        |
|----------------------|---------------------------------------------------|------------------------------|----------------------------------------------------------|-----------------------------|
| Brewers spent grains | Steam explosion and amyl-glucosidase treatment    | 74.2%                        | Xylitol                                                  | Swart et al. (2021)         |
| Miscanthus           | Extrusion and autohydrolysis                      | 32.5%                        | Cellulose microfibrils and biogas                        | Lan et al. (2021)           |
| Paper mulberry       | Acetic acid pretreatment and enzymatic hydrolysis | 40.72%                       | Amino-acids and monosaccharides                          | (Zhao et al., 2022a, 2022b) |
| Rice and corn bran   | Enzymatic hydrolysis                              | 32 and 33.9%                 | Polyphenols                                              | Mehta et al. (2022)         |
| Sugarcane bagasse    | Steam explosion                                   | 29.62%                       | ethanol, cellulose nanofibrils, and lignin nanoparticles | Pereira et al. (2021)       |
| Wheat straw          | Steam explosion                                   | 91%                          |                                                          | Álvarez et al. (2017)       |

XOS is also incorporated into hydrogel structures to improve gel strength and better conserve compounds such as vitamins or probiotics. Microparticles of alginate containing 3% XOS increased the survival rate of encapsulated *Lactobacillus acidophilus* during refrigerated storage and simulated digestion by increasing the gel strength and reducing the pore size of the capsule (Martins et al., 2020). In another study, XOS influenced the rheological properties, contributing to forming a gel network with high viscosity to protect the encapsulated vitamin complex (A + D + E) based on pectin and lentil protein (Mekala et al., 2022).

### 3.4. Perspectives and opportunities

At present, most XOS production methods still face drawbacks. Although enzymatic hydrolysis provides greener and more efficient production, it requires biomass pretreatment and downstream decolorization. Additionally, XOS-related products are not widely present in the market, so future research should focus on the following aspects: i) optimization of the enzymatic hydrolysis and purification processes in a biorefinery concept of valorization of lignocellulosic biomass; ii) development of food, medicines, feed, and health products containing XOS to increase its market presence and further investigate its bioactivities.



Agro-industrial wastes represent an ideal source for obtaining XOS due to their low cost and high availability. The recalcitrance of the material remains a challenge, so biomass fractionation technologies should be focused on improving the sustainability of processes, preferably those that are easy to operate and under continuous mode in the context of a circular bioeconomy. More research should be conducted at a pilot-scale and industrial level to improve the feasibility of the production processes.

#### 4. Conclusion

The bibliometric analysis of XOS research showed that it is a growing and global research field with many well-established concepts and emerging technologies that direct toward new potential industrial solutions and applications. Globally, 68 countries are engaged in XOS research, being China the most engaged country, followed by the USA, Japan, and Brazil. The field of gut microbiota showed to be both a consolidate and a trend topic. Based on the most cited documents, enzymatic hydrolysis of agro-industrial wastes is a current focus of XOS research, as well as XOS separation and purification, which still need further study to improve the purity and lower the cost. These studies may have a strong impact and lead to cost reduction for XOS production from agro-industrial wastes, pre-treatment technologies, and enzyme engineering in a biorefinery concept, as well as a better understanding of the health effects of XOS regarding the microbiota and immunomodulation.

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#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

No data was used for the research described in the article.

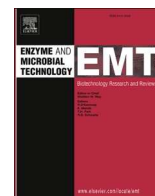
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## Review

## Recombinant chimeric enzymes for lignocellulosic biomass hydrolysis

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## ABSTRACT

Chimeras with improved properties can be designed by synergistically incorporating multiple proteins into one peptide chain. Such design enhances hydrolysis efficiency and lowers enzyme production costs for numerous applications, particularly in the fields of lignocellulosic biomass hydrolysis, plant-based industries, food additives, waste treatment, and biofuels. In this minireview, we summarize a brief, common approach to construct recombinant enzymes, especially in the *Pichia pastoris* host. We present the design strategies and the desired properties of chimeras and provide examples of recent studies in the area of lignocellulosic biomass hydrolysis. Subsequently, we describe the progress in structure prediction tools that facilitate the design of chimeric proteins toward identifying the best combinations of fusion partners and linkers.

## 1. Introduction

The application of enzymes in various industrial processes is well established. Enzyme-catalyzed reactions occur at moderate temperatures, are stereospecific, and eliminate the use of toxic reagents, which is particularly beneficial for the synthesis of biomolecules for food or pharmaceutical purposes [1].

The advent of purification techniques and genetic engineering improved enzymatic processes applied in the manufacture of biofuels, resins, processed meats, cheeses, juices, oils, and alcoholic beverages, among other products [2]. Special attention has been given to the hydrolysis of lignocellulosic materials: these structures are varied and complex, so their complete degradation requires a coordinated action of an enzymatic complex [3].

Several vegetables are grown in large quantities in the agroindustry and generate massive amounts of wastes. The diverse constitution of these wastes opens up numerous application opportunities for the development of new products in the food, chemical and pharmaceutical industries. Increasing knowledge about biopolymers, willingness to develop new bioproducts, and the fact that agro-industrial residues constitute an abundant source of lignocellulosic biomass have intensified efforts to develop enzyme cocktails that can deconstruct and convert these materials in an economically viable way [4–6].

## 2. Lignocellulosic enzymes

Lignocellulosic materials are the major constituents of plant biomass and consist mainly of three macromolecules [7]: cellulose (40–50 %), hemicellulose (heteropolysaccharide derived from monosaccharides, such as arabinose, xylose, mannose, and rhamnose) (20–30 %), and lignin (4-hydroxyphenylpropanoids aromatic blocks from hydroxycinnamyl, coniferyl and sinapyl alcohols) (10–15 %) [8], all of which can be hydrolyzed to products of interest. A smaller portion of the biomass comprises pectin, proteins, waxes, and extracts. The amount of each component varies with the biomass source, part, and age [7].

Cellulosic lytic complex is responsible for the hydrolysis of the main polysaccharide in the cell wall, cellulose. Endo-1,4- $\beta$ -D-glucanase (EC 3.2.1.4) randomly hydrolyzes cellulose chains, releasing cellobiose, which is hydrolyzed to glucose by action of  $\beta$ -glucosidase or cellobiase (EC 3.2.1.21). Cellobiose dehydrogenase is an auxiliary enzyme that oxidizes cellobiose, and cellobiose phosphorylase catalyzes the reversible phosphorylation of cellobiose into  $\alpha$ -D-glucose-1-phosphate and D-glucose. These enzymes activate other hydrolytic cellulases by relieving their inhibition by cellobiose. Exoglucanases have affinity for the crystalline sites along external cellulose chains. They include the 1,4- $\beta$ -D-glucan glucohydrolase (EC 3.2.1.74), which releases glucose from 1,4- $\beta$ -D-glucans and slowly hydrolyze cellobiose, and 1,4- $\beta$ -D-glucan cellobiohydrolase (CBH) (EC 3.2.1.91), which releases cellobiose from 1,4-

Aminoacids abbreviations: Ala, A, alanine; Glu, E, glutamic acid; Gly, G, glycine; Lys, K, lysine; Pro, P, proline; Ser, S, serine.

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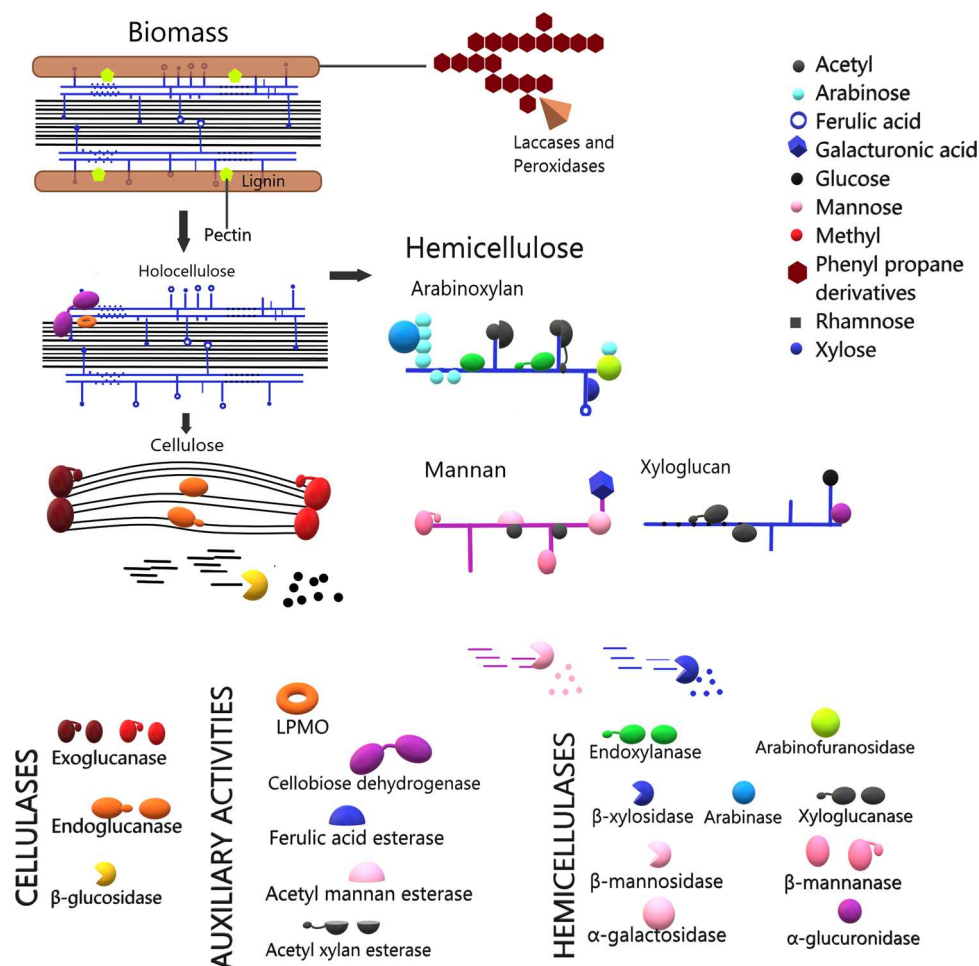


Fig. 1. Lignocellulosic biomass enzymatic lytic complex.

$\beta$ -glucans from the reducing- (CBH I) or nonreducing ends (CBHII). Moreover, CBH can hydrolyze highly crystalline cellulose [9–11]. LPMOs (lytic polysaccharide monooxygenases) also aid crystalline cellulose degradation. These enzymes are described as metalloenzymes (EC 1.14.99) and break glycosidic bonds by oxidizing C1 and C4 or both [12, 13].

Delignification of the phenylpropane polymeric structures involves laccases (EC 1.10.3.2), which oxidize phenolic compounds and aromatic amines. Lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (EC 1.11.1.13) are able to oxidize lignin at the phenolic and non-phenolic aryl-ether positions, respectively [14].

Mannans, which are hemicellulose polysaccharides, are degraded by the synergistic action of three enzymes: (1)  $\beta$ -mannanase (EC 3.2.1.78) acts on the main chain; (2)  $\beta$ -mannosidase (EC 3.2.1.25) releases mannose from the non-reducing ends; (3)  $\beta$ -glucosidase (EC 3.2.1.21) releases monosaccharides cleaving  $\beta$ -D-1,4-glucopyranose in the final non-reducing region of the oligosaccharides that are released from glucomannan and galactoglucomannan cleaved by (1)  $\beta$ -mannanase. Acetyl mannan esterase (EC 3.1.1.6) and  $\alpha$ -galactosidase (EC 3.2.1.22) cleave the acetyl group bonds [2,15].

Xylan, also a hemicellulose polysaccharide, has a complex and heterogeneous nature, so its enzymatic hydrolysis is more complicated as compared to the enzymatic hydrolysis of other polysaccharides. Different enzymes are required depending on the specific type of xylan and on the desired product. Arabinofuranosidases, esterases, endoxylanases, and xylosidases are among the enzymes involved in xylan depolymerization [11]. However, as substituents are removed, xylan becomes less soluble and forms aggregates that hinder and further delay

its degradation. The simultaneous removal of these substituents and cleavage of the glycosidic bonds of the main xylan chain increase the degradation rate. Therefore, mixtures of enzymes with synergistic action are desirable and have been the target of intense research to understand their mode of action and optimize enzymes loadings [11,16].

Endo-1,4- $\beta$ -xylanases (EC 3.2.1.8) cleave glycosidic bonds between xylose units that constitute the main chain, releasing xylo-oligosaccharides. However, to improve xylan depolymerization, other enzymes that act on the side chains are needed: acetyl xylan esterase (EC 3.1.1.6) removes the O-acetyl groups from positions 2, 3, or both in  $\beta$ -D-xylopyranosyl residues;  $\alpha$ -glucuronidase (EC 3.2.1.131) hydrolyzes  $\alpha$ -1,2 bonds between glucuronic acid and  $\beta$ -D-xylopyranosyl residues found in glucoxylans; arabinase removes L-arabinose from positions 2, 3, or both in  $\beta$ -D-xylopyranosyl residues; and ferulic acid esterase (EC 3.1.1.-) and p-coumaric acid esterase (EC 3.1.1.-) cleave ester bonds from the xylan side chains.  $\beta$ -1,4-D-xylosidases (EC 3.2.1.37) hydrolyze small xylo-oligosaccharides and xylobiose, releasing xylose [17,18].

The potential application of lignocellulosic enzymes (Fig. 1) includes bioconversion of agro-industrial residues into biofuels and production of high value added products, such as prebiotics (xylo-oligosaccharides) and sweeteners (xylitol); juice clarification; beverage consistency and animal feed digestibility improvements [2,16,19].

### 3. Heterologous expression

Enzymatic hydrolysis is crucial for biofuel production and lignocellulosic biomass conversion: it is a controlled and selective process that demands less energy and milder conditions than acid hydrolysis.

However, costs for enzymes production are still a hurdle. Great effort has been made to increase efficiency of enzyme cocktails [2].

To meet industrial needs, the employed enzymes must present certain properties such as stability over a wide range of pH and temperature, high specific activity, and resistance to cations. In addition, enzyme production needs to be efficient, environmentally friendly, and easily reproducible. Generally, native enzymes do not meet these demands because their production rates are low, which is incompatible with industrial processes. Therefore, gene expression tools are necessary to produce recombinant enzymes with better activity, selectivity, and stability [20].

The data available from sequencing, crystallography, molecular dynamics, and computational design provide all the essential information to efficiently produce recombinant enzymes with distinct properties. In this way, a specific process can be optimized for a specific substrate, which is an important strategy to deconstruct the biomass completely and obtain the industrial products of interest while reducing costs and saving resources [2,19].

As the most engineered prokaryote, *Escherichia coli* is the first host cell to be considered because it is well characterized, and its genetics is easy to manipulate. Nevertheless, expression in *E. coli* poses countless problems such as linker sequence degradation in multi-domain proteins, production of low levels of soluble proteins, and poor secretion ability, which makes this microorganism unsuitable for large-scale production [21]. *Zymomonas mobilis* is also an amenable host for expression of enzymes secreted to the periplasma or extracellularly. Just like *E. coli*, part of the expressed enzymes is intracellular. However, the fusion of signal peptides allows improved secretion into the media [22].

Eukaryotic microorganisms that express heterologous enzymes are more attractive because they offer additional advantages over other expression systems: they allow post-translational modifications, grow under high cell densities, and provide high protein secretion into the medium. Furthermore, they are toxin-free and are generally recognized as safe (GRAS) organisms [23]. The yeast secretion pathway generates favorable disulfide bridges and glycosylation, which increases enzyme stability and functionality. Glycosylation, which typically connects the catalytic domain with the carbohydrate-binding module region, offers protection against protease action and increases affinity for the substrate. This paves the way for more active and thermotolerant protein engineering [24].

*Saccharomyces cerevisiae* is the most engineered host due to high productivity, well established genetic tools and high tolerance to inhibitors, while *Kluyveromyces marxianus* possess the desirable thermotolerance [25], and *Saccharomyces pastorianus* the improved enzyme activity [26]. However, *Pichia pastoris* has become one of the most popular hosts for heterologous expression systems because this species produces structurally and functionally correct proteins [27]. *P. pastoris* grows rapidly in minimal medium and efficiently secretes recombinant protein, thereby allowing for savings with culture media and protein purification. Its genome is simple to manipulate, and commercial expression kits involving inductive or constitutive promoters are available [20]. Moreover, to optimize the downstream process, additional sequences, such as a polyhistidine or antibody recognition peptide sequence, may be inserted in the C-terminal peptide of the integrative plasmids used in the expression systems, so the recombinant protein can be detected and isolated with purification kits. These sequences do not affect the protein structure or biological function [28].

The success of the *Pichia pastoris* expression system was initially attributed to the inductive promoter methanol oxidase  $P_{AOX1}$ , the first isolated enzyme to be used for vector development and genetic manipulation protocols. Albeit effective, expression is induced only upon addition of methanol, which is flammable and poses serious health risks, especially when operated on a large scale. On the other hand, constitutive promoters enable protein synthesis to occur simultaneously to cell growth, since they are correlated to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene regulation. GAPDH (EC 1.2.1.12) is constantly

expressed at high levels because it participates in the glycolysis and gluconeogenesis pathways [20,29].

Given the recent advances in the development of expression systems, constitutive expression promoters are widely employed. They avoid the issues arising from the use of methanol as an expression inducer, simplifies the process, and reduces costs. These systems are stable and efficient, thus elevating protein production at the transcriptional level [20,27,29].

Not only thermal stability, but also high production and high catalytic performance are extremely relevant for the biotechnological and industrial application of enzymes. Many cellulosic and hemicellulosic enzyme genes produced by various fungi and bacteria have been cloned and expressed in *Pichia pastoris* [30–34]. Also, from an industrial viewpoint, understanding enzymatic synergism is essential to accelerate biomass conversion and reduce the required amount of enzyme and hydrolysis time. These factors significantly affect production costs [2].

Despite all the efforts to isolate and to express efficient enzyme genes, the costs associated with enzyme technologies remain a major obstacle [24]. One strategy to overcome this issue is to design multifunctional enzymes with synergistic catalytic activities. Rational protein design enables the creation of polypeptides with multiple catalytic domains and specificity for two or more substrates [35]. Multifunctional enzymes have numerous advantages. First, they simplify the expression path, facilitating metabolic pathways. Second, several reactions are regulated simultaneously in a single polypeptide chain. Thus, chimeric proteins have been developed to reduce production costs and to improve the performance of catalytic domains [35–37].

#### 4. Chimeric proteins

Three billion years of evolution have selected excellent catalytic proteins. However, these proteins are generally not optimal for an industrial application. Even though enzymes of extremophiles with appropriate activity for a specific process can be searched, the nature has selected these enzymes to act in a complex mixture of molecules, either inside the cells or in the environment where these organisms live, rather than in industrial bioreactors. To obtain all the desired properties, more advanced optimization is necessary [1]. In addition to the production of recombinant enzymes, another approach has been used: proteins with different characteristics have been coupled by genetic fusion at the translational level. This approach generates multifunctional enzymes in a single polypeptide chain, which facilitates regulatory mechanisms and optimizes enzymatic performances [38].

Although mixtures of enzymes are widely employed in the industry, many studies have reported that chimeric enzymes perform better than enzyme cocktails. A chimera that catalyzes consecutive reactions can immediately transfer the intermediate to the next active site in the corresponding enzyme. Consequently, the intermediate is consumed before its distribution in the solution, which increases reaction efficiency [36,37,39]. Apart from their synergistic effect and easier production, two or more fused proteins may have their physical and chemical properties combined, such as temperature and pH profile. As a result, fused proteins have equally stable and active biocatalysis under the same operational conditions [38,40].

Various multi-domain proteins, including cellulosomes, occur naturally [41,42]. The availability of sequential data has increased dramatically, which has heightened awareness of the complexity to which the mechanisms of genetic rearrangement have evolved. As a consequence, multimodular proteins, the chimeras, make up a large proportion of an organism's protein complement [43]. Inspired by nature, bioengineers have been developing methods that mimic natural processes to produce chimeric proteins by synthetic domain fusion [36,41,44]. The proximity between the catalytic domains of fused enzymes improves enzymatic performance as compared to the activities of the parent enzymes. Chimeric proteins are built for several purposes, including greater stability, expression efficiency, affinity, and specificity. Creating

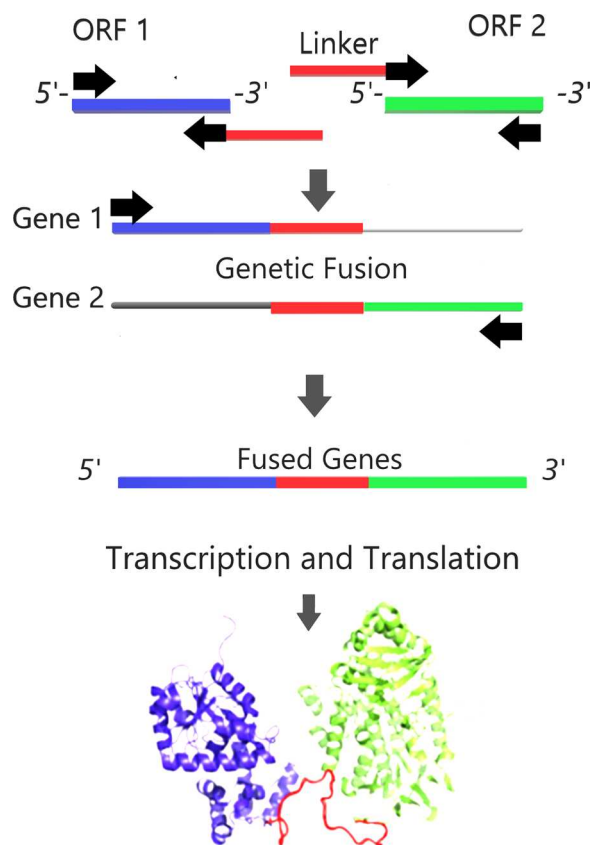


**Fig. 2.** Tandem fusion schematic illustration of the construction of a chimera by PCR overlap (black arrows indicate primer orientation).

bifunctional enzymes to act on lignocellulosic materials has proven to be a practical option to optimize enzymes that are involved in depolymerization [44].

Although different strategies exist, some general rules must be followed to provide adequate functionality to the proteins. Regardless of the protein fusion procedure, these rules include translation efficiency; the order of the fused structures depending on molecular size, structure and thermostability; and fusion flexibility [36,38]. Furthermore, a key factor to succeed in creating chimeras is to achieve compatible catalytic activities in relation to the physical-chemical requirements (e.g. optimal pH and temperature) of the individual proteins [44].

Recently, numerous chimeras have been developed to optimize lignocellulosic biomass hydrolysis. A laccase/glucanase chimera developed using domain insertion was 20 % more active on sugarcane bagasse as compared to the mixture of these enzymes [45]. An endoxylanase/endomannanase chimera built by tandem fusion displayed synergistic activity in hemicellulose hydrolysis [39]. A xylosidase/arabinofuranosidase chimera obtained by tandem fusion released 30 % more xylose [42]. A laccase/endoxylanase built by domain insertion provided to the endoxylanase domain a significant increase in thermostability and resistance to proteolytic attack [44]. The fusion of two complementary enzymes, xylanase and xylosidase, resulted in a chimera with higher optimum temperature and thermostability for the xylosidase domain, which enhanced xylose production as compared to the parent enzymes [35].

Synthetically fused proteins are widely applied for process optimization. Three strategies are used to promote domain fusion: tandem fusion (end-to-end), domain insertion, and post-translational conjugation. Computational prediction of the structures follows the progress of these strategies. Even though the aforementioned strategies focus on the fusion of two proteins, they are also applicable for multicomponent systems.

#### 4.1. Tandem fusion

Tandem fusion is the predominant method used to synthetically join domains to build chimeric proteins (Fig. 2). In tandem fusion, two or more sequences that encode proteins (Open Reading Frames - ORFs) are connected end-to-end, with or without a linker sequence, to produce a single polypeptide chain with bi or multifunctional characteristics [36, 38]. This method is preferable because it is the least dependent on the structural knowledge of proteins, so it requires the use of special equipment less frequently. Albeit simpler, this method demands that several parameters be met to adapt the structural arrangement and to ensure chimera functionality [35,36].

Factors that determine the success of protein fusion include spacing between modules, individual receptor structures and their correlation, and linker size and complexity. The first gene to be fused must be free of the stop codon, and the last gene must not contain the signal peptide or the ribosome binding site. The order in which genes are inserted is extremely important because it affects the chimera's conformation, structure, and physical-chemical properties [38]. The N-terminal residue, referring to the free amine group ( $-NH_2$ ) at the start of the polypeptide chain, plays a decisive role in protein half-life. Therefore, the gene of the most thermostable protein should be placed in the N-terminal position to make the next protein more thermally stable [46].

Direct fusion works for cases in which the N-terminal and C-terminal (the end of the polypeptide chain terminated by a free carboxyl group ( $-COOH$ )) of the ligand sequences are flexible and do not have a regular secondary structure or a catalytic function. Nevertheless, if these terminals are essential for catalytic function or are not flexible or long enough to avoid steric hindrance, a short amino acid sequence (the linker) must be added to bind the proteins and to improve conformation and bioactivity. An optimized linker improves the levels of expression, corrects conformation, minimizes steric influences due to proximity of the catalytic domains, and maximizes module flexibility [40,47].

##### 4.1.1. Linker

Natural multi-domain proteins consist of two or more domains that are joined by a linker sequence. This sequence of peptides connects the modules and helps to maintain inter-domain cooperative interaction and to preserve bioactivity. They can adopt varied secondary structures, such as  $\alpha$ -helix,  $\beta$ -strand, coil, and turns. Helix, which is rigid and stable, and coil, which is more flexible, are the most common structures. Knowledge about natural linkers is relevant to rationally design recombinant linkers to synthetically fuse recombinant enzymes [41,47, 48].

Rational linker design started with the development of the LINKER software [49], which used an online server to automatically generate binding sequences of determined length to link proteins. The program searches through a library of loops derived from the Brookhaven Protein Databank (PDB) and suggests the most favorable loop for the input. Subsequently, new automatic prediction tools from a database of sequences extracted from natural multi-domain proteins (e.g., LinkerDB) were developed to provide candidates that respected the protein sequence, length, secondary structure, and PDB code [47], engendering the SynLinker, the most updated (<http://bioinfo.bti.a-star.edu.sg/synlinker>). In addition, many databases are available and can be accessed; for example, iGEM ([http://parts.igem.org/Protein\\_domains/Linker](http://parts.igem.org/Protein_domains/Linker)) and the Center for Integrative Bioinformatics at the University of Amsterdam (<http://www.ibi.vu.nl/programs/linkerdbwww/>).

The properties of natural linkers, like length, hydrophobicity, amino acids, and secondary structure, serve as a reference for the design of empirical linkers for recombinant proteins [48]. Linkers are usually categorized by their length, amino acid composition, hydrophobicity, secondary structure, sensitivity to proteases, and solubility. However, the most comprehensive and prevalent way to classify linkers is based on flexibility, rigidity, and sensitivity to proteases [36,41]. Table 1 lists some examples of linkers that are used in chimeric hemicellulosic

**Table 1**

Examples of linkers and their purposes in hemicellulosic chimeric proteins.

| Example                        | Type              | Sequence                          | Function                       | Ref. |
|--------------------------------|-------------------|-----------------------------------|--------------------------------|------|
| Endoxylglucanase               | Flexible          | (GGGG)                            | Flexibility                    | [50] |
| Endoxylanase-xylosidase        | Natural, flexible | <i>Thermotoga petrophila</i> 0899 | Steric hindrance prevention    | [35] |
| Endoxylanase-mannase           | Rigid             | (EAAAK) <sub>3</sub>              | Improves thermostability       | [39] |
| Endoxylglucanase               | Flexible          | (GGSGG)                           | Flexibility                    | [51] |
| Xylosidase-arabinofuranosidase | Flexible          | (GGGGG...N)                       | Steric hindrance prevention    | [42] |
| Xylosidase-arabinosidase       | Flexible          | (SAGSSAAGSGSG)                    | Improve stability and activity | [52] |

proteins produced by tandem fusion.

#### 4.1.2. Flexible linker

Flexible linkers consist of small sequences that are rich in glycine and alanine. This type of linker is applied when the merged domains require more freedom of mobility or interaction. The presence of polar amino acids (such as serine, glutamic acid, and lysine) increases stability in aqueous solvents through hydrogen bond formation [48]. The most often employed flexible linkers appear in the form of (Gly)<sub>n</sub> and (Gly-Gly-Gly-Gly-Ser)<sub>n</sub>, where “n” is the number of residues. This “n” is often lower than six, and the linkers form a loop [41,48]. The use of linkers where n = 3 promotes greater catalytic activity, and the Michaelis constant K<sub>m</sub> value is lower as compared to a rigid linker [39]. Short glycine-rich sequences allow greater mobility without disturbing enzyme conformation. In addition, short sequences make proteolytic attack less likely during protein expression and purification [50,51].

Although glycine-rich sequences provide the necessary interdomain flexibility, monotonous ligands (GGGG or GGGGS or GGGSGGGGS) can limit the catalytic activity. Furthermore, glycine-rich regions may decrease the level of expression by creating mRNA with stable secondary structure, and codons that are rich in guanidine (G) and cytosine (C) (GGN for glycine and GCN for alanine, where N can be any nucleotide base) may be unacceptable for some microorganisms [53].

However, some studies on the optimization of peptide ligands have suggested that addition of alanine and serine affects the flexibility of these ligands and can increase the enzymatic activity in different ways: alanine accelerates folding, and serine slows down unfolding [52]. Combining small amino acids like alanine and serine with glycine-rich sequences and increasing the sequence length can overcome these problems, thereby maintaining flexibility and increasing catalytic activity. As noted in Table 1, proteins that require more distance between the domains to avoid steric hindrance and to increase catalytic activity were constructed with longer ligands. Nevertheless, proteases can cleave longer linkers more easily [54].

In short, flexible linkers are sequences that are generally rich in small and/or polar amino acids. These linkers promote good flexibility and solubility and are suitable to fuse domains that require freedom of movement or interaction. Although these linkers do not have a rigid structure, they can passively maintain the distance between the domains, helping to adjust the length of the fused proteins and to elicit their adequate folding and biological activity [48].

#### 4.1.3. Rigid linker

While flexible linkers are advantageous because they passively connect the catalytic domains, hence allowing the modules to be somewhat mobile, the lack of rigidity or insufficient spacing between the domains can be a limitation. In these cases, establishing a fixed distance between the domains can ensure the maintenance of the independent activities of each enzyme [48]. Numerous natural linkers have α-helix structure [47], which is stabilized by Glu<sup>−</sup> Lys<sup>+</sup> bridges. These linkers can act as rigid spacers to inhibit interactions between the domains. This type of linker was discovered in proteases and pyruvate dehydrogenases [48].

On the basis of these natural ligands, empirical linkers A(EAAAK)<sub>n</sub> (where 2 < n < 5) have been developed and compared to flexible

**Table 2**

Summaries of linker classes and characteristics.

| Linker    | Advantage                                | Characteristics                          | Example                                           |
|-----------|------------------------------------------|------------------------------------------|---------------------------------------------------|
| Flexible  | Interactions and spacing between domains | Rich in small or hydrophilic amino acids | (G) <sub>n</sub><br>(GGGGS) <sub>n</sub>          |
| Rigid     | Fixed distance between domains           | α-helix structure                        | (EAAAK) <sub>n</sub>                              |
| Cleavable | Domain separation                        | Cleaved by proteases or solvents         | (XP) <sub>n</sub><br>Protease-sensitive sequences |

linkers of the same length, showing to separate the domains more efficiently [55]. Another type of rigid linker bearing a proline-rich sequence, (XP)<sub>n</sub>, where X can be an alanine, a lysine, or a glutamine, has been developed. The presence of proline increases rigidity by favoring an α-helix structure, the length of which is adjusted to achieve the ideal distance between the domains. Apart from having a circular and solid structure, proline contains an amide group that lacks hydrogen bonds, which prevents interactions with other amino acids [47]. Studies comparing (GGGGS)<sub>n</sub> and (EAAAK)<sub>n</sub> linkers (where 2 < n < 4) have shown that the rigid linker provides greater thermostability [39] but lower catalytic activity [39,53].

#### 4.1.4. Cleavable linker

The two groups of linkers presented above consist of stable peptide sequences that preferably will not be cleaved. Stable ligands promote a longer half-life; however, as mentioned earlier, some problems must be overcome, including distance between the domains, reduced levels of expression, and decreased activity. Under these circumstances, ligands that can be cleaved by solvents or proteases can be introduced to release the fused domains in vivo, which eliminates the issue of steric impediment and increases protein bioactivity [48].

A well-studied process uses the reversible nature of disulfide bridges, reduced in vivo. After protein expression, this type of linker allows the domains to be separated and hence recover their individual characteristics, such as bioactivity [56]. In this way, protease-sensitive sequences can be incorporated into linkers and then be cleaved by a specific protease, thus activating the fused protein at a specific site. MEROPS is a specific database of protease-sensitive sequences, so it is an important tool to design this type of linker [57]. Unlike flexible and rigid linkers, there are no reports on the use of cleavable binders to construct lignocellulosic chimeras.

To sum up, linkers can adopt different structures and perform diverse functions to fulfill the requirements of the fused domains, as summarized in Table 2. Flexible linkers are generally rich in small or hydrophilic amino acids (e.g. glycine and serine), which promote interdomain flexibility. Rigid linkers are preferable when a defined spacing between the domains is required. Both flexible and rigid linkers are stable and do not allow the joined proteins to separate, which contrasts with cleavable



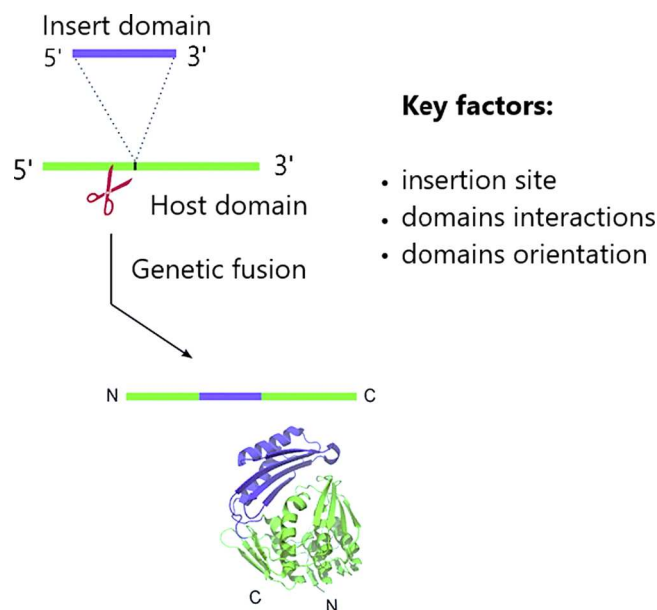


Fig. 3. Domain insertion schematic illustration.

linkers, that are adopted when the recover the individual characteristics of the fused proteins is necessary. A rational choice should be based on the properties of the ligands and fused protein. The rapid development of protein biotechnology has made the design of linkers for protein fusion increasingly important. A better understanding of their structures and functions will facilitate the incorporation of linkers to construct stable bioactive recombinant fused proteins for various applications [48].

#### 4.2. Domain insertion

Domain insertion exists naturally in about 9% of multidomain proteins, especially those belonging to  $\alpha/\beta$  classes [41]. The main advantage of domain insertion is that it allows the insertion of only the catalytic domain, and not the entire protein, in the genomic sequence of another enzyme. This method results in a molecule with lower weight when compared to the molecule that would be obtained by tandem fusion; also, reduces the metabolic stress generated by insertion of a heterologous gene in the host organism and enables the chimera to have more access and penetrability in complex substrates, such as the lignocellulosic biomass [36].

This technique, illustrated in Fig. 3, is more complicated than tandem fusion because it requires structural knowledge of the proteins to find where the domain can be inserted without interfering in the conformation and integrity [45]. In other words, the domain must be inserted in places (such as loops and turns) that are not directly involved with the enzymatic activity [36,41]. Computational methods that can predict how the insertion of a segment can impact protein conformation are often used, e.g., GROMACS [44,45] and SCHEMA [36]. Basically, these programs read the protein structure to identify which interactions between residues may break upon segment insertion [41]. However, conformational prediction does not avoid the issue of steric impediment resulted from the proximity of domains with the consequent decrease in catalytic activity [36,38].

When this method was used for the first time, it involved inserting the domain of a  $\beta$ -lactamase into a protein binding to maltodextrin, which affected the active site of lactamase positively and increased its stability [38]. Rational design based on computational methods allowed a xylanase to be inserted on the surface of a laccase loop, to yield a more accessible domain [44]. Improved stability at acid pH and temperature was achieved by combining the properties of an acidophilic and a

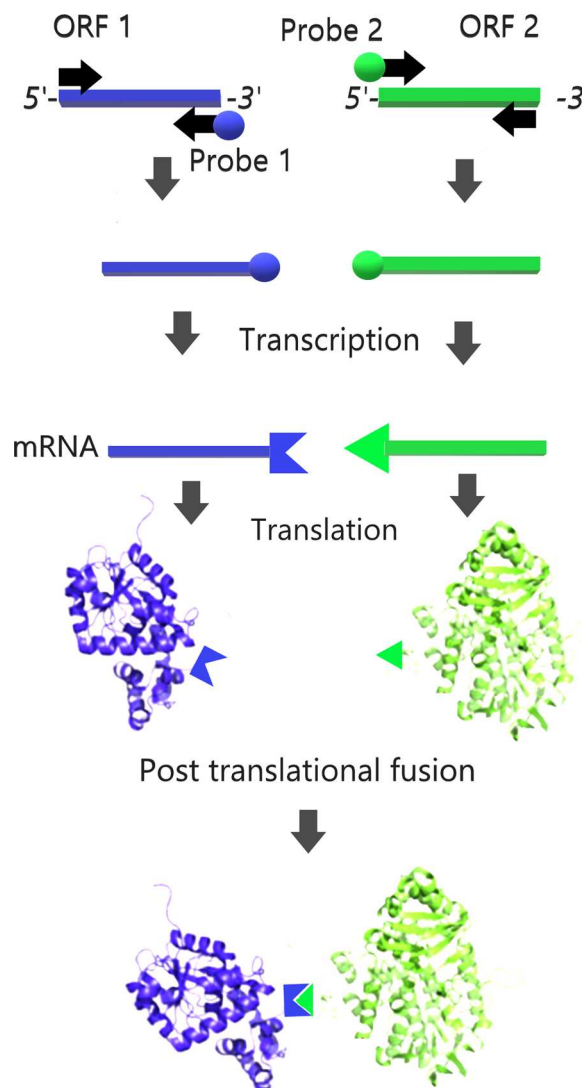


Fig. 4. Post-translational insertion schematic illustration (black arrows indicate primer orientation).

thermophilic endoglucanase [58].

Besides the insertion of domains, other regions can be transferred to the target domain for physical-chemical improvements. Modules from members of the family 10 of the glycoside hydrolases (GH 10) have stood out for this purpose. When the homologous regions of endoxylanases that are responsible for binding with the substrate are known, the exchange of a module for another module from a member with more desirable characteristics, such as thermotolerance [59] and alkali-tolerance [60], can result in a chimera with improved properties with unaffected or improved activity.

#### 4.3. Post-translational conjugation

Another strategy to create fused proteins is to join the individual proteins after their expression. Although the two strategies presented in the previous items represent the most popular methods to produce chimeric proteins, in some cases failures in expression or conformation may occur, impairing biological activity. Post-translational union allows the modules to be paired after they are expressed in optimized hosts, to bypass the limitation of the genetic fusion cloning steps [41].

This post-translational step is also called protein splicing, in analogy with the splicing of RNA introns, and it does not require any co-factor or auxiliary enzyme [38]. Protein splicing can be modulated by mutation

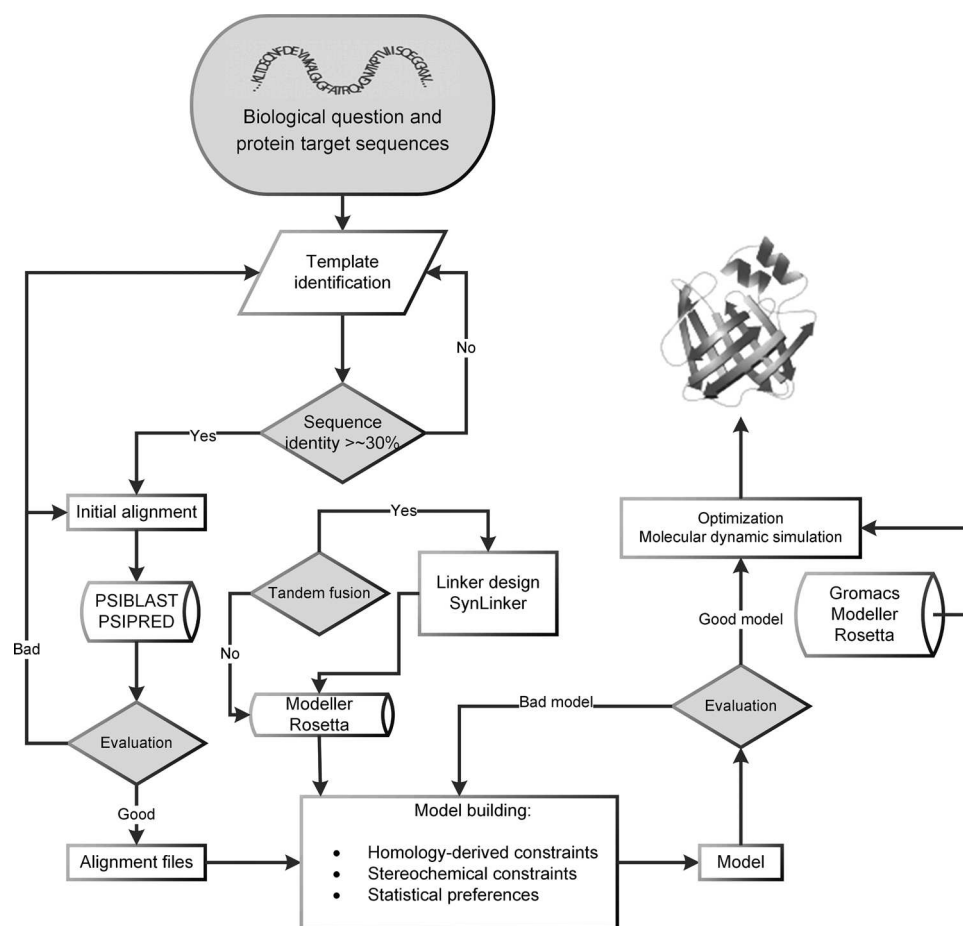


Fig. 5. Flowchart for protein modelling: scheme of the methods used for modeling a chimera.

and converted into highly specific protein self-cleavage and binding reactions, which are useful tools in protein engineering [61].

Proteins in their native conformations can be conjugated by means of a minimum binding tail, which avoids refolding steps. Such methods add small “tags” at the protein N- and C- terminal, in the same way as in the first stage of tandem fusion (amplification of the protein gene coupled to the linker sequence) [62]. For example, sortase-catalyzed transacylation reactions, or “sortagging”, allows proteins to be labelled with small probes (<2 kDa), then site-specific conjugation takes place [63]. Chemical reactions based on non-specific crosslinking via amines and sulfhydryl also enable conjugation, but their products are heterogeneous because the reaction is not controlled [62] (Fig. 4).

## 5. Structure prediction

Protein structures are more conserved than their sequences. A protein that presents 50 % homology has 90 % of its C $\alpha$  atoms positioned similarly, with a variation of 3 Å. This ensures successful structural homology modeling by means of bioinformatic tools [54]. A structure can be predicted by means of a known model structure and an alignment map of amino acid sequences (Fig. 5). Bioinformatic tools like PSI-BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>) [64] allow the target sequence to be aligned with a database of proteins of known structure, to provide the best candidates for modeling. One example is UCSF Chimera (<http://www.rbvi.ucsf.edu/chimera/>), a program for interactive structure visualization and analysis, including load density and sequence alignment. This tool has an interface for the MODELLER software [65], which is a three-dimensional prediction program of protein structures by homologous comparison based on the PDB file of the model sequence and

the file of sequences alignment between the model and the target. Molecular dynamics simulations can be developed using the GROMACS algorithm [66] or GROMOS [67]. Fusion Protein MODELLER [68] is a tool that has been especially developed to model fused proteins, defining regions for flexible linkers between two or more rigid domains.

Predicting chimeric proteins constructed using domain insertion is cinematically more complicated than predicting chimeric proteins built using tandem fusion. The ROSETTA software is one of the tools that help to locate spatial conformational impediment and to identify hotspot residues in the protein-protein interface [69]. This method is applied for *de novo* predictions of the structure of proteins generated by domain insertion [41].

Unexpected consequences like failure of conformation, low level of expression, or impaired activity can happen mainly due to limited knowledge of the structure-function relationship in these proteins. Spatial orientation and interaction between the fused parts are still largely unknown and are often overlooked when new chimeric proteins are designed. Developing computer programs for specifically predicting structure and rational design is an imperative direction, as well as expanding a database of linkers containing new types of linkers with varied properties and more extensive study mechanisms about the linker functions on protein solubility, stability, and activity. Synthetic biology tools, including codon optimization, can be used to overcome problems of expression level. All these available tools contribute to a more rational construction of proteins for the most diverse purposes [39,41,48].

## 6. Conclusion

The diverse industrial use of recombinant enzymes has led to the creation of relevant chimeric enzymes to reduce production costs and

improve the characteristics of the enzymes. In all the methods discussed here, the most critical criteria are maintaining protein conformation and inhibiting overall interference, which in some cases can be achieved with the right choice of the linker sequence. Bioinformatic tools are important to the development of chimeras, the right choice of partners, structure prediction, and molecular simulations. Simplifying and improving large-scale processes involving synergic enzymatic reactions constitute one of the greatest challenges to overcome efficiency issues and production costs of bioenergy and value added biocompounds.

### Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Synergism for xylo-oligosaccharides, $\rho$ -coumaric and ferulic acid production, and thermostability modulation of GH 62 $\alpha$ -L-arabinofuranosidase

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## ABSTRACT

GH 62  $\alpha$ -L-arabinofuranosidases are a group of enzymes that preferably hydrolyze L-arabinose residues of arabinoxylans. Albeit the least studied, these enzymes have gained attention because they exert synergistic action with other xylanolytic enzymes for arabinoxylan depolymerization. This is the first study on the thermodynamic and kinetic parameters, structural prediction, and metal ion effect on the thermostability of a GH 62 arabinofuranosidase subfamily 1. The optimum enzymatic conditions are pH 4.8 and 42 °C, and the presence of  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  ions enhanced the thermostability and activity. The synergism of this arabinofuranosidase with a GH 10 endoxylanase increased the production of xylo-oligosaccharides 2.3 times with additional coproduction of 0.82 mM of ferulic acid and 0.09 mM of  $\rho$ -coumaric acid released from corn hull arabinoxylan (4% w/v). These results are relevant to developing an enzymatic formulation that is economically feasible for industrial applications, especially for prebiotic and antioxidant compounds production in the food and pharmaceutical sectors from sources rich in arabinoxylan, especially agro-industrial wastes of grains husks and peels.

## 1. Introduction

Arabinoxylans (AX) are the main hemicellulosic group found in grasses and grains. They are composed of a xylose backbone, with  $\alpha$ -L-arabinofuranoside residues in the C (O)-3 or -2 positions of the xylose backbone, cross-linked to ferulic acid and  $\rho$ -coumaric acid via an ester linkage. The side chains form covalent bonds with cellulose and lignin, molding a complex that surrounds the cellulose fibers (Zhang et al., 2014). Ferulic acid (FA) and  $\rho$ -coumaric acid (PCA) presence in arabinoxylans confers antioxidant properties and are used in the pharmaceutical and food industries due to their anti-inflammatory and antimicrobial properties, respectively (Cobs, 2020).

The enzymatic hydrolysis of arabinoxylan, especially those from agricultural wastes (grain husks, hulls, and peels) represents an

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economical and sustainable way for industrial applications (Yuwang et al., 2018), such as the production of xylo-oligosaccharides (XOS). XOS are xylose short-chains (with 2–6 xylose units) prebiotic oligosaccharides recognized as functional ingredients for food and pharmaceutical formulations. XOS obtainment from arabinoxylan enzymatic hydrolysis of agricultural wastes is appealing due to the positive effects on microbiota regulation, treating dysbiosis, preventing infections, reducing gut constipation, and facilitating digestion and nutrient absorption (Kumar et al., 2021), as well as antioxidant potential (Valls et al., 2018).

The L-arabinofuranosyl  $\alpha(1 \rightarrow 2)$  and  $\alpha(1 \rightarrow 3)$  bonds in non-reducing polysaccharides are cleaved by  $\alpha$ -L-arabinofuranosidase (EC 3.2.1.55). The GH 62 family solely contains arabinofuranosidases that preferably hydrolyze L-arabinose residues attached to the side chain of arabinoxylans, in contrast to most GH 43 and GH 51 enzymes, which show a preference for  $\alpha(1 \rightarrow 5)$  arabino-oligosaccharide bonds (Kaur et al., 2015). Furthermore, structural analysis of GH 62 arabinofuranosidase reported it potentially accommodates a feruloyl group attached to arabinose from the arabinoxylan side chains. This means GH 62 can release feruloyl L-arabinose residues (Tonozuka and Tanaka, 2017), which are efficient antioxidants that can be used in food, medicine and cosmetics (Lin et al., 2014). However, this family has been the least studied (Kaur et al., 2015).

Phylogenetic analysis has indicated that four GH 62 subfamilies exist, but information about whether this division represents distinctions in enzymatic properties is scarce. The first studies on GH 62 structural determination and characterization were only published in 2014 (Wilkins et al., 2017). To deepen our understanding of GH 62 enzymes, this study presents the thermodynamic and biochemical characterization of the GH 62  $\alpha$ -L-arabinofuranosidase from *Aspergillus fumigatus*, and the synergism for enhancement in ferulic acid (FA), p-coumaric acid (PCA), and xylo-oligosaccharides (XOS) release. Our previous study reported the synergistic effect of GH 51 arabinofuranosidase and GH 10 endoxylanase for XOS production (Martins et al., 2020). To date, however, there are no previous studies on the application of GH 62 enzymes to improve hemicellulose fraction processing in the food industry.

## 2. Materials and methods

### 2.1. Material

Corn seed hull was donated by Milhão Ingredients (Goianira, Brazil). Composition characterization followed the NREL methodology (Sluiter et al., 2005, 2008) and is described in Supplemental Table S1. Corn hull arabinoxylan was extracted with 5% KOH as described elsewhere (de Figueiredo et al., 2017). Wheat arabinoxylan was purchased from Megazyme Ltd. (Wicklow, Ireland). All other chemicals were reagent grade.

### 2.2. Protein prediction and bioinformatic analysis

The Afua\_2g12770 gene (Gene ID 3513529) was analyzed by using the PSI-BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>), Clustal Omega tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), and InterProScan Sequence Search (<http://www.ebi.ac.uk/Tools/pfa/iprscan>). The putative N- and O-glycosylation sites were located by using NetNGlyc1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and NetOGlyc3.1 (<http://www.cbs.dtu.dk/services/NetOGlyc/>), respectively. The structural model by the comparative modeling used MODELLER9 v12 and was validated with PROCHECK. Polar hydrogens were added, and Gasteiger partial charges were assigned. Charges were computed using ANTECHAMBER Protein-ligand docking was carried out by using Autodock Vina and visualized with UCSF Chimera 1.14. The 3D structures of feruloyl arabinobiose (CID 6438898) and xylotetraose (CID 10230811) were downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov>). The docking grid box centered on the conserved catalytic glutamic acid residue ( $85 \times 80 \times 60$  Å), and the poses were ranked by their predicted binding energy and inspected based on the correct geometry between catalytic residues and substrates.

### 2.3. Expression of recombinant gene

The pGAPZ $\alpha$ A vector (Invitrogen™, USA) was used for cloning, sequencing, and expressing the Afua\_2g12770  $\alpha$ -L-arabinofuranosidase (Gene ID 3513529). The Afua\_2g12770 recombinant gene (948 pb, without signal peptide and TAG codon) was synthesized by GenOne (Brazil) and inserted between the *Eco*RI and *Xba*I sites. *Escherichia coli* DH5 $\alpha$  was heat shock-transformed and cultivated at 37 °C and 200 rpm in low-salt Luria-Bertani (LB) medium with 100  $\mu$ g/mL Zeocin (Thermo Fisher Scientific™, USA), to propagate the recombinant plasmid. The plasmid containing the gene was extracted, linearized with the *Bsp*HI enzyme (5' ... T<sup>+</sup>CATGA ... 3', Thermo Scientific™, USA), and transformed into electrocompetent *Komagataella pastoris* X-33 by electroporation (1500 V, 5 ms). The transformants were selected for Zeocin resistance on YPDS plates containing 100  $\mu$ g/mL Zeocin according to the transformation protocol No. 4308 915.545–03/2004 (Eppendorf). Gene insertion was confirmed by PCR, by using the 5' GAP forward and 3' AOX1 reverse primers recommended by Invitrogen (2010). The PCR conditions were 30s at 95 °C, 30 cycles of 30s denaturation at 95 °C, 60s hybridization at 53 °C, and 90s elongation at 68 °C, followed by a final 5 min extension at 68 °C, using Taq DNA polymerase (Sinapse Inc.™, Brazil).

### 2.4. Enzyme production, purification, and activity

Small-scale enzyme production was performed in 50 mL of YDP (in a 250-mL Erlenmeyer) incubated at 30 °C and 250 rpm for 96 h, as recommended by Invitrogen (2010). The culture supernatant containing the secreted enzyme was concentrated 100 times by ultrafiltration (Amicon Ultra-15, Merck KGaA, Germany) with a molecular weight cut-off of 10 kDa. Then, the enzyme was purified with the HisPur kit (Thermo Scientific™, USA), as recommended (equilibration buffer: 10 mM imidazole; wash buffer: 25 mM imidazole; elution buffer: 250 mM imidazole. All buffers contained 10 mM PBS pH 7.4). The resin column was equilibrated with 2 resin-bed volumes of equilibration buffer, centrifuged at 700 g 2min. The sample was mixed to the equilibration buffer, so the total volume was 2 resin-bed volumes. The sample was incubated 30 min at 4 °C on an end-over-end rocking platform. The flow through was collect

after centrifugation at 700 g 2 min 4 °C, and the resin was washed with 2 resin-bed volumes wash buffer twice, centrifuged at 700 g 2 min 4 °C. One resin-bed volume of elution buffer was added twice and centrifuged at 700 g 2 min 4 °C, and the histidine tagged protein was analyzed by SDS-PAGE. The protein content was determined by using bovine albumin (Sigma-Aldrich, USA) as the standard (Lowry et al., 1951). After purification, the buffer was exchanged by ultrafiltration (concentrated 20 times) and dilution in 500 µL of 10 mM Tris-HCl buffer pH 7.4 and 150 mM NaCl, which afforded a final protein concentration of 1 mg/mL. The molecular mass under denaturing conditions was determined by SDS-PAGE gel electrophoresis with a gel system (Bio-Rad Laboratories), 4% acrylamide stacking gel (pH 6.8), and 12% separating gel (pH 8.8). The proteins were visualized by staining with Coomassie brilliant blue G-250 (Thermo Scientific™, USA) (Laemmli, 1970).

The arabinofuranosidase activity was measured by adding 10 µL of 10 mM *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside (*p*NP-A) to 65 µL of 100 mM McIlvaine buffer (variable pH), 20 µL of water (or 50 mM  $\text{Ca}^{2+}$  or  $\text{Mn}^{2+}$ ), and 5 µL of 0.01 µg/µL purified enzyme at 45 °C for 30 min. The reaction was stopped with 50 µL of 1 M  $\text{Na}_2\text{CO}_3$ , and the absorbance was read at 405 nm (Sakamoto and Kawasaki, 2003).

## 2.5. Biochemical characterization and kinetic parameters

A Central Composite Rotational Design (CCRD) with two factors, three repetitions in the central point condition, and four axial points, evaluated pH from 2.6 to 5.4 and temperature from 25.9 to 54.1 °C (Supplemental Table S3). The statistical analysis was performed with the Protimiza Experimental Design software (Rodrigues and Iemma, 2014). The kinetic parameters ( $K_m$  and  $V_{max}$ ) were examined by incubating the diluted enzyme for 30 min in McIlvaine buffer containing 0–4 mg/mL wheat arabinoxylan (arabinose/xylose = 38:62) under the optimum temperature and pH conditions. The Graphpad Prism Software version 9 was used to compute the parameters. Enzyme stability at different pH was studied by incubating the enzyme in McIlvaine buffer at pH ranging from 3.0 to 8.0 at 4 °C for 24 h, and the relative activity was measured at the optimum conditions.

## 2.6. Thermostability, salt tolerance, and metal ion effects

The effect of metal ions and additives was evaluated by adding different metal ions and surfactants (1, 5, or 10 mM) to the enzymatic reaction. Salt tolerance was evaluated by incubating 0.1 mg/mL purified enzyme in McIlvaine buffer pH 4.8 containing 0–3 M NaCl at 4 °C overnight. The residual activity was measured under the optimum conditions.

The purified enzyme was thermally treated in a thermocycler at 50, 60, or 70 °C for 4 h; aliquots were removed every 20 min and immediately cooled at 4 °C for at least 1 h to ensure that all the samples had sufficient time for the reversibly denatured protein to be converted back to its native active form, while the irreversibly denatured form remained inactive. At the end of the thermal treatment, the enzymatic assay of the aliquots was carried out at 45 °C for 30 min, and the relative activities were expressed as a percentage of the original activities, to calculate the thermodynamic parameters (Saqib and Siddiqui, 2018).

## 2.7. Circular dichroism

The far-UV CD spectrum was measured between 190 and 260 nm in 20 mM phosphate buffer pH 6.0 at 25 °C on a spectrophotometer Jasco J-810 (Jasco Inc, Tokyo, Japan) equipped with a thermostatic Peltier system with constant nitrogen flow; a cuvette with 1-mm path length and protein concentration of 0.05 mg/mL were employed. The spectrum was measured at a rate of 1 nm/min four times, and ellipticity was reported as the mean residue molar ellipticity ( $\theta$ , deg.cm<sup>2</sup> dmol<sup>-1</sup>). The secondary structure was computed with BesStSel (<https://bestsel.elte.hu/index.php>).

## 2.8. Xylo-oligosaccharides and ferulic acid release

The synergistic cooperation degree of the equimolar mixture of Af afu62 and GH 10 *Papiliotrema flavescens*  $\beta$ -1,4-endoxyranase Xyn10cf (Martins et al., 2020) was calculated by the release of reducing sugars (Miller, 1959) and xylo-oligosaccharides production after incubation of 1% (w/v) wheat arabinoxylan (Megazyme, Ireland) with 10 U/µL Af afu62 and 30 U/µL Xyn10cf during 12 h at 40 °C and pH 5.0. The synergic degrees were calculated as the ratio between XOS content produced by the simultaneous activity of

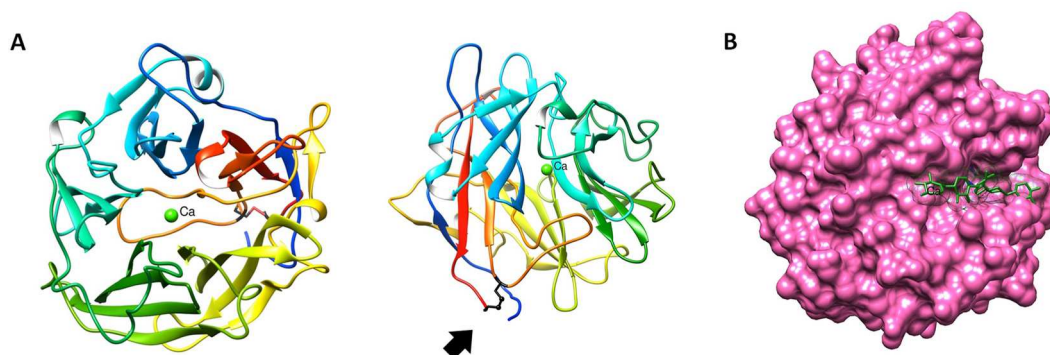


**Fig. 1.** Af afu62 structural homology model. A disulfide bridge is pointed by a black arrow and a calcium atom is shown in green (A); Enzyme surface illustrating the catalytic cleft and interaction with arabinoxypentose (B). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

both enzymes and the sum of the activities of each enzyme separately (Gonçalves et al., 2012).

The XOS, PCA, and FA release were tested with corn hull arabinoxylan (4% w/v) incubated with 10 U/μL Af\_afu62 and 30 U/μL Xyn10cf at pH 5.0 for 48 h at 40 °C. For control, the arabinoxylan was incubated under the same conditions except for the addition of enzymes. The hydrolysate was filtered (0.22 μm membrane) and subjected to high-performance liquid chromatography (HPLC) sugar analysis. The XOS, PCA, and FA quantification followed the methodology described elsewhere (Brenelli et al., 2018; Martins et al., 2020).

### 3. Results and discussion

#### 3.1. Structural prediction of Af\_afu62

Sequence analysis confirmed the conserved domain of the GH 62\_1 family (pfam03664, cl14647 superfamily), characterized by the five-blade β-propeller structure illustrated in Fig. 1. Af\_afu62 showed two conserved aspartic acid residues, D45 (general base) and D160 (pKa modulator), and one glutamic acid residue, E212 (general acid), which together form the catalytic triad. GH 62 enzymes have a calcium atom located in the center of the structure, held by a histidine residue (Tonozuka and Tanaka, 2017). The corresponding histidine in Af\_afu62 is H281, and it has been proposed as forming the catalytic nucleus together with the triad (Kaur et al., 2015).

From the alignment phylogeny (Table 1), we selected *Coprinopsis cinerea* (CcAbf62A) 5B6S, *Podospora anserina* 4N2Z (complexed with α-L-arabinofuranose), and *Mycothermus thermophilus* 4PVI to model the Af\_afu62 structure. We computed interactions between the amino acid residues within a 4 Å distance (Fig. 1). Cysteine residues 298 and 332 (C-terminal) are appropriately positioned for disulfide bridge formation. The residues of the catalytic triad D45, D160, and E212 are highlighted in yellow, and H281 is highlighted in blue (Supplemental Table S2).

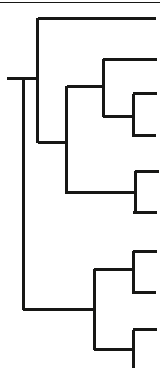
The purified enzyme had a 7.4-fold purification factor and the purification yield was about 47%. SDS-PAGE gel (Fig. 2a) confirmed the molecular mass of ~37 kDa predicted for the purified enzyme, which was expected after the addition of the 6x-polyhistidine tag and post-translational modifications (O-glycosylation at positions 25, 26, 136, 143, 257, 265, and 271). Two peaks emerged in the far-UV CD spectrum (Fig. 2b): a positive one at 195 nm and a negative one at 215 nm, typical of proteins rich in β-sheets (Sreerama and Woody, 2004). Secondary structure determination estimated an index of 44% extended antiparallel β-sheets and 11% α-helices, consistent with the predicted structure presented in Fig. 1.

According to the literature (Maehara et al., 2014), α-L-arabinofuranose is accommodated by subsite -1, and catalytic cleavage occurs between subsites -1 and +1 (Fig. 3a), which are very conserved among the GH 62 family of enzymes. Xylopentose interacts with subsites +4NR, +3NR, +2NR, +1R, and +2R, starting from the non-reducing (NR) to the reducing (R) terminal. Also, two tyrosine residues, Y 67 and Y 97 (highlighted in pink in Supplemental Table S2) accommodate a ferulate residue between the arabinose C5–O5 atoms (Hashimoto et al., 2011; Tonozuka and Tanaka, 2017). Two aspartic acid residues and one glutamic acid residue form the catalytic triad. A calcium ion is found on the central axis of the five blades, linked to a conserved histidine residue. This histidine residue appears to compose the catalytic nucleus together with the triad, and, according to the literature, the presence of Ca<sup>2+</sup> or Mg<sup>2+</sup> causes small changes in the enzymatic activity (Tonozuka and Tanaka, 2017).

#### 3.2. Biochemical characterization

The optimum pH lies between 4.0 and 5.0, and the optimum temperature is between 37 and 45 °C (Fig. 4). A similar result was reported by Pérez and Eyzaguirre (2016), who also reported the low activity of GH 62 enzymes against p-nitro-phenyl-α-arabinofuranoside substrate and high specificity for arabinoxylan. The positive coefficient of the interaction between pH and temperature indicated that, when these independent variables are combined, they affect the enzymatic activity depending on their

**Table 1**  
PSI-BLAST Homology of the Afua\_2g12770 with proteins from other species.

| Phylogeny                                                                           | PDB ID       | Organism                              | Taxonomy          | Query Cover | E Value            | % Identity |
|-------------------------------------------------------------------------------------|--------------|---------------------------------------|-------------------|-------------|--------------------|------------|
|  | 4NII         | <i>Ustilago maydis</i>                | Smut fungi        | 92%         | 3e <sup>-60</sup>  | 39.49%     |
|                                                                                     | 3WMY         | <i>Streptomyces coelicolor</i>        | Gram+ bacteria    | 93%         | 6e <sup>-67</sup>  | 41.27%     |
|                                                                                     | 6F1J         | <i>Talaromyces pinophilus</i>         | Ascomycete        | 94%         | 1e <sup>-72</sup>  | 43.89%     |
|                                                                                     | 6CC7         | <i>Thermoithelavioides terrestres</i> | Ascomycete        | 94%         | 2e <sup>-65</sup>  | 40.13%     |
|                                                                                     | 4O8N         | <i>Streptomyces thermoviolaceus</i>   | Gram+ bacteria    | 94%         | 9e <sup>-72</sup>  | 40.75%     |
|                                                                                     | 5UBJ         | <i>Aspergillus nidulans</i>           | Ascomycete        | 97%         | 3e <sup>-65</sup>  | 42.73%     |
|                                                                                     | <b>Query</b> | <b><i>Aspergillus fumigatus</i></b>   | <b>Ascomycete</b> | <b>-</b>    | <b>-</b>           | <b>-</b>   |
|                                                                                     | 5B6S         | <i>Coprinopsis cinerea</i>            | Basidiomycete     | 94%         | 7e <sup>-170</sup> | 69.94%     |
|                                                                                     | 4N2Z         | <i>Podospora anserina</i>             | Ascomycete        | 94%         | 3e <sup>-156</sup> | 64.35%     |
|                                                                                     | 4PVI         | <i>Mycothermus thermophilus</i>       | Ascomycete        | 92%         | 3e <sup>-142</sup> | 60.75%     |

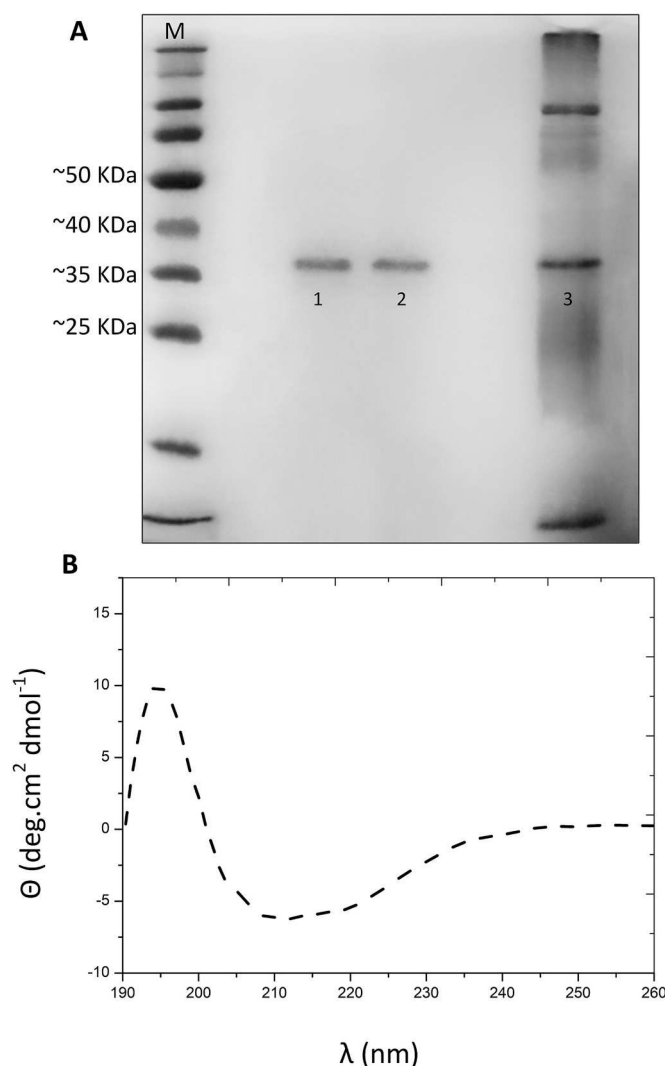


**Fig. 2.** SDS-page gel showing the molecular weight standard (M), the purified Af\_afu62 (lanes 1 and 2), and the culture supernatant of *P. pastoris* (lane 3) (A). Far-UV CD spectrum of purified Af\_afu62 (B).

value to generate a response (Supplemental Table S4). We validated the model by setting the pH and temperature values to 4.8 and 42 °C, and the experimental value of  $70.487 \pm 1.813 \text{ U}/\mu\text{L}$  is within the predicted value range.

The enzymatic kinetics followed the model proposed by Michaelis-Menten. The purified enzyme has  $K_m$  and  $k_{cat}$  of  $1.37 \pm 0.3 \text{ mg/mL}$  and  $47.82 \pm 2.15 \text{ s}^{-1}$ , respectively (at pH 4.8 and 42 °C). Despite little information about the kinetic parameters of GH 62 arabinofuranosidases, Table 2 gathers the parameters reported for wheat arabinoxylan. Af\_afu62 displays better catalytic efficiency than GH 62 from most of the cited bacteria and fungi, including *Mycothermus thermophilus* 4PVI, which is one of the models for Af\_afu62 structural prediction. We also observed lower  $k_{cat}$  and  $K_m$  as compared to *Aspergillus nidulans*. The catalytic efficiency ( $k_{cat}/K_m$ ) of Af\_afu62 was superior to all species, except for arabinofuranosidases from *Aspergillus nidulans*. The kinetic parameters of the 4PVI GH 62\_1 subfamily are the only ones available for this subfamily.

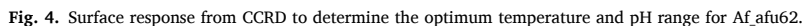
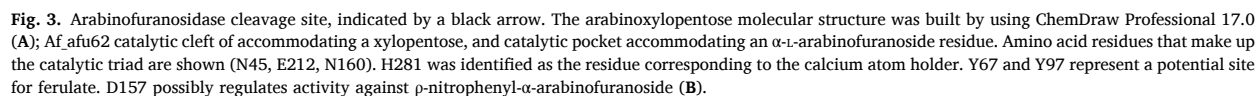
### 3.3. Influence of salt and pH on enzyme stability

The purified Af\_afu62 is stable at pH (Fig. 5a) from 4.0 to 6.0, following the studies indicating that arabinofuranosidases from fungi have optimum pH in the acid region, usually between pH 4.0 and 5.0 (Patel et al., 2015; Terrone et al., 2020). Increasing pH to above 7.0 decreases activity by 30% probably because groups ionize, altering enzyme configuration. Af\_afu62 preserved activity after 24h incubation at pH from 4.0 to 6.0.

Incubation with 0.5 M NaCl activated the purified enzyme (140% of control activity, Fig. 5b). At 1 M and at 3 M NaCl, 92 and 54% enzymatic activity is retained, suggesting that Af\_afu62 is a halotolerant enzyme. Good content of acidic amino acids, especially glutamate, may provide greater stability in the presence of higher salt concentrations (Oren, 2003; Terrone et al., 2020).

The Af\_afu62 stability in acidic conditions and the presence of salt indicate that this enzyme presents desirable properties for





GH 62 thermodynamics and how metal ions affect the GH 62 thermodynamic parameters have not been studied yet. However, Long et al. (2020) reported an increased thermostability of GH 62 from *Eupenicillium parvum* after they added calcium to the enzymatic reaction. Calcium-dependent thermostability has been reported for xylanases, glucosidases, and other arabinofuranosidases. This thermostability is partly explained by the catalytic domain structural stability provided by calcium. Studies have also reported that calcium, located in the central channel of the five-fold  $\beta$ -propeller structure, does not affect the specific GH 62 enzymatic activity (Long et al., 2020; Siguier et al., 2014; Wang et al., 2014). Nevertheless, herein  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  significantly increased the Afafu62

**Table 2**

Comparison of the kinetic parameters and catalytic efficiencies (kcat /Km ratios) of GH 62 arabinofuranosidases from several microorganisms made by using wheat arabinoxylan as substrate.

| Organism                  |           | PDB  | Sub-family | Wheat arabinoxylan     |                                     |                                  | Reference               |
|---------------------------|-----------|------|------------|------------------------|-------------------------------------|----------------------------------|-------------------------|
|                           |           |      |            | K <sub>m</sub> (mg/mL) | k <sub>cat</sub> (s <sup>-1</sup> ) | k <sub>cat</sub> /K <sub>m</sub> |                         |
| <i>A. fumigatus</i>       | Fungus    | -    | 62_1       | 1.37 ± 0.3             | 47.82 ± 2.15                        | 34.85                            | This study              |
| <i>A. nidulans</i>        | Fungus    | 5UBJ | 62_2       | 3.78 ± 1.05            | 361.74                              | 95.69                            | Contesini et al. (2017) |
| <i>A. nidulans</i>        | Fungus    | -    | 62_2       | 2.3                    | 178                                 | 77.39                            | Wilkens et al. (2016)   |
| <i>E. parvum</i>          | Fungus    | -    | 62_2       | 45.46 ± 2.44           | 42.04 ± 2.05                        | 0.92                             | Long et al. (2020)      |
| <i>G. zeae</i>            | Fungus    | -    | 62_2       | 3.4 ± 0.3              | 27.7 ± 0.7                          | 8.2                              | Sarch et al. (2019)     |
| <i>N. haematococca</i>    | Fungus    | -    | 62_2       | 9.2 ± 1.2              | 56.3                                | 6.1                              | Sarch et al. (2019)     |
| <i>S. reilianum</i>       | Fungus    | -    | 62_2       | 4.0 ± 0.6              | 13 ± 0.5                            | 3.3                              | Sarch et al. (2019)     |
| <i>S. coelicolor</i>      | Bacterium | 3WMY | 62_2       | 7.3                    | 18 ± 2                              | 2.5                              | Maehara et al. (2014)   |
| <i>S. thermophilum</i>    | Fungus    | 4PVI | 62_1       | 3.66 ± 0.82            | 0.27                                | 0.07                             | Kaur et al. (2015)      |
| <i>S. thermophilum</i>    | Fungus    | -    | 62_2       | 9.38 ± 1.98            | 0.86                                | 0.09                             | Kaur et al. (2015)      |
| <i>S. thermoviolaceus</i> | Bacterium | 408O | 62_2       | 12.0 ± 5.0             | 180.0 ± 65.0                        | 15.0                             | Wang et al. (2014)      |

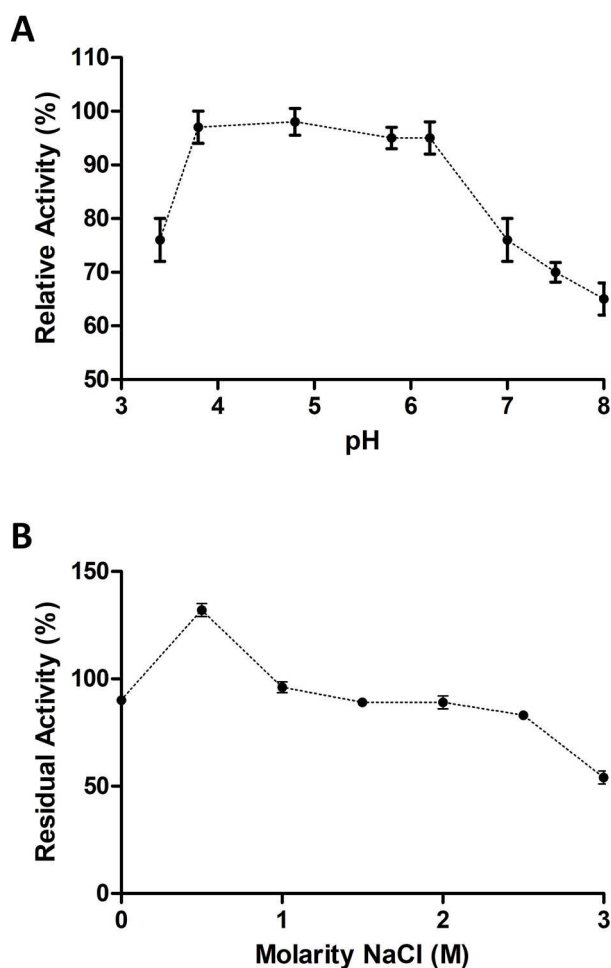


Fig. 5. pH stability (A) and the effects of salt concentration and (B) after overnight incubation.

enzymatic activity. Fig. 6 illustrates the effects of other chemicals.

Cofactor activation enhances the catalytic power of enzymes, and the economic suitability of any enzyme for industrial applications is judged by its thermodynamic parameters (Abd El Aty et al., 2018). Table 3 shows that the cofactors Ca<sup>2+</sup> and Mn<sup>2+</sup>, besides improving the activity, increased the residual activity (Fig. 7), half-life time  $t_{1/2}$  from 78 to 210 min, at 50 °C. Over 4-h-exposure is necessary to reduce its initial activity to 10%, and the presence of the cofactors increased the D value from 258 to 697 min at 50 °C.

Large and positive values of both energies of deactivation  $E_d$  and enthalpy of denaturation  $\Delta H_d$  indicate high enzyme thermostability. The results in Table 3 also show that the  $E_d$  and  $\Delta H_d$  values increase in the presence of cofactors. Negative entropy of

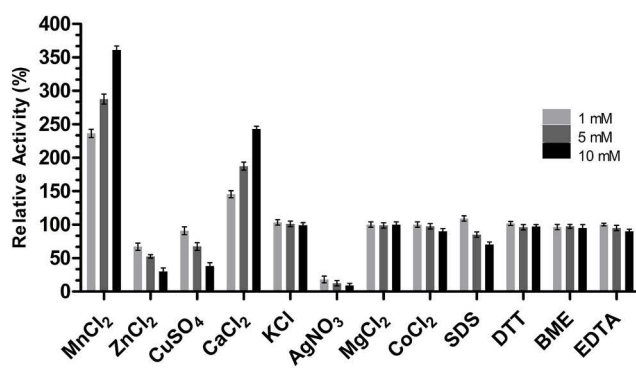


Fig. 6. Influence of metal ions or additives at 1, 5, or 10 mM on enzymatic activity.

**Table 3**  
Af\_afu62 thermodynamic parameters.

|                        | $k_d$ (min <sup>-1</sup> ) | $R^2$ | $t_{1/2}$ (min) | D value (min) | $\Delta G$ (kJ/mol) | $\Delta H$ (kJ/mol) | $\Delta S$ (J/mol.K) | Ed (kJ/mol) |
|------------------------|----------------------------|-------|-----------------|---------------|---------------------|---------------------|----------------------|-------------|
| <b>Control</b>         |                            |       |                 |               |                     |                     |                      |             |
| 50 °C                  | 0.0089                     | 0.836 | 77.882          | 258.717       | 103.044             | 7.516               | -295.616             | 10.203      |
| 60 °C                  | 0.0102                     | 0.850 | 67.956          | 225.744       | 105.940             | 7.433               | -295.683             |             |
| 70 °C                  | 0.0111                     | 0.835 | 62.446          | 207.440       | 108.963             | 7.350               | -296.119             |             |
| <b>Ca<sup>2+</sup></b> |                            |       |                 |               |                     |                     |                      |             |
| 50 °C                  | 0.0037                     | 0.933 | 187.337         | 622.320       | 105.403             | 10.330              | -294.207             | 13.016      |
| 60 °C                  | 0.0046                     | 0.911 | 150.684         | 500.562       | 108.146             | 10.247              | -293.859             |             |
| 70 °C                  | 0.0049                     | 0.934 | 141.459         | 469.915       | 111.296             | 10.163              | -294.718             |             |
| <b>Mn<sup>2+</sup></b> |                            |       |                 |               |                     |                     |                      |             |
| 50 °C                  | 0.0033                     | 0.944 | 210.045         | 697.753       | 105.710             | 13.625              | -284.962             | 16.311      |
| 60 °C                  | 0.0040                     | 0.975 | 173.287         | 575.646       | 108.533             | 13.541              | -285.131             |             |
| 70 °C                  | 0.0047                     | 0.985 | 147.478         | 489.912       | 111.415             | 13.458              | -285.463             |             |

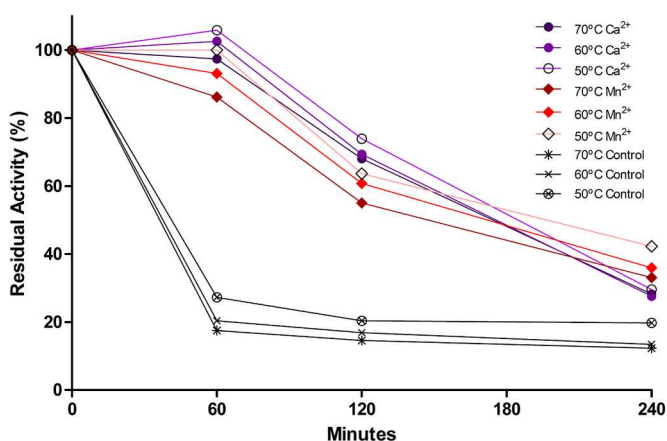


Fig. 7. Residual activity of Af\_afu62 after thermal treatment at 50, 60, or 70 °C without cofactor (Control) and in the presence of 5 mM Ca<sup>2+</sup> or Mn<sup>2+</sup>.

denaturation  $\Delta S_d$  values indicates a slightly less disordered transition state compared to the native enzyme, thereby highlighting reasonable thermostability at high temperatures (50, 60, and 70 °C) (Bisswanger, 2017; Soares da Silva et al., 2018). Negative values at all temperatures also indicate that aggregation takes place during thermal inactivation (Abd El Aty et al., 2018).

The enzyme incubated with Ca<sup>2+</sup> or Mn<sup>2+</sup> has slightly increased Gibbs free energy of denaturation  $\Delta G_d$  at all the temperatures, along with a significant increase in  $t_{1/2}$ , D,  $E_d$ , and  $\Delta H_d$ . These results show that Ca<sup>2+</sup> and Mn<sup>2+</sup> enhance the Af\_afu62 thermostability at temperatures ranging from 50 to 70 °C. An increase in  $\Delta G_d$  means an increase in denaturation resistance and hence increased thermostability (Bisswanger, 2017; Souza et al., 2015).

### 3.5. Synergism for xylo-oligosaccharides and ferulic acid production

The release of reducing sugars was enhanced by 5% with the association of 30 U/ $\mu$ L Xyn10cf and 10 U/ $\mu$ L Af\_afu62, and total XOS (X2-X6) production increased by 42.6% (1058 mg/L) for wheat arabinoxylan 1% w/v (Table 4). The synergistic activity of Xyn10cf and



Af\_afu62 on agricultural residue was tested for the ability to release XOS, PCA, and FA. Even without an esterase, the combination of both enzymes released higher concentrations of readily soluble PCA and FA from the alkaline extraction of arabinoxylan due to further degradation of the material since higher XOS concentration was also achieved (Zhang et al., 2014). It was found that Xyn10cf (only) produced 2.43 g/L XOS, 0.62 mM FA (120 mg/L), and 0.06 mM PCA. The co-incubation of Xyn10cf with Af\_afu62 produced 5.67 g/L XOS, 0.82 mM (160 mg/L), and 0.09 mM PCA. GH 10 endoxylanase exhibit greater catalytic versatility than GH 11 endoxylanase, being less hampered by the presence of substituents along the xylan backbone. However, a high degree of branching along the backbone may impair xylan degradation by hindering the formation of the enzyme-substrate complex. Therefore, the simultaneous removal of arabinose residues improves the catalytic efficiency of Xyn10cf (Faulds et al., 2006). At the same time, the arabinose content was higher with co-incubation. Hence, arabinose-substituted xylo-oligosaccharides generated by Xyn10cf showed to be better substrates for Af\_afu62, being able to release more arabinose. Xylotetraose was not detected in this assay, similar to results from the literature (Brandt et al., 2020; Rahmani et al., 2019), where no X4 was found in the enzymatic reaction, indicating that either no X4 is formed during the reaction or, less likely, a very rapid turnover of X4 takes place.

These results highlight the functionality of the GH 62 enzymes for arabinoxylan debranching and xylo-oligosaccharide production, when these enzymes act synergistically with endoxylanases, with an improvement of PCA and FA release, whose ester linkages were cleavage by the alkaline pretreatment for the arabinoxylan extraction (Cobs, 2020).

#### 4. Conclusion

The Af\_afu62 structure reveals conserved residues in the catalytic site and suggests greater, if not exclusive, specificity for arabinoxylan. According to residual activity assays, the presence of the cofactors  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  improved the thermoresistance and increased the half-life time by 2.4 times. The synergism assays for xylo-oligosaccharides,  $p$ -coumaric acid, and ferulic acid production from alkali-treated agricultural residue demonstrated the biotechnological potential of using GH 62 for prebiotics and natural antioxidants production. These results provide a basis for further development of novel enzymatic routes for applications in the food and pharmaceutical industries.

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#### CRedit authorship contribution statement

**Manoela Martins:** Conceptualization, Methodology, Investigation, Formal analysis, Validation, Writing – original draft. **Robson Tramontina:** Methodology, Investigation, Formal analysis. **Fábio Márcio Squina:** Resources, Supervision. **Taísa Magnani Dinamarco:** Resources, Supervision, Writing – review & editing. **Rosana Goldbeck:** Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Table 4**

Synergistic hydrolysis of wheat and corn hull arabinoxylan by Xyn10cf and Af\_afu62 for XOS and FA release.

|                               | Af_afu62              | Xyn10cf               | Xyn + Abf              | Degree of synergy |
|-------------------------------|-----------------------|-----------------------|------------------------|-------------------|
| <b>Wheat arabinoxylan</b>     |                       |                       |                        |                   |
| Reducing sugars               | 31.44 $\mu\text{mol}$ | 95.05 $\mu\text{mol}$ | 133.25 $\mu\text{mol}$ | 1.05              |
| Xylose (X1)                   | 0                     | 0                     | 0                      |                   |
| Xylobiose (X2)                | 0                     | 303.23                | 304.78                 |                   |
| Xylotriose (X3)               | 0                     | 318.88                | 393.6                  |                   |
| Xylotetraose (X4)             | 0                     | 0                     | 0                      |                   |
| Xylopentaose (X5)             | 0                     | 99.07                 | 253.89                 |                   |
| Xylohexaose (X6)              | 0                     | 20.71                 | 105.72                 |                   |
| Total XOS                     | 0                     | 741.89 mg/L           | 1057.99 mg/L           | 1.43              |
| <b>Corn hull arabinoxylan</b> |                       |                       |                        |                   |
|                               |                       |                       |                        | <b>Control</b>    |
| Arabinose                     | 1.31 g/L              | 0                     | 3.37 g/L               | 0                 |
| Ferulic acid                  | 0.27 mM               | 0.62 mM               | 0.82 mM                | 0.22              |
| $p$ -coumaric acid            | 0.02 mM               | 0.06 mM               | 0.09 mM                | 0                 |
| Total XOS                     | 0                     | 2.43 g/L              | 5.67 g/L               | 0                 |

## Data availability

No data was used for the research described in the article.

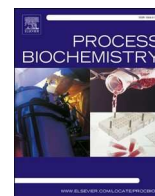
## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2022.102469>.

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# Novel bi-functional thermostable chimeric enzyme for feasible xylo-oligosaccharides production from agro-industrial wastes

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## ABSTRACT

Arabinoxylan is a sustainable source for xylo-oligosaccharide production, as agro-industrial wastes are an abundant and the industrial interest for these prebiotics is raising due to their bioactivities for food and cosmetics. However, enzyme production costs and thermostability still represent a drawback. The main enzymes required for hydrolysis are endoxylanase and arabinofuranosidase, where GH 10 endoxylanase presents great thermostability and activity, and GH 62 enzymes are the arabinofuranosidases with the highest specificity but poor thermostability. In this sense, the fusion of these enzymes can generate a more stable enzyme with multiple catalytic domains in a single batch. The present study designed a bifunctional enzyme combining a GH 10 endoxylanase and a GH 62 arabinofuranosidase to enhance activity and thermostability, thus increasing the feasibility of xylo-oligosaccharides production from wastes. The chimera showed superior specificity for arabinoxylan and halotolerance than the parental enzymes and higher efficiency than commercial cocktail. Thermostability improved especially for the arabinofuranosidase module, which increased 10 °C, enabling a process at higher temperatures and lower enzyme loads, which increases efficiency and decreases contamination. Calcium and manganese enhanced thermostability and activity. Xylobiose and xylotriose yields improved by 25%, and up to 7 g/L XOS was achieved from corn hull arabinoxylan hydrolysis.

## 1. Introduction

Arabinoxylans (AX) are the principal group of hemicellulose found in cereal grains, located in the cell walls of starchy endosperm, aleurone, bran tissues, and in the husks, whose concentration varies depending on the tissue and grain. For example, arabinoxylan content in wheat bran is 20.9%, whereas in rye grain is as much as 30% [1]. They are composed of a chain of D-xylopyranosyl residues joined by  $\beta$  - 1,4 glycosidic bonds, with  $\alpha$ -L-arabinofuranoside residues in the C (O) - 3 or - 2 positions of the xylose backbone (Fig. 1A). The side groups form covalent bonds with cellulose and lignin, molding a complex that surrounds the cellulose fibers. These strong bonds with lignin and cellulose make their separation difficult while maintaining their integrity [2]. Large quantities of grains are cultivated in the agroindustry and generate tons of agro-industrial residues. The particular interest in AX polymers is due to the potential to obtain prebiotic xylo-oligosaccharides (XOS) [3].

Endo-1,4- $\beta$ -xylanases (EC 3.2.1.8) cleave the glycosidic bonds in the

arabinoxylan (AX) backbone, releasing lower-grade xylose and XOS (Fig. 1B). GH 10 endoxylanases require only two unsubstituted xylopyranosyl residues between branches. It results in higher shorter-chain XOS production, especially xylobiose and xylotriose, which shows the greatest prebiotic potential [4]. However, to optimize the production of linear XOS, other enzymes that act on the AX side chains are necessary. The family GH 62 is composed of  $\alpha$ -L-arabinofuranosidases with almost exclusive specificity by  $\alpha$ (1 $\rightarrow$ 2) and  $\alpha$ (1 $\rightarrow$ 3) bonds of L-arabinofuranosyl residues of hemicellulose [5]. The simultaneous substituents removal and glycosidic bonds cleavage increases the degradation rate (Fig. 1B). Thus, synergistic enzymes are desirable and under intense investigation to understand the mode of action and optimize enzyme mixtures and concentrations [6,7]. Several studies have already confirmed the synergistic effect of the mixture of endoxylanases and arabinofuranosidases for XOS production [3,4,8,9].

From an industrial point of view, the enzymatic synergism allows accelerating the activity for biomass conversion, reducing the amount of

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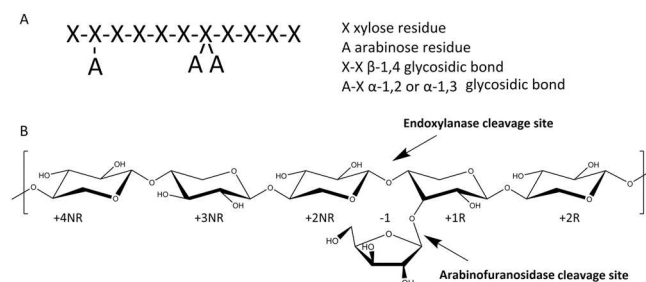


**Fig. 1.** Schematic representation of arabinoxylan structure and cleavage sites of GH 10 endoxylanase and GH 62 arabinofuranosidase.

enzyme needed, the hydrolysis time, and production costs [10]. Although industries widely use enzymatic mixtures, several studies report chimeric enzymes present superior performance [11]. A common method to synthetically join catalytic domains is tandem fusion. Two or more sequences encoding proteins connected end-to-end produce a single polypeptide chain with multiple functions. Short sequences compose the linkers among the domains to provide mobility freedom and avoid steric impediments. The most used linkers are in the form (Gly-Gly-Gly-Ser)n, where “n” is the number of residues (<6), forming a loop. Short and flexible linkers can promote higher catalytic activity, greater mobility without unsettling the conformation of enzymes, and decrease the probability of proteolytic attack during protein expression and purification [12].

In addition to the synergistic effect and greater ease of production, the catalysis of the fused enzymes presents similar stability and activity under the same conditions [13,14]. This is because the N-terminal position plays a decisive role in protein half-life and thermostability. Placing the most thermostable protein in the N-terminal position can increase the thermostability of the next linked protein. Additionally, the final structure of the whole molecule sets the stability under different pH and salt conditions to guarantee success in creating chimeric enzymes [15,16]. The proximity between the catalytic domains of fused enzymes often increases enzymatic performance compared to the activities of separate enzymes [17].

Several chimeras were developed to enhance the hydrolysis efficiency of lignocellulosic biomass, but none was designed with the principal goal of optimizing XOS production. The GH 10 endoxylanase (EC 3.2.1.8) of *Papiliotrema flavescens* (Xyn10cf) showed high thermostability and synergy with arabinofuranosidases for short-chain XOS production, especially xylobiose and xylotriose [8]. GH 62 arabinofuranosidase (ED 3.2.1.55) of *Aspergillus fumigatus* (Af\_afu62) presented high specificity for arabinoxylan and the synergism with GH 10 endoxylanase improved xylotetraose production by 42.6%; however, its thermostability is still a drawback [18]. Thus, this study expressed a chimera by tandem fusion of a thermostable GH 10 endoxylanase of *P. flavescens* and GH 62 arabinofuranosidase of *A. fumigatus* using a flexible linker (GGGGS)<sub>2</sub> to obtain a bifunctional protein with enhanced hydrolytic power and thermostability. This strategy aimed to blend the biochemical characteristics and enhance the half-life of the less thermostable enzyme (Af\_afu62), increasing the catalytic performance. The results from biochemical characterization, thermostability, and synergic effects confirmed the goal of enhancing arabinoxylan specificity and the rate of catalysis to produce XOS from agro-industrial wastes.

## 2. Material and methods

### 2.1. In silico molecular modeling

The 3D structure of chimera Xyn10cf+Af\_afu62 was built by RoseTTAFold [19]. The predicted structure was evaluated by the GDT score, Ramachandran's plot, and angstrom error estimate. These results

were further evaluated by PROCHECK [20]. The 3D structures of feruloyl arabinobiose (CID 6438898) and xylotetraose (CID 10230811) were downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). Polar hydrogens were added, and Gasteiger partial charges were assigned. Charges were computed using ANTECHAMBER [21]. AutoDock Vina was used for protein–ligand docking, and the results were analyzed using UCSF Chimera [22,23].

### 2.2. Expression and purification of recombinant chimeric gene

The gene sequences of Xyn10cf GH 10 endoxylanase from *Papiliotrema flavescens* (GenBank Gene ID: A120248.1, without a signal peptide and TAG codon), the flexible linker [GGGSGGGGS], Af\_afu62 GH 62 arabinofuranosidase from *Aspergillus fumigatus* (GenBank Gene ID: Afu\_2G12770, 948 pb, without a signal peptide and TAG codon) were optimized using the Codon Optimization Tool (<https://www.novoprolabs.com/tools/codon-optimization>), synthesized by GenOne (Rio de Janeiro, Brazil), and inserted into the pGAPZα vector (Invitrogen, Carlsbad, CA, USA) between *EcoRI* and *XbaI* sites with 6x His-tag and α-factor peptide signal. The synthetic gene sequence can be found in GenBank with the accession number OM867846.

*Escherichia coli* DH5α was heat-shock transformed and cultivated at 37 °C and 200 RPM in low-salt Luria-Bertani (LB) medium with 100 µg/mL Zeocin. The plasmid containing the chimeric gene Xyn10cf+Af\_afu62 was propagated in *E. coli*, extracted, and linearized with FastDigest PstI (5'-T<sup>+</sup>CATGA.3', Thermo Scientific™, USA) according to the instructions. Electrocompetent *Komagataella pastoris* X-33 was prepared according to the transformation protocol No. 4308 915.545 –03/2004 (Eppendorf) and transformed by electroporation (1500 V, 5 ms). Transformants were selected for Zeocin resistance on YPDS plates containing 100 µg/mL Zeocin. Ten colonies of resistant transformants were selected after three days of incubation at 30 °C and transferred to another YPDS plate with the same concentration of Zeocin. The presence of the insert was confirmed by PCR using the sequences of the primers 5' pGAP forward and 3' AOX1 reverse recommended by the Invitrogen cloning manual.

Small-scale enzyme production of the PCR-positive colonies was performed in 50 mL YDP (in 250 mL Erlenmeyer) at 30 °C and 250 RPM for 96 h. The supernatant was concentrated by ultrafiltration (Amicon Ultra15, Merck KGaA, Germany) using a molecular weight cutoff of 10 kDa. The enzyme was purified using the HisPur kit (Thermo Scientific™, USA), as recommended. Proteins were visualized on 12% SDS-PAGE for the separation gel and 4% for the stacking gel [24] and stained with Coomassie blue G-250 (Thermo Scientific™, USA). Protein content was determined using bovine albumin (Sigma–Aldrich, USA) as a standard [25]. After purification, the protein was diluted to 1 mg/mL in 10 mM Tris-HCl buffer 150 mM NaCl pH 7.4. For enzymatic assays, the protein was diluted to a final concentration of 0.01 mg/mL.

The parental recombinant enzymes GH 10 endoxylanase and GH 62 arabinofuranosidase were also produced and purified according to the same methodology.

### 2.3. Circular dichroism (CD)

Far-UV CD spectrum was measured between 195 and 250 nm in phosphate buffer 20 mM pH 6.0 at 25 °C using a spectrophotometer Jasco J-810 (Jasco Inc, Tokyo, Japan) equipped with a thermostatic Peltier system with constant nitrogen flow, using a cuvette of 1 mm and protein concentration of 0.05 mg/mL and a rate of 1 nm/min. The ellipticity was reported as the mean residue molar ellipticity (θ, deg.cm<sup>2</sup>dmol<sup>−1</sup>). The secondary structure was computed with BesStSel (<https://bestsel.elte.hu/index.php>) [26].

### 2.4. Biochemical characterization and kinetic parameters

The optimum temperature and pH were determined by a central



composite rotational design (CCRD) with two factors, 3 repetitions in the central point condition and 4 axial points [27], evaluating the enzymatic activity of endoxylanase and arabinofuranosidase separately using the substrates xylan of beechwood and  $\rho$ -nitrophenyl- $\alpha$ -L-arabinofuranoside, respectively. The temperature varied from 50 °C to 80 °C for endoxylanase and from 36 °C to 64 °C for arabinofuranosidase, and the pH varied from 3.4 to 6.2.

The kinetic parameters ( $K_m$  and  $V_{max}$ ) were examined by incubating the diluted enzyme for 30 min in McIlvaine buffer containing 0–4 mg/mL wheat arabinoxylan (Megazyme, Ireland) under the optimal conditions determined for temperature and pH. The parameters were calculated using GraphPad Prism Software version 9.

## 2.5. Effects of metal ions, inhibitors, pH, and salt concentration

The effect of various metal ions ( $Na^+$ ,  $K^+$ ,  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Ni^{2+}$ ,  $Ag^{3+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ , and  $Co^{2+}$ ) and many inhibitors [EDTA, dimethyl sulfoxide (DMSO), Tween-20,  $\beta$ -mercaptoethanol, DTT, and sodium dodecyl sulfate (SDS)] was analyzed by incubating the purified chimera in 50 mM solutions of varying concentrations (1, 5, and 10 mM) for 1 h before the enzymatic assay.

The pH stability of the enzyme was studied by incubating in McIlvaine buffer pH ranging from 3.0 to 8.0 for 24 h at 4 °C. Salt tolerance was evaluated by incubating the enzyme with NaCl concentrations ranging from 0 to 3 M for 24 h at 4 °C. After 24 h, the relative activities were measured at the optimum conditions for enzymatic assays.

## 2.6. Thermostability and thermal shift assays

Thermal treatment of the purified chimera was carried out in a thermocycler, scheduling each treatment at a controlled temperature of 50, 60, and 70 °C for 4 h. Aliquots were taken every 20 min and cooled at 4 °C for a minimum of 1 h to ensure that the reversibly denatured protein was converted back to its native active form, while the irreversibly denatured form remained inactive. At the end of the thermal treatment, the enzymatic assays of all the aliquots were carried out at 65 °C for 30 min, and the relative activities were expressed as a percentage of the original activities to calculate the thermodynamic parameters [28].

The melting temperatures of the chimera Xyn10cf+Af\_afu62 and the parental enzymes were assayed by differential scanning fluorimetry (DSF) using a real-time PCR instrument Eppendorf MasterCycler Realplex 4, 560–580 nm detection filter. Five microliters of enzyme preparations (1–5  $\mu$ g), 4  $\mu$ L of 50X stock solution of SYPRO® Orange (Supelco, Merck, S5692), 1, 5 and 10 mM  $CaCl_2$  or  $MnCl_2$ , and McIlvaine buffer to complete 12.5  $\mu$ L were pipetted into the wells of a qPCR plate and sealed. The temperature was raised from 25 °C to 95 °C at a step of 0.1 °C/s. The fluorescence was monitored by the qPCR system, and the  $T_m$  was calculated from the inflection points of the transition curve [Fluorescence =  $f(T)$ ], which was computed as the maximum of the first derivative  $[d(\text{Fluorescence})/d(T)]$ .

## 2.7. Synergism for XOS production from agro-industrial wastes

The hydrolytic power of the chimera analyzed the XOS production from arabinoxylan extracted from soy and corn husks (see 2.8.2). Soluble concentrations of arabinoxylan (0.4–4.6% w/w) were incubated in McIlvaine buffer pH 5.0 with 10  $\mu$ L/mL of diluted chimera (final protein concentration of 0.01 mg/mL) and 5 mM  $MnCl_2$ . The results were compared to the yields obtained from hydrolysis using 10  $\mu$ L/mL of Shearzyme endoxylanase (NS50030- Novozymes®), final protein concentration at the saturation point of 0.165 mg/mL) under the optimum conditions previously determined (pH 5.0 and 50 °C, data not shown).

The synergic efficiency of the chimera and the parental enzyme mixture was estimated after 12 h hydrolysis of 1% wheat arabinoxylan (arabinose:xylose ratio 33.5:62, Megazyme, Ireland) at 40 °C and pH

5.0. The synergism was calculated by the ratio of xylo-oligosaccharides released by the chimera and the parental enzyme equimolar mixture, according to Eq. (1).

$$D = \frac{Y_c}{Y_{mix}} \text{ or } \frac{Y_{mix}}{Y_x + Y_a} \quad (1)$$

Where D is the degree of synergy,  $Y_c$  is the total xylo-oligosaccharides produced by the chimera;  $Y_{mix}$  the total xylo-oligosaccharides produced by the equimolar mixture of the parental enzymes Af\_afu62 and Xyn10cf; and  $Y_x$  and  $Y_a$  are the total xylo-oligosaccharides produced by Xyn10cf and Af\_afu62, respectively.

## 2.8. Analytical methods

### 2.8.1. Enzymatic assays

The endoxylanase activity was assayed with 25  $\mu$ L of 4% wheat arabinoxylan or beechwood xylan (Megazyme, Ireland), 60  $\mu$ L of 100 mM McIlvaine buffer (variable pH), 10  $\mu$ L of Milli-q (or 50 mM  $Ca^{2+}$  or  $Mn^{2+}$ ) and 5  $\mu$ L of the diluted enzyme (0.01 mg/mL). The tests were conducted at different temperatures for 10 min [29]. The reducing sugars were analyzed by DNS [30], with one xylanase unit (U/ $\mu$ L) being the amount of enzyme needed to produce 1  $\mu$ mol of reducing sugars per minute.

The activity of arabinofuranosidase was measured with 10  $\mu$ L of 10 mM  $\rho$ -nitrophenyl- $\alpha$ -L-arabinofuranoside ( $\rho$ NP-A) in 65  $\mu$ L of 100 mM McIlvaine buffer, 20  $\mu$ L of water or 50 mM cofactor  $Ca^{2+}$  or  $Mn^{2+}$ , and 5  $\mu$ L of the diluted enzyme (0.01 mg/mL). After incubation at 45 °C for 30 min, the reaction was stopped by adding 50  $\mu$ L of 1 M  $Na_2CO_3$ . The concentration of  $\rho$ -nitrophenol was measured at 405 nm, with one unit (U/ $\mu$ L) of  $\alpha$ -L-arabinofuranosidase defined as the amount of enzyme required to release 1  $\mu$ mol of  $\rho$ -nitrophenol per min [31].

### 2.8.2. Biomass characterization, hemicellulose extraction and XOS quantification

Milhão Ingredients (Goianira, Goiás, Brazil) and Oleoplan S.A. (Veranópolis, Rio Grande do Sul, Brazil) kindly donated the corn and soy hulls, respectively. Corn hull (pericarp fiber) was washed several times with hot distilled water to remove residual starch and then centrifuged. The starch and the corn hulls were dried at 50 °C overnight and weighed. Cellulose, hemicellulose, and lignin characterization followed the NREL/TP-510-42619 procedure [32,33]. Protein was determined as the total nitrogen content by the Kjeldahl method using the  $N \times 6.25$  conversion factor according to the AOAC 984.13 method. Xylooligosaccharides were quantified as described elsewhere [8].

Xylan extraction used 5% KOH and a solid ratio of 1:10 at 121 °C for 30 min [18,34]. The samples were centrifuged at 5000g for 20 min, and the supernatant was vacuum filtered, neutralized with acetic acid to pH 5.0–5.5, and precipitated with 3 volumes of cold ethanol. After an overnight precipitation at 4 °C, the solid fraction was separated by centrifugation at 5000g for 20 min, washed once with 50% ethanol 0.5% EDTA [34,35], centrifuged, rinsed with Milli-Q®, centrifuged again, dried at 50 °C overnight, and diluted in McIlvaine buffer pH 5.0.

### 2.8.3. Statistical analysis and graphical software

All experiments were conducted in triplicate, except CCDR. One-way ANOVA and Tukey post hoc tests were conducted using OriginPro 8.5 software. Graphics were developed using GraphPad Prism 5.

## 3. Results and discussion

### 3.1. Structure prediction and molecular docking

The quality of the predicted model of the chimera was evaluated based upon Ramachandran's plot, QMEAN score, and confidence based on the GDT score (1.0 good; 0.0 bad). The model presented a confidence of 0.84 and a span of  $1^{-656}$ . The 3D model showed > 95% residues in

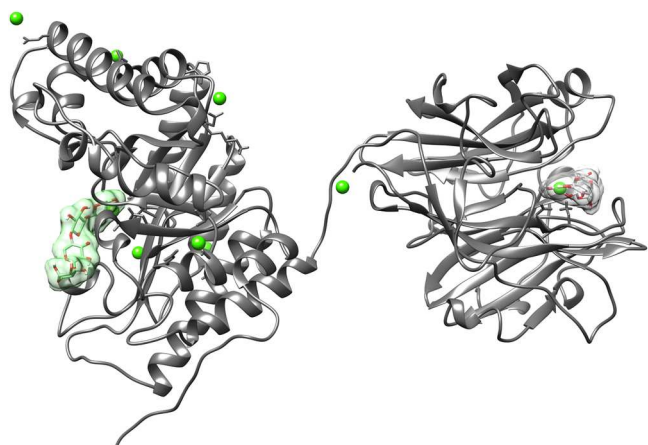


**Fig. 2.** Chimera structure and substrate binding sites. Endoxylanase Xyn10cf module on the left, docked with xylohexaose and arabinofuranosidase Af\_afu62 module on the right, docked with feruloyl arabinobiose. Calcium ions putative locations are represented as green balls.

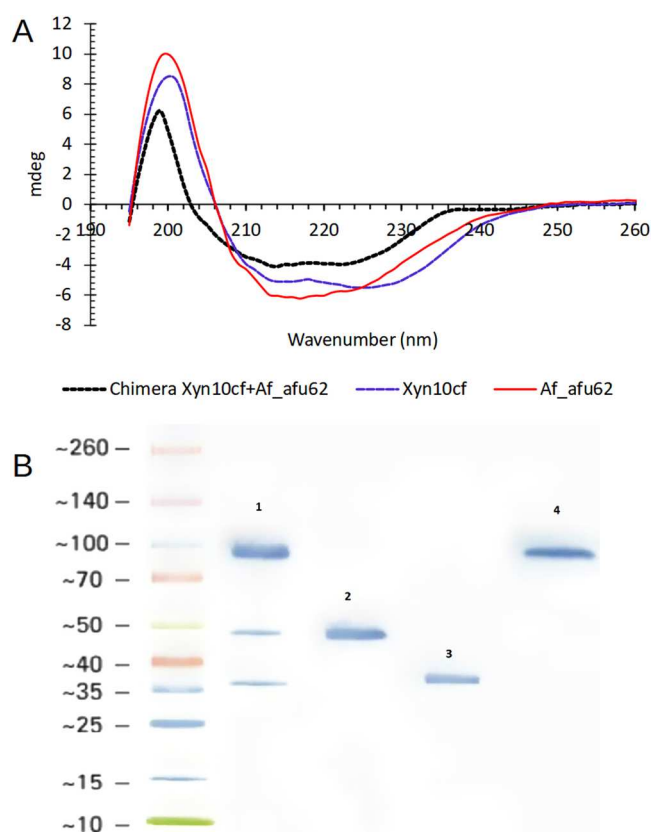


**Fig. 3.** (A) CD spectrum of Xyn10cf+Af\_afu62 and parental enzymes; (B) SDS-page of crude supernatant of chimera enzyme (1), purified Xyn10cf (2), purified Af\_afu62 (3), purified chimera (4).

Ramachandran's favorable region and a QMEAN score lower than 2. The arabinofuranosidase domain displayed the typical 5-bladed  $\beta$ -propeller structure found in GH 62 enzymes, with the central axis occupied by a calcium ion [36]. The endoxylanase domain also accommodate 7  $\text{Ca}^{2+}$  ions in the central loops of helix-loop-helix structures, suggesting that calcium may contribute to GH 10 ( $\beta/\alpha$ )<sub>8</sub> stabilization [37] (Fig. 2).

### 3.2. Recombinant expression and circular dichroism

The contours of the CD spectra of Xyn10cf, Afua\_2g12770, and the chimera Xyn10cf+Af\_afu62 are shown in Fig. 3A. The CD spectra of Xyn10cf and Xyn10cf+Af\_afu62 exhibit double minima signatures at approximately 210 and 222 nm, which indicate a significant  $\alpha$ -helix content. The lower molar ellipticity values at these minima indicate that Xyn10cf presents a higher helical content than the chimera, which also contains significant amounts of other secondary structures. The CD spectrum of Af\_afu62 presents two main bands at 200 (positive) and 210 (negative) and lacks the "double minima signature", indicating that this protein contains a high amount of  $\beta$ -sheet content and poor helical content. These results are consistent with the expected structure of the ( $\alpha/\beta$ )<sub>8</sub> motif for GH 10 endoxylanase and the 5-bladed  $\beta$ -propeller motif for GH 62 arabinofuranosidase. The chimera presented a helical content of 22.65%, consistent with the predicted structure. The parental Xyn10cf and Af\_afu62 presented a helical content of 42.5% and 10.9%, respectively.

SDS-page gel confirms the molecular mass of around 85 kDa (between 70 and 100 kDa bands), which is coherent with the sums of the molecular masses of parental Af\_afu62 (~37 kDa) and Xyn10cf (~48 kDa), poly-histidine tag, and flexible linker. The crude supernatant extract shows little degradation of the recombinant chimera (Fig. 3B). The enzyme yield was of 10.4 mg/L after 96 h, while the cultures of the parental enzymes yielded 15 mg/L for Xyn10cf and 4 mg/L for Af\_afu62. Even though Xyn10cf separately resulted in higher enzyme concentration, the mixture of the cultures would result in dilution and improper proportion of the enzymes. Further research will be conducted to optimize fermentation conditions to scale up the production and improve protein yields.

### 3.3. Biochemical characterization

CCRD showed good reproducibility under the central point conditions for both endoxylanase (65 °C and pH 4.8) and arabinofuranosidase activities (50 °C and pH 4.8). The analysis of the regression coefficients showed that the linear and quadratic terms for temperature and pH are significant at the 95% confidence level ( $p < 0.05$ ), except for the linear term for temperature (Tables A.1 and A.2). Data analysis for multiple regression led to a second-order polynomial model, whose validity was verified by analysis of variance (ANOVA) for the response surface construction (Fig. 4). The positive interaction coefficient of pH and temperature indicates that the combination of both independent variables influences enzymatic activity. The endoxylanase moiety presented optimum conditions between pH and temperature 4.8–6.0 and 60–78 °C, and the arabinofuranosidase was between 4.0 and 5.5 and 50–66 °C. The predicted values were validated at pH and temperature of 5.3 and 70 °C for endoxylanase activity and 4.8 and 55 °C for arabinofuranosidase. The experimental values of  $98.004 \pm 1.813 \text{ U}/\mu\text{L}$  and  $91.201 \pm 3.630 \text{ U}/\mu\text{L}$ , respectively, for endoxylanase and arabinofuranosidase, were within the predicted value range of the model.

The CCRD of both parental enzymes are presented in the Tables A.3 and A.4. The parental Af\_afu62 enzyme showed a narrow optimum temperature range of 37–42 °C against the 50–66 °C displayed by the chimera Af\_afu62 module, with an average increase of 46%.

Regarding the optimum pH and temperature for the entire chimeric protein, an intersection area was observed in the response surface where both catalytic domains presented the highest activity at pH 4.5 and 5.0 and temperatures from 60° to 65°C. Therefore, it can be assumed that the physicochemical characteristics of Xyn10cf and Af\_afu62 merged with the fusion. At the same time, the optimum temperature range of Xyn10cf did not decrease and maintained between 60 °C and 78 °C after fusion.

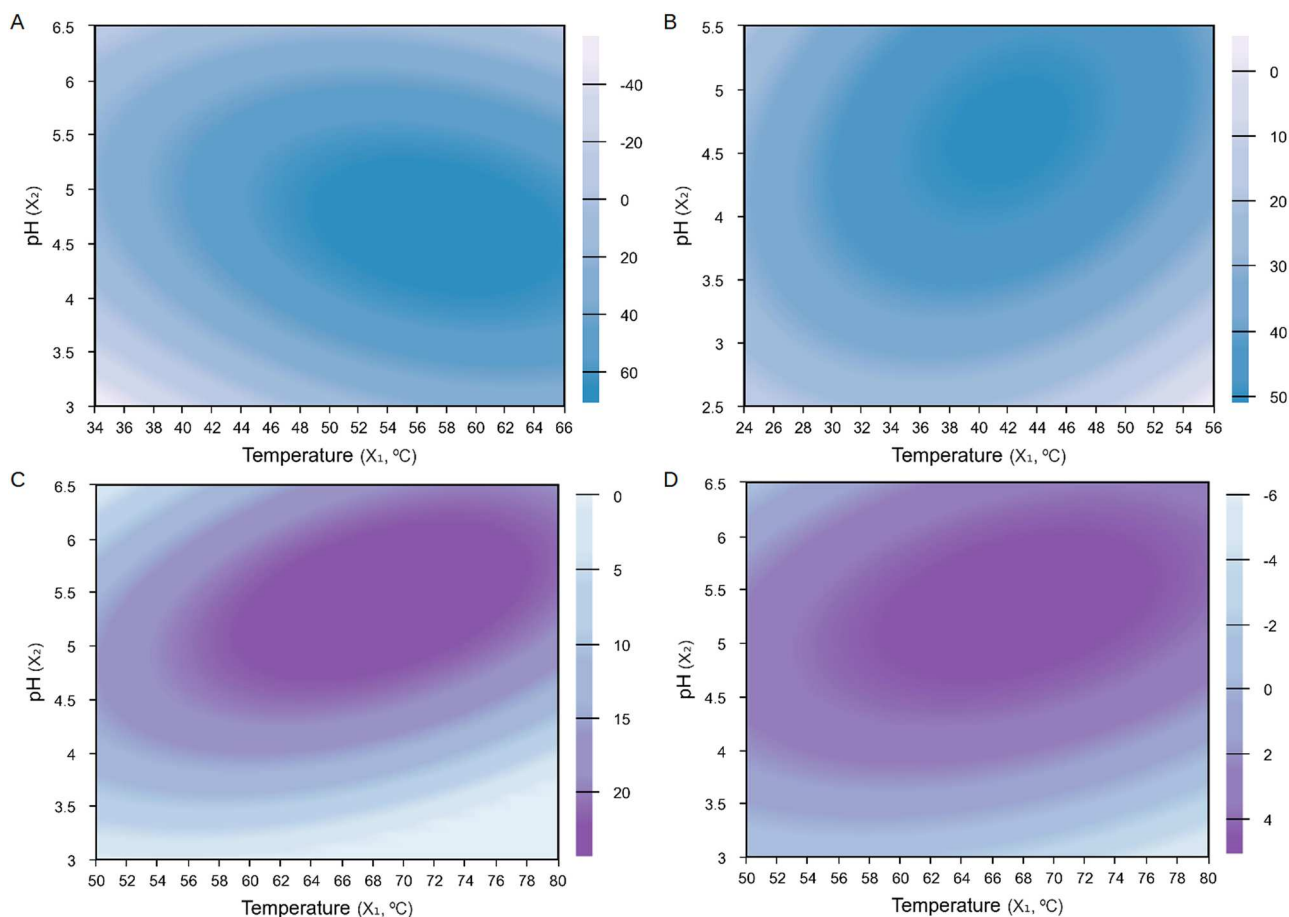


**Fig. 4.** Surface response to optimal conditions of pH and temperature determined for (A) chimeric arabinofuranosidase, (B) parental arabinofuranosidase, (C) chimeric endoxylanase, and (D) parental endoxylanase.

**Table 2**

Kinect parameters and degree of synergy for XOS production, where “Mix” stands for the equimolar mixture of the parental enzymes Af\_afu62 and Xyn10cf.

|                   | Af_afu62 | Xyn10cf | Mix     | Chimera |
|-------------------|----------|---------|---------|---------|
| $k_m$             | 1.372    | 4.969   | –       | 0.745   |
| $v_{max}$         | 1.435    | 1.190   | –       | 1.462   |
| $k_{cat}/k_m$     | 209.111  | 47.917  | –       | 392.483 |
| Xylose (X1)       | 0        | 0       | 0       | 44.54   |
| Xylobiose (X2)    | 0        | 303.23  | 304.78  | 351.06  |
| Xylotriose (X3)   | 0        | 318.88  | 393.6   | 523.23  |
| Xylopentaose (X4) | 0        | 0       | 0       | 0       |
| Xylopentose (X5)  | 0        | 99.07   | 253.89  | 94.46   |
| Xylohexaose (X6)  | 0        | 20.71   | 105.72  | 99.35   |
| Total XOS (mg/L)  | 0        | 741.89  | 1057.99 | 1068.1  |
| Arabinose (mg/L)  | 471.64   | 0       | 940.76  | 997.20  |
| Degree of synergy |          |         | 1.43    | 1.01    |

### 3.4. Kinetic parameters of commercial arabinoxylan

The chimera Xyn10cf+Af\_afu62 presented a  $v_{max}$  value 1.23 times higher than that of the parental Xyn10cf and 1.02 times higher than that of Af\_afu62. The  $k_m$  value was 45.77% and 85.02% lower than Af\_afu62 and Xyn10cf, respectively. The  $k_{cat}$  value was also enhanced, and therefore, the catalytic efficiency ( $k_{cat}/k_m$ ) of Xyn10cf+Af\_afu62 was 1.88 and 8.2 times higher than that of parental Af\_afu62 and Xyn10cf, respectively (Table 2). The catalytic efficiency of chimeras designed with short flexible linkers was also discussed by Guo et al. [38], where  $k_{cat}/k_m$  of the chimera designed with (GGGGS)<sub>2</sub> linker enhanced 1.53 times.

### 3.5. Effects of metal ions, chemicals, salt, and pH

The chimera Xyn10cf+Af\_afu62 displayed high relative activity in the range of pH 4.0–7.5, maintaining more than 90% maximum activity (Fig. 5). Compared to the parental enzymes, the chimera displayed superior behavior at pH values above 7.5 than Af\_afu62 but was inferior to Xyn10cf, again confirming that the physicochemical characteristics merged. Also, Xyn10cf+Af\_afu62 displayed elevated halotolerance, maintaining 82% maximum activity with 3 M NaCl (Fig. 5). The stability in salty and alkali media is especially important for arabinoxylan hydrolysis since its extraction usually uses alkaline methods whose neutralization prior to ethanol precipitation produces elevated quantities of salts and acetate [39].

The effects of metal ions and chemicals on the enzymatic activity of the chimera and parental enzymes were also analyzed (Fig. 5). The chimera Xyn10cf+Af\_afu62 and the parental Xyn10cf were significantly stimulated by  $Mn^{2+}$ ,  $Ca^{2+}$ ,  $Co^{2+}$ , and  $Mg^{2+}$ . BME and DTT, which are disulfide bond reducing agents known to prevent the oxidation of sulfhydryl groups, significantly enhanced their activity, indicating the presence of a reduced thiol group of cysteine. The activities of the chimera and both parental enzymes were suppressed by  $Zn^{2+}$ ,  $Cu^{2+}$ , and  $Ag^{3+}$ , which complex with reactive protein groups, affecting the catalytic site. Lower concentrations of SDS (1 and 5 mM) enhanced the catalytic activity, but 10 mM SDS strongly inhibited the enzymes, suggesting that hydrophobic interactions are essential to maintaining the protein structures. EDTA suppressed the activities of the chimera Xyn10cf+Af\_afu62 and parental enzymes, indicating that they require a cofactor for its activation [40].

$Mn^{2+}$  and  $Ca^{2+}$  were the ions that displayed positive effects for both



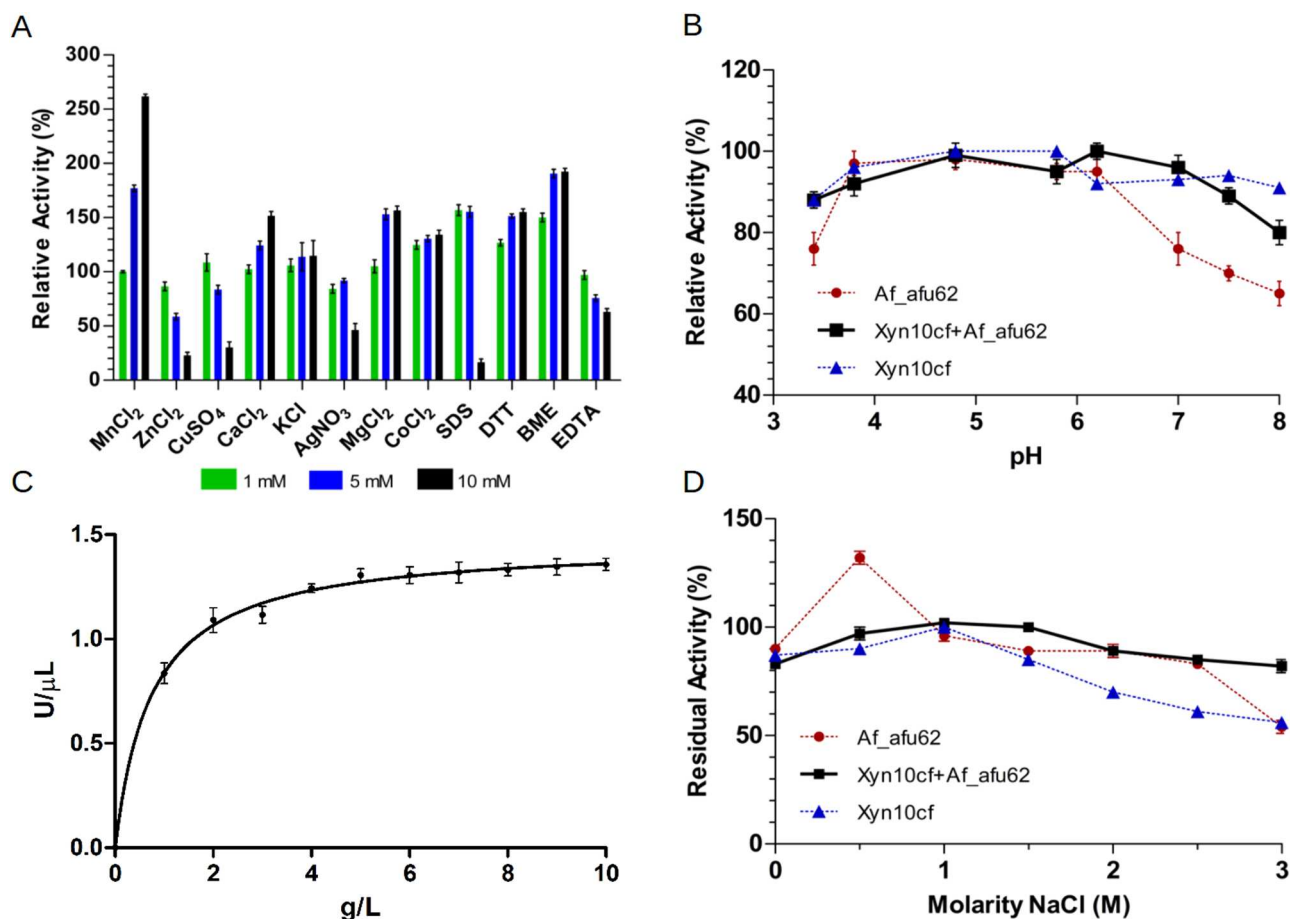


Fig. 5. Characterization of chimeric enzyme Xyn10cf+Af\_Afu62: (A) Metal and inhibitor effects, (B) pH stability, (C) kinetic parameters, and (D) salt tolerance.

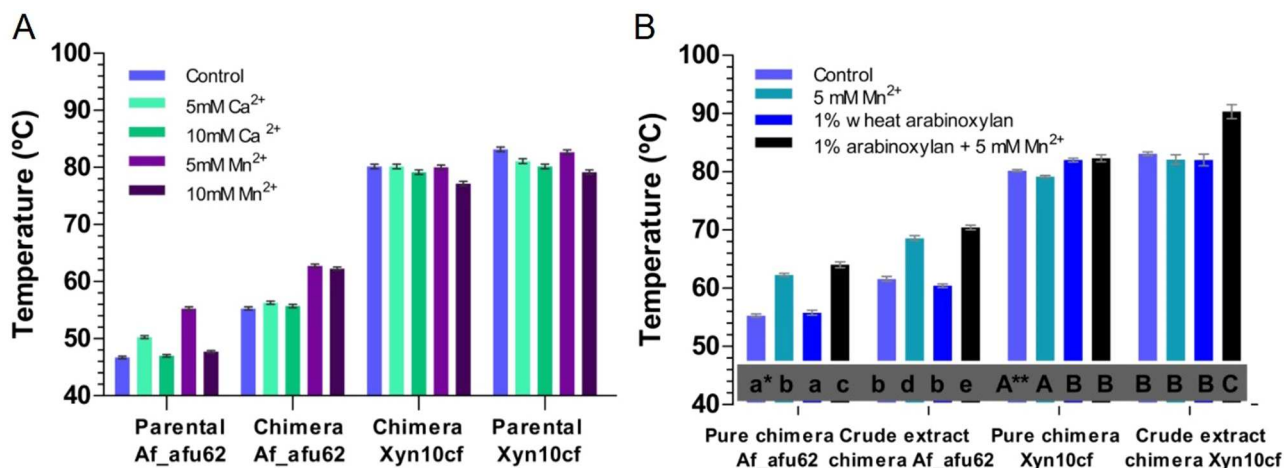


Fig. 6. Melting temperatures of the chimera and parental enzymes: cofactors Mn<sup>2+</sup> and Ca<sup>2+</sup> ions influence (A); arabinoxylan and Mn<sup>2+</sup> influence (B). (\*different letters mean significant differences among the Af\_afu62 melting temperatures; \*\*different capital letters mean significant differences among the Xyn10cf melting temperatures).

parental (endoxylanase and arabinofuranosidase) and chimeric enzymes. Several studies have reported the influence of these ions on the thermostability of many enzymes. GH 10 from *Thermotoga thermarum* retained nearly 100% of its residual activity after incubation at 85 °C for 2 h with 5 mM Ca<sup>2+</sup> and more than 50% after 90 °C for 1 h [41], and the calcium-binding site of GH 10 from *Rhodothermus marinus* was shown to play a role in thermostability [37]. GH 62 arabinofuranosidase from *Eupenicillium parvum* also enhanced thermostability after the addition of

Ca<sup>2+</sup> [42].

### 3.6. Thermal shift

Differential scanning fluorometry (DSF) was used to analyze the changes in thermostability of the chimeric and parental enzymes, as well as the effects of Ca<sup>2+</sup> and Mn<sup>2+</sup> ions. DSF is a useful technique to investigate multidomain proteins, and it is possible to visualize the

**Table 1**

Thermodynamic parameters of chimera Xyn10cf+Af\_afu62 and parental enzymes in the presence of 5 mM  $\text{Ca}^{2+}$  or 5 mM  $\text{Mn}^{2+}$  (no ion for control) after thermal treatment at 50, 60, and 70 °C.

| Chimera Xyn10cf+Af_afu62           | $k_d$ ( $\text{min}^{-1}$ ) | $R^2$  | $t_{1/2}$ (min) | D-value (min) | $\Delta G_d$ (kJ/mol) | $\Delta H_d$ (kJ/mol) | $\Delta S_d$ (J/mol.K) | $E_d$ (kJ/mol) |
|------------------------------------|-----------------------------|--------|-----------------|---------------|-----------------------|-----------------------|------------------------|----------------|
| <b>Control</b>                     |                             |        |                 |               |                       |                       |                        |                |
| 50 °C                              | 0.0033                      | 0.9515 | 222.260         | 697.753       | 105.710               | 34.378                | -220.740               | 37.065         |
| 60 °C                              | 0.0042                      | 0.9918 | 146.899         | 548.235       | 108.398               | 34.295                | -222.431               |                |
| 70 °C                              | 0.0074                      | 0.9968 | 99.463          | 311.160       | 110.120               | 34.212                | -221.210               |                |
| <b><math>\text{Ca}^{2+}</math></b> |                             |        |                 |               |                       |                       |                        |                |
| 50 °C                              | 0.0012                      | 0.9993 | 577.623         | 1918.821      | 108.428               | 46.620                | -191.268               | 49.306         |
| 60 °C                              | 0.0020                      | 0.9897 | 346.574         | 1151.293      | 110.453               | 46.536                | -191.854               |                |
| 70 °C                              | 0.0035                      | 0.9861 | 198.042         | 657.881       | 112.256               | 46.453                | -191.761               |                |
| <b><math>\text{Mn}^{2+}</math></b> |                             |        |                 |               |                       |                       |                        |                |
| 50 °C                              | 0.0012                      | 0.9061 | 577.623         | 1918.821      | 108.428               | 42.576                | -203.780               | 45.263         |
| 60 °C                              | 0.0021                      | 0.9694 | 330.070         | 1096.469      | 110.318               | 42.493                | -203.585               |                |
| 70 °C                              | 0.0032                      | 0.9637 | 216.608         | 719.558       | 112.512               | 42.410                | -204.288               |                |
| <b>Parental Xyn10cf</b>            |                             |        |                 |               |                       |                       |                        |                |
| <b>Control</b>                     |                             |        |                 |               |                       |                       |                        |                |
| 50 °C                              | 0.0009                      | 0.9479 | 770.164         | 2558.428      | 109.201               | 51.102                | -179.790               | 53.788         |
| 60 °C                              | 0.0014                      | 0.9950 | 495.105         | 1644.704      | 111.441               | 51.018                | -181.366               |                |
| 70 °C                              | 0.0029                      | 0.9923 | 239.016         | 793.995       | 112.792               | 50.935                | -180.262               |                |
| <b><math>\text{Ca}^{2+}</math></b> |                             |        |                 |               |                       |                       |                        |                |
| 50 °C                              | 0.0028                      | 0.9960 | 247.553         | 822.352       | 106.151               | 29.217                | -238.075               | 31.904         |
| 60 °C                              | 0.0038                      | 0.9764 | 182.407         | 605.943       | 108.675               | 29.134                | -238.753               |                |
| 70 °C                              | 0.0056                      | 0.9940 | 123.776         | 411.176       | 110.915               | 29.051                | -238.566               |                |
| <b><math>\text{Mn}^{2+}</math></b> |                             |        |                 |               |                       |                       |                        |                |
| 50 °C                              | 0.0016                      | 0.9818 | 433.217         | 1439.116      | 107.655               | 27.899                | -246.808               | 30.586         |
| 60 °C                              | 0.0025                      | 0.9595 | 277.259         | 921.034       | 109.835               | 27.816                | -246.192               |                |
| 70 °C                              | 0.0031                      | 0.9811 | 223.596         | 742.769       | 112.602               | 27.733                | -247.325               |                |

$k_d$  = first order rate denaturation constant;  $t_{1/2}$  = half-life time; D-value = decimal reduction (time required to reduce residual activity to 10%);  $\Delta H_d$  = activation enthalpy of denaturation;  $\Delta G_d$  = activation Gibbs free energy of denaturation;  $\Delta S_d$  = activation entropy of denaturation;  $E_d$  = activation energy of denaturation

transition state of each domain in the enzyme. The goal of this assay was to show the impact of fusing the catalytic domains on thermostability and investigate the cofactor influence (Fig. 6).

The chimera Xyn10cf+Af\_afu62 displayed two melting points, one related to the arabinofuranosidase domain and the second to the endoxylanase (the most thermostable). Researchers revealed that the integration order of the two enzymes results in different properties of the chimera, where the most thermostable should be N-terminal [38]. A higher  $T_m$  for the arabinofuranosidase domain was observed in all the assays, with an increase of 10 °C, while the endoxylanase domain displayed a lower  $T_m$  than the parental Xyn10cf, with a reduction of 3 °C, probably due to protein aggregation [37]. Additionally, the presence of 5 mM  $\text{Ca}^{2+}$  and especially  $\text{Mn}^{2+}$  enhanced  $T_m$  for Xyn10cf+Af\_afu62 and parental Af\_afu62 up to 6.8 °C. The  $T_m$  of the chimera endoxylanase domain did not suffer significant change with 5 mM  $\text{Ca}^{2+}$  or  $\text{Mn}^{2+}$ , while the parental Xyn10cf displayed 4 °C reduced  $T_m$  in the presence of the ions. The chimera showed a superior tolerance and affinity for the ions than the parental enzymes. Despite the small reduction in  $T_m$  for the endoxylanase module, the overall thermostability improvement for the arabinofuranosidase domain and cofactor concentration up to 5 mM overcame this drawback. The flexible linkers are often related to improved kinetic parameters but reduced thermostability, while rigid linkers may improve thermostability and reduce the substrate specificity [38]. However, this present study showed the opposite as, although Xyn10cf didn't present significant changes in thermostability, Af\_afu62 thermostability improved 1.2 times.

Significant differences in  $T_m$  were found for the crude supernatant and purified chimeric enzyme, as presented in Fig. 6. The crude supernatant presented  $T_m$  values for arabinofuranosidase and endoxylanase modules of 61.6 °C and 88.1 °C, respectively, against 55.3 °C and 83.2 °C from the purified chimeric enzyme. Additionally, the presence of 1% wheat arabinoxylan and 5 mM  $\text{Mn}^{2+}$  increased  $T_m$  to 70.4 °C and 90.3 °C (arabinofuranosidase and endoxylanase, respectively), since hydrophobic interactions of the substrate-binding site play a role in the thermostability of GH 10 [43–45] and the  $\beta$ -propeller structure of GH 62 stabilizes the arabinofuranosyl substituent through hydrogen bonding and hydrophobic interactions [46].

### 3.7. Thermostability

Table 1 presents the thermodynamic parameters of the fused and parental enzymes under the influence of 5 mM  $\text{Mn}^{2+}$  or  $\text{Ca}^{2+}$ . Our previous study already reported the positive effects of  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  on Af\_afu62 thermostability [18]. The same treatment was therefore applied to the parental endoxylanase Xyn10cf and compared to the chimera results. The cofactors impaired the thermostability of parental Xyn10cf by decreasing half-life time ( $t_{1/2}$ ) from 770 to 247 min at 50 °C and energy of denaturation ( $E_d$ ) from 53 to 30 kJ/mol. This result is in accordance with the thermal shift analyses (Section 3.6).

All thermodynamic parameters of the chimera increased in the three scenarios (control, 5 mM  $\text{Ca}^{2+}$  and 5 mM  $\text{Mn}^{2+}$ ). The enthalpy of denaturation ( $\Delta H_d$ ) expresses the total energy required to denature an enzyme, related to the  $E_d$  value (energy of denaturation). Higher values of  $E_d$  and  $\Delta H_d$  indicate higher thermostability. Additionally, high positive Gibbs free energy ( $\Delta G_d$ ) values indicate an increase in denaturation resistance [47,48].  $\Delta G_d$  computed a slight increase for the chimera Xyn10cf+Af\_afu62 and parental Af\_afu62 incubated with  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  at all temperatures, along with the significant enhancement of  $t_{1/2}$ , D value,  $E_d$ , and  $\Delta H_d$ . On the other hand, there was an expression decrease in the  $\Delta G_d$  and  $\Delta H_d$  values of Xyn10cf incubated with  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$ . In conclusion,  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  enhanced the thermostability of Xyn10cf+Af\_afu62 in the temperature range of 50–70 °C.

Although the chimera displayed a lower  $E_d$  value than parental endoxylanase, the presence of  $\text{Mn}^{2+}$  and  $\text{Ca}^{2+}$  increased stability, represented by the increase in  $E_d$ , D-value, and  $t_{1/2}$  values. The chimera D-value (time required to decrease residual activity to 10%) at 50 °C increased from 697 to 1918 min with 5 mM  $\text{Ca}^{2+}$ , increasing  $E_d$  from 37.1 to 49.3 kJ/mol, closer to the Xyn10cf control value of 53.4 kJ/mol. Therefore, the drop in thermostability from the increase in protein flexibility and  $\beta$ -sheet content after fusion may be overcome with cofactors in the hydrolysis reaction.

### 3.8. Synergism analysis and XOS production from natural substrates

The synergism assay of parental enzymes and chimera was carried

**Table 3**

Chemical composition of corn and soy hulls agro-industrial wastes.

| %             | Corn hull         | Soy hull          |
|---------------|-------------------|-------------------|
| Cellulose     | 15.06 ± 2.90      | 41.72 ± 0.90      |
| Hemicellulose | 40.33 ± 2.3117.83 | 29.36 ± 2.3119.71 |
| Xylose        | ± 2.1011.5 ± 0.20 | ± 2.105.65 ± 0.20 |
| Arabinose     |                   |                   |
| Lignin        | 2.76 ± 1.30       | 9.88 ± 1.30       |
| Extractives   | 22.92 ± 2.90      | 9.67 ± 0.22       |
| Starch        | 15.03 ± 3.40      | –                 |
| Protein       | 2.61 ± 0.30       | 12.10 ± 1.00      |
| Ashes         | 0.88 ± 0.10       | 0.47 ± 0.10       |

out at 40 °C, a temperature at which the three enzymes (the two parental enzymes and the chimera enzyme) are fully stable. The degree of synergism consisted of the ratio between the chimera activity and the sum of activities of the parental enzymes' equimolar mixtures, as shown in Table 2. Despite the small increase of 1% in total XOS production, xylobiose and xylotriose yields, which present higher prebiotic potential, improved 15.5% and 33%, respectively.

Table 3 presents the composition of each biomass. Soy hull hemicellulose is constituted mainly by arabinose and xylose units (~84%), followed by glucuronic acid and acetyl groups. Corn hull presented a higher hemicellulosic content; ~73% of its hemicellulose was characterized as arabinose and xylose polymers, and the remaining 27% constituted galactose, mannose, acetyl groups, and uronic acid units. Corn hull presented a higher arabinose content, 11.5%, and soy hull, 5.6%.

The extraction with 5% KOH resulted in an arabinoxylan purity (based only on the content of xylose and arabinose) of 73.4% for soy hull and 75.1% for corn hull. Increasing the alkali concentration increased the degradation of arabinoxylan, which reduced the purity of the extracted material. Additionally, a higher alkali concentration increases the extraction of glucans from corn hull that goes along arabinoxylan, which is also classified as 4-O-methyl-glucuronarabino-xylan acetate [49].

The positive interaction between substrate concentration and temperature revealed the best conditions for the efficient conversion of arabinoxylan into xylooligosaccharides. Higher conversion yields (arabinoxylan to XOS) were obtained for low substrate loads: 50% conversion was obtained with a 0.4% solid load (Fig. 7). However, higher XOS concentrations were obtained using a higher substrate load: 6.97 g/L and 3.33 g/L XOS were obtained at 60 °C with 4% arabinoxylan from corn and soy hulls, respectively (Fig. 6). These results are comparable to the recent literature: 5.3 g/L XOS were obtained from 10% wheat bran by a recombinant endoxylanase [50], 4.1 g/L XOS from sugarcane straw

were produced by the synergic mixture of Xyn10cf and a commercial GH 51 arabinofuranosidase (*Aspergillus niger*) [8], and 11 g/L XOS were obtained from hydrothermal treatment of corn stover, which required additional purification steps to remove impurities generated due to biomass degradation [51].

Comparing the yields of the bifunctional chimera and the Shearzyme xylanase at a 1% substrate load, 2.40 g/L (from corn) and 1.37 g/L (from soy) XOS were obtained at 60 °C using the bifunctional chimera, against 0.46 g/L (from corn) and 0.84 g/L (from soy) using Shearzyme xylanase at 50 °C. The higher amount of arabinose in corn hull may directly influence the hydrolysis yields. The low yield obtained with Shearzyme xylanase indicates the hindrance brought by the arabinose side chains, impairing xylanase activity. However, the chimeric enzyme was able to increase the hydrolysis yield 5.22 times, proving the synergy between arabinofuranosidase and endoxylanase for the efficient depolymerization of arabinoxylan from lignocellulosic biomass.

A potential drawback for the industrial production of chimeric enzymes is the cleavage of the linker, leading to protein degradation and reduction of catalysis power, or loss of activity in one of the domains due to improper conformation [52]. Also, the ratio between the enzymes required to improve the yields of XOS in different biomass may differ from an equimolar mixture [53]. However, both the equimolar mixture and the chimeric enzyme proved the synergy between Xyn10cf and Af\_afu62. Based on the literature [54–56], producing chimeric enzymes could be more advantageous than producing single enzymes separately due to the kinetic advantages presented by chimeras over a simple mixture of parental enzymes, representing a positive influence on the cost of enzymes [52].

#### 4. Conclusion

To reduce the costs of enzyme production for XOS production, this novel bifunctional chimera combining the GH families 10 and 62 presented good thermostability and higher substrate specificity in comparison to the parental enzymes. The cofactors  $Mn^{2+}$  and  $Ca^{2+}$  improved the activity and thermostability. The synergism of the endoxylanase and arabinofuranosidase domains improved the production of xylobiose and xylotriose, and up to 7 g/L XOS was obtained after hydrolysis of arabinoxylan from agro-industrial wastes at 60 °C. The molecular fusion of synergic enzymes is a promising strategy to produce more efficient and thermostable enzymes and reduce operational costs.

#### CRediT authorship contribution statement

Manoela Martins: Conceptualization, Methodology, Investigation,

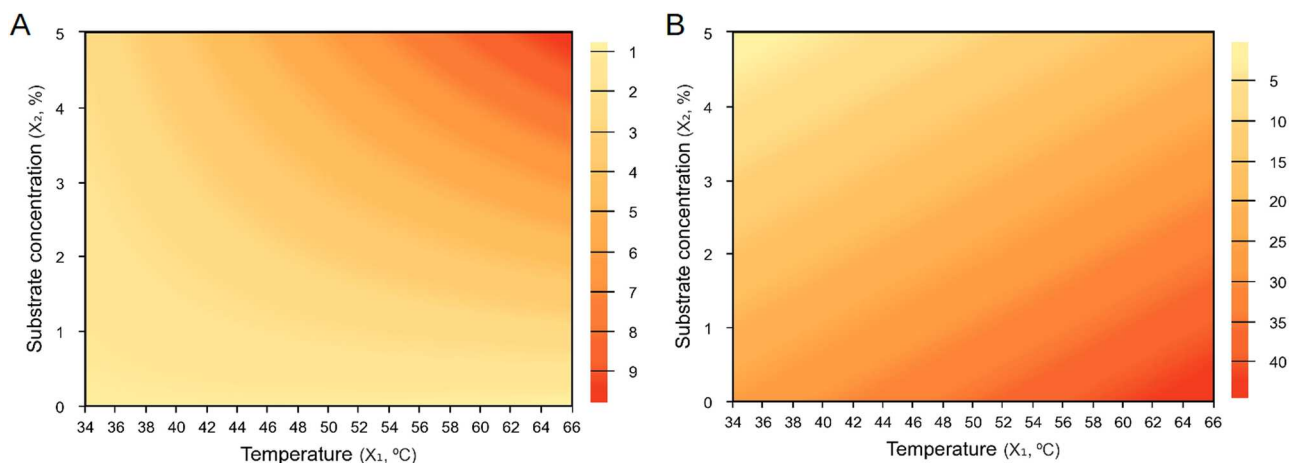


**Fig. 7.** Effect of temperature and corn hull arabinoxylan concentration (1–5% w/v) on (A) total xylooligosaccharides production (g/L) and (B) arabinoxylan conversion yields into xylooligosaccharides after 48 h hydrolysis.

Formal analysis, Validation, Writing – original draft preparation. **Marcos Feliipe Silva**: Methodology, Formal analysis. **Taísa Magnani Dinamarco**: Resources, Supervision, Writing – review & editing. **Rosana Goldbeck**: Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.procbio.2022.09.018](https://doi.org/10.1016/j.procbio.2022.09.018).

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# Thermostability Enhancement of GH 62 $\alpha$ -L-Arabinofuranosidase by Directed Evolution and Rational Design

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Supporting Information

**ABSTRACT:** GH 62 arabinofuranosidases are known for their excellent specificity for arabinoxylan of agroindustrial residues and their synergism with endoxylanases and other hemicellulases. However, the low thermostability of some GH enzymes hampers potential industrial applications. Protein engineering research highly desires mutations that can enhance thermostability. Therefore, we employed directed evolution using one round of error-prone PCR and site-saturation mutagenesis for thermostability enhancement of GH 62 arabinofuranosidase from *Aspergillus fumigatus*. Single mutants with enhanced thermostability showed significant  $\Delta\Delta G$  changes ( $<-2.5$  kcal/mol) and improvements in perplexity scores from evolutionary scale modeling inverse folding. The best mutant, G205K, increased the melting temperature by 5 °C and the energy of denaturation by 41.3%. We discussed the functional mechanisms for improved stability. Analyzing the adjustments in  $\alpha$ -helices,  $\beta$ -sheets, and loops resulting from point mutations, we have obtained significant knowledge regarding the potential impacts on protein stability, folding, and overall structural integrity.

**KEYWORDS:** arabinoxylan, error-prone PCR, protein evolution, site-directed mutagenesis

## INTRODUCTION

In the agroindustrial field,  $\alpha$ -L-arabinofuranosidases (E.C.3.2.1.5.) synergistically play a prominent role with other hemicellulases to ensure an effective degradation of hemicellulose to obtain a wide range of bioproducts, including medicinal substances, dietary fiber, ferulic acid, and fermentable sugars for bioethanol. GH 62  $\alpha$ -L-arabinofuranosidases catalyze the hydrolysis of the terminal nonreducing  $\alpha$ -1,2, and  $\alpha$ -1,3-L-arabinofuranoside substitution of  $\alpha$ -L-arabinofuranoside such as arabinoxylan, the primary hemicellulosic group in grain and grasses.<sup>1,2</sup> The synergistic effect of GH 62 arabinofuranosidase and endoxylanase is a sustainable route for obtaining xylooligosaccharides from agroindustrial residues. We recently designed a bifunctional chimera to improve arabinoxylan specificity and degree of synergy to enhance xylooligosaccharides production from corn and soy husks.<sup>3,4</sup>

Despite the high specificity and synergy for arabinoxylan depolymerization, GH 62 arabinofuranosidases present a drawback for industrial applications. Their high flexibility and low  $\alpha$ -helix content compared to other hemicellulases, such as GH10 endoxylanase,<sup>3</sup> result in lower thermostability and half-life at temperatures above 50 °C, which could hamper industrial processes where higher hydrolysis temperatures are necessary to speed up reaction rate, reduce microbial contamination, and increase substrate solubility.<sup>5</sup> One solution to overcome this drawback is engineering novel enzymes with enhanced thermostability by directed evolution or rational design. To this date, there are no reports of a GH 62 arabinofuranosidase re-engineered specifically to improve thermostability.

Directed evolution is an approach that simulates Darwinian evolution by molecular biology techniques to rapidly evolve peptides and proteins into new forms with improved properties for specific applications. We can create millions of mutants in a single experiment and subject them to high-throughput screening to identify those with the enhanced desired properties. Researchers have successfully employed this approach to improve the catalytic power of GH51 arabinofuranosidases by one-round error-prone PCR<sup>6</sup> and site-directed mutagenesis.<sup>5</sup> Random mutagenesis by error-prone PCR has the advantage of enhanced probability of finding unexpected beneficial mutations in different regions of a protein, while the primary benefit of site-directed mutagenesis is that the diversity in a library is focused on a location of the protein known or thought to be essential for function or stability. Furthermore, we can explore mutations not likely to occur by error-prone PCR random mutagenesis by site-directed mutagenesis.<sup>7,8</sup>

On the other hand, rational design based on protein structure helps identify hotspots for mutation by predicting the effect of mutations on structural stability, reducing workload. One strategy for identifying thermostabilizing mutations involves the comparison of more thermostable proteins with less stable proteins and identification of amino acid sequence

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GC enhancer [PCR conditions: 98 °C for 3 min, 30 cycles of denaturation at 98 °C for 10 s, annealing for 30 s (variable temperatures), extension at 72 °C for 150 s, and a final extension at 72 °C for 5 min]. The annealing temperatures for each primer pair are listed in Table 1. We digested the amplified fragments using DpnI at 37 °C for 1.5 h. Successful PCRs were purified using the PureLink Quick Gel Extraction and Zymo Cleanup and phosphorylated. The phosphorylation reaction used about 400 ng of DNA, 2  $\mu$ L of T4 DNA Ligase, and 1  $\mu$ L of T4 PNK, incubated 1 h at 37 °C. Then, the DNA was purified (Zymo Cleanup), ligated, and transformed to NiCo21(DE3) for screening.

**Purification of High-Activity Variants.** We overexpressed the high-activity variants in 10 mL of LB medium and obtained the soluble extracts as described above. The supernatant was concentrated 20 times by ultrafiltration (Amicon Ultra-15, Merck KGaA, Germany) with a molecular weight cutoff of 10 kDa and loaded onto HisPur Ni-NTA Spin Columns (Thermo Scientific, USA), following the recommended protocol (equilibration buffer: 10 mM imidazole; wash buffer: 25 mM imidazole; elution buffer: 250 mM imidazole. All buffers had 10 mM sodium phosphate (pH 7.4) and 300 mM NaCl). We dialyzed the fractions using 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl buffer to remove imidazole. The enzyme concentration was determined using the DC Protein assay (Bio-Rad Laboratories Inc., USA) and BSA as standard. The molecular mass under denaturing conditions was determined by SDS-PAGE gel electrophoresis, using NuPAGE 4–12%, bis-tris gel, and MOPS SDS Running Buffer (Thermo Scientific, USA). The proteins were visualized by staining with Coomassie brilliant blue R-250.

**Biochemical Assays.** Detecting arabinofuranosidase activity in the crude supernatant was determined by enzymatic hydrolysis of wheat arabinoxylan (Megazyme International Ireland, Wicklow, Ireland). In short, 10  $\mu$ L of culture supernatant was added to a 90  $\mu$ L reaction volume containing 1% (w/v) wheat arabinoxylan and 5 mM  $\text{MnCl}_2$  in McIlvaine buffer pH 5.0 (unless otherwise specified), in triplicate. The released arabinose was detected using the L-arabinose/D-galactose assay kit (Megazyme International Ireland, Co., Wicklow, Ireland).

We evaluated the thermostability of the mutants by incubating the samples in the absence of the substrate at a determined temperature for various periods. The initial activity at 42 °C was considered 100% activity. Next, we assessed residual activity at 42 °C after thermal treatment from 45 to 65 °C and pH 5.0 using a thermocycler gradient (Mastercycler nexus X2-Eppendorf SE). We aliquoted every 30 min for 4 h and immediately cooled them at 4 °C for at least 1 h to ensure sufficient time for the reversibly denatured protein to convert back to its native active form, while the irreversibly denatured remained inactive. At the end of the thermal treatment, we conducted an enzymatic assay of the aliquots at 42 °C for 30 min. We computed the relative activities as a percentage of the original activities to calculate the thermodynamic parameters.<sup>13</sup>

$T_{50}^{60}$  refers to the temperature at which the enzyme activity drops to 50% of its initial value after an incubation time of 60 min. We quantified the enzymatic activity of the mutants and WT by measuring the residual activity of the enzyme after incubation at different temperatures for 60 min.

The enzyme half-life ( $t_{1/2}$ ) was calculated as  $\ln(2)/k_{\text{inact}}$  from linear plots of  $\ln(\text{residual activity})$  versus time (min) to obtain the inactivation rate constant  $k_{\text{inact}}$  ( $\text{min}^{-1}$ ). The half-life was the time elapsed for the enzyme activity to decline to half of its initial level at 50 °C ( $t_{1/2}^{50}$ ) and 60 °C ( $t_{1/2}^{60}$ ).

We calculated the energy of denaturation  $E_d$  (kJ/mol) from the linearization of the Arrhenius plot ( $\ln(k_{\text{inact}})$  versus  $1/T$ ), according to eq 1

$$\ln(k_{\text{inact}}) = \ln(A_0) - \frac{E_d}{R} \times \frac{1}{T} \quad (1)$$

where  $k_{\text{inact}}$  is the inactivation rate constant,  $A_0$  is the Arrhenius constant,  $E_d$  is the energy of denaturation,  $R$  is  $8.314 \times 10^3$ , and  $T$  is the absolute temperature (kelvin).

**Determination of  $k_{\text{cat}}$  and  $K_M$ .** For determination of the Michaelis–Menten constants  $K_M$  and  $k_{\text{cat}}$ , the activity assay was performed at 45 °C pH 5.0 for 15 min using substrate concentrations from 0 to 20  $\mu$ M wheat arabinoxylan and  $1 \times 10^{-8}$   $\mu$ M of enzyme concentration. The reported values are the average of triplicate experiments.

**Enzyme Denaturation.** We assayed the melting temperatures of the thermoresistant variants by differential scanning fluorimetry (DSF) using the Protein Thermal Shift Dye Kit (Thermo Fisher Scientific Inc., USA). We pipetted 12.5  $\mu$ L of enzymes preparations (1–5  $\mu$ g), 2.5  $\mu$ L of Diluted Protein Thermal Shift Dye (8 $\times$ ), and 5  $\mu$ L of Protein Thermal Shift Buffer into the wells of a qPCR plate and sealed it, raising the temperature from 25 to 95 °C at a step of 0.1 °C/s. We computed the  $T_m$  from the inflection points of the transition curve [fluorescence =  $f(T)$ ] as the maximum of the first derivative [ $d(\text{fluorescence})/d(T)$ ].

We determined the denaturation temperatures of the variants by differential scanning calorimetry (DSC) (N-DSC II instrument, Calorimetry Sciences, Provo, UT). The purified enzyme samples (0.5 mg/mL) were dialyzed against 100 mM NaCl and 50 mM Tris/HCl buffer pH 7.0, and the dialysis buffer was used as a reference solution. Samples were analyzed at a heating rate of 1 °C per minute over a temperature range of 30–90 °C. The denaturation temperature was defined as the temperature at maximum molar heat capacity. Data analysis was carried out with GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

**Bioinformatic Analysis.** We compared the deduced amino acid sequence from nucleotide sequencing to that from the WT sequence using Clustal  $\Omega$  and BLAST algorithms. We aligned the amino acid sequences of Af\_afu62 and templates from the PDB library using UCSF Chimera.<sup>14</sup>

We used the Hotspot Wizard 3.0 server (<https://loschmidt.chemi.muni.cz/hotspotwizard>) to find hotspots for thermostability enhancement and undertake calculations of  $\Delta\Delta G$ , which indicates the change in stability caused by a point mutation. We calculated the hotspots by sequence consensus and the  $\Delta\Delta G$  as the difference between the minimal  $\Delta G$  of the WT structure and the minimal  $\Delta G$  of the mutant. Negative  $\Delta\Delta G$  values indicate increased stability.<sup>15</sup>

Then, we used the ESM-IF1 language to calculate the inverse folding and to predict the functional effects of sequence variation. The ESM-IF1 inverse folding model predicts protein sequences from their backbone atom coordinates. For mutational effect predictions with ESM-IF1, we scored mutations using the masked-marginal likelihood ratio between the mutant and WT amino acids.<sup>16</sup>

**Computational Simulations.** We performed MD simulations using the Amber20 package.<sup>17</sup> We used the crystal structure of GH 62 from *Coreopsis cinerea* (PDB code: 5B6S, resolution: 1.7 Å, chain: A)<sup>18</sup> as the basis for constructing six different systems using MODELER.<sup>19</sup> These systems included the WT (1) arabinofuranosidase GH 62 from *A. fumigatus* (Af\_afu62) and five single mutants' molecular systems: (2) S127G, (3) S188F, (4) G205K, (5) T256G, and (6) Q271T. Before the MD simulations, we determined the protonation states of the residues at a pH of 5.0 using the empirical propKa program.<sup>20</sup> The systems were parametrized using the all-atom force fields CHARMM36m<sup>21</sup> and solvated using the TIP3P explicit solvation model in a truncated octahedral water box.<sup>22</sup> A cutoff distance of 20.0 Å was employed to establish the separation between the simulation box wall and the solvated atoms within the system. We added calcium and chloride ions for electro-neutralization. The minimization process involved five steps during which the water molecules, hydrogens, ions, and the entire protein structure were subjected to minimization using the steepest descent and conjugate gradient methods. This approach, as described previously,<sup>23</sup> also included the gradual relaxation of restraints. Subsequently, the entire system underwent a series of 14 heating steps. We conducted the initial heating phase under constant volume conditions for 5 ps (0 to 5 K). Then, from the second to the seventh, we achieved a gradual temperature increase from 5 to 313.5 K throughout 50 ps. Upon reaching the seventh heating stage, the temperature was 313.5 K until the 14th step. We conducted a further 2 ns of MD to achieve density



equilibration of the system under 1 bar pressure while maintaining a temperature of 313.5 K. All covalent bonds involving hydrogen atoms were kept at a fixed length using SHAKE.<sup>24</sup> We implemented a distance constraint to limit the separation among histidine, crystallographic water, and calcium ions during the equilibration stage. We initially set the limit at approximately 15.0 kcal and gradually reduced it. The purpose of this constraint was to ensure the proper coordination of calcium in the GH 62 active site.

We conducted a total of 200 ns of MD for each system using the isothermal–isobaric (NPT) ensemble. To ensure the accuracy and consistency of our results, we replicated our MD simulation systems. We analyzed the rmsd values of WT and mutant structures over a trajectory of 200 ns, focusing on the heavy atom's backbone of the enzyme. Additionally, RMSF, cavity analysis, and secondary structure analysis were all performed for the last 50 ns of the simulation for a more accurate comparison between the systems after equilibration.

We analyzed the volume variation of the catalytic pocket using MDpocket.<sup>25</sup> The initial detection used Fpocket, which served as a benchmark for generating the required input pocket file for the subsequent analysis of MD trajectories under MDpocket. The primary objective of our investigation was to analyze the effects of point mutations in the proximity of the calcium coordination region adjacent to the active site, which is widely recognized for its importance to the protein conformation, particularly the catalytic pockets.<sup>26</sup>

We performed a secondary structure analysis using the Define Secondary Structure of Proteins (DSSP) algorithm<sup>27</sup> to examine the changes in the secondary structural components within the systems.

**Statistical Analysis.** All data represent the mean  $\pm$  standard deviation of three independent replicates. We performed unpaired and two-tailed *t* tests ( $p < 5\%$ ) using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). We computed Pearson and Spearman's coefficients from the correlation between the experimental data (melting temperature) and ESM-IF1 final scores. Pearson's coefficient measures the linear relation between 2 variables, where  $-1$ ,  $0$ , and  $1$  indicate perfect negative, none, and perfect positive correlation, respectively. Spearman's rank measures the monotonic relationship between the two variables, capturing non-linear correlation not computed by Pearson, where  $-1$  indicates perfect negative correlation,  $0$  indicates no relation, and  $1$  indicates perfect positive correlation.

## ■ RESULTS

**Directed Evolution for Selecting Thermostable Mutants.** Typically, low mutation frequencies are better to avoid deleterious mutations masking the effects of beneficial ones.<sup>8</sup> Consequently, we conducted one round of error-prone PCR to introduce, on average, 1–2 mutations per kb in the *Af\_afu62* gene. Three mutant libraries using error-prone PCR were generated. A low initial DNA concentration (less than 100 ng) resulted in a high mutation frequency (9–16 mutations/kb), including deletions and insertions causing frame shifts, precluding the proper expression of the enzyme. Decreasing the  $Mn^{2+}$  concentration to 50  $\mu M$  and increasing the initial target DNA concentration to 500 ng displayed less than 50% inactive mutants and an overall mutation rate of 1.8/kb.

We cloned the variants into NiCo21(DE3) and cultivated individual colonies in 96-well microplates. After induction of secretion of *Af\_afu62* with 2.5 mM IPTG at 30 °C for 24 h, we separated the cells by centrifugation and used the crude supernatant of the cultures to quickly evaluate the thermostability of mutants exhibiting higher arabinofuranosidase activity than the WT. After screening a total of 350 colonies, the mutants with initial activity higher than  $0.5(\mu - \sigma)_{WT}$  after 30 min of incubation at 50 °C were sequenced, where  $\mu_{WT}$  and  $\sigma_{WT}$  are the average and standard error of WT activity.

Notably, beneficial mutations were at nonconserved residues (with high mutability probability) in loops at the surface of the enzyme: S127A (tct  $\rightarrow$  gct), S188Y (tct  $\rightarrow$  tat), G205D (ggt  $\rightarrow$  gat); T256S (acc  $\rightarrow$  tcc); and Q271H (cag  $\rightarrow$  cat) (Table S2). Solvent-exposed residues or residues far from the active cleft were the top candidates for stabilizing mutations to avoid triggering allosteric effects that might alter the entire protein structure and substrate specificity.

Then, we analyzed consensus sequence hotspots using the Hotspot Wizard 3.0 server. We considered mutants with a predicted  $\Delta\Delta G$  (folding free energy)  $\leq -2.5$  kcal/mol as significant.<sup>28</sup> The five positions identified by the error-prone PCR experiment were among the 19 calculated hotspots (Table S1). These positions presented a calculated  $\Delta\Delta G < -2.5$  kcal/mol. Consequently, we selected these positions for the site saturation experiments to analyze if other amino acids at these locations might yield higher stability. Degenerate NNK mutagenic primers were constructed for each position to create a site-saturation library by inverse PCR. We screened 70 colonies from each of the five positions for thermostability-enhanced mutants to cover the theoretically possible variants (Figure 1A).

**Site-Directed Saturation Library Analysis.** The process consisted of initial enzymatic activity without pre-exposure to heat treatment and residual activities after incubation at different temperatures for up to 120 min. We only selected mutants with initial activity higher than  $0.5(\mu - \sigma)_{WT}$  for heat treatment and residual activity assay after 120 min exposure at 55 °C (Figure 1B), where  $\mu_{WT}$  and  $\sigma_{WT}$  are the average and standard error of WT activity. Mutants with residual activity above  $1.5(\mu - \sigma)_{WT}$  were overexpressed for further biochemical characterization.

**Characterization of Thermostability-Enhanced Mutants.** We purified high-activity-displaying variants for enzyme characterization to understand the structure–stability relation. We verified the purity of *Af\_afu62* and its variants by SDS-Page analysis (Figure S1). The molecular weights of the protein samples were around 37 kDa, as expected based on previous characterization.<sup>4</sup> *Af\_afu62* and its variants presented similar purity profiles and protein concentrations. The mutations S127G, S188F, G205K, T256G, and Q271T consistently improved thermostability, as measured when wheat arabinoxylan was the substrate. G205K had significantly more residual activity at 50 and 60 °C and  $T_{50}^{60}$  (temperature at which the enzyme activity drops to 50% after 60 min) increased to 64.7 °C, as presented in Table 2 and Figure S2. G205K doubled the decimal-reduction time at 50 °C and improved 89.9% half-life time by 89.9% at 50 °C. The energy of denaturation increased by 41.3%. As a comparison, the GH 62 from *G. zeae* and *N. hematococca* lost 78 and 99% activity, respectively, after 45 min incubation at 50 °C.<sup>29</sup> Similarly, SthAbf62A from *Streptomyces thermoviolaceus* presented optimum temperature at 55 °C but lost 60% activity after 60 min of incubation at 60 °C.<sup>30</sup>

All variants presented higher optimal reaction temperatures than the WT, which showed optimal temperatures below 45 °C (pH 5.0). The results showed that the mutants had optimal activities around 50 °C except for G205K and S188F, which showed optimal results at higher temperatures (53 °C) (Figure 2A and Table 2). Per the  $T_{50}^{60}$  values, all mutants showed a significant reduction in activity at temperatures above 56 °C. Concerning the main significant improvements, the mutants

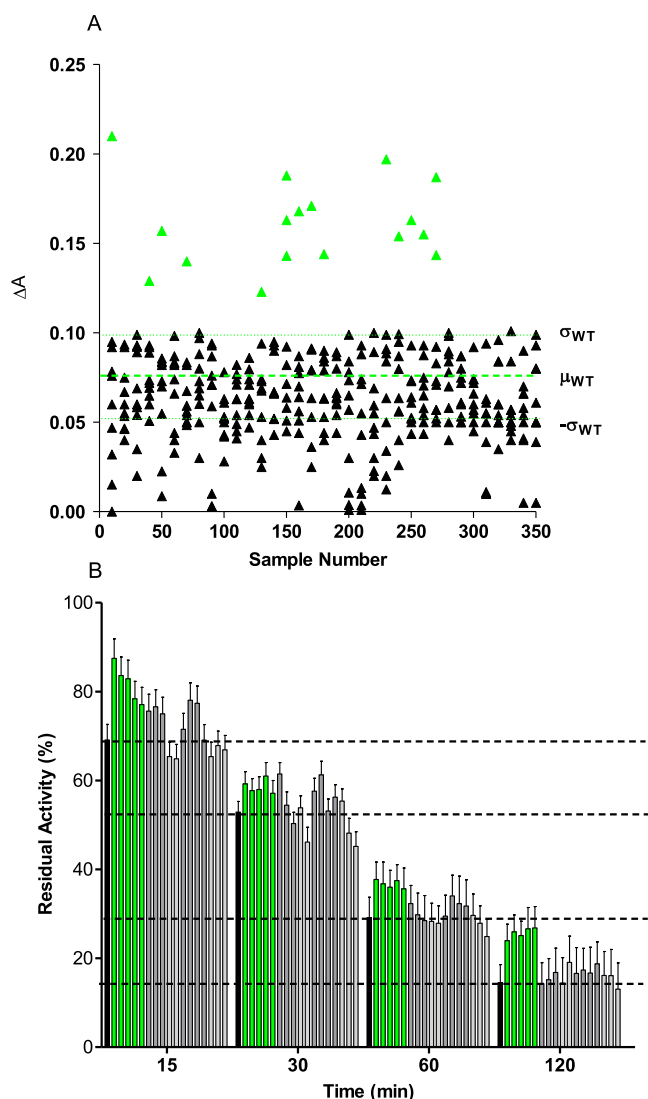


**Figure 1.** Screening results of the site-saturation mutagenesis library. (A) Initial activity of 350 clones at 50 °C for 30 min. The bold dashed line represents the average and standard error of the WT initial activity. Mutants activities in green were above  $0.5(\mu - \sigma)_{WT}$  and were selected for the second step (B), where mutants with residual activity above  $1.5(\mu - \sigma)_{WT}$  were sequenced.

exhibit a wide range of optimal pH values (4–6), differing from the WT with an optimum pH of 4.6 (Figure 2B).

The mutants showed a significant increase ( $p < 0.05$ ) of 12% in the arabinose content after 48 h incubation at 42 °C than WT (which released 3.39 mg/mL arabinose). The mutants at S127G (3.79 mg/mL arabinose) and T256G (3.63 mg/mL arabinose) also presented significant differences. The variants,

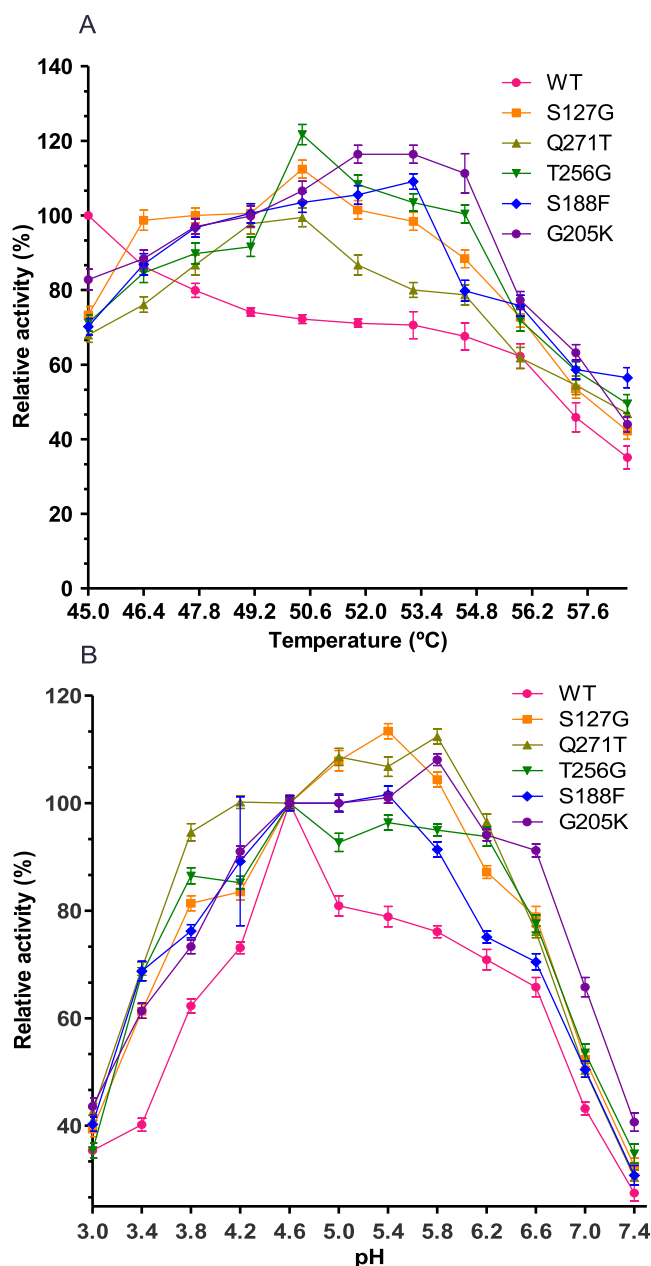


**Figure 2.** (A) Optimal reaction temperature for the WT and selected mutants. (B) Optimal reaction pH for the WT and selected variants.

therefore, showed more versatility than WT, working at optimal activity over a wide range of operating conditions and being able to further hydrolyze wheat arabinosyl over a 48 h of incubation (Figure 3A).

**Table 2.** Thermodynamic and Kinetic Parameters of Af<sub>afu62</sub> WT and Variants for Wheat Arabinosyl at 45 °C pH 5.0 for 15 min

|       | $T_{50}^{60}$    | $R^2$ | $K_m$ ( $\mu$ M) | $k_{cat}$ ( $\times 10^{10}$ min <sup>-1</sup> ) | $R^2$ | $k_{cat}/K_m$ ( $\times 10^9$ $\mu$ M <sup>-1</sup> min <sup>-1</sup> ) |
|-------|------------------|-------|------------------|--------------------------------------------------|-------|-------------------------------------------------------------------------|
| WT    | 59.42 $\pm$ 0.20 | 0.993 | 23.22 $\pm$ 6.02 | 5.95                                             | 0.954 | 2.41                                                                    |
| S127G | 63.19 $\pm$ 0.47 | 0.986 | 16.33 $\pm$ 5.69 | 4.58                                             | 0.963 | 2.81                                                                    |
| S188F | 61.80 $\pm$ 0.56 | 0.979 | 13.27 $\pm$ 4.77 | 3.52                                             | 0.952 | 2.65                                                                    |
| G205K | 64.72 $\pm$ 0.76 | 0.993 | 15.90 $\pm$ 5.08 | 4.55                                             | 0.967 | 2.86                                                                    |
| T256G | 61.43 $\pm$ 0.77 | 0.977 | 15.32 $\pm$ 4.83 | 3.75                                             | 0.967 | 2.45                                                                    |
| Q271T | 63.21 $\pm$ 0.56 | 0.984 | 16.61 $\pm$ 5.95 | 4.08                                             | 0.961 | 2.46                                                                    |

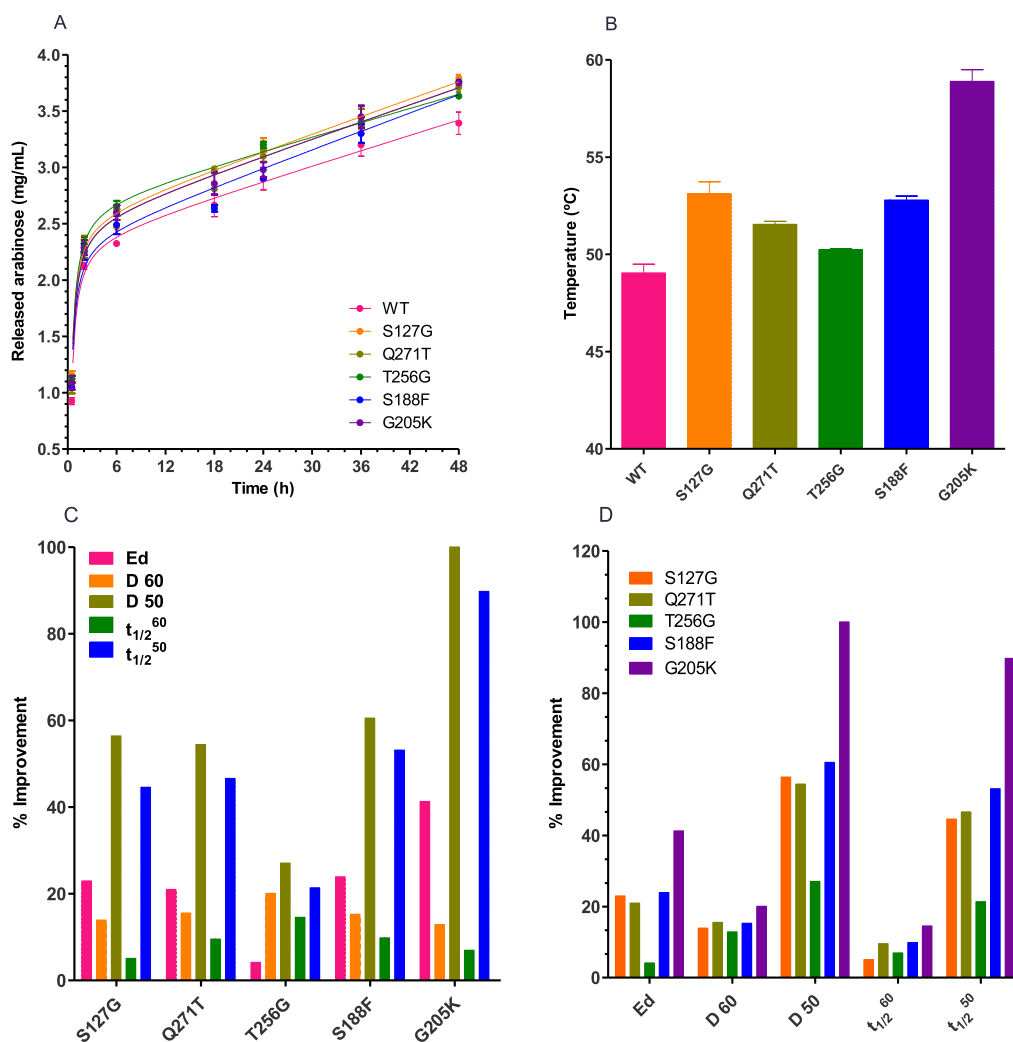


**Figure 3.** (A) Released arabinose content after 48 h hydrolysis at 42 °C. (B) Melting temperatures for purified WT and variants. The first derivative of the fluorescence emission is a function of temperature. (C) Thermodynamic parameters of WT and its variants:  $E_d$  (kJ/mol),  $D_{60}$  (min),  $D_{50}$  (min),  $t_{1/2}^{60}$  (min),  $t_{1/2}^{50}$  (min). (D) Percentage of improvements in thermodynamic parameters compared to WT.

Table 2 displays the kinetic parameters of WT and its variants at 45 °C and pH 5.0. The  $K_M$  values of variants were lower than that of the WT, suggesting heightened substrate affinity for the variants. Nevertheless, upon considering the 95% confidence interval and standard error, we found no significant difference between  $K_M$  and  $v_{max}$  values of WT and variants ( $p < 0.05$ ). Af<sub>afu62</sub> showcases superior catalytic efficiency of other GH 62 enzymes, even in the absence of comprehensive kinetic parameter data for GH 62 arabinofuranosidases, specially for GH 62 subfamily 1. This is evident when contrasting Af<sub>afu62</sub> with GH 62 subfamilies 1 and 2 across various bacteria and fungi, including *Mycothermus thermophilus*, which presented optimum activity at 50 °C and pH 5.0. Notably, the kinetic parameters for the GH 62\_1 subfamily from *Scytalidium thermophilus* represent the sole available data for this specific subfamily (see Table S5). For an in-depth analysis of the kinetic parameters of Af<sub>afu62</sub> and other GH 62 enzymes, readers are encouraged to refer to our previous work.<sup>4</sup>

The catalytic efficiency ( $k_{cat}/K_M$ ) of Af<sub>afu62</sub> outperformed that of all species, except for AnAbf62A from *Aspergillus nidulans*, which contains a carbohydrate binding module and optimum temperature between 60 and 65 °C.<sup>31,32</sup> However,

AnAbf62A also presented its melting temperature at 60 °C and no residual activity data were available for comparison.

Regarding the thermodynamic parameters, the mutants showed significantly higher energy of denaturation ( $E_d$ ) than the WT, indicating enhanced thermoresistance. The increase in  $D_{50}$  and  $t_{1/2}$  is consistent with the melting temperatures presented in Figure 3B,C. T256G showed the lowest improvement in thermodynamic parameters and did not present a significant improvement in melting temperature determined by DSF compared to WT. S127G, S188F, and Q271T melting temperatures were statistically higher than WT (Figure 4D). The G205K variant demonstrated noteworthy enhancements in both experimental assays compared to other variants and the WT, evidenced by a substantial increase in the melting temperature from 49 to 58 °C. These findings align well with the denaturation temperatures determined through DSC, as illustrated in Figure S3. It is essential to highlight that the denaturation temperatures determined by DSF tend to underestimate actual melting temperatures due to the influence of the substrate and cofactors present in the medium. Notably, the thermostability of the  $\beta$ -propeller structure in GH 62 is intricately tied to hydrophobic interactions within the substrate-binding site. This structural stability is further

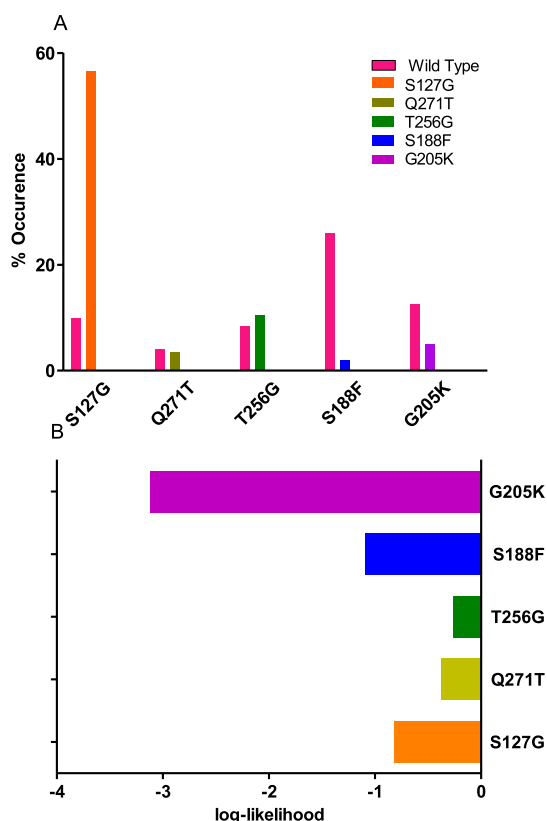


**Figure 4.** (A) Probability of occurrence of amino acids at the selected residues, where the pink column represents the WT amino acid at each selected residue. (B) Score of log-likelihood for mutant sequences according to the WT Af<sub>afu62</sub> structure.

reinforced by hydrogen bonding and hydrophobic interactions facilitated by the arabinofuranosyl substituent.<sup>30</sup>

**Mutational Landscape Analysis.** We computed the frequency of the amino acids based on 200 sequences comparison and the mutational landscape of each mutated position on the Hotspot Wizard 3.0 server, a tool designed for interactive selection of protein engineering targets (Figure 4A). This analysis helped compare with the experimental finds, revealing that glycine showed higher occurrence in position 127, coherent with the mutation S127G. The occurrence of aspartic acid at position 205 is 10%, and we identified the mutation G205D in the random mutagenesis library. On the other hand, the occurrence of lysine is 2% at position 205 (G205K). Threonine accounts for 4% of occurrences in position 271 (Q271T). We also identified the mutations S188T, T256A, and G205N in the site-saturated mutagenesis library. These mutations exhibit the highest occurrence rates at their respective positions, with percentages of 37, 54, and 55%, respectively. We did not characterize them due to their comparatively lower level of improvement in comparison to S188F, T256G, and G205K.

Thermophilic proteins have more hydrophobic and charged amino acids and fewer uncharged polar residues.<sup>33</sup> In our study, G205K presented the highest melting temperature and improved thermodynamic parameters. One interpretation is that the higher hydrophobicity and positive charge of the lysine (K) residue enabled a more compact configuration. Moreover, G205K, which replaced a smaller amino acid (glycine, G) with a bulkier residue (lysine, K), might build up a steric hindrance effect, increasing the interaction between residues and

improving enzyme stabilization. Similarly, upon mutation of serine 188 to phenylalanine (S188F), this hydrophobic residue contains a benzene ring and could also introduce a bulkier side chain. A similar functional mechanism could be attributed to the S188T mutation since threonine also presents a bulkier side chain than serine and is less hydrophilic.<sup>34</sup> As expected, the most negative log-likelihoods from ESM-IF1 scores were computed for G205K (−3.12) and S188F (−1.08), indicating improved stability (Figure 4B).

We calculated the correlation values between the mutant melting temperatures and the ESM-IF1 scores. The data showed a Pearson correlation coefficient of −0.98 ( $p = 0.0007$ ) and a Spearman correlation coefficient of −0.90 ( $p = 0.0833$ ). This strong negative correlation indicates that lower ESM-IF values correspond to higher melting temperatures. Although a medium/strong negative correlation was substantial, which indicates an adequate fit between the ESM-IF values and the experimental data, further investigation is needed due to lack of statistical significance for nonparametric correlation (Figure S4).

In this study, the mutations S127G (log-likelihood −0.82) and T256G (log-likelihood −0.26, the lowest stability improvement) also showed improved thermostability, even though T256G did not significantly improve melting temperature compared to the WT. Since glycine is more flexible regarding adopting backbone torsion angles, these substitutions for glycine may have improved thermostability by releasing steric strains from unfavored dihedral angles.<sup>35</sup> Threonine's hydrophilic nature and display of the presence of an OH group on its side chain may contribute to additional hydrogen bond formation with surrounding residues. The mutation Q271T might result in the formation of an additional hydrogen bond, between residues T271 and T272, and residues R269 and T272. Also, Q271T (log-likelihood −0.38) mutation increases the possibility of additional hydrogen bonds with water molecules.<sup>36</sup> Despite the significant insights acquired from the computational and experimental analyses described previously, the details regarding the molecular intricacies arising from these mutations in the dynamics of protein structure were further investigated with MD simulations.

**Molecular Dynamics Simulations.** We investigated the behavior of six distinct systems by 250 ns MD simulations. All systems exhibited similar rmsd time histories throughout the simulations (Supporting Information, Figure S5 and Table S3) albeit with minor variations. The RMSF also behaved similarly regarding the overall fluctuation profile.

However, we observed notable differences in the fluctuation patterns of specific regions and residues among the systems. The RMSF profiles (Figure 5A) underscore these differences. Consistently observed fluctuations emerged around residues F99 and L286 in all mutations, which were absent in the WT system. These fluctuations occurred within the  $\beta 6$ – $\beta 7$  (Blade II) and  $\beta 19$  (Blade V) regions, respectively.

The  $\beta 6$ – $\beta 7$  (Blade II) region, previously identified as a xylotriase subsite, plays a role in restricting a curved cylindrical subcavity that leads to the active site of the enzyme. Furthermore, the movement of  $\beta 19$ , which crosses the inner portion of the enzyme, impacts both the catalytic cavity and calcium coordination. This region encompasses a known disulfide bond linking  $\beta 18$  and  $\beta 19$ , while a connecting loop obstructs the lower part of the tunnel.<sup>26</sup>



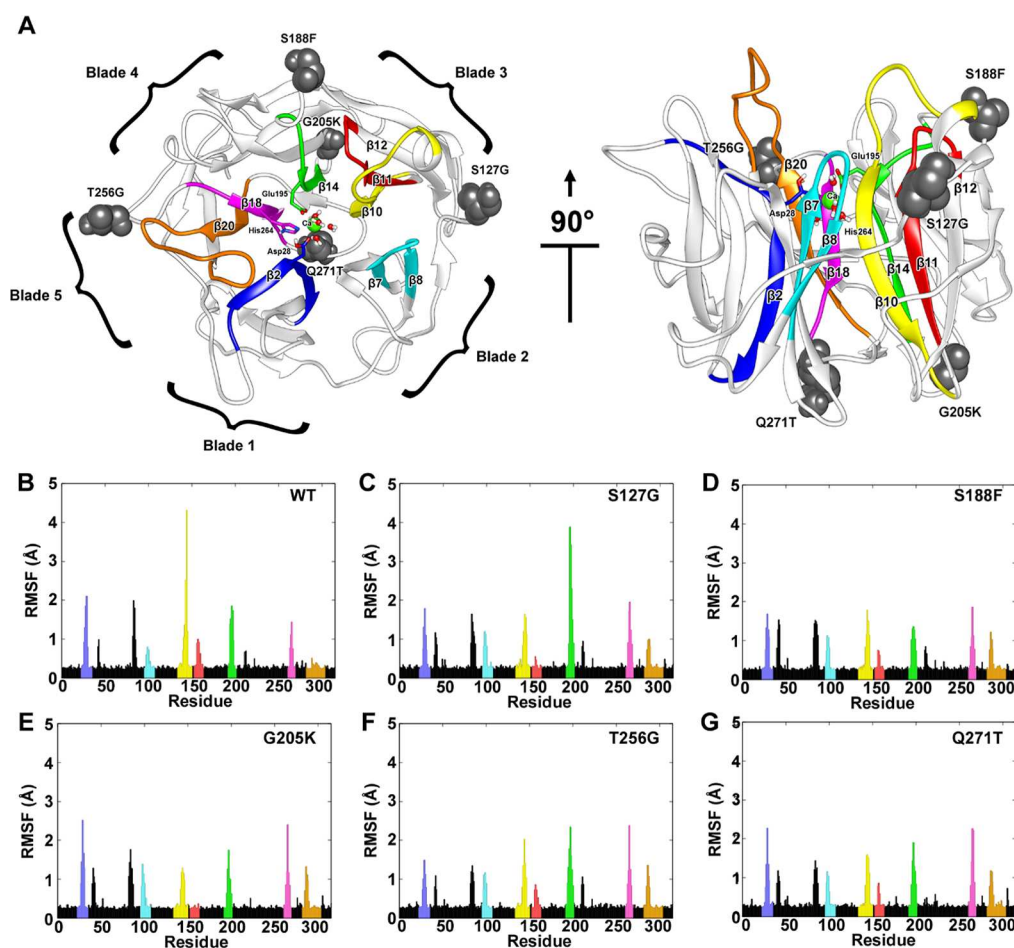


**Figure 5.** Location of the point mutations and the impacts on the protein dynamics. (A) Representation of Af<sub>afu62</sub> enzyme and the location of the point mutations. The RMSF of the (B) WT, (C) S127G, (D) S188F, (E) G205K, (F) T256G, and (G) Q271T were calculated based on the enzyme backbones over the last 50 ns simulations at 300 K.

Notable fluctuations characterized the G205K system, with residue D28 within the  $\beta_2$  region (Blade I), one of the residues forming the catalytic triad and exhibiting the highest fluctuation. Additionally, both the WT and Q271T systems displayed fluctuations above 2 Å within the  $\beta_2$  region. The residue H264 exhibited the highest fluctuation in the S188F, T256G, and Q271T mutations with a common fluctuation observed in S157 across these systems. Among all systems, residue E195 in the S127G mutant showed the largest fluctuations. Contrastingly, the WT system exhibited more fluctuation at residue D143, located between  $\beta_8$  and  $\beta_9$  (Figure 5B).

An intriguing observation is the occurrence of these fluctuations in the protein regions, which are distant from the mutation sites. This orthogonal effect occurs at locations common to various mutations or unique to a specific mutation. Such differences in the fluctuation patterns may be associated with the distinct thermostability profiles observed experimentally. It also suggests that the impact of point mutations extends beyond their immediate vicinity, potentially influencing the overall conformational dynamics and protein stability.<sup>38</sup>

**Secondary Structure Analysis.** The results of the MD simulations indicate that the point mutations did not induce significant alterations in protein folding or disrupt local hydrogen bonding within the protein. The overall fold of a five-bladed  $\beta$ -propeller, consisting of four antiparallel  $\beta$ -strands

in each blade, characteristic of GH 62 enzymes, remained preserved.<sup>26,37</sup> Nonetheless, we observed subtle variations in secondary structure in specific regions, as depicted in Figure 6A. These differences are attributed to the influence of the mutations on local conformational dynamics and may have implications for the functional properties of the enzyme. Although the global structural fold remains intact, these localized variations in the secondary structure highlight the potential impact of the mutations on specific regions of the enzyme.

We observed that certain regions exhibited extended secondary structures compared with the WT system. During the MD simulations, we noticed changes in secondary structures depending on the specific mutation (Figure 6A). This finding aligns with the observed subtle fluctuations in the RMSF analysis as some motifs correlate with these fluctuations. To further investigate differences in the behavior of secondary structures, we examined their characteristics during the last 50 ns of the trajectories (Figure 6A,B). In some systems, we observed a loss of configurations associated with beta-sheet regions. Notably, in the S127G system, we observed an overall increase of approximately 5% in the secondary structure of the enzyme with 4.1% attributed to beta-sheet motifs (Figure 6B). We observed proportional reductions in turn and coil regions, particularly with a more notable decrease in turn regions. This behavior was consistently seen across all mutations, with

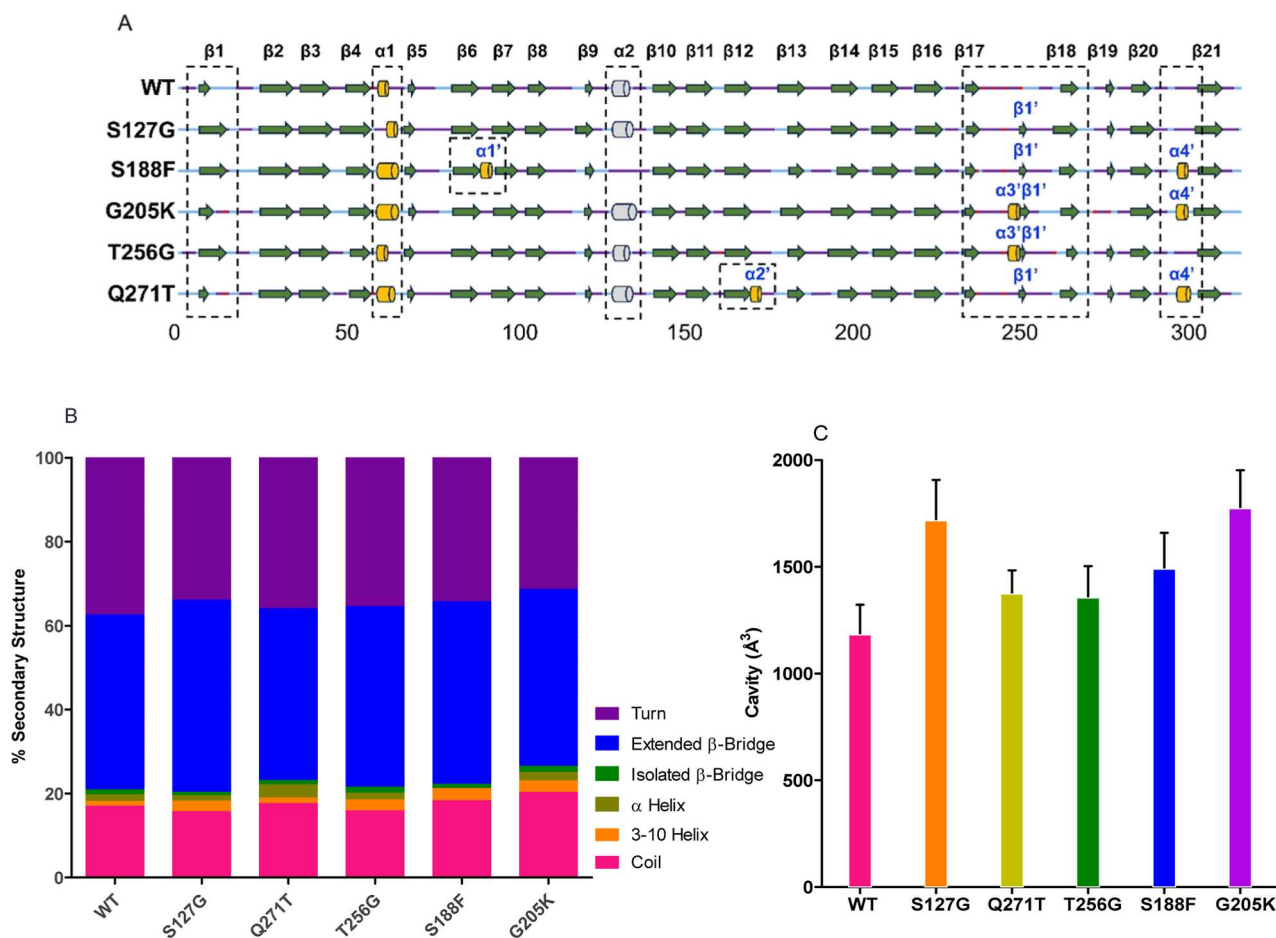


**Figure 6.** Secondary Structure and Cavity analysis obtained from the last 50 ns of MD simulation of WT and mutations. (A) Alterations in secondary structures obtained from the mean structure. (B) DSSP analysis of trajectories with values are expressed as a percentage of the distributions of all the residues. (C) Cavity analysis of the catalytic site.

G205K exhibiting the fewest turn regions among the systems. A higher proportion of secondary structure content has been associated with enhanced thermal stability for other enzymes.<sup>39,40</sup>

Analysis of the secondary structure patterns during the last 50 ns of the trajectories indicates that all mutations exhibited a higher percentage of secondary structure motifs than the percentage of turns and coil regions of the WT enzyme (Figure 6B). This suggests that the point mutations induced a shift toward a more structured conformation characterized by a higher proportion of secondary structure elements.

The higher number of secondary structure motifs observed in the mutations may indicate improved thermostability, as corroborated by the experimental evidence. Furthermore, the observed changes in the secondary structure could correlate to the subtle fluctuations observed during the MD simulations, particularly that of the  $\beta$ 19 and  $\beta$ 20 regions surrounding residue D143. These findings suggest a potential connection among alterations in secondary structure, local residue fluctuations, and overall enzymatic stability.

Regarding the residue fluctuations associated with differences in secondary structure, we identified four regions of interest within the enzyme. These regions include residues D28–F99, H264, and L286. The first, notably involving D28, which is part of the catalytic triad, does not exhibit significant fluctuations in the S127G, S188F, and T256G mutations. This region corresponds to  $\beta$ 2 (Blade II). The second region,

involving H264, is situated between  $\beta$ 17 and  $\beta$ 18, representing a loop region that connects Blade IV to Blade V.

The fluctuations observed around residues F99 and L286, which are consistently higher in all mutations compared to those of the WT, are particularly interesting. These residues are in regions involved in forming the entrance to the binding site, specifically, the  $\beta$ 6–7 (Blade II) and  $\beta$ 19 (Blade V) regions. Notably, the loop between  $\beta$ 19 and  $\beta$ 20 encompasses a small helix, and our analysis suggests that the increased fluctuation observed in this region may be associated with its ability to modulate the size of the substrate-binding cavity.

**Cavity Analysis.** To assess the potential impact of system dynamics on the catalytic site, we performed cavity analysis for the last 50 ns of the simulations. Our analysis unveiled significant changes in the size of the catalytic cavity across all mutations, with volumes exceeding those of the WT system (Figure 6C). Despite variances in the catalytic pocket's dynamics among the systems, the G205K mutation exhibited larger volume expansion relative to the WT. Considering the cavity volume fluctuations, the S127G, S188F, and G205K mutations displayed similar behaviors characterized by elevated volume fluctuations (Figure S6 and Table S4). Particularly, the S127G mutation exhibited the greatest mean deviation, indicating increased flexibility between closed and open states during the MD simulations. Conversely, the T256G and Q271T mutations demonstrated behavior comparable to that of the WT system.

Among all of the systems analyzed, only the Q271T mutation exhibited reduced flexibility in the cavity, suggesting a more rigid substrate-binding site. In contrast, the other systems displayed greater mobility in the opening and closing movements of the enzyme's binding site than the WT system. This increased mobility implies a potentially more dynamic and adaptable nature of the binding site in these systems, allowing for conformational changes that facilitate substrate binding and catalysis.

Although the volume of the cavities provides valuable insights for distinguishing between these systems, it is crucial to emphasize the distinctions in cavity shapes (Figure S7). The variations in shape and size of the cavities could be attributed to the  $\alpha$ -helix formed between  $\beta$ 19 and  $\beta$ 20 and the varying degrees of flexibility of this region. This loop is associated with the opening and closing movements of the catalytic cavity. Cavity shapes that are more elongated or deeper may indicate better substrate adjustments within the catalytic site, even at higher temperatures. Therefore, the computational analysis of MD simulations provides insights into the dynamic behavior of the mutated systems. These findings shed light on the dynamic nature of these regions and their potential implications for substrate binding and catalytic cavity modulation.

## DISCUSSION

We identified five key residues affecting the thermostability of GH 62 arabinofuranosidase from *A. fumigatus* by directed evolution. G205K amino acid substitution showed the best improvement and ESM-IF1 score, increasing the melting temperature from 49 to 58 °C and improving the energy of denaturation by more than 40%. We identified conservative mutations in the selected mutants, which indicated their evolutionary importance. The selected variants can be templates for new directed evolution rounds to combine and find new potential sites that play a role in enzyme stability. Also, ESM-IF1 inverse folding modeling can predict residues for mutation, saving time and labor in rounds of library screening from direct evolution experiments. The effects caused by other mutations such as Q271T, S127G, T256G, and S188F may be related to the creation of additional hydrogen bonds, released steric strains, and increased hydrophobicity. Further detailed insights obtained through MD simulations revealed significant regional and residue-based differences in the fluctuation patterns of the enzyme. The residues D28, F99, H264, and L286, exhibited distinct fluctuation behaviors that could impact substrate binding and catalytic cavity modulation. Simultaneously, the catalytic cavity in all mutated systems showed significant changes in size compared to the WT. Also, despite the point mutations not causing significant alterations in the overall protein fold, there were subtle variations in secondary structures that could affect the functional properties of the enzyme. These findings contribute to our understanding of how point mutations influence the structural dynamics and stability of the enzyme, shedding light on its functional implications.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.3c08019>.

Additional results details, amino acid sequence analysis, rmsds, and cavity analysis; and mutant screening and

MD simulations, DSSP analysis, and cavity analysis (PDF)

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## Notes

The authors declare no competing financial interest.

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# Integrated biorefinery for xylooligosaccharides, pectin, and bioenergy production from orange waste

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Abstract: Orange waste is one of the most underutilized biowastes. It accounts for 50% of the fruit weight after juice extraction, presenting an environmental problem due to inappropriate disposal. Industrial waste exploitation is a sustainable way to obtain bioproducts and bioenergy. However, the efficiency of chemical treatments and enzymatic cocktails is still a bottleneck in these processes. One potential route to reduce costs is to design consecutive extraction and hydrolysis stages to yield more products from different fractions of the same feedstock, reducing the use of time and bulk materials. This study assessed the economic performance of an orange processing waste (OPW) biorefinery for xylooligosaccharides (XOS), pectin, and bioenergy, involving three aspects: (1) experimental procedures, (2) a simulation approach, and (3) optimization tools. The optimization of hydrothermal treatment for pectin extraction decreased material costs by more than 60% and preserved the xylan content for xylooligosaccharide production by enzymatic hydrolysis. This study analyzed different biorefinery capacities, and solid waste exploitation for heat and steam generation, improving sustainability. The production cost per kg of high-purity pectin and xylooligosaccharides is 3.67 \$ with an efficient solvent recovery and self-sufficiency for standard power usage. These results proved the economic feasibility and low environmental impact of the OPW biorefinery. © 2023 Society of Industrial Chemistry and John Wiley & Sons Ltd.

*Supporting information may be found in the online version of this article.*

Key words: antioxidant potential; bioeconomy; circular economy; energy analysis; SuperPro designer; prebiotics

## Introduction

Orange processing waste (OPW), composed of fruit peels and bagasse (~50% of the fruit wet weight), is a prospective feedstock for the coproduction of bioenergy and bioactive compounds in an integrated biorefinery.<sup>1–3</sup> The biggest citrus enterprises, such as Citrosuco in Brazil, can process more than 60 million boxes (2.4 million tons) of fruit in one season.<sup>4</sup> The large amounts of OPW induce soil contamination because traditional management involves landfill disposal and incineration, which may cause leachate production and serious organic pollution due to the high demand for biological oxygen for its decomposition.<sup>1,5</sup> In recent years, studies have focused on new processes for OPW valorization through the production of biofuels and high-value compounds, such as essential oil, cellulose, pectin, lactic acid, xylooligosaccharides (XOS), and phenolic compounds.<sup>1,2,5–8</sup>

Global Market Insights reported that citrus fruits have been the prime source of hydrocolloid pectin because of their active phytochemical content, high degree of esterification, gelling properties, and elastic characteristics at elevated temperatures. Even though the food industry demands a high level of pectin production for use as a gelling agent and as an additive in bio-packing manufacturing, industrial pectin production is quite energy consuming due to the need for large quantities of water and ethanol, and because of the utility requirements (electricity, heating, cooling). Pectin manufacturers are also offering clean-label ingredients for food manufacturers because natural ingredients are perceived to be healthier and better for the planet. Consumers lean toward plant-based ingredients instead of animal-based products with less processed ingredients, thus driving the market's growth. The pectin global market is forecast to increase from approximately 103 990 tons and USD 957.70 million in 2023 to USD 1261.20 million by 2028, at a compound annual growth rate (CAGR) of 5.66% during the forecast period (2023–2028).<sup>2,9,10</sup>

Likewise, the CAGR projection for the prebiotics market is 9.71% over the next 5 years. The rising popularity of functional foods and beverages comes from the increasing health consciousness of consumers, which drives the demand for prebiotics as sugar substitutes and probiotic growth stimulators. The ban on antibiotics in the animal feed industry also influences this market sector.<sup>10</sup> Xylooligosaccharides (XOS) have a remarkable potential as a functional food ingredient as they have advantages over other prebiotics. Xylooligosaccharides are also stable in response to differences in temperature and pH, and have favorable organoleptic properties. The global XOS market

expectation is to achieve ~\$130 million by 2025.<sup>11</sup> The enzymatic hydrolysis of xylan (hemicellulose) is the most sustainable way to produce high purity XOS with prebiotic potential.<sup>12</sup> However, XOS production is still more expensive than other prebiotics because of the costs related to alkalis used during pretreatments (generally NaOH or KOH) and enzymatic cocktails. Various approaches are being developed to use renewable feedstocks and integrated processes for biorefineries.<sup>13</sup>

Increasing consumer awareness of the harmfulness of artificial ingredients and processed and unnatural food products, along with government regulations favoring the use of natural additives in food, is propelling the growth of the market for natural ingredients like pectin and xylooligosaccharides.<sup>10</sup> Understanding the economic effectiveness and determinants of the production cost is essential to evaluate industrial capacities and innovative technologies to meet commercial demands. The software used for process simulation and techno-economic analysis is SuperPro Designer, which has been used successfully to develop and improve innovative technologies.<sup>14,15</sup> This software uses mass and energy balances to decide equipment size and to estimate the capital investment, unit cost, and revenues, environmental impact, bottlenecks, and potential areas for improvement.

Despite the vast literature reporting the use of OPW as a source of bioproducts, most studies are still at an early stage of development. There is still a lack of studies assessing economic feasibility on a large scale, especially for XOS production, because of the low amount of xylan in OPW. Other authors have reported techno-economic analysis of OPW biorefineries for pectin and biogas production<sup>1</sup> and the profitability of XOS production from different types of industrial wastes.<sup>16</sup>

A critical factor for the greater profitability of the OPW biorefinery is the market prices of value-added products, such as XOS. Scientific papers have not yet reported the integrated use of OPW for XOS and other high-value products, which can help to increase the viability of the biorefinery, nor did other research identify the drivers for production costs and OPW biorefinery's financial performance under varied configurations (treatment conditions, product purity, and solvent recycling). To address this gap, this study assessed energy and cost efficiency to develop a process to utilize OPW for pectin, XOS, and biogas through process optimization and simulation models. We organized this study into (1) determination of material composition; (2) experimental design for pectin and xylan extraction; (3) xylan enzymatic hydrolysis for XOS production; (4) scenario analysis using SuperPro Designer software.

## Material and methods

### Raw material composition and processing

The Vita Citrus orange juice manufacturer (Ribeirão Preto, Brazil) donated the orange processing waste (OPW), which we dried in an air-circulating oven at 50 °C for 24 h and grounded. We removed the free sugars by washing twice for 1 h at 50 °C using a solid concentration of 50 g L<sup>-1</sup>. Cellulose, hemicellulose, ash, extractives, and lignin determination followed the National Renewable Energy Laboratory (NREL) procedures.<sup>17,18</sup>

### Pectin extraction

This study extracted pectin from the free-sugar OPW, conserving the hemicellulosic integrity in the residual solids, analyzing the conditions of pectin extraction by a central composite rotatable design (CCRD) with three pivotal points and two axial points, 20 g L<sup>-1</sup> citric acid (pH 2.0 ± 0.2), and a solid ratio of 1:40. The independent variables were temperature (70–130 °C), and incubation time (1–70 min). The Protimiza Experimental Design tool<sup>19</sup> was used for statistical analysis. After extraction, the acidic suspension was centrifuged (9000 g, 10 min, 4 °C) and the supernatant was filtered. The filtrate was treated with 1 volume of ethanol, the pH was adjusted to 3.5 (pH of minimum pectin solubility) with KOH, and incubated overnight at 4 °C for pectin precipitation. We collected the pectin-rich extract by centrifugation (9000 g, 10 min) and dried and expressed the yield of the pectin-rich extract as its weight per 100 g of sugar-free OPW. A rotary evaporator recovered the ethanol (at 50 °C), and HPLC determined the chemical composition of the ethanol-free liquid fraction.<sup>20</sup> Pectin characterization followed the spectrophotometric method.<sup>21</sup>

### Xylan extraction and enzymatic hydrolysis

The remaining solids from pectin extraction were washed with water to remove the citric acid, and were centrifuged (9000 g, 10 min). The experiments for xylan extraction analyzed KOH concentration between 2.5% and 15% and temperatures between 90 °C and 120 °C during 30–120 min using a solid ratio of 1:10. The material was filtered (Whatman paper #1) and neutralized with 0.1 volume of acetic acid to pH ~5.5 (color change seen). Xylan was precipitated with 1 volume of ethanol overnight at 4 °C. Then, the xylan was centrifuged (9000 g, 20 min), washed with 50% ethanol and 0.5% ethylenediaminetetraacetic acid (EDTA), centrifuged again, and dried overnight at 50 °C.<sup>22</sup>

Enzymatic hydrolysis occurred in acetate buffer at pH 5.0 with 10 mL L<sup>-1</sup> Shearzyme endoxylanase (NS50030,

Novozymes, Bagsvaerd, Denmark) which represents a final protein concentration of 0.165 mg mL<sup>-1</sup>, at 50 °C for 48 h, followed by heat inactivation at 98 °C for 5 min. Then, the samples were filtered (0.22 µm Millex PVDF membrane, Merck, Darmstadt, Germany) and analyzed for XOS composition and antioxidant potential.

### Analytical procedures

The antioxidant capacity of XOS was evaluated with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and phosphate-molybdenum free-radical capture methods, using Trolox as standard.<sup>23,24</sup> The phenolic compounds were quantified using the Folin–Ciocalteu method as described elsewhere, with gallic acid as standard.<sup>25</sup> The XOS quantification followed a methodology described elsewhere.<sup>20</sup> This study used the reducing sugar method to measure the endoxylanase activity<sup>26,27</sup> and the Lowry method for protein quantification.<sup>28</sup>

### Biorefinery simulation

SuperPro Designer v11 (Intelligen, Scotch Plains, NJ, USA) software simulated technical and economic parameters and environmental assessment aspects of bioenergy generation, pectin extraction, and XOS production. The software provided the international cost of the equipment (see Supporting Information), assuming that the plant operates 200 batches per year. The process has four main areas, namely (1) OPW feedstock washing and the combined heat and power (CHP) plant; (2) pectin extraction; (3) pectin precipitation; (4) xylan extraction and enzymatic hydrolysis (see Supporting Information).

### Orange processing waste feedstock washing and CHP plant

A typical capacity of an extractor for medium-sized fruit in an industry of orange juice is 5–7 tons of fruit per hour, corresponding to about 2500 L of juice and ~2.5–3.5 tons of OPW generated per hour. One extractor therefore generates at least 60–84 tons of OPW in 24 h.<sup>29,30</sup> Based on the average amount of OPW generated by medium-sized plants, this study adopted an amount of 100 tons of OPW as input.

The first step is to reduce the particle size and separate the free sugars. After grinding, the OPW was washed with warm water (50 °C). The first screw-pressing machine collected the first fraction of free sugars. Then, the material was washed with warm water and pressed again. One batch generates 67.3 tons of sugar-free OPW (85% humidity) and ~13 tons of sugars. The sugars go to the anaerobic digester in the CHP plant, and evaporation recovers 19.7 m<sup>3</sup> of water (see Supporting Information). The evaporator returns the water at

feverish temperatures (98 to 100 °C), which heats the washing water. This heat exchange provides warm water for the washing steps without needing an electric heater, thus saving energy.

The CHP plant runs 7800 h per year in continuous mode. The organic waste streams from the processes go to the reactor for biogas production in an anaerobic digester, performed at mesophilic conditions of 35 °C in a semi-continuous flow stirred reactor for 600 h and 0.01 kW m<sup>-3</sup> of power for heating and stirring. The reaction conversion efficiency is 90% for proteins and sugars and 50% for cellulose, hemicellulose, pectin, and lignin.<sup>31</sup> After digestion, the biogas is compressed and burnt in a gas-turbine generator. The turbine propels the compressor and the generator to produce power. During electricity generation, steam-generation equipment captures the heat from the exhaust stream (450 °C) through an input of air and water. The plant can use or sell the produced power and heat: 4.5 tons per batch of digestate can be sold as fertilizer.<sup>14</sup>

## Pectin extraction

Washed and grounded OPW (85% humidity) was mixed with citric acid (20 g L<sup>-1</sup> final acid concentration) at 99 °C for 70 min (10 min setup, 80 m<sup>3</sup> working volume). Solids were separated by vacuum filtration and washed with water. Then, reverse osmosis recovered the water. Impurities were removed by a granular activated carbon adsorber (GAC) (18 min bed contact, binding capacity of 9.14 mg g<sup>-1</sup>), removing HMF, furfurals, and some flavonoids. Then, the column was washed with NaOH and water. Two columns were operating in a staggered mode so that there would always be one column being loaded while the other would be washing. Based on the chemical composition of pectin in Table 3, the following yields were adopted: 70% solubilization efficiency; 10% degradation of hemicellulose, cellulose, and pectin into furfural, HMF (hydroxymethylfurfural), and formic acid, respectively; and 5% conversion of hemicellulose into XOS. This step produces 2.85 tons of soluble pectin (see Supporting Information) and 11 tons of lignocellulosic biomass (with 40% moisture).

## Pectin precipitation

Four scenarios evaluated the energy requirements and ethanol consumption. These scenarios involved four concentrations of the pectin-rich stream (0, 30, 50, and 70% evaporation). After evaporation, pectin solids were precipitated with 500 mL L<sup>-1</sup> ethanol for 12 h and separated by vacuum filtration. A drum dryer separated the ethanol from the remaining monosaccharides, XOS, and citric acid.

Each batch produced 3 tons of 95% pectin solids, and 99% of the consumed alcohol was recovered by distillation, along with 48 m<sup>3</sup> of water (see Supporting Information).

Citric acid recovery was computed by precipitation and crystallization. Purification started by adding Ca (OH)<sub>2</sub> for every three flow volumes to precipitate calcium citrate, separated by vacuum filtration. The filtrate went to XOS purification, and the calcium citrate was acidified with dilute sulfuric acid to precipitate calcium sulfate (gypsum). Another filter separated the gypsum from the citric acid solution, which was crystallized using an evaporator/crystallizer. Then, filtration separated the crystals.<sup>32</sup>

## Xylan extraction and enzymatic hydrolysis

Eleven tons of lignocellulosic biomass (40% moisture) was mixed with 25 g L<sup>-1</sup> KOH at 99 °C for 120 min and 100 g L<sup>-1</sup> solid concentration in a stirred reactor (80 m<sup>3</sup> working volume). Then, vacuum filtration separated the solids, and 4.68 tons of cake (rich in cellulose) went to the CHP plant. An evaporator concentrated the filtrate (rich in xylan). Here, four scenarios were analyzed to reduce the usage of ethanol and energy: 0, 30, 50, and 70% evaporation. In Scenario 1, the xylan precipitated without prior evaporation. Scenario 1 had no evaporator but required an additional vacuum filter, a drum dryer, and a vertical tank. Scenario 2 (30%) and Scenario 3 (50%) needed an evaporator system and an additional drum dryer. Scenario 4 (70%) required only the evaporator.

After evaporation, KOH was neutralized with an equimolar concentration of acetic acid, forming CH<sub>3</sub>COOK. The extract was transferred to a tank and mixed with 1 volume of ethanol for 12 h. Then, a solution of 50% ethanol and 0.5% EDTA washed ~1 ton of xylan. The xylan was then washed with water and stored. A distillation system recovered the solvents (ethanol and water).

Enzymatic hydrolysis used 20 g L<sup>-1</sup> xylan in acetate buffer pH 5.0 at 50 °C for 48 h with 10 mL L<sup>-1</sup> Shearzyme endoxylanase (NS50030, Novozymes). After hydrolysis, filtration removed the solids. The hydrolysate was mixed with the stream containing the filtrate from pectin extraction (74 kg XOS) and stored in a tank (600 m<sup>3</sup>) and 900 L h<sup>-1</sup> of hydrolysate was concentrated 10 times by ultrafiltration using a reverse osmosis membrane, which removed monosaccharides too. Then, a PBA (Phenylboronic acid) column, equipped with dextran resin, removed the buffer, enzyme, and impurities. We set the loading flow rate to 12-bed volumes/min and 2000 g L<sup>-1</sup> dextran resin binding capacity. The PBA columns process 1570 L per batch. Finally, XOS is dried using a rotary dryer to achieve 98.84% purity



and 0.5% moisture, representing 825 kg per batch. Reverse osmosis recovers 54 m<sup>3</sup> of water.

## Cleaning in place

The simulations also computed the 'cleaning in place' (CIP) to wash the equipment. It happens in a closed circuit of detergents and rinsing solutions, removing residues and bacteria. The operating temperature varied based on the type of waste and the equipment. The prewash used cold water to

remove coarse residues. Then, an alkaline cleaning solution (2.0% NaOH) removed residues such as proteins and fats, followed by 30 min acid cleaning to remove mineral residues and rinsing with water to remove the chemicals. The CIP water in the water recovery system was not computed.

## Economic and environmental analysis

For the economic assessment, the plant cost (PC) is the sum of the total plant indirect cost (TPIC, engineering,

**Table 1. Studies on the use of orange processing waste using different technologies and main products.**

| Products                           | Technologies                                                                              | Feasibility study and cost estimate                                                                                                                                                                                                                                                                                                                                                               | Eco-footprint                                                                                                                                                                                                        | Reference  |
|------------------------------------|-------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| XOS, pectin, power, steam          | Citric acid extraction, KOH extraction, enzymatic hydrolysis, CHP plant                   | The use of residual solids and free sugars for biogas production circumvents the need for pretreatment before anaerobic digestion. The integrated process reduced the net unit cost of pectin and XOS production. Optimization of the scenarios for pectin and xylan extraction reduced more than 50% of bulk material cost. XOS production is workable only if associated with other bioproducts | Recyclability of solvents and reduction in all environmental impact categories were successfully achieved.                                                                                                           | This study |
| Essential oil, pectin, phenols     | Solar hydro-distillation of essential oils and phenols, HCl extraction                    | The distillation time was reduced by more than 30%, with no need for an added source of energy. The investment cost is 28.5% higher. However, the system does not need other expenses                                                                                                                                                                                                             | There was no significant difference between the oils' quality of solar extraction (green solution) and conventional methods.                                                                                         | 49         |
| Essential oil, pectin, biogas      | Steam distillation, citric acid extraction, anaerobic digestion                           | Only high scales can be applied in a biorefinery context. The energy assessment of the proposed OPW biorefinery demonstrates the need to reduce the thermal needs of the process. Thus, a deep heat integration study must be performed.                                                                                                                                                          | Not discussed                                                                                                                                                                                                        | 1          |
| Mucic acid, biogas, digestate      | Anaerobic digestion; pectin extraction with citric acid, mucic acid production simulation | Biogas and mucic acid production costs were reduced by 36% increasing the OPW flow. Mucic acid production demands 42% of the capital investment of the OPW biorefinery.                                                                                                                                                                                                                           | The liquid releases of mucic acid production have high negative impacts and should be studied carefully                                                                                                              | 50         |
| Limonene                           | Solvent extraction                                                                        | Not discussed                                                                                                                                                                                                                                                                                                                                                                                     | Bio-based cyclopentylmethylether and 2-methyl tetrahydrofuran are promising alternatives for replacing hexane in the extraction of limonene. Recyclability of solvents conducted.                                    | 51         |
| Citric acid, limonene, animal feed | Microwave extraction, solid state fermentation                                            | Not discussed                                                                                                                                                                                                                                                                                                                                                                                     | The sensible solution would be only the limonene extraction. The essential oil extraction can be realized as an add-on in current juice facilities, while dried residual OPW should be directly used as animal feed. | 7          |

and construction) and the total plant direct cost (TPDC, equipment buys costs, installation costs, process piping, instrumentation, electrical, building, yard improvement, and auxiliary facilities costs). The contractor's fee and contingency were calculated as 5% and 8% of the PC and constituted the direct fixed capital cost of the plant. The biorefinery operated 200 batches a year. The main operational costs include labor, facility, and utility (see Supporting Information). The data were acquired from the SuperPro Designer database and literature.<sup>7,14,16,33</sup> The environmental impacts were calculated through emissions from inputs, products, and waste streams.

## Results and discussion

Brazil is the leading nation for citrus fruit processing, accounting for 50% of the world's total. Oranges correspond to 80% of the total processed citrus fruits.<sup>34</sup> In 2023, Brazilian production was estimated at 16.75 billion ton of fruits, 80% of which was designated for juice production. São Paulo state accounts for around 80% of the national production, and harvesting happens all year round.<sup>35</sup> The 'Pera Rio' orange (*Citrus sinensis* (L.) Osbeck) is the most cultivated type and is ideal for juices due to its sweet taste and the fact that it

**Table 2. Composition of OPW fractions (values are percentages %).**

|                    | Sugar-free OPW | Extracted pectin* | Residue from pectin extraction | Extracted xylan | Residue from xylan extraction |
|--------------------|----------------|-------------------|--------------------------------|-----------------|-------------------------------|
| Pectin             | 40.5±2.5       | 81.2±1.4          | 11.2±1.0                       | 10.0±0.8        | 15.4±0.8                      |
| Hemicellulose      | 14.8±0.2       | 4.9±0.2           | 30.1±2.9                       | 69.0±1.2        | 8.0±0.1                       |
| Cellulose + starch | 39.0±2.4       | 14.3±0.3          | 56.7±3.2                       | 21.0±1.0        | 76.6±1.1                      |
| Ashes              | 1.0±0.0        | -                 | -                              | -               | -                             |
| Lignin             | 1.2±0.0        | -                 | -                              | -               | -                             |
| Protein            | 3.2±0.1        | -                 | -                              | -               | -                             |
| Fats               | 2.3±0.1        | -                 | -                              | -               | -                             |
| Total              | 102.5±2.6      | 100.4±0.7         | 97.1±3.1                       | 101.0±1.0       | 101.0±1.0                     |

\*Under optimized conditions.

**Table 3. Pectin yields, hemicellulose recovery, and components of hydrothermal assays (CCRD matrix).**

| Assay                                 | 1     | 2     | 3     | 4     | 5     | 6     | 7    | 8     | 9     | 10    | 11    |
|---------------------------------------|-------|-------|-------|-------|-------|-------|------|-------|-------|-------|-------|
| °C                                    | 80    | 120   | 80    | 120   | 70    | 130   | 100  | 100   | 100   | 100   | 100   |
| Min                                   | 10    | 10    | 60    | 60    | 35    | 35    | 1    | 70    | 35    | 35    | 35    |
| Final pH                              | 2.42  | 2.36  | 2.42  | 2.35  | 2.40  | 2.33  | 2.4  | 2.43  | 2.4   | 2.4   | 2.4   |
| Pectin yield (%)                      | 6     | 12    | 13.0  | 42.0  | 7.0   | 39.0  | 12.0 | 42.0  | 32.0  | 34.0  | 33.0  |
| Pectin purity (%)                     | 40    | 58.0  | 40.0  | 58.0  | 35.0  | 57.0  | 20.0 | 81.0  | 61.0  | 60.0  | 59.0  |
| Pectin recovery (%)                   | 4.20  | 12.1  | 9.0   | 42.3  | 4.2   | 38.1  | 4.0  | 58.5  | 33.9  | 34.9  | 33.8  |
| Pectin in remaining solids (%)        | 21.0  | 23.0  | 22.0  | 9.0   | 25.0  | 11.0  | 24.0 | 13.0  | 16.0  | 16.0  | 16.0  |
| Hemicellulose in remaining solids (%) | 10.1  | 11.9  | 10.7  | 7.6   | 8.6   | 12.5  | 16.7 | 15.9  | 13.7  | 14.1  | 14.0  |
| Xylobiose (g/L)                       | 0.327 | 0.275 | 0.339 | 0.446 | 0.431 | 0.453 | -    | 0.347 | 0.341 | 0.341 | 0.344 |
| Xylotriose (g/L)                      | 0.334 | 0.266 | 0.343 | 0.398 | 0.414 | 0.436 | -    | 0.349 | 0.339 | 0.340 | 0.339 |
| Xylohexaose (g/L)                     | -     | -     | -     | 0.200 | -     | -     | -    | -     | -     | -     | -     |
| Xylopentaose (g/L)                    | -     | -     | -     | 0.133 | -     | -     | -    | -     | -     | -     | -     |
| Xylohexaose (g/L)                     | -     | -     | -     | 0.125 | -     | -     | -    | -     | -     | -     | -     |
| Total XOS (g/L)                       | 0.660 | 0.541 | 0.683 | 1.301 | 0.845 | 0.889 | 0    | 0.695 | 0.680 | 0.681 | 0.681 |
| Arabinose (g/L)                       | 0     | 0     | 0.025 | 0.342 | 0     | 0.107 | 0    | 0.122 | 0.015 | 0.015 | 0.015 |
| HMF (g/L)                             | 0     | 0.007 | 0     | 0.01  | 0     | 0.009 | 0    | 0.007 | 0     | 0     | 0     |
| Furfural (g/L)                        | 0     | 0     | 0     | 0.019 | 0     | 0.015 | 0    | 0     | 0     | 0     | 0     |
| Acetic acid (g/L)                     | 0.03  | 0.034 | 0.034 | 0.03  | 0.043 | 0.03  | 0    | 0.033 | 0.03  | 0.03  | 0.03  |
| Formic acid (g/L)                     | 0     | 0.021 | 0.02  | 0.03  | 0     | 0.022 | 0    | 0.016 | 0     | 0     | 0     |

contains few seeds. The primary season for harvesting is from May to October. The off-season produces a small quantity of 'Pera Rio' oranges and other species to meet market needs,

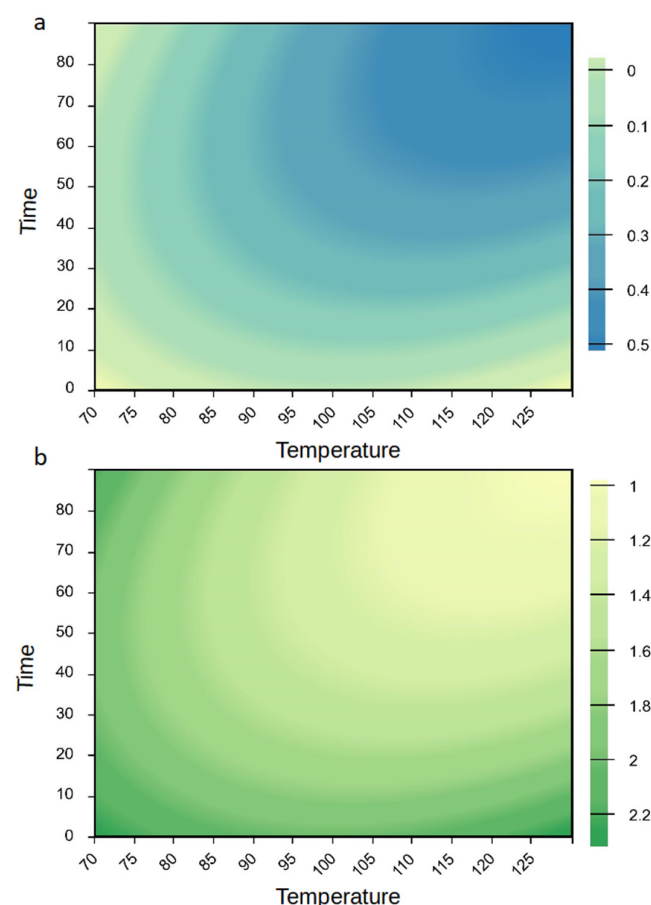


Figure 1. Response surface plots of central composite rotatable design for pectin extraction (a) and xylan recovery (b) on the temperature and time of extraction.

such as 'Charmute' orange and 'Natal' oranges.<sup>35,36</sup> This guarantees the orange supply all year round for continuous industrial processing and export, which also entails ongoing generation of orange waste. The waste has no commercial value unless dried, texturized, and sold as fertilizer or animal feed.<sup>37</sup>

Studies have proposed alternatives for a more sustainable industrial processing of OPW, focusing on economic and environmental aspects related to OPW biorefinery and novel green technologies to reduce the eco-footprint of OPW upgrading. Table 1 compares the present proposal and recent studies from 2017 to 2023. This proposal estimated energy reduction and the reuse of reagents and investigated different economic scenarios. Other research reported the feasibility of OPW for pectin and biogas production<sup>1</sup> and the techno-economic analysis of producing xylo-oligosaccharides industrial wastes.<sup>16</sup> Here, the integrated use of OPW for pectin, XOS, and bioenergy was analyzed to add value and reduce the eco-footprint of OPW biorefinery.

### Orange processing waste composition

The chemical characterization showed that  $81 \pm 1.9\%$  of OPW wet weight was moisture, and  $50 \pm 3.4\%$  of OPW dry weight was free sugars. The free sugars were composed of glucose (38.5%), fructose (37.2%), and sucrose (22.3%). >90% of the total free sugars were separated in the first washing. Table 2 presents the chemical composition of washed and dried OPW, indicating that OPW was adequate for pectin extraction. The remaining solids were poor in lignin and rich in cellulose and hemicellulose. Nevertheless, chemical variations may occur due to fruit maturity, growth conditions, and equipment adjustments for orange juice extraction.<sup>1</sup>

**Table 4. XOS composition from xylan enzymatic hydrolysis, antioxidant ability, and total phenols.**

| Solid concentration (g L <sup>-1</sup> ) | 10     | 20     | 30     | 40     |
|------------------------------------------|--------|--------|--------|--------|
| Xylose                                   | 0.343  | 0.349  | 0.348  | 0.352  |
| Xylobiose (x2)                           | 0.319  | 0.334  | 0.329  | 0.339  |
| Xylotriose (x3)                          | 0.320  | 0.326  | 0.309  | 0.341  |
| Xylotetraose (x4)                        | 3.522  | 6.690  | 1.820  | 1.976  |
| Xylopentaose (x5)                        | 0.117  | -      | -      | -      |
| Xylohexaose (x6)                         | 1.524  | 1.807  | 0.497  | 1.736  |
| Total XOS (g/L)                          | 5.802  | 9.157  | 2.955  | 4.391  |
| XOS (mg/mg xylan)                        | 0.840  | 0.663  | 0.107  | 0.212  |
| % conversion                             | 84.10% | 72.68% | 21.23% | 10.72% |
| μg gallic acid eq                        | 52.33  | 104.62 | 154.32 | 193.80 |
| μM Trolox eq                             | 3803   | 20279  | 11587  | 15279  |



## Pectin extraction

Pectin extractions were assayed as shown in Table 3. The solid ratio of 1:40 was adopted according to preliminary assays (data not shown) and the literature.<sup>2</sup> Although higher ratios may favor pectin extraction due to lower viscosity, the excessive use of solvents leads to higher processing costs and autohydrolysis due to over-dilution.<sup>2</sup> In this study, the integrity of the remaining solids was the main factor for its further use for XOS production.

Assay 8 (100 °C and 70 min) showed the highest purity (81%) and lowest material degradation, releasing 0.7 mg/mL XOS (Table 3). This assay showed superior efficiency compared to other studies,<sup>38</sup> with a pectin recovery of 58.5%. Assays 2 and 4 supplied 58% pectin and released 0.54 and 1300 mg/mL XOS, respectively. The higher XOS concentration in assay 4 was due to the acidic autohydrolysis of the hemicellulose (70.35% conversion). However, higher temperatures (>120 °C) and longer incubation times resulted in more acid pH due to intense biomass degradation and production of formic acid, HMF, furfural, and acetic acid, as presented in Table 3. The mathematical models were obtained through second-order polynomial equations ( $p < 0.05$ ).

Figure 1 shows the effects of temperature and incubation times for pectin extraction (a) and xylan recovery (b), with  $R^2$  values of 0.98 and 0.95, respectively. Xylan recovery decreased at temperatures above 105 °C and incubation time above 50 min. Table 2 presents the hemicellulose content in residual solids. No studies have analyzed the liquid fraction

(which holds XOS) and residual solids (lignocellulose) that remain after pectin extraction for comparison with these results. Citric acid is cheaper than other organic acids for pectin extraction.<sup>39</sup> Citric acid improves economic feasibility through recycling and presents a low environmental footprint.<sup>40</sup>

## Xylan extraction and enzymatic hydrolysis

The purest xylan was obtained with 25 g L<sup>-1</sup> KOH at 100 °C for 120 min. Xylose was the main monosaccharide in the alkali-extracted material (>1 g L<sup>-1</sup>). Glucose, galactose, and mannose were detected with more than 50 g L<sup>-1</sup> KOH (~0.005 g L<sup>-1</sup>); arabinose content increased with increasing KOH concentrations (0.04 g L<sup>-1</sup> with 50 g L<sup>-1</sup> KOH and 0.07 g L<sup>-1</sup> with 100 g L<sup>-1</sup> KOH). Higher KOH concentrations did not increase the extracted weight mass (~30% of the first mass). The remaining solids contained 76% cellulose and 8% hemicellulose. These results are consistent with earlier studies: Pereira *et al.* (2022)<sup>41</sup> reported that the largest xylan solubilization (80.81% for the orange bagasse waste) occurred with KOH in comparison with NaOH and hydrogen peroxide. The characteristics of each biomass, especially chemical composition, influence the best hemicellulose solubilization conditions.

The extracted material was neutralized with acetic acid before ethanol was added. Some methodologies recommend the addition of 10 volumes<sup>42</sup> or three volumes<sup>43</sup> to precipitate xylan. However, higher ethanol volumes reduced the purity

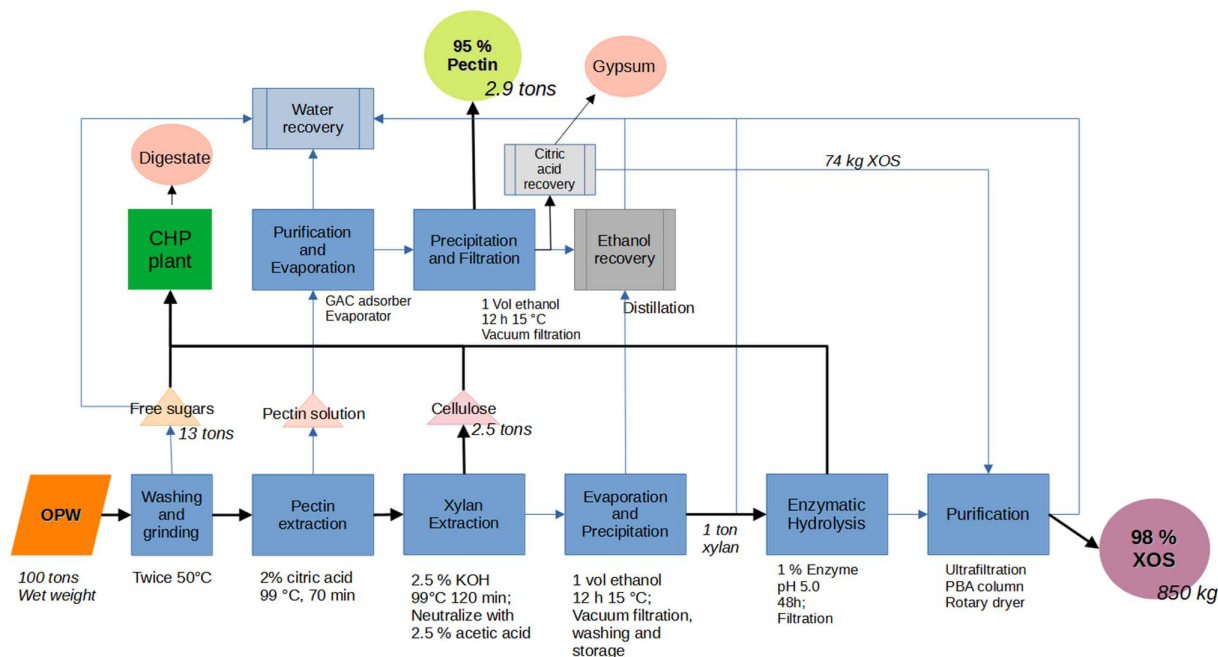


Figure 2. Biorefinery flowchart.

from 69% (1 ethanol volume) to 15% (3 ethanol volumes) due to increased arabinose content in the material.

Later, the enzymatic hydrolysis yielded  $9.16 \text{ g L}^{-1}$  of XOS, which stands for a conversion of 72% (Table 5). The hydrolysis product contained  $0.32 \text{ g L}^{-1}$  of monosaccharides. The main oligosaccharide was xylotetraose (x4), which constituted ~74% of the total XOS. The hydrolysate with higher XOS concentration presented higher antioxidant activity. A considerable fraction of phenolic compounds ( $\mu\text{g}$  gallic acid equivalent) was also detected (Table 4) and contributed to the antioxidant potential of the material.

## Process simulation

Figure 2 shows the flowchart of the biorefinery, where 13 tons of free sugars are collected from 100 tons OPW (wet basis). Each batch produces 2.9 tons of 95% pectin and 825 kg of XOS (from 1 ton of extracted xylan). The remaining solids

from all stages go to the CHP plant, and a new batch begins every 38 h (see Supporting Information).

Figure 3(a) shows that Scenario 1 for pectin precipitation, with no evaporation, resulted in the highest usage of ethanol, steam, and chilled water. In Scenario 1, a cooling system is necessary before precipitation because of the elevated inlet temperature. Scenario 2 (30% evaporation) resulted in the lowest power consumption, and Scenario 3 (50% evaporation) resulted in the highest power and cooling water consumption. Scenario 4 (70% evaporation) resulted in the lowest ethanol, steam, and cooling water consumption but presented the best equipment investment and payback time. Evaporation above 50% also led to a viscous solution, which would be difficult to process in an industrial operation. The total capital investment calculation adopted Scenario 2, based on its energy and ethanol consumption.

When considering citric acid recovery, 92% of the citric acid could be recycled. This process could generated 158.38 kg

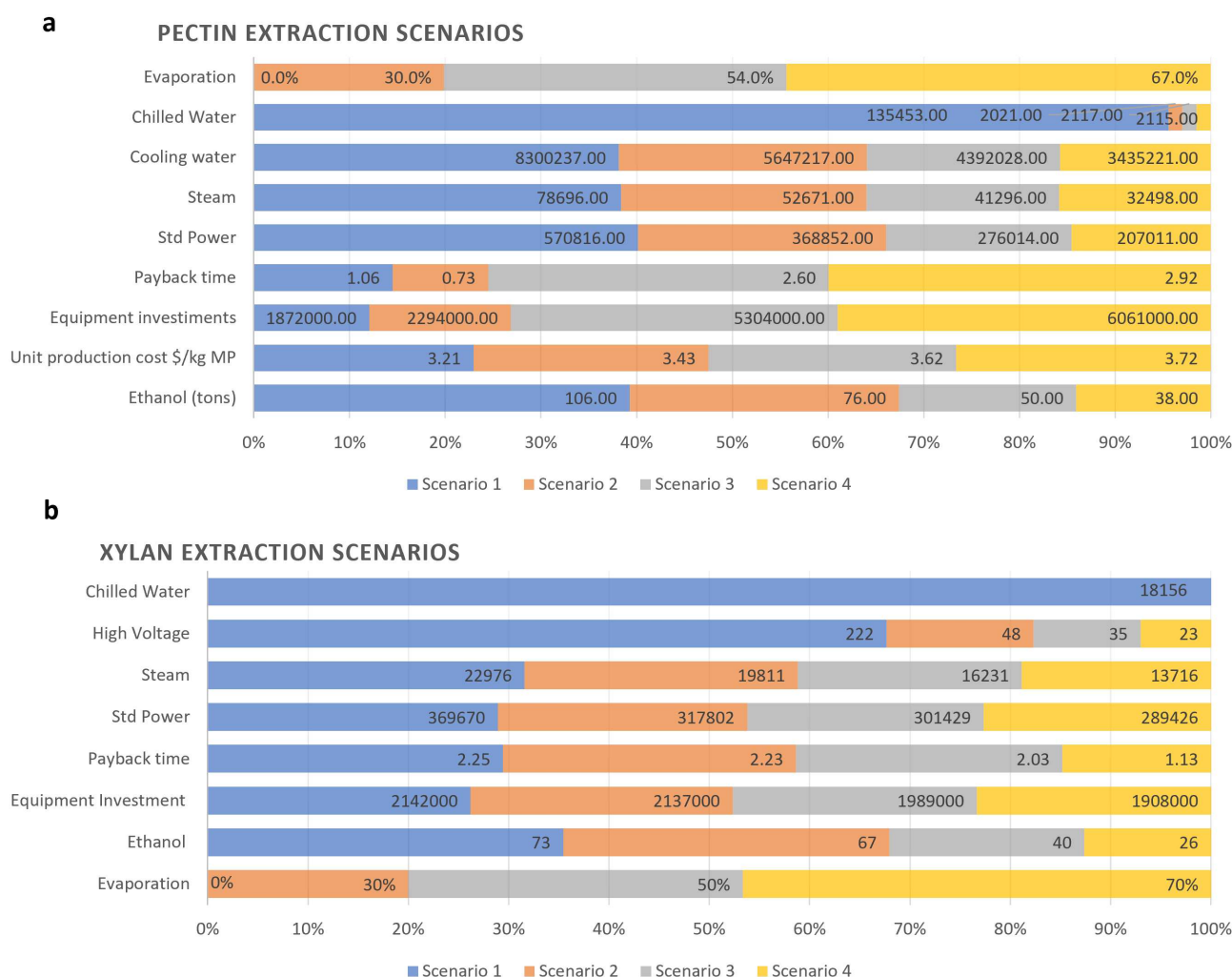


Figure 3. Economic scenarios for precipitation of pectin (a) and xylan (b) on solvent evaporation before ethanol precipitation.

**Table 5. Power and solvent consumption and recovery; environment properties, wastes, and emissions.**

| Power consumption           | Total cost (\$)     | CHP plant revenue (\$) | Final cost (\$)       | Savings |
|-----------------------------|---------------------|------------------------|-----------------------|---------|
| Std power                   | 75 400.00           | 403 540.00             | 328 270.00            |         |
| Steam                       | 102 756.00          | 14 516.00              | 88 240.00             | 14.13   |
| Cooling water               | 262 196.00          |                        |                       |         |
| Chilled water               | 644.00              |                        |                       |         |
| High voltage                | 1374.00             |                        |                       |         |
| <b>Solvents recovery</b>    | <b>Ethanol</b>      | <b>Citric acid</b>     | <b>Water</b>          |         |
| Consumed                    | 19 641 358.00       | 23 282.00              | 38 914 146.00 kg year |         |
| Recovered                   | 19 122 780.35       | 20 661.81              | 33 428 184.50 kg year |         |
| Recovery yield              | 97.36%              | 88.75%                 | 85.90%                |         |
| Total consumption           | 518 577.65          | 2620.19                | 5 485 961.50 kg year  |         |
| Total cost                  | \$114.09            | \$1179.08              | \$10971.92            |         |
| Savings                     | \$4207.01           | \$9297.82              | \$66 856.37           |         |
| <b>Environment property</b> | <b>In (kg year)</b> | <b>Out (kg year)</b>   | <b>% reduction</b>    |         |
| TOC                         | 3 963 358           | 3 663 278              | 7.57                  |         |
| COD                         | 14 076 171          | 13 314 481             | 5.41                  |         |
| BOD                         | 1 440 397           | 1 166 563              | 19.01                 |         |
| TKN                         | 22 959              | 22 959                 | 0.00                  |         |
| TDS                         | 1 991 781           | 1 476 244              | 25.88                 |         |
| VDS                         | 1 580 563           | 1 072 947              | 32.12                 |         |
| <b>Aqueous waste</b>        | <b>kg per batch</b> | <b>kg per year</b>     | <b>%</b>              |         |
| HMF                         | 0.53                | 105                    | 0.00                  |         |
| Impurities                  | 181.56              | 36 386                 | 0.12                  |         |
| NaOH                        | 2287.03             | 457 406                | 1.54                  |         |
| Phosphoric acid             | 30.5                | 6099                   | 0.02                  |         |
| Sulfuric acid               | 3.76                | 753                    | 0.00                  |         |
| Water                       | 145 692             | 29 138 398             | 98.30                 |         |
| Total                       | 148 206.1           | 29 641 295             | 100.00                |         |
| <b>Emissions</b>            | <b>kg per batch</b> | <b>kg per year</b>     | <b>%</b>              |         |
| CO <sub>2</sub>             | 27 328.92           | 5 465 784              | 6.18                  |         |
| N <sub>2</sub>              | 313 509.9           | 62 702 700             | 70.94                 |         |
| O <sub>2</sub>              | 63 987.95           | 12 797 808             | 14.48                 |         |
| H <sub>2</sub> O            | 37 134.87           | 7 427 266              | 8.40                  |         |
| Acetic acid                 | 0.89                | 159                    | 0.00                  |         |
| Sulfuric acid               | 1.64                | 329                    | 0.00                  |         |
| Total                       | 441 961.6           | 88 393 558             | 100.00                |         |

Abbreviations: BOD, biological oxygen demand; COD, chemical oxygen demand; HMF, hydroxymethylfurfural; TDS, total dissolved solid; TKN, total kjeldahl nitrogen; TOC, total organic carbon; VDS, volatile dissolved solids.

of gypsum, a soft mineral filler that has applications as fertilizer, a constituent of drywalls, and in the production of bioplastics.<sup>44</sup> However, citric acid recovery increased the final unit production cost by \$0.40 per kg.

For xylan precipitation, Fig. 3(b) presents the four scenarios that we analyzed in order to reduce the

consumption of ethanol and energy. Scenario 4 (70% evaporation) showed the lowest investment, payback time, ethanol, and energy consumption. Unlike pectin, with high viscosity, the xylan-rich extract can have its water content evaporated up to 70% without disturbing the next operations in the biorefinery.

## Combined heat and power plant and environment analysis

Anaerobic digestion is one of the most promising techniques for biofuel production because of its easy implementation, versatility, low investment requirements, and appealing alternative for waste management, minimizing the environmental impacts of inappropriate disposal. One of the worthwhile applications of OPW is anaerobic digestion (AD) and biogas production due to its high energy content, abundance, and availability. Nevertheless, pretreatment is necessary before AD to reduce the antioxidant compounds, D-limonene, and other components that reduce biogas yields.<sup>45</sup> This study circumvented the need for pretreatment as it used the waste solids (free sugars and cellulose) from the biorefinery stages, improving profitability and sustainability.

The gas turbine generator runs continuously and can produce 4 035 402 kW-h per year, saving 403 540 \$ per year. (0,10 \$/kW-h) along with 14 516 \$ per year from the steam generation (see Supporting Information), and 4.5 tons per batch of digestate can be sold at 70 \$ per ton, generating an annual credit of 70 000 \$ per year. Using digestate as a biofertilizer in orange crops can reduce the environmental impact of orange production and agrochemicals. It is another way to increase the sustainability of food processing.<sup>46</sup> Table 5 shows the total power consumption and savings. The CHP plant supplies 14.13% of savings related to steam costs, and there is a credit of \$328 270 from power generation. This biorefinery may therefore present self-sustainability for standard power usage. Table 5 also shows that the plant can recover 85.90% water, 97.36% ethanol, and 88.75% citric acid.

The designed process showed reductions in all environmental properties, as presented in Table 5, decreasing

the organic pollution that may accrue from OPW's inadequate disposal. Carbon dioxide emissions correspond to 6% of total emissions (27 metric tons of CO<sub>2</sub> per 100 tons OPW, or 6 kg per kg of products). This value is below the reported in the literature for pectin production from fruit wastes, accounted for 9.69 kg CO<sub>2</sub> per kg pectin,<sup>47</sup> and supports the low environmental impact of the proposed process.

The environmental impact is directly proportional to the biorefinery complexity, but even more complex biorefineries showed better environmental performance than the disposition of OPW in landfills.<sup>46</sup> The valorization of OPW contributes to reducing greenhouse gas emissions, water releases, and fossil fuel use because the impact categories related to these aspects are the lowest in the biorefinery.<sup>45,46</sup> Future experiments must implement a sensitivity analysis to confirm if the extraction of essential oil and phenolic compounds from the washed OPW (before the pectin extraction stage) is also feasible.

## Techno-economic evaluation

Figure 4 presents the cost composition, where bulk materials account for 13%. SuperPro pricing models estimated the facility-dependent and utility costs, based on the US market proportional to the purchased equipment cost. These may be overestimated as no adjustment factor was applied to the prices provided by the software.<sup>33</sup> Potassium hydroxide accounts for 49% of the material cost, and the enzyme accounts for 9%. Strategies to reduce KOH and enzyme load were crucial to decrease the production cost, even though KOH still represents half the total bulk material cost.

The overall net unit production cost is 3.67 \$ per kg of products (pectin and XOS, Table 6). Figure 5 shows the cost

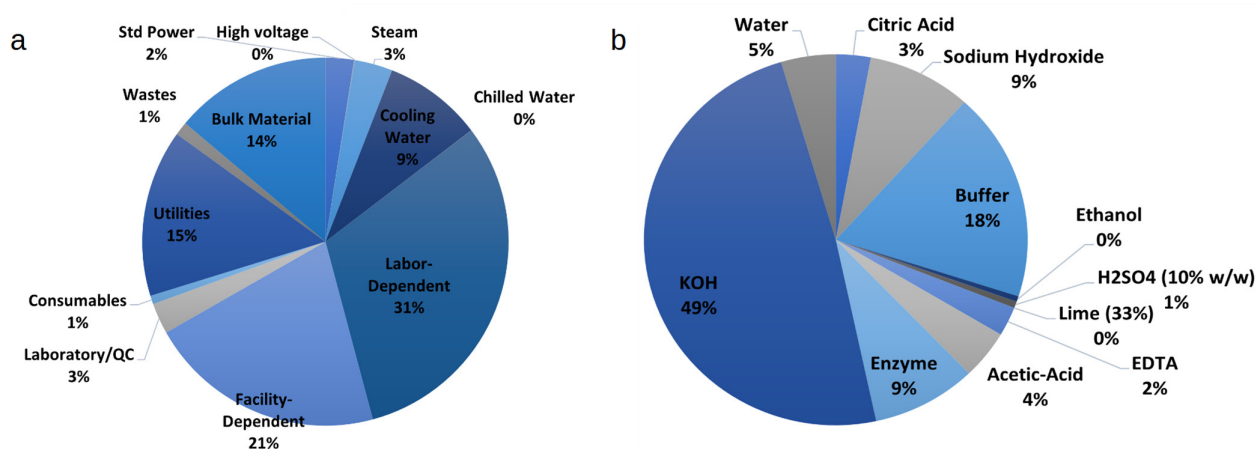


Figure 4. Biorefinery cost composition (a). Bulk material cost composition (b).

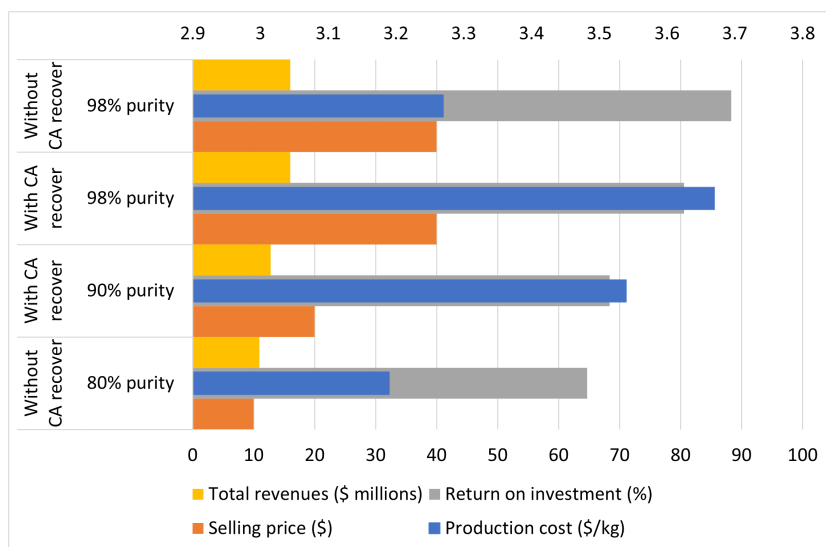


Figure 5. Cost analysis and investment return according to xylooligosaccharide purity and citric acid recovery.

**Table 6. Executive summary of biorefinery.**

| Executive summary                          |                       |
|--------------------------------------------|-----------------------|
| Total capital investment                   | \$20 005 000.00       |
| Charged capital investment                 | \$10 142 000.00       |
| Net operating cost                         | 2 793 918.00 \$ year  |
| Total revenues                             | 16 031 000.00 \$ year |
| Total credits/savings                      | 93 027.00 \$ year     |
| Batch size                                 | 3810.81 kg MP         |
| Cost basis annual rate                     | 762 160.00 kg MP year |
| Net unit production cost                   | 3.67 \$ per kg MP     |
| Unit production revenue                    | 55.00 \$ per kg MP*   |
| Gross margin                               | 82.67%                |
| Return on investment                       | 80.60%                |
| Payback time                               | 1.24 year             |
| IRR (after taxes)                          | 38.01%                |
| Net present value (NPV) (at 7.0% interest) | 15 217 750.00 \$      |
| *15 \$ per kg pectin and 40 \$ per kg XOS. |                       |

analysis for different XOS purities, with and without citric acid recovery: 98% purity XOS without citric acid recovery showed an 88.3% investment return, and 80% XOS showed the lowest production cost, \$3.19 per kg; however, it has the lowest selling price and investment return (64.66%). Nevertheless, these results are superior to recent techno-economic studies, which showed a production cost of 9.80 \$ per kg pectin<sup>47</sup> and 3.10 \$ per kg 95% pure XOS.<sup>16</sup> The current market price for pectin is \$15 per kg<sup>48</sup> and \$25–\$50 per kg for XOS (80–98% purity).<sup>11</sup> These values prove

the economic feasibility and profitability of the proposed biorefinery.

## Conclusion

This analysis of the factors affecting the cost on a large scale contributes to understanding the potential use of OPW in the food industry in a way that is self-sufficient in energy and that has a low impact on the environment. Process design reduced energy and solvent requirements by more than 60%. These results are especially relevant for orange processing industries to increase profitability. Future research should investigate the impact of biomass seasonality and the implementation of the proposed biorefinery into existing orange processing plants to reduce initial capital investment. In short, researchers are diversifying OPW exploitation to detour landfilling disposal and its environmental damage while increasing the marketability of bioproducts and other value-added compounds. In this sense, the studies should consider all OPW fractions to obtain a wider diversity of products, creating integrated biorefineries to end up with food ingredients, materials, and bioenergy. In all cases, solvent and water recycling and reuse are critical to reduce the environmental footprint and production costs.

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