UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

LILIANE PIRES ANDRADE

ALTERNATIVE STRATEGIES TO IMPROVE THE SACCHARIFICATION EFFICIENCY OF PRETREATED SUGARCANE BAGASSE

ESTRATÉGIAS ALTERNATIVAS PARA MELHORAR A EFICIÊNCIA DA SACARIFICAÇÃO DO BAGAÇO DE CANA-DE-AÇÚCAR PRÉ-TRATADO

CAMPINAS
2023

LILIANE PIRES ANDRADE

ALTERNATIVE STRATEGIES TO IMPROVE THE SACCHARIFICATION EFFICIENCY OF PRETREATED SUGARCANE BAGASSE

ESTRATÉGIAS ALTERNATIVAS PARA MELHORAR A EFICIÊNCIA DA SACARIFICAÇÃO DO BAGAÇO DE CANA-DE-AÇÚCAR PRÉ-TRATADO

Thesis presented to the Biology Institute of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Molecular and Morphofunctional Biology, in the area of Biochemistry.

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Biologia Molecular e Morfofuncional, na Área de Bioquímica

Orientador: DR. MÁRIO TYAGO MURAKAMI

Coorientador: DR. GEORGE JACKSON DE MORAES ROCHA

ESTE ARQUIVO DIGITAL CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA LILIANE PIRES ANDRADE, E ORIENTADA PELO PROF. DR. MÁRIO TYAGO MURAKAMI.

CAMPINAS
2023

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

An24a
Andrade, Liliane Pires, 1985-
Alternative strategies to improve the saccharification efficiency of pretreated sugarcane bagasse / Liliane Pires Andrade. – Campinas, SP : [s.n.], 2023.

Orientador: Mário Tyago Murakami.
Coorientador: George Jackson de Moraes Rocha.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Sacarificação. 2. Bagaço de cana. 3. Processos de fabricação. 4. Pré-tratamento. 5. Biorrefinaria. I. Murakami, Mário Tyago, 1981-. II. Rocha, George Jackson de Moraes. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Informações Complementares

Título em outro idioma: Estratégias alternativas para melhorar a eficiência da sacarificação do bagaço de cana-de-açúcar pré-tratado
Palavras-chave em inglês:
Saccharification
Bagasse
Manufacturing processes
Pretreatment
Biorefinery
Área de concentração: Bioquímica
Titulação: Doutora em Biologia Molecular e Morfofuncional
Banca examinadora:
Mário Tyago Murakami [Orientador]
Priscila Oliveira de Giuseppe
Adilson Roberto Gonçalves
César Rafael Fanchini Terrasan
Maria de Lourdes Teixeira de Moraes Polizeli
Data de defesa: 29-06-2023
Programa de Pós-Graduação: Biologia Molecular e Morfofuncional

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0003-3569-9029
- Currículo Lattes do autor: http://lattes.cnpq.br/9875065822420268

Campinas, 29 de junho de 2023.

COMISSÃO EXAMINADORA

Prof. Dr. Mário Tyago Murakami

Profa. Dra. Priscila Oliveira de Giuseppe

Prof. Dr. Adilson Roberto Gonçalves

Prof. Dr. César Rafael Franchini Terrasan

Profa. Dra. Maria de Lurdes Teixeira de Moraes Polizeli

Os membros da Comissão Examinadora acima assinaram a Ata de defesa, que se encontra no processo de vida acadêmica do aluno.

Ata da defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa de Pós-Graduação em Biologia Molecular e Morfofuncional da Unidade do Instituto de Biologia da Unicamp.

Dedicatória

Com amor ao Rodolfo, que
embarcou nesta jornada mesmo
consciente dos grandes desafios.

Agradecimentos

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001 - (Processo 88887.468496/2019-00) e do Conselho Nacional de Desenvolvimento Científico e Tecnológico - Brasil (CNPq) - (Processo 157043/2018-5).

Meus sinceros agradecimentos ao meu orientador, Dr. Mário Murakami, por tornar possível essa jornada especial, além de fornecer orientação durante todo o meu doutorado e por me apoiar a buscar novos desafios.

Ao meu coorientador, Dr. George Rocha, pelo grande apoio em cada etapa, pelas discussões enriquecedoras e sábios ensinamentos, por todo carinho e incentivo frente aos desafios enfrentados ao longo dessa jornada.

Ao Professor Simon McQueen-Mason (in memoriam) por ter acreditado no meu potencial e me convidado a executar parte deste doutorado em seu laboratório após nos conhecermos.

À Letícia Zanphorlin, pelo grande apoio para que este doutorado se tornasse realidade, por todas as contribuições científicas, carinho e amizade.

Ao Rodolfo, meu grande amor, incentivador, amigo e companheiro de jornada, que esteve paciente e resiliente ao meu lado durante todo o doutorado, que me fez sorrir quando queria chorar e suportou a pandemia a um oceano de distância.

Aos meus pais, José Maria e Sueli, por todo amor, carinho, ensinamentos e torcida em todas as etapas da minha vida e, principalmente, por me ensinarem a voar e acreditar nos meus sonhos.

Aos meus amigos de jornada Adriana, Ana, Camila, Cíntia, Elaine, Fernando, Giovana, Graciela, Isabela, José Augusto, Juliana, Karina, Laurisa, Natália, Nayere, Paula, Rafael, Rosangela e Sondess pelo suporte, torcida, conselhos, incentivos e por estarem ao meu lado nos momentos difíceis. Mas, principalmente, por todos os momentos alegres que compartilhamos e por me motivarem a nunca desistir dos meus objetivos.

À minha irmã Barbara, avós, tias, tios, primas, primos, sogra, cunhados e toda a minha família que mesmo à distância, me incentivaram, torceram e confiaram.

Aos colaboradores do Laboratório Nacional de Biorrenováveis que me apoiaram, tiraram dúvidas, foram prestativos, colaborativos e me incentivaram ao longo do doutorado Em especial à Camila, Carlos Alberto, Carlos Driemeier, Daniel, Daniele, David, Eduardo Couto,

Eduardo Ribeiro, Emerson, Evandro, Flávio, Gabriela, Juliana, Lívia, Lúcia, Márcia, Mayko, Priscila, Raquel, Rodrigo, Rebeca, Renan, Tatiane e Viviane.

Aos pesquisadores Alexandra Lanot e Leonardo Gomez pelos valiosos ensinamentos, assim como a todo o time do Centro de Novos Produtos Agrícolas da Universidade de York, em especial a Carla, Federico, Giovanna, Juliana, Katrin e Rachel que me receberam de braços abertos em plena pandemia e me apoiaram para que eu cumprisse meu objetivo.

Aos colegas de laboratório, em especial à Amanda, Atílio, Beto, Bruno, Djanira, Isabela, Fabricia, Fernan, Josiane, Letícia, Marcele, Mayara, Plínio, Renan, Ricardo, Rosa, Thiago e Vandy, por compartilharem a rotina tornando nosso local de trabalho um ambiente alegre, pelo suporte e pela paciência com minhas dúvidas.

Aos pesquisadores que dedicaram tempo de suas agendas para enriquecer este trabalho, em especial ao Daniel Atala (Bioprocess Improvement), Lisbeth Thygesen (Universidade de Copenhague), Lívia Brenelli (Cargill), Maria Isabel Berto (ITAL), Maria Isabel Rodrigues (Protimiza), Paulo Mantelatto (Consultor), Robson Tramontina (ECRA Biotec) e Roberto Ruller (UNESP).

Ao Centro Nacional de Pesquisa em Energia e Materiais por propiciar um ambiente com infraestrutura ímpar para a pesquisa.

Ao Programa de Pós-Graduação em Biologia Molecular e Morfofuncional da UNICAMP, em especial à Andréia, Beatriz e Marco Antônio pelo suporte constante com os assuntos acadêmicos.

Àqueles que conheci ao longo da vida e que de alguma forma me transformaram para que eu tivesse o necessário para realizar esse trabalho.

À Deus por me capacitar e colocar as pessoas certas no momento certo em meu caminho.

Resumo

Dentre as estratégias de implementação de uma economia de base biológica e ambientalmente sustentável destacam-se as biorrefinarias de biomassa lignocelulósica. Para o desenvolvimento das biorrefinarias, vários fatores são fundamentais como a seleção apropriada da biomassa, passando pelo desenvolvimento de soluções para o escalonamento do pré-tratamento, aliado a uma sacarificação enzimática mais eficiente que promova uma redução de custos associada a maior concentração de açúcar, até a adoção de engenharia genética e metabólica com vistas a aumentar os rendimentos dos bioprodutos de interesse. Enfim, o desenvolvimento de biorrefinarias em escala industrial possui grandes desafios a serem resolvidos como os custos operacionais e de capital, imaturidade técnica e dificuldades de escalonamento. Sendo assim, esta tese teve como objetivo avançar em dois grandes desafios, sendo o primeiro tecnológico e o segundo científico, visando em aumentar a eficiência da sacarificação da biorrefinaria de cana-de-açúcar. O primeiro está relacionado com a transformação da matéria-prima ao submetê-la ao refino mecânico após o pré-tratamento em processos realizados em escala piloto. O segundo buscou a identificação de enzimas com potencial para futuras modificações genéticas que promovam o aumento da eficiência do secretoma de *Trichoderma reesei* RUT-C30 engenheirado (Br_TrR03). O refino mecânico demonstrou influenciar a digestibilidade enzimática independentemente do método de pré-tratamento e ao refinar bagaço de cana-de-açúcar pré-tratado por explosão a vapor (190°C por 10 min) melhorou em 8% a eficiência de sacarificações conduzidas com 10% de sólidos (p/p) e carga enzimática de 10 $FPU.gPTB^{-1}$. As imagens de microscopia eletrônica de varredura sugerem que esta melhora foi promovida pela exposição das fibrilas da parede celular e delaminação interna das fibras, que pode ter refletido numa maior porosidade e intumescimento das mesmas. Em seguida, as enzimas selecionadas para este estudo: endoglucanases (*Bacillus subtilis* GH5R1 e *Humicola insolens* GH45), endoxilanase (*Kalmanozyma brasiliensis* GH11), expansina (*Bacillus subtilis*) e LPMOs (*Kitasatospora papulosa* AA10 e *Termobia domestica* AA15) tiveram seu sinergismo com os coquetéis Br_TrR03 e Cellic® CTec2 avaliado por uma plataforma automatizada em ensaios com baixo teor de sólidos. A formulação resultante da combinação do coquetel Br_TrR03 e a endoglucanase BsGH5R1 foi a mais efetiva em promover sacarificação e redução do tamanho das partículas de bagaço de cana-de-açúcar pré-tratado. Estes resultados demonstram o potencial de se utilizar refino mecânico de alta consistência associado ao pré-tratamento por explosão a vapor e assim como, a importância de avaliar enzimas que possuem ação sinérgica com secretomas microbianos para aumentar a produtividade da sacarificação enzimática.

Abstract

Among the strategies for implementing a biobased and environmentally sustainable economy, the lignocellulosic biorefineries are promising alternatives. For the development of lignocellulosic biorefineries, several factors are essential, such as the appropriate selection of biomass, development of solutions for the pretreatment scaleup, efficient enzymatic hydrolysis reducing the cost while increasing the sugar release, and adopting genetic and metabolic engineering to increase bioproduct yields. Industrial biorefinery development faces significant challenges to be solved, such as high capital and operating costs, technical immaturity, and difficulties in scaling up. Therefore, this thesis aimed to advance in two important challenges, the first technological and the second scientific, intending to increase saccharification efficiency in the sugarcane biorefinery. First related to substrate transformation using mechanical refining associated with mild pretreatment at the pilot scale. The second focused on identifying potential enzymes for future genetic modifications to increase the efficiency of the engineered *Trichoderma reesei* RUT-C30 secretome (Br_TrR03). Mechanical refining influences enzymatic digestibility independently of the pretreatment method. Its association with steam-exploded pretreated sugarcane bagasse at 190°C for 10 min improved the efficiency by 8% in saccharifications conducted with 10% (w/w) solids and enzyme load of 10 FPU.gPTB^{-1}. The scanning electron microscopy images suggest that the improvement observed is promoted by the exposure of cell wall fibrils and internal delamination, which could be reflected in higher porosity and swellability. Afterward, the selected target enzymes: endoglucanases (*Bacillus subtilis* GH5R1 and *Humicola insolens* GH45), endoxylanase (*Kalmanozyma brasiliensis* GH11), expansin (*Bacillus subtilis*), and LPMOs (*Kitasatospora papulosa* AA10 and *Termobia domestica* AA15) had their synergism with the cocktails Br_TrR03 and Cellic® CTec2 evaluated at low solids by an automated platform. The blend combining Br_TrR03 and the endoglucanase, BsGH5R1, was the most effective in promoting saccharification and particle size reduction of pretreated sugarcane bagasse. These results demonstrate the potential of using high-consistency mechanical refining associated with steam explosion pretreatment and the importance of evaluating enzymes that have synergistic action with microbial secretomes to increase the productivity of enzymatic saccharification.

Summary

Thesis outline

This work has been divided into four chapters. The **Chapter 1** contains a general introduction of how lignocellulosic biomass composition and structure results in a complex and recalcitrant barrier to developing effective and economically viable biorefineries and presents an overview of key proposed strategies to overcome this challenge. **Chapter 2** presents a manuscript (*in preparation*) in which this work contributed to the knowledge advance about the application and bottlenecks of applying high-consistency mechanical refining associated with mild pretreatment in sugarcane biorefineries. The **Chapter 3** presents a manuscript (*in preparation*) where a strategy to identify candidates for future genetic modifications of an engineered *Trichoderma reesei* RUT-C30 strain was investigated by enzyme supplementation aiming to enhance the liquefaction and hydrolysis efficiency. The concluding remarks of this work and perspectives are presented in the **Chapter 4**.

Chapter 1

Introduction

Overcoming the recalcitrant barrier from lignocellulosic biomass

The latest IPCC report shows Earth's temperature has risen 1.1 °C. Even the new or enhanced countries' commitment may not be enough to attend the Paris Agreement of impeding the temperature increase of 1.5 °C, the Nationally Determined Contributions (NDCs). It has triggered an unparalleled degree of climate change in recent history. To meet the presented environmental needs, the coming decades expect biorefineries to complement or replace petroleum refineries, maximizing the value of lignocellulose biomass (LCB) by producing sustainable bioenergy, biofuels, chemicals, and materials (IPCC Working Group III, 2023).

LCB is abundantly accessible worldwide and includes forest and wood processing residues, agricultural and agro-industrial residues, part of municipal solid waste, herbaceous energy crops, and short-rotation trees. It predominantly comprises three main polymers: 40–50% of cellulose, 25–35% of hemicellulose, and 15–20% of lignin, with trace amounts of acetyl groups, minerals, and phenolic substituents. Its origin affects the quantity and quality of the biomass constituents (Figure 1) (Hernández-Beltrán et al., 2019).

Cellulose is the main structural component consisting of a linear glucose chain linked by β-1,4 glycosidic bonds forming microfibrils held together by hydrogen bonds, creating a rigid and resistant structure with a degree of polymerization (DP) as high as 8,000 (Fengel and Wegener, 1989). Crystallinity contributes to the challenge of breaking it down into monomers sugars by chemicals and enzymes. As a minor part of the macromolecule, there are also amorphous regions, more susceptible to reactions (Martín et al., 2022)

Hemicellulose is a heterogeneous mixture of branched polysaccharides of 5- and 6-carbon monosaccharide units and uronic acids. It is interconnected with cellulose microfibrils and is essential in maintaining the plant cell's structural integrity. With relatively low DP, the amorphous structure of hemicellulose varies considerably across different plant species and has higher reactivity than cellulose, resulting in hydrolysis even under mild conditions. This characteristic is particularly essential in lignocellulose biorefining (Martín et al., 2022; Ruiz et al., 2023).

Lignin is a three-dimensional aromatic polymer of phenylpropanoid units, namely p-coumaryl, coniferyl, and sinapyl alcohols; depending on the biomass source, the relative

composition and structure can vary significantly. It is linked to hemicellulose via the covalent bonds forming the lignin carbohydrate's structure. This complex bonding acts as a strengthening agent. It provides additional rigidity to the cell wall, making it highly resistant to degradation, hampering the challenge of being converted into value-added products. Recently, the efficient utilization of lignin was raised as an opportunity to enhance biorefineries' economic and environmental sustainability significantly (Khan et al., 2022).

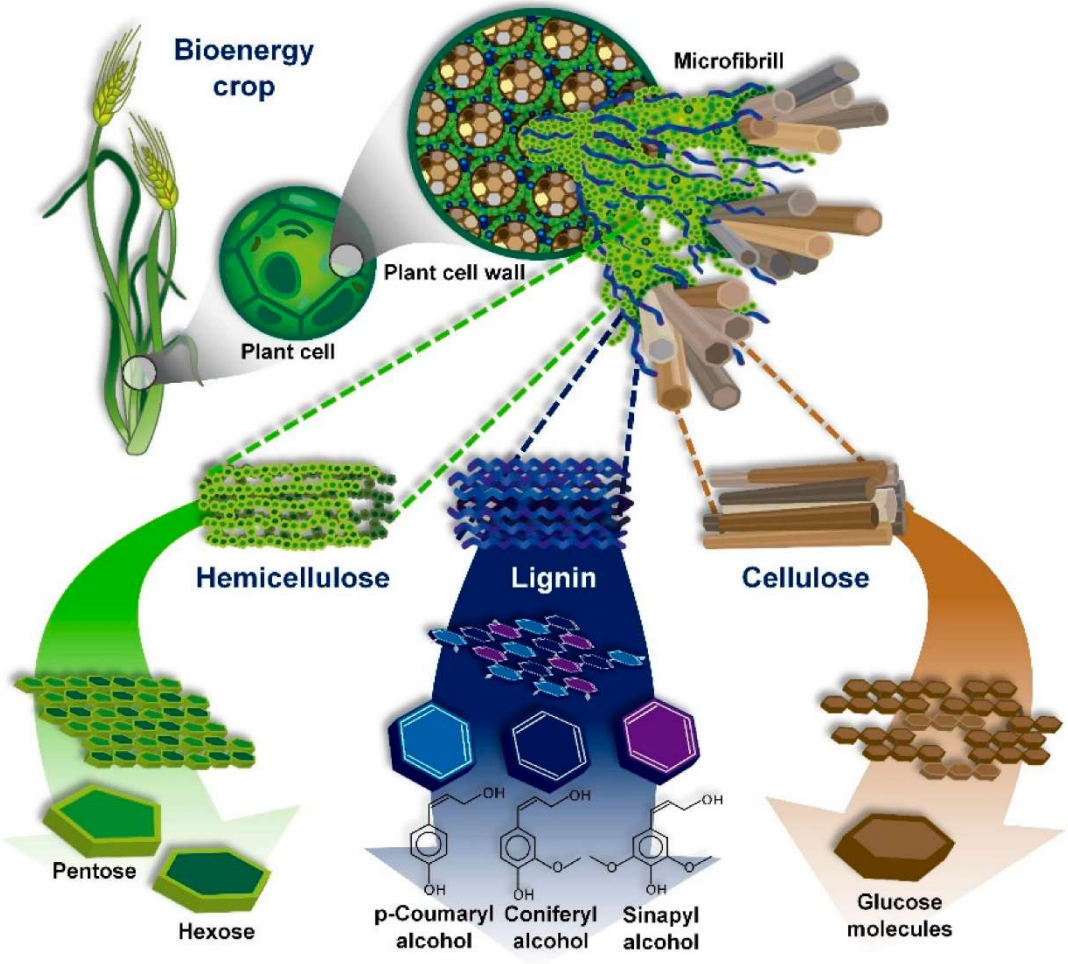


Figure 1: Schematic structure of lignocellulose. Source: Hernández-Beltrán et al. (2019)

The challenge of developing an efficient and sustainable process for biomass utilization is due to this complex and recalcitrant structure. Aiming to achieve this goal, the biochemical route has been considered the most suitable to be implemented and studied. Under this strategy, LCB conversion can be carried out in three steps: pretreatment, hydrolysis, and fermentation (Saini et al., 2022). High enzyme accessibility to cellulose is crucial in this route, many studies have been conducted to enhance it by breaking the lignin seal and disrupting the

cellulose crystalline structure (Martín et al., 2022). For example, crops' genetic engineering to reduce lignin content or alter cell wall composition (García et al., 2023; Zheng et al., 2023), pretreatments to loosen the complex and rigid cell wall structure (Espirito Santo et al., 2020; Hernández-Beltrán et al., 2019; Ruiz et al., 2020), and development of enzymatic cocktails to enhance enzymatic hydrolysis (Adsul et al., 2020; Chylenski et al., 2017; Du et al., 2020). Still, further research is needed to optimize these methods and strategies to realize their full potential as biorefinery building blocks.

Irrespective of the route, biochemical or thermochemical, the pretreatment usually precedes biomass conversion, aiming to reduce the recalcitrant biomass structure by diminishing LCB crystallinity, solubilizing hemicellulose, increasing the porosity and surface area, and breaking the lignin bond. Several pretreatment approaches have been developed, including physical, chemical, physicochemical, and biological methods (Figure 2). Works on different LCBs have shown that the success of pretreatment is feedstock-dependent, which means, that the results of a given method can diverge according to the evaluated biomass (Martín et al., 2022; Saini et al., 2022).

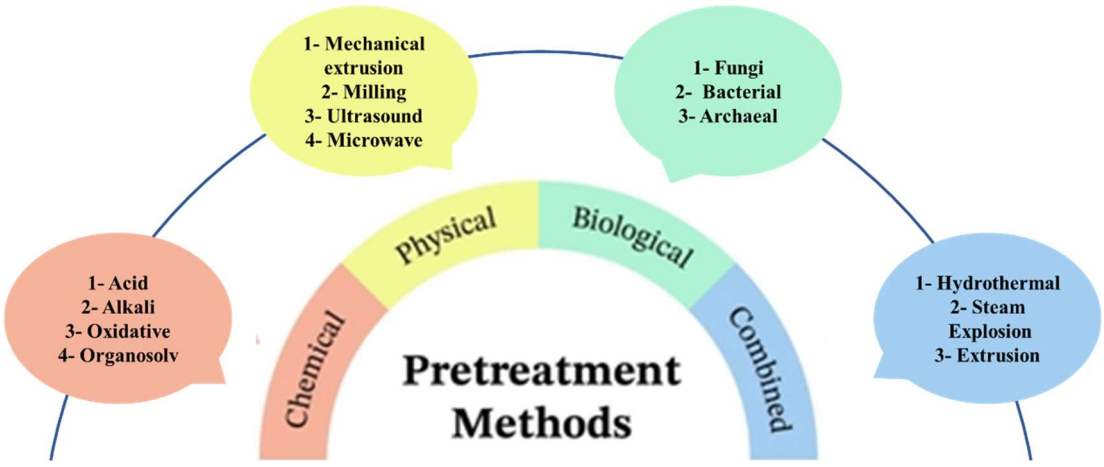


Figure 2: Pretreatment methods. Physical generally involving mechanical forces to break down the biomass structure and increase the surface area. Biological uses microorganisms to secrete enzymes that directly modify the structure before it undergoes further processing. Chemical comprises using its agents to alter the surface structure, mainly by the solubilization of hemicellulose and/or lignin.

Among the different approaches, hydrothermal pretreatment has been reported as having the uppermost potential to be scaleup and facilitate cellulose digestibility, and pentose recovery, with the advantage of producing hydrolysates with fewer inhibitors for fermentation. It is a thermochemical process in which moist feedstocks go for a specific time under elevated temperatures, either alone or with chemical additives. It typically is conducted in acidic

conditions, either due to the release of organic acids from the biomass or by adding acids. The acids' action mainly solubilizes the hemicellulose, while most cellulose and lignin components remain in the solid biomass after pretreatment. Hemicellulose removal reduces the recalcitrant structure and enhances the lignocellulosic biomass's surface area while improving cellulose's enzymatic digestibility (Martín et al., 2022; Santucci et al., 2015; Shiva et al., 2022).

Use of mechanical refining to increase sugar yield

As mentioned previously, biomass recalcitrance is the primary barrier to generating sugars with reduced costs which can be fermented to sustainable fuels, chemicals, and bioproducts. Mechanical refining has shown the potential to overcome it and increase biomass conversion efficiency substantially when implemented after a mild conventional pretreatment (Arce and Kratky, 2022; Batalha et al., 2015; Corbett et al., 2020, 2018; De Assis et al., 2018; Jones et al., 2017, 2013; Koo et al., 2011; Wang et al., 2018).

The mechanical refining process includes three mechanisms to significantly modify fiber morphology: fibrillation, delamination, and fiber cutting. Fibrillation creates fibrils on the surface by peeling fragments from the fiber, while delamination swells and loosens the fibers internally from repeated compression and decompression. Finally, fiber cutting occurs due to the shear action of the refiner plate, and its magnitude varies depending on the apparatus and refining conditions (Corbett et al., 2020). Defining the most effective refining mechanism for enzymatic saccharification will allow the efficient utilization of this method in lignocellulosic biorefineries.

Although mechanical refining has been employed to augment paper properties, studies have shown that similar physical effects can also improve the lignocellulosic biomass reactivity for enzymatic hydrolysis. It has already been extensively optimized for paper manufacturing with a commercially proven technology, but its optimization for fiber reactivity is still in the early stage. The research in this field aims to elucidate its impact on fiber reactivity characteristics and enhance fibers' digestibility towards enzymatic hydrolysis (or other chemical reactions) while minimizing energy consumption (Nagl et al., 2022; Wang et al., 2018).

Therefore, before considering mechanical refining for biochemical conversion, assessing its relevance in enhancing enzymatic conversion under industrially relevant conditions for specific biomass processes and evaluating the refining reactivity is necessary. As demonstrated by Corbett et al. (2020) the enhancement of refining reactivity relies on several

factors, such as biomass type, pretreatment severity, and enzyme load, among others. (Park et al., 2016) proposed that further research focused on techno-economic analysis must be conducted to comprehend the refining advantages. Notably, the utilization of mechanical refining, with reduced pretreatment severity, may result in comparable carbohydrate conversion rates to a process lacking refining. Thus, the economic benefits could arise from the inhibitor amounts reduction and the use of less expensive reactor metallurgy rather than an increase in carbohydrate conversion.

The high costs of enzymatic hydrolysis at high solids

Cellulase-mediated biorefinery from lignocellulosic biomass has been recognized as the most appropriate strategy to depolymerize the polysaccharides (Da Silva et al., 2020; Sun et al., 2023). Working synergistically and simultaneously as versatile machinery, a battery of enzymes cleaves the bonds of complex biomass polysaccharides into sugar monomers (Nargotra et al., 2023). The cellulose is broken down into glucose mainly by the action of three enzymes: endoglucanases, cellobiohydrolases (CBH I and II), and β-glucosidases, in addition to auxiliary enzymes: lytic polysaccharide monooxygenases (LPMO), carbohydrate-binding modules (CBM) bacterial expansins-like; and fungal swollenin (Figure 3 A). Due to the complexity, varied, and highly branched of the hemicelluloses, its depolymerization is facilitated through the collaborative action of several enzymes, including xyloglucanases, mannanases, arabinofuranosidases, and feruloyl esterases (Figure 3 B).

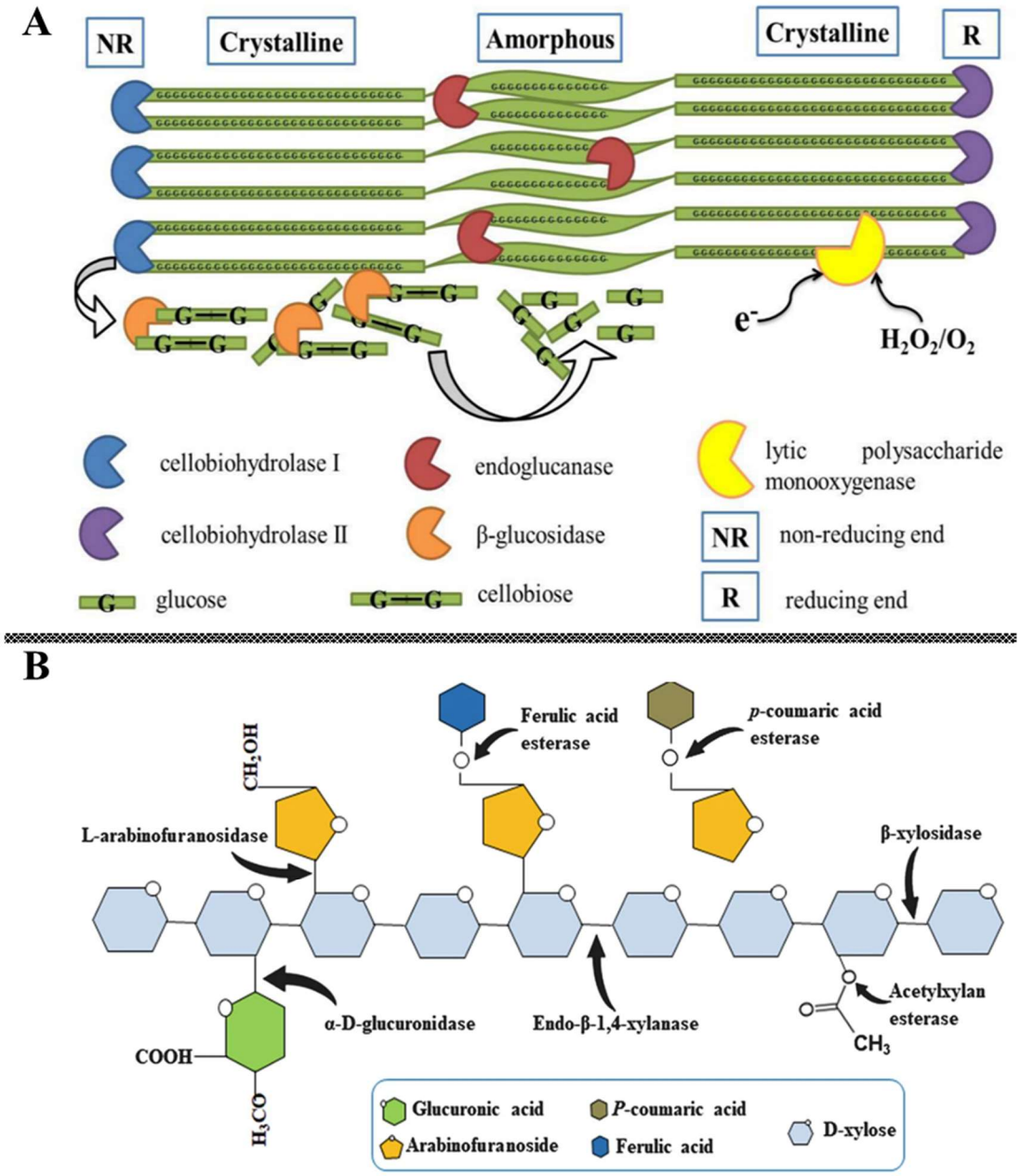


Figure 3: Mode of action of the enzymes involved on lignocellulose degradation. A) Celulose: endoglucanases cleave the glycosidic bonds on in the amorphous region increasing the availability of reducing and non-reducing chain ends. Cellobiohydrolases act on these ends of the chain producing cellodextrins and cellobiose. β-glucosidases convert cellobiose and other low molar mass oligosaccharides into glucose. Lytic polysaccharide monooxygenases are copper-dependent enzymes that oxidatively cleave the crystalline region of polysaccharides. **B) Hemicellulose:** endo-β-1,4-xylanases randomly cleave the main chain of xylan. β-xylosidases release xylose from the reducing end of the backbone. Accessory enzymes, such as α-arabinofuranosidase and α-glucuronidase, remove the substituents arabinose and methyl glucuronic acid groups, respectively. Ferulic acid esterase cleaves the ester linkage between ferulic acid and arabinose residues, while the *p*-coumaric acid esterase cleaves the one between arabinose and p-coumaric acid. The chain is deacetylated by acetylxylan esterase. Figure adapted from Mamo (2020 and Nargotra et al. (2023).

In recent years, some industrial plants have been launched in different countries aiming to produce cellulosic ethanol through enzymatic hydrolysis of biomass. However, most of them have encountered challenges in scaling up the pretreatment stage, and enzymes cost remains a significant factor affecting the final product price. Consequently, the competitiveness of this technology in the biofuels and biochemicals market has been negatively impacted (Da Silva et al., 2020). Working at high-solids enzymatic saccharification (loads >15%) has been proposed to overcome it and attend the distillation viability target of 40 g/L ethanol. It is also desirable for the industrial downstream of other biochemicals (Sun et al., 2023). Therefore, enzymatic hydrolysis with high solids loading presents technical bottlenecks seemingly related to the system's low free water, such as long hydrolysis time, low fermentable sugar yields, slow liquefaction, which compels the use of high enzyme loads to reduce the negative effects (van der Zwan et al., 2017).

(Sun et al., 2023) have proposed that this bioprocess comprises multi-scale mass transfer and multi-phase reactions and that its accomplishment is intrinsically dependent on the interaction of both (Figure 4), and classified the challenges of working in "high-solids" into three aspects: Firstly, the slurry's poor fluidity and high viscosity result in non-homogeneous concentration profiles within hydrolysis reactors. The increased slurry viscosity makes mixing difficult and severely limits the convective transfer of enzymes in the system. Secondly, the slurry typically contains single and agglomerated LCB particles with variable degrees of intergranular and internal pores. This porous structure impacts the interface characteristics of biomass particles and water distribution state, which may adversely affect enzyme diffusion and adsorption transfer onto the substrates. Finally, difficulties in matching mass transfer and enzymatic reaction in a synergistic multi-enzyme system may lead to an unbalanced rate or dynamic process, thereby reducing sugar production.

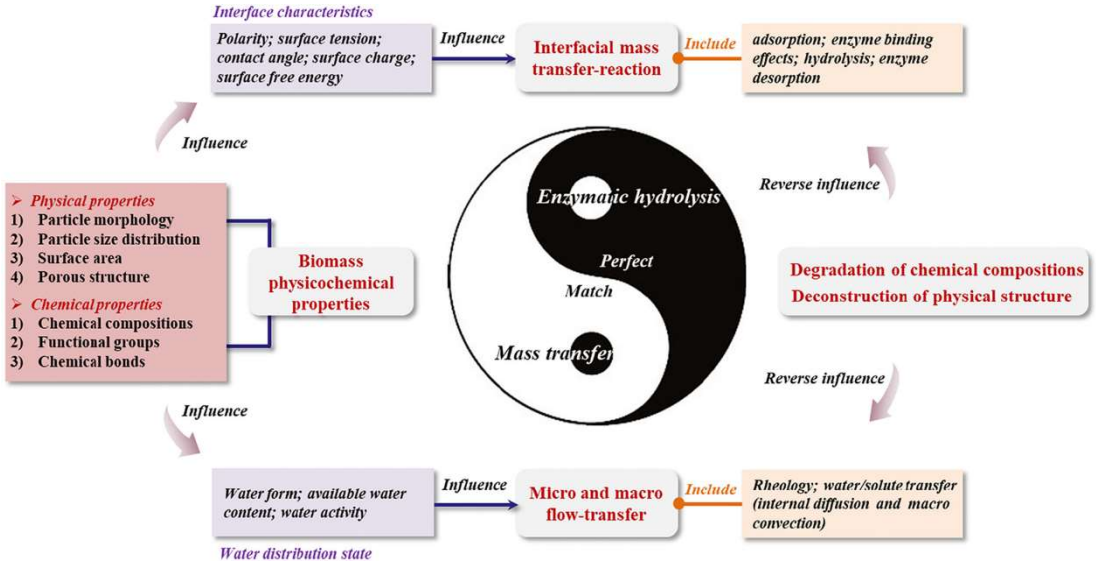


Figure 4: Schematic diagram of the flow interactions between the multi-scale mass transfer and multi-phase reactions during enzymatic hydrolysis. Source: Sun et al. (2023)

Intense research has been conducted to development of strategies to overcome the limitations imposed by the mass transfer of enzymatic reactions at high solids loadings (Du et al., 2020; Espinheira et al., 2022; Mukasekuru et al., 2020a; Shiva et al., 2022). They have shown that they must address several issues instead of being isolated, and could be synthesized to: 1) Development of innovative hydrolysis reactors and pre-treatment methods to reduce the LCB structural barriers, increase convection/diffusion transfer, improve fluidity, and decrease surface water constraints in order to create a more favorable mass transfer environment. 2) Formulation of new enzyme cocktails with efficient, low-cost additives/auxiliary enzymes that simultaneously maximize rapid achievement of industrially relevant sugar titers and particle liquefaction. 3) Bioprocess development to improve flowability and reduce water constraints and hydrolysis inhibitions, such as the fed-batch approach, process integration, detoxification, lignin modification, etc. In summary, the emerging technologies to surpass these challenges must also address the feasibility of industrial-scale applications, evaluating the capital costs and energy consumption aiming at a viable lignocellulosic sugar platform.

Insights into enzyme-mediated lignocellulose liquefaction

A potential solution for handling the high viscosities and yield stresses of concentrated lignocellulose slurries is implementing a liquefaction stage comparable to the approach used in starch-based biorefineries (Nóra Szijártó et al., 2011a). Studies have demonstrated that lignocellulose slurries' viscosity or yield stress decreases through various mechanisms during

enzymatic hydrolysis. These include material dilution, particle fragmentation, and modification of interparticle interactions, rheological regime, substrate concentration, and enzyme-substrate specificity (Roche et al., 2009; Skovgaard et al., 2014; Thygesen et al., 2014; van der Zwan et al., 2020, 2017). While most of the previous works have investigated liquefaction from the perspective of the rheological behavior and particle modifications applying commercial cocktails (Chandrasekar et al., 2021; Chen et al., 2019; Fockink et al., 2017; Lu et al., 2010; Mukasekuru et al., 2020b; Roche et al., 2009), (Nóra Szijártó et al., 2011b) was the first to propose the evaluation looking out for the critical enzyme(s) responsible for the process efficacy, which means a fast viscosity reduction independent of sugar monomer release.

Ever since, several works (Gourlay et al., 2018; Skovgaard et al., 2014; Nára Szijártó et al., 2011) have related the viscosity reduction to the action of endoglucanases that cleaves cellulose into oligosaccharides with a lower degree of polymerization and increasing LCB flowability. Moreover, (Skovgaard et al., 2014) added that xylanase also enabled liquefaction by xylan solubilization and improvement of the accessibility of the substrate for other enzymes. Cellobiohydrolases' role in particle fragmentation has been proposed to work synergically with endoglucanases (Walker et al., 1992). However, recently, some studies have reported that they can fragment cellulose and reduce the viscosity when evaluated individually (Jeoh et al., 2013; van der Zwan et al., 2020).

Moreover, since the classification of the redox enzymes as auxiliary activities enzymes (Corrêa et al., 2019), the LPMOs were introduced in industrial cocktails because of their capacity to work synergically with cellulases and hemicellulases oxidizing the crystalline surface of polysaccharides (Cannella and Jørgensen, 2014; Smuts et al., 2023; Velasco et al., 2021). Their enzymatic conversion enhancement has been attributed to their ability to improve the accessibility of other carbohydrate-active enzymes (CAZymes) to the most recalcitrant parts of the LCB. However, their role in enzyme-mediated liquefaction remains indeterminate.

Expansins-like is another auxiliary activity, which appears to weaken the cell wall without releasing soluble sugars and could play a significative role in LCB degradation and has been called "amorphogenesis inducers" (Arantes and Saddler, 2010). Alongside LPMOs, they enhance the enzymatic hydrolysis by loosening the biomass crystalline regions, facilitating enzyme action into the polysaccharide's glycosidic bonds (Ding et al., 2022; Zhang et al., 2021).

Thus, considering the relevance of enhancing the saccharification efficiency of lignocellulosic biorefinery, this study investigated two alternative approaches to expanding the current strategies to achieve it for sugarcane bagasse as raw material. One was the mechanical

refining associated with mild pretreatment to improve fiber reactivity characteristics and sugar release. The other was the identification of candidates for future genetic modifications of the engineered *Trichoderma reesei* RUT-C30 secretome capable of enhancing the liquefaction and hydrolysis efficiency simultaneously.

References

Adsul, M., Sandhu, S.K., Singhania, R.R., Gupta, R., Puri, S.K., Mathur, A., 2020. Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels. Enzyme Microb Technol. https://doi.org/10.1016/j.enzmictec.2019.109442

Andrade, L.P., Crespim, E., de Oliveira, N., de Campos, R.C., Teodoro, J.C., Galvão, C.M.A., Maciel Filho, R., 2017. Influence of sugarcane bagasse variability on sugar recovery for cellulosic ethanol production. Bioresour Technol 241, 75–81. https://doi.org/10.1016/j.biortech.2017.05.081

Arantes, V., Saddler, J.N., 2010. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol Biofuels 3, 4. https://doi.org/10.1186/1754-6834-3-4

Arce, C., Kratky, L., 2022. Mechanical pretreatment of lignocellulosic biomass toward enzymatic/fermentative valorization. iScience 25. https://doi.org/https://doi.org/10.1016/j.isci.2022.104610

Baral, P., Jain, L., Kurmi, A.K., Kumar, V., Agrawal, D., 2020. Augmented hydrolysis of acid pretreated sugarcane bagasse by PEG 6000 addition: a case study of Cellic CTec2 with recycling and reuse. Bioprocess Biosyst Eng 43, 473–482. https://doi.org/10.1007/s00449-019-02241-3

Batalha, L.A.R., Han, Q., Jameel, H., Chang, H. min, Colodette, J.L., Borges Gomes, F.J., 2015. Production of fermentable sugars from sugarcane bagasse by enzymatic hydrolysis after autohydrolysis and mechanical refining. Bioresour Technol 180, 97–105. https://doi.org/10.1016/j.biortech.2014.12.060

Bischof, R.H., Ramoni, J., Seiboth, B., 2016. Cellulases and beyond: The first 70 years of the enzyme producer Trichoderma reesei. Microb Cell Fact. https://doi.org/10.1186/s12934-016-0507-6

Cannella, D., Jørgensen, H., 2014. Do New Cellulolytic Enzyme Preparations Affect the Industrial Strategies for High Solids Lignocellulosic Ethanol Production? Biotechnol. Bioeng 111, 59–68. https://doi.org/10.1002/bit.25098/abstract

Chandrasekar, M., Joshi, L., Krieg, K., Chipkar, S., Burke, E., Debrauske, D.J., Thelen, K.D., Sato, T.K., Ong, R.G., 2021. A high solids field-to-fuel research pipeline to identify interactions between feedstocks and biofuel production. Biotechnol Biofuels 14. https://doi.org/10.1186/s13068-021-02033-6

Chen, H.Z., Liu, Z.H., 2017. Enzymatic hydrolysis of lignocellulosic biomass from low to high solids loading. Eng Life Sci. https://doi.org/10.1002/elsc.201600102

Chen, X., Crawford, N., Wang, W., Kuhn, E., Sievers, D., Tao, L., Tucker, M., 2019. Kinetics and Rheological Behavior of Higher Solid (Solids >20%) Enzymatic Hydrolysis Reactions Using Dilute Acid Pretreated, Deacetylation and Disk Refined, and Deacetylation and Mechanical Refined (DMR) Corn Stover Slurries. ACS Sustain Chem Eng 7, 1633–1641. https://doi.org/10.1021/acssuschemeng.8b05391

Chen, X., Shekiro, J., Pschorn, T., Sabourin, M., Tao, L., Elander, R., Park, S., Jennings, E., Nelson, R., Trass, O., Flanegan, K., Wang, W., Himmel, M.E., Johnson, D., Tucker, M.P., 2014. A highly efficient dilute alkali deacetylation and mechanical (disc) refining process for the conversion of renewable biomass to lower cost sugars. Biotechnol Biofuels 7. https://doi.org/10.1186/1754-6834-7-98

Chylenski, P., Forsberg, Z., Ståhlberg, J., Várnai, A., Lersch, M., Bengtsson, O., Sæbø, S., Horn, S.J., Eijsink, V.G.H., 2017. Development of minimal enzyme cocktails for hydrolysis of sulfite-pulped lignocellulosic biomass. J Biotechnol 246, 16–23. https://doi.org/10.1016/j.jbiotec.2017.02.009

Corbett, D.B., Knoll, C., Venditti, R., Jameel, H., Park, S., 2020. Fiber fractionation to understand the effect of mechanical refining on fiber structure and resulting enzymatic digestibility of biomass. Biotechnol Bioeng 117, 924–932. https://doi.org/10.1002/bit.27258

Corbett, D.B., Venditti, R., Jameel, H., Park, S., 2018. Effect of Mechanical Refining Energy on the Enzymatic Digestibility of Lignocellulosic Biomass. Ind Eng Chem Res 57, 14648–14655. https://doi.org/10.1021/acs.iecr.8b02932

Corrêa, T.L.R., Júnior, A.T., Wolf, L.D., Buckeridge, M.S., Dos Santos, L.V., Murakami, M.T., 2019. An actinobacteria lytic polysaccharide monooxygenase acts on both cellulose and xylan to boost biomass saccharification. Biotechnol Biofuels 12. https://doi.org/10.1186/s13068-019-1449-0

Da Silva, A.S.A., Espinheira, R.P., Teixeira, R.S.S., De Souza, M.F., Ferreira-Leitão, V., Bon, E.P.S., 2020. Constraints and advances in high-solids enzymatic hydrolysis of lignocellulosic biomass: A critical review. Biotechnol Biofuels. https://doi.org/10.1186/s13068-020-01697-w

De Assis, T., Huang, S., Driemeier, C.E., Donohoe, B.S., Kim, C., Kim, S.H., Gonzalez, R., Jameel, H., Park, S., 2018. Toward an understanding of the increase in enzymatic hydrolysis by mechanical refining. Biotechnol Biofuels 11, 1–11. https://doi.org/10.1186/s13068-018-1289-3

de Lima, E.A., Mandelli, F., Kolling, D., Matsusato Souza, J., de Oliveira Filho, C.A., Ribeiro da Silva, M., Lobo de Mesquita Sampaio, I., Lopes Junqueira, T., Ferreira Chagas, M., Teodoro, J.C., de Morais, E.R., Murakami, M.T., 2022. Development of an economically competitive Trichoderma-based platform for enzyme production: Bioprocess optimization, pilot plant scale-up, techno-economic analysis and life cycle assessment. Bioresour Technol 364. https://doi.org/10.1016/j.biortech.2022.128019

Ding, S., Liu, X., Hakulinen, N., Taherzadeh, M.J., Wang, Yaru, Wang, Yuan, Qin, X., Wang, X., Yao, B., Luo, H., Tu, T., 2022. Boosting enzymatic degradation of cellulose using a fungal expansin: Structural insight into the pretreatment mechanism. Bioresour Technol 358. https://doi.org/10.1016/j.biortech.2022.127434

Du, J., Liang, J., Gao, X., Liu, G., Qu, Y., 2020. Optimization of an artificial cellulase cocktail for high-solids enzymatic hydrolysis of cellulosic materials with different pretreatment methods. Bioresour Technol 295. https://doi.org/10.1016/j.biortech.2019.122272

Du, J., Zhang, F., Li, Y., Zhang, H., Liang, J., Zheng, H., Huang, H., 2014. Enzymatic liquefaction and saccharification of pretreated corn stover at high-solids concentrations in a horizontal rotating bioreactor. Bioprocess Biosyst Eng 37, 173–181. https://doi.org/10.1007/s00449-013-0983-6

Du, L., Ma, L., Ma, Q., Guo, G., Han, X., Xiao, D., 2018. Hydrolytic boosting of lignocellulosic biomass by a fungal lytic polysaccharide monooxygenase, AnLPMO15g from Aspergillus niger. Ind Crops Prod 126, 309–315. https://doi.org/10.1016/j.indcrop.2018.10.029

Espinheira, R.P., Rocha, V.A.L., Guimarães, T.M., Oliveira, C.A., de Souza, M.F., Domont, G.B., Nogueira, F.C.S., Teixeira, R.S.S., Bon, E.P. da S., Silva, A.S.A. da, 2022. *Aspergillus awamori* endoglucanase-rich supernatant enhances lignocellulosic biomass liquefaction in high-solids enzymatic hydrolysis. Biochem Eng J 183. https://doi.org/10.1016/j.bej.2022.108448

Espirito Santo, M.C., Fockink, D.H., Pellegrini, V.O.A., Guimaraes, F.E.G., deAzevedo, E.R., Ramos, L.P., Polikarpov, I., 2020. Physical techniques shed light on the differences in sugarcane bagasse structure subjected to steam explosion pretreatments at equivalent combined severity factors. Ind Crops Prod 158. https://doi.org/10.1016/j.indcrop.2020.113003

Fengel, D., and Wegener, G. (1989). Wood – Chemistry, Ultrastructure, Reactions. Berlin, Germany: De Gruyter.

Fockink, D.H., Urio, M.B., Sánchez, J.H., Ramos, L.P., 2017. Enzymatic Hydrolysis of Steam-Treated Sugarcane Bagasse: Effect of Enzyme Loading and Substrate Total Solids on Its Fractal Kinetic Modeling and Rheological Properties. Energy and Fuels 31, 6211–6220. https://doi.org/10.1021/acs.energyfuels.7b00818

Fonseca, L.M., Parreiras, L.S., Murakami, M.T., 2020. Rational engineering of the Trichoderma reesei RUT-C30 strain into an industrially relevant platform for cellulase production. Biotechnol Biofuels 13. https://doi.org/10.1186/s13068-020-01732-w

García, J.M., Molina, C., Simister, R., Taibo, C.B., Setten, L., Erazzú, L.E., Gómez, L.D., Acevedo, A., 2023. Chemical and histological characterization of internodes of sugarcane and energy-cane hybrids throughout plant development. Ind Crops Prod 199. https://doi.org/10.1016/j.indcrop.2023.116739

Gharehkhani, S., Sadeghinezhad, E., Kazi, S.N., Yarmand, H., Badarudin, A., Safaei, M.R., Zubir, M.N.M., 2015. Basic effects of pulp refining on fiber properties - A review. Carbohydr Polym. https://doi.org/10.1016/j.carbpol.2014.08.047

Ghose, T.K., 1987. Measurement of cellulase activities. Pure and Applied Chemistry 59, 695–702.

Gomez, L.D., Whitehead, C., Barakate, A., Halpin, C., McQueen-Mason, S.J., 2010. Automated saccharification assay for determination of digestibility in plant materials. Biotechnol Biofuels 3, 1–12. https://doi.org/10.1186/1754-6834-3-23

Gourlay, K., Hu, J., Arantes, V., Andberg, M., Saloheimo, M., Penttilä, M., Saddler, J., 2013. Swollenin aids in the amorphogenesis step during the enzymatic hydrolysis of pretreated biomass. Bioresour Technol 142, 498–503. https://doi.org/10.1016/j.biortech.2013.05.053

Gourlay, K., van der Zwan, T., Shourav, M., Saddler, J., 2018. The potential of endoglucanases to rapidly and specifically enhance the rheological properties of micro/nanofibrillated cellulose. Cellulose 25, 977–986. https://doi.org/10.1007/s10570-017-1637-7

Gupta, V.K., Kubicek, C.P., Berrin, J.G., Wilson, D.W., Couturier, M., Berlin, A., Filho, E.X.F., Ezeji, T., 2016. Fungal Enzymes for Bio-Products from Sustainable and Waste Biomass. Trends Biochem Sci 41, 633–645. https://doi.org/10.1016/j.tibs.2016.04.006

Hernández-Beltrán, J.U., Hernández-De Lira, I.O., Cruz-Santos, M.M., Saucedo-Luevanos, A., Hernández-Terán, F., Balagurusamy, N., 2019. Insight into pretreatment methods of lignocellulosic biomass to increase biogas yield: Current state, challenges, and opportunities. Applied Sciences (Switzerland). https://doi.org/10.3390/app9183721

Himmel, M.E., Abbas, C.A., Baker, J.O., Bayer, E.A., Bomble, Y.J., Brunecky, R., Chen, X., Felby, C., Jeoh, T., Kumar, R., McCleary, B. V., Pletschke, B.I., Tucker, M.P., Wyman, C.E., Decker, S.R., 2017. Undefined cellulase formulations hinder scientific reproducibility. Biotechnol Biofuels 10. https://doi.org/10.1186/s13068-017-0974-y

IPCC Working Group III, 2023. Climate Change 2023: Sixth Assessment Report (AR6), Diriba Korecha Dadi. Panmao Zhai, Interlaken.

Jeoh, T., Santa-Maria, M.C., O'Dell, P.J., 2013. Assessing cellulose microfibrillar structure changes due to cellulose action. Carbohydr Polym 97, 581–586. https://doi.org/10.1016/j.carbpol.2013.05.027

Jones, B.W., Venditti, R., Park, S., Jameel, H., 2017. Optimization of pilot scale mechanical disk refining for improvements in enzymatic digestibility of pretreated hardwood lignocellulosics. Bioresources 12, 4567–4593. https://doi.org/10.15376/biores.12.3.4567-4593

Jones, B.W., Venditti, R., Park, S., Jameel, H., Koo, B., 2013. Enhancement in enzymatic hydrolysis by mechanical refining for pretreated hardwood lignocellulosics. Bioresour Technol 147, 353–360. https://doi.org/10.1016/j.biortech.2013.08.030

Jørgensen, H., Vibe-Pedersen, J., Larsen, J., Felby, C., 2007. Liquefaction of Lignocellulose at High-Solids Concentrations. Biotechnol Bioeng 96, 862–870.

Ju, X., Bowden, M., Engelhard, M., Zhang, X., 2014. Investigating commercial cellulase performances toward specific biomass recalcitrance factors using reference substrates. Appl Microbiol Biotechnol 98, 4409–4420. https://doi.org/10.1007/s00253-013-5450-4

Junior, A.T., Dolce, L.G., de Oliveira Neto, M., Polikarpov, I., 2015. Xanthomonas campestris expansin-like X domain is a structurally disordered beta-sheet macromolecule capable of synergistically enhancing enzymatic efficiency of cellulose hydrolysis. Biotechnol Lett 37, 2419–2426. https://doi.org/10.1007/s10529-015-1927-9

Khan, M.U., Usman, M., Ashraf, M.A., Dutta, N., Luo, G., Zhang, S., 2022. A review of recent advancements in pretreatment techniques of lignocellulosic materials for biogas

production: Opportunities and Limitations. Chemical Engineering Journal Advances. https://doi.org/10.1016/j.ceja.2022.100263

Koo, B., Park, J., Gonzalez, R., Jameel, H., Park, S., 2019. Two-stage autohydrolysis and mechanical treatment to maximize sugar recovery from sweet sorghum bagasse. Bioresour Technol 276, 140–145. https://doi.org/10.1016/j.biortech.2018.12.112

Koo, B.W., Treasure, T.H., Jameel, H., Phillips, R.B., Chang, H.M., Park, S., 2011. Reduction of enzyme dosage by oxygen delignification and mechanical refining for enzymatic hydrolysis of green liquor-pretreated hardwood. Appl Biochem Biotechnol 165, 832–844. https://doi.org/10.1007/s12010-011-9301-4

Kristensen, J.B., Felby, C., Jørgensen, H., 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol Biofuels 2. https://doi.org/10.1186/1754-6834-2-11

Leal Silva, J.F., Mariano, A.P., Maciel Filho, R., 2021. Less severe reaction conditions to produce levulinic acid with reduced humins formation at the expense of lower biomass conversion: Is it economically feasible? Fuel Communications 9, 100029. https://doi.org/10.1016/j.jfueco.2021.100029

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275. https://doi.org/https://doi.org/10.1016/S0021-9258(19)52451-6

Lu, Y., Wang, Y., Xu, G., Chu, J., Zhuang, Y., Zhang, S., 2010. Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass. Appl Biochem Biotechnol 160, 360–369. https://doi.org/10.1007/s12010-008-8306-0

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E., 2008. How biotech can transform biofuels. Nat Biotechnol 26, 169–72. https://doi.org/10.1038/nbt0208-169

Mamo, G., 2020. Alkaline Active Hemicellulases, in: Advances in Biochemical Engineering/Biotechnology. Springer, pp. 245–291. https://doi.org/10.1007/10_2019_101

Martín, C., Dixit, P., Momayez, F., Jönsson, L.J., 2022. Hydrothermal Pretreatment of Lignocellulosic Feedstocks to Facilitate Biochemical Conversion. Front Bioeng Biotechnol. https://doi.org/10.3389/fbioe.2022.846592

Modenbach, A. a., Nokes, S.E., 2013. Enzymatic hydrolysis of biomass at high-solids loadings – A review. Biomass Bioenergy 56, 526–544. https://doi.org/10.1016/j.biombioe.2013.05.031

Monclaro, A.V., Gorgulho Silva, C. de O., Gomes, H.A.R., Moreira, L.R. de S., Filho, E.X.F., 2022. The enzyme interactome concept in filamentous fungi linked to biomass valorization. Bioresour Technol 344. https://doi.org/10.1016/j.biortech.2021.126200

Mukasekuru, M.R., Kaneza, P., Sun, H., Sun, F.F., He, J., Zheng, P., 2020a. Fed-batch high-solids enzymatic saccharification of lignocellulosic substrates with a combination of additives and accessory enzymes. Ind Crops Prod 146. https://doi.org/10.1016/j.indcrop.2020.112156

Muraleedharan, M.N., Zouraris, D., Karantonis, A., Topakas, E., Sandgren, M., Rova, U., Christakopoulos, P., Karnaouri, A., 2018. Effect of lignin fractions isolated from different biomass sources on cellulose oxidation by fungal lytic polysaccharide monooxygenases. Biotechnol Biofuels 11. https://doi.org/10.1186/s13068-018-1294-6

Nagl, M., Haske-Cornelius, O., Bauer, W., Csarman, F., Ludwig, R., Nyanhongo, G.S., Guebitz, G.M., 2022. Towards a better understanding of synergistic enzyme effects during refining of cellulose fibers. Carbohydrate Polymer Technologies and Applications 4. https://doi.org/10.1016/j.carpta.2022.100223

Nargotra, P., Sharma, V., Lee, Y.C., Tsai, Y.H., Liu, Y.C., Shieh, C.J., Tsai, M.L., Dong, C. Di, Kuo, C.H., 2023. Microbial Lignocellulolytic Enzymes for the Effective Valorization of Lignocellulosic Biomass: A Review. Catalysts. https://doi.org/10.3390/catal13010083

Novozymes, 2010. Fuel ethanol application sheet Cellic® CTec2 and HTec2-Enzymes for hydrolysis of lignocellulosic materials.

Olejnik, K., Fabijanska, A., Pelczynski, P., Stanislawska, A., 2019. Controllability of low-consistency chemical pulp refining process. Comput Chem Eng 128, 468–476. https://doi.org/10.1016/j.compchemeng.2019.06.024

Park, J., Jones, B., Koo, B., Chen, X., Tucker, M., Yu, J.H., Pschorn, T., Venditti, R., Park, S., 2016. Use of mechanical refining to improve the production of low-cost sugars from lignocellulosic biomass. Bioresour Technol 199, 59–67. https://doi.org/10.1016/j.biortech.2015.08.059

Ramos, L.P., da Silva, L., Ballem, A.C., Pitarelo, A.P., Chiarello, L.M., Silveira, M.H.L., 2015. Enzymatic hydrolysis of steam-exploded sugarcane bagasse using high total solids and low enzyme loadings. Bioresour Technol 175, 195–202.

Rocha, G.J. de M., Nascimento, V.M., Gonçalves, A.R., Silva, V.F.N., Martín, C., 2015a. Influence of mixed sugarcane bagasse samples evaluated by elemental and physical-chemical composition. Ind Crops Prod 64, 52–58. https://doi.org/10.1016/j.indcrop.2014.11.003

Rocha, G.J.M., Gonçalves, A.R., Nakanishi, S.C., Nascimento, V.M., Silva, V.F.N., 2015b. Pilot scale steam explosion and diluted sulfuric acid pretreatments: Comparative study aiming the sugarcane bagasse saccharification. Ind Crops Prod 74, 810–816.

Rocha, G.J.M., Martín, C., da Silva, V.F.N., Gómez, E.O., Gonçalves, A.R., 2012. Mass balance of pilot-scale pretreatment of sugarcane bagasse by steam explosion followed by alkaline delignification. Bioresour Technol 111, 447–52. https://doi.org/10.1016/j.biortech.2012.02.005

Roche, C.M., Dibble, C.J., Stickel, J.J., 2009. Laboratory-scale method for enzymatic saccharification of lignocellulosic biomass at high-solids loadings. Biotechnol Biofuels 2, 1–11. https://doi.org/10.1186/1754-6834-2-28

Rodrigues, M.I., Iemma, A.F., 2014. Experimental design and process optimization, Experimental Design and Process Optimization. https://doi.org/10.1201/b17848

Ruiz, H.A., Conrad, M., Sun, S.N., Sanchez, A., Rocha, G.J.M., Romaní, A., Castro, E., Torres, A., Rodríguez-Jasso, R.M., Andrade, L.P., Smirnova, I., Sun, R.C., Meyer, A.S., 2020. Engineering aspects of hydrothermal pretreatment: From batch to continuous operation, scale-up and pilot reactor under biorefinery concept. Bioresour Technol. https://doi.org/10.1016/j.biortech.2019.122685

Ruiz, H.A., Sganzerla, W.G., Larnaudie, V., Veersma, R.J., van Erven, G., Shiva, Ríos-González, L.J., Rodríguez-Jasso, R.M., Rosero-Chasoy, G., Ferrari, M.D., Kabel, M.A., Forster-Carneiro, T., Lareo, C., 2023. Advances in process design, techno-economic assessment and environmental aspects for hydrothermal pretreatment in the fractionation of biomass under biorefinery concept. Bioresour Technol. https://doi.org/10.1016/j.biortech.2022.128469

Saini, J.K., Himanshu, Hemansi, Kaur, A., Mathur, A., 2022. Strategies to enhance enzymatic hydrolysis of lignocellulosic biomass for biorefinery applications: A review. Bioresour Technol 360. https://doi.org/10.1016/j.biortech.2022.127517

Sant'Ana da Silva, A., Fernandes de Souza, M., Ballesteros, I., Manzanares, P., Ballesteros, M., P. S. Bon, E., 2016. High-solids content enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse using a laboratory-made enzyme blend and commercial preparations. Process Biochemistry 51, 1561–1567.

Santucci, B.S., Maziero, P., Rabelo, S.C., Curvelo, A.A.S., Pimenta, M.T.B., 2015. Autohydrolysis of Hemicelluloses from Sugarcane Bagasse During Hydrothermal Pretreatment: a Kinetic Assessment. Bioenergy Res 8, 1778–1787. https://doi.org/10.1007/s12155-015-9632-z

Shi, Y., Chen, K., Long, L., Ding, S., 2021. A highly xyloglucan active lytic polysaccharide monooxygenase EpLPMO9A from Eupenicillium parvum 4-14 shows boosting effect on hydrolysis of complex lignocellulosic substrates. Int J Biol Macromol 167, 202–213. https://doi.org/10.1016/j.ijbiomac.2020.11.177

Shiva, Climent Barba, F., Rodríguez-Jasso, R.M., Sukumaran, R.K., Ruiz, H.A., 2022. High-solids loading processing for an integrated lignocellulosic biorefinery: Effects of transport phenomena and rheology – A review. Bioresour Technol. https://doi.org/10.1016/j.biortech.2022.127044

Skovgaard, P.A., Thygesen, L.G., Jørgensen, H., Cardona, M., Tozzi, E., Mccarthy, M., Siika-Aho, M., Jeoh, T., 2014. The role of endoglucanase and endoxylanase in liquefaction of hydrothermally pretreated wheat straw. Biotechnol Prog 30, 923–931. https://doi.org/10.1002/btpr.1893

Smuts, I.E., Blakeway, N.J., Rose, S.H., den Haan, R., Viljoen-Bloom, M., van Zyl, W.H., 2023. Supplementation of recombinant cellulases with LPMOs and CDHs improves consolidated bioprocessing of cellulose. Enzyme Microb Technol 164. https://doi.org/10.1016/j.enzmictec.2022.110171

Sun, C., Meng, X., Sun, F., Zhang, J., Tu, M., Chang, J.S., Reungsang, A., Xia, A., Ragauskas, A.J., 2023. Advances and perspectives on mass transfer and enzymatic hydrolysis in the enzyme-mediated lignocellulosic biorefinery: A review. Biotechnol Adv. https://doi.org/10.1016/j.biotechadv.2022.108059

Szijártó, Nára, Horan, E., Zhang, J., Puranen, T., Siika-Aho, M., Viikari, L., 2011a. Thermostable endoglucanases in the liquefaction of hydrothermally pretreated wheat straw. Biotechnol Biofuels 4. https://doi.org/10.1186/1754-6834-4-2

Szijártó, Nóra, Siika-aho, M., Sontag-Strohm, T., Viikari, L., 2011b. Liquefaction of hydrothermally pretreated wheat straw at high-solids content by purified Trichoderma enzymes. Bioresour Technol 102, 1968–1974. https://doi.org/10.1016/j.biortech.2010.09.012

Thygesen, L.G., Thybring, E.E., Johansen, K.S., Felby, C., 2014. The mechanisms of plant cell wall deconstruction during enzymatic hydrolysis. PLoS One 9. https://doi.org/10.1371/journal.pone.0108313

van der Zwan, T., Hu, J., Saddler, J.N., 2017. Mechanistic insights into the liquefaction stage of enzyme-mediated biomass deconstruction. Biotechnol Bioeng 114, 2489–2496. https://doi.org/10.1002/bit.26381

van der Zwan, T., Sigg, A., Hu, J., Chandra, R.P., Saddler, J.N., 2020. Enzyme-Mediated Lignocellulose Liquefaction Is Highly Substrate-Specific and Influenced by the Substrate Concentration or Rheological Regime. Front Bioeng Biotechnol 8. https://doi.org/10.3389/fbioe.2020.00917

Velasco, J., de Oliveira Arnoldi Pellegrini, V., Sepulchro, A.G.V., Kadowaki, M.A.S., Santo, M.C.E., Polikarpov, I., Segato, F., 2021. Comparative analysis of two recombinant LPMOs from *Aspergillus fumigatus* and their effects on sugarcane bagasse saccharification. Enzyme Microb Technol 144. https://doi.org/10.1016/j.enzmictec.2021.109746

Vermaas, J. v., Petridis, L., Qi, X., Schulz, R., Lindner, B., Smith, J.C., 2015. Mechanism of lignin inhibition of enzymatic biomass deconstruction. Biotechnol Biofuels 8, 1–16. https://doi.org/10.1186/s13068-015-0379-8

Walker, L.P., Wilson, D.B., Irvin, D.C., McQuire, C., Price, M., 1992. Fragmentation of cellulose by the major Thermomonospora fusca cellulases, Trichoderma reesei CBHI, and their mixtures. Biotechnol Bioeng 40, 1019–1026. https://doi.org/10.1002/bit.260400905

Wang, Z., Dien, B.S., Rausch, K.D., Tumbleson, M.E., Singh, V., 2018. Fermentation of undetoxified sugarcane bagasse hydrolyzates using a two stage hydrothermal and mechanical refining pretreatment. Bioresour Technol 261, 313–321. https://doi.org/10.1016/j.biortech.2018.04.018

Weiss, N.D., Felby, C., Thygesen, L.G., 2019. Enzymatic hydrolysis is limited by biomass-water interactions at high-solids: Improved performance through substrate modifications. Biotechnol Biofuels 12. https://doi.org/10.1186/s13068-018-1339-x

Westereng, B., Cannella, D., Wittrup Agger, J., Jørgensen, H., Larsen Andersen, M., Eijsink, V.G.H., Felby, C., 2015. Enzymatic cellulose oxidation is linked to lignin by long-range electron transfer. Sci Rep 5. https://doi.org/10.1038/srep18561

Yang, B., Wyman, C.E., 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol Bioeng 94, 611–617. https://doi.org/10.1002/bit.20750

Yuan, H., Song, X., Guan, R., Zhang, L., Li, X., Zuo, X., 2019. Effect of low severity hydrothermal pretreatment on anaerobic digestion performance of corn stover. Bioresour Technol 294. https://doi.org/10.1016/j.biortech.2019.122238

Zhang, J., Tuomainen, P., Siika-aho, M., Viikari, L., 2011. Comparison of the synergistic action of two thermostable xylanases from GH families 10 and 11 with thermostable cellulases

in lignocellulose hydrolysis. Bioresour Technol 102, 9090–9095. https://doi.org/10.1016/j.biortech.2011.06.085

Zhang, P., Su, R., Duan, Y., Cui, M., Huang, R., Qi, W., He, Z., Thielemans, W., 2021. Synergy between endo/exo-glucanases and expansin enhances enzyme adsorption and cellulose conversion. Carbohydr Polym 253. https://doi.org/10.1016/j.carbpol.2020.117287

Zheng, Y., Zhao, X., Lin, W., Yong, Q., Huang, C., 2023. Revealing the performance of phenoxyethanol-acid pretreatment on the intercellular structure of bamboo and its enzymatic hydrolysis. Ind Crops Prod 199. https://doi.org/10.1016/j.indcrop.2023.116663

Zou, X., Liang, L., Shen, K., Huang, C., Wu, T., Wu, Y., Fang, G., 2021. Alkali synergetic two-step mechanical refining pretreatment of pondcypress for the fiber with intact 3D structure and ultrahigh cellulose accessibility fabrication. Ind Crops Prod 170, 113741. https://doi.org/10.1016/j.indcrop.2021.113741

Chapter 2

Type of chapter: Research Article
Current status: In preparation

A pilot scale evaluation of high-consistency mechanical refining to improve enzymatic saccharification of low severity pretreated sugarcane bagasse

Andrade, L. P.[a,b], Rodrigues, M. I.[c], Rocha, G. J. M.[a,*] and Murakami, M. T.[a,*]

[a]Brazilian Biorenewables National Laboratory (LNBR), Brazilian Center for Research in Energy and Materials, Campinas, SP, Brazil
[b]Postgraduate Program in Functional and Molecular Biology (BFM), Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil
[c]Protimiza Consulting and Training in Experimental Design and Process Optimization, Campinas, SP, Brazil

*Correspondence to mario.murakami@lnbr.cnpem.br and george.rocha@lnbr.cnpem.br

Abstract

The pulp and paper industry has extensively used mechanical refining to improve cellulose fiber properties. Recently, its use has been investigated to overcome lignocellulose recalcitrance and enhance the enzymatic saccharification yields. The present work investigates the enzymatic hydrolysis of high-consistency mechanical refining associated with pretreated sugarcane bagasse hydrothermally, or steam exploded at 180 and 190°C for 10 min, respectively, both at a pilot scale. The saccharifications were conducted at the laboratory with 10% (w/w) solid content and an enzyme load of 10 $FPU.gPTB^{-1}$. Interestingly, mechanical refining affected the enzymatic digestibility, depending on the pretreatment type. Despite the positive impact of mechanical refining on the saccharification of both pretreated materials, higher effectiveness was observed in the steam-exploded bagasse, with an increase of 8%. Scanning electron microscopy reveals that the improvement observed in the enzymatic saccharification of steam-exploded bagasse is promoted by the exposure of cell wall fibrils and internal delamination, which reflects higher porosity and swellability. These results evidence an opportunity for applying mechanical refining associated with steam explosion pretreatment for the utilization of sugarcane bagasse, advancing the current knowledge about the application and challenges of high-consistency mechanical refining in lignocellulosic biorefineries.

Keywords: Sugarcane bagasse, mechanical refining, pilot scale, enzymatic saccharification, fiber morphology.

Introduction

The natural recalcitrance of lignocellulosic biomass is a major challenge for biorefineries (Corbett et al., 2020), and the combination of thermochemical processes followed by mechanical refining has been proposed as a promising route to enhance biomass enzymatic saccharification (Chen et al., 2014; Koo et al., 2019; Zou et al., 2021).

Mechanical refining is an established technology in the pulp and paper industry and is widely used to improve fiber properties (Gharehkhani et al., 2015; Olejnik et al., 2019). The main refining mechanisms are (i) external fibrillation - creating fibrils on the fiber surface; (ii) internal delamination - swelling of fibers and loosening of internal structures; and (iii) fiber cutting - fiber shortening due to the shear action can be observed in different lengths, depending on the apparatus and refining conditions (Park et al., 2016). Understanding which of these mechanisms provides the best scenario for enzymatic saccharification and the critical fiber properties to be modified by mechanical refining will enable more efficient use of this approach in lignocellulosic biorefineries (Arce and Kratky, 2022; Corbett et al., 2018; De Assis et al., 2018).

According to Arce and Kratky, (2022) and Park et al., (2016), it is expected that with the implementation of mechanical refining, biomass recalcitrance, and process complexity can be significantly reduced due to the following reasons: (a) increased sugar recovery with reduced enzyme dosages; (b) significant reduction in the pretreatment severity; and (c) ease of integration with any type of thermochemical processes. Moreover, mechanical refining is a commercially proven technology in process streams of approximately 1500 tons per day of biomass. However, the effectiveness of mechanical refining seems to vary mainly according to biomass type, pretreatment severity, enzyme formulation, and dosage, among other factors (Jones et al., 2017). Therefore, understanding the potential of mechanical refining in enhancing enzymatic conversion under industrially relevant conditions for specific biomass conversion routes is strictly necessary to assess its relevance and applicability.

It is particularly promising that mechanical refining might promote efficient biomass saccharification under low severity pretreatment, resulting in less sugar degradation, low amounts of inhibitors during fermentation, and less complex equipment for pretreatment, a true engineering challenge in any lignocellulosic biorefinery. In this context, this work aimed to investigate the effect of high-consistency mechanical refining associated with low severity pretreatments on the enzymatic saccharification of sugarcane bagasse.

Experimental

Pretreatment at pilot scale

Two pretreatment conditions were carried out with sugarcane bagasse (SCB) at the Pilot Process Development Plant (PPDP) from Brazilian Biorenewables National Laboratory (LNBR), hydrothermal or steam explosion. Before the pretreatment, SCB was processed in the Dry-Cleaning System from PPDP, where the air flows against the material, promoting its separation from the non-structural inorganics (stones, pieces of iron and steel, and sand). Hydrothermal pretreatment was performed in a 350 L Hastelloy steel 276 batch reactor from the Pope Scientific® using 20 kg (dry basis) of SCB at a solid:liquid ratio of 1:10. The material was added to the reactor when the water temperature was close to 100 °C, taking 15 min to reach 180 °C, with a plateau time of 15 min. After the pretreatment, the liquid fraction was separated using a 100 L Hastelloy steel 276 Nutsche Filter from Pope Scientific®, and the remaining solid fraction (pretreated bagasse) from the eight batches was homogenized and stored in a cold room. Steam explosion pretreatment was performed at a feed rate of 10 $kg.h^{-1}$ and a residence time of 15 min at 190°C in a stainless-steel continuous reactor from AdvancedBio®, and the resulting pretreated bagasse was homogenized and stored in a cold room. Both pretreated bagasse had their chemical characterization and mass balance of the process determined accordingly to Rocha et al., (2015a), and the severity parameter (R_0) was calculated using the following equation (Wang et al., 2018):

$$R_0 = t * exp\frac{(T-100)}{14.75} \quad (1)$$

Equation 1. Calculation of severity factor. **t** is the pretreatment reaction time (min), and **T** is the reaction temperature (°C).

High-consistency mechanical refining at pilot scale

High-consistency mechanical refining was applied on the pretreated bagasse using a 12-in continuous disc refiner model 12-1C from Andritz®. The disc refiner comprises two vertical disks with a three-zone fine, one rotates while the other remains stationary. The refining effect was evaluated by a Central Composite Rotatable Design – CCRD (2^2 factorial design with 4 trials under the axial conditions and 3 repetitions at the central point) totaling 11 trials

(Table 1) (Rodrigues and Iemma, 2014), and the variables investigated were the gap width (GW) and disc rotation (DR). In the terminology of the tests, RB means ‘‘refined bagasse’’, and the number represents the experiment that was performed as part of the design. The RB9 investigation represents the center point (CP) repeated in four independent assays. For each experimental condition, the GW (from 1 to 2.5 mm) and DR (from 1500 to 3000 RPM) used were adjusted, and 5 kg of pretreated bagasse with a moisturizing average of 65% was fed by a screw at a speed of 20 rpm, and a dilution water flow of 400 mL/min resulted in a high-consistency refined pulp with 15% of solids. The material was collected at the bottom of the refiner. The resulting refined pretreated sugarcane bagasse was homogenized and stored at -10 °C. The chemical composition of the refined samples was assumed to be the same as the pretreated biomass presented in the above section.

Table 1: The central composite rotatable design conditions for investigating the high-consistency mechanical refining.

Experiment	Gap Width (mm)	Disc Rotation (RPM)
RB1	1,22 (-1)	1718 (-1)
RB2	2,28 (+1)	1718 (-1)
RB3	1,22 (-1)	2782 (+1)
RB4	2,28 (+1)	2782 (+1)
RB5	1 (-1.41)	2250 (0)
RB6	2,5 (+1.41)	2250 (0)
RB7	1,75 (0)	1500 (-1.41)
RB8	1,75 (0)	3000 (+1.41)
RB9 (CP)	1,75 (0)	2250 (0)

The mathematical modeling of the resulting CCRD was carried out based on the function cellulose conversion (%) after the enzymatic saccharification. Data were analyzed via Protimiza Experiment Design Software (http://experimental-design.protimiza.com.br). The effect on the response function of all individual process variables and their binary interactions was calculated for each selected reaction time during a saccharification time course of 6, 24, 30, 48, and 72 hrs using the confidence level of 95% (p = 0.05).

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on an INSPECT F50 from a Thermo Fischer Scientific field-emission scanning electron microscope operating at 5 kV. Fiber

samples were vacuum-dried before analysis. Samples were analyzed coated with a thin layer of gold (20 nm) in a sputtering BAL-TEC SCD 050.

Enzymatic saccharification of refined pretreated bagasse

Enzymatic saccharification assays were performed with refined pretreated bagasse and the commercially available blend Cellic® CTec2, and cellulase activities were determined according to Ghose (1987). The tests were performed with 10% of total solids, an enzyme load of 10 FPU.gPTB^{-1}, 50°C, pH 5 (100 mM sodium citrate buffer solution), and 150 rpm. Over 72 hrs, aliquots were taken at a pre-determined time to monitor the sugar release. The samples were subjected to glucose analysis using the D-Glucose Assay Kit. Alternatively, the carbohydrates were measured by high-performance liquid chromatography (HPLC). The cellulose conversion was calculated using Equation 2, and hemicellulose conversion by Equation 3.

$$\eta = \frac{M_{Glucose} * f}{M_{Cellulose}} * 100 \quad (2)$$

Equation 2. Calculation of glucan conversion into monomeric glucose. $\mathbf{M_{Glucose}}$ is the mass of glucose present in the hydrolysate, and $\mathbf{M_{Cellulose}}$ is the initial mass of cellulose present in the pretreated bagasse. *f* is the conversion factor of glucose into cellulose (*f* = 162/180 = 0.9).

$$\eta = \frac{M_{Arabinose+Xylose} * f}{M_{Hemicellulose}} * 100 \quad (3)$$

Equation 3. Calculation of xylan conversion into monomeric xylose and arabinose. $\mathbf{M_{Xylose + Arabinose}}$ is the mass of xylose and arabinose present in the hydrolysate, and $\mathbf{M_{Hemicelulose}}$ is the initial mass of hemicellulose present in the pretreated bagasse. *f* is the conversion factor of xylose and arabinose into hemicellulose (*f* = 132/150 = 0.88).

Analysis of carbohydrate, organic acids, and furan concentrations

HPLC was used to quantify glucose, xylose, arabinose, cellobiose, acetic acid, formic acid, succinic acid, and lactic acid present in the enzymatic hydrolysate and chemical composition samples. The chromatographer was the 1260 Infinity from Agilent, equipped with a refractive index detector and an Aminex HPX 87H column (300 x 7.8 mm) and pre-column (30 x 4.6 mm) at 35ºC with a flow of 0.6 mL.min^{-1} of 5 mM sulfuric acid solution eluted isocratically.

Furfural and hydroxymethylfurfural (HMF) present in the hydrolysates and compositional analysis samples were quantified in the same HPLC equipped with a UV-visible detector at 274 nm, an Acclaim C18 3 µm column (4.6 x 150 mm) at 25°C with a flow of 0.8 $mL.min^{-1}$ and acetonitrile:water solution (1:8 *v/v*) containing 1% acetic acid.

The samples were homogenized and filtered through a Millex syringe filter with a diameter of 13 mm, 0.22 µm (for enzymatic hydrolysates), and 0.45 µm (for chemical composition samples). Sample quantification was performed by external calibration.

Results and Discussion

Chemical composition of raw and pilot-scale pretreated sugarcane bagasse

The major goal in the pretreatment step is to enable the efficient recovery of carbohydrates and lignin concomitant with the low production of enzyme and microbial inhibitors. Through the low severity conditions investigated in this work, we pursue to attend to these demands, maximizing the hemicellulose recovery and minimizing HMF and furfural formation.

.

Table 2: Compositional analysis of raw sugarcane bagasse (SCB), HPTB – hydrothermally pretreated bagasse (180 °C for 15 min), and SEPTB – steam-exploded pretreated bagasse (190 °C for 10 min) based on the insoluble solids content (IS) and soluble solids content (SS).

Sample	Severity	Insoluble Solids (%)	Cellulose (%, IS)	Hemicellulose (%, IS)	Lignin (%, IS)	Ash (%, IS)	Extractive (%, IS)	Soluble Solids (%)	Glucose Mono (%, SS)[a]	Glucose Oligo[b] (%, SS)[a]	HMF (%, SS)[a]	Xylose Mono (%, SS)[a]	Xylose Oligo[b] (%, SS)[a]	Furfural (%, SS)[a]	Acetic Acid (%, SS)[a]
SCB	n.a.	n.a.	42.1 ± 0.3	30.5 ± 0.1	24.4 ± 1.1	1.2 ± 0.1	3.51 ± 0.08	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
HPTB	3.53	63.2±0.8	60.1±0.8	15.1±0.2	24.5± 1.3	1.1±0.1	n.a.	36.8± 0.8	0.1	4.5	0.2	6.2	39.5	0.6	2.4
SEPTB	3.65	65.6± 0.1	59.9±1.0	9.2±0.3	28.3±0.6	2.8 ± 0.1	n.a.	34,4± 0.1	0.2	6.9	0.6	12.5	29.9	1.6	3.7

n.a. = not applied; [a] = these concentrations are from single assays; [b] = oligo concentration was determined by the difference of sugar amount after the liquor laboratory acid hydrolysis from the previous.

The chemical composition of raw sugarcane bagasse, hydrothermally and steam-exploded pretreated bagasse (Table 2) follow the previous work Rocha et al. (2015b), and it is possible to observe significant differences in the contents of the solid fractions when comparing raw and pretreated biomasses. These differences are mainly due to the solubilization of hemicellulose and cellulose, and lignin condensation during the pretreatment reactions. Hemicellulosic polymers were mostly solubilized to C5 oligomers in both pretreatments (hydrothermal and steam explosion), with a minor formation of degradation compounds such as furfural and acetic acid. Whereas for cellulose, as expected, minor solubilization and degradation were observed. Another meaningful change in the lignocellulose structure after the pretreatment is the fragmentation and deposition of lignin on the cellulosic fibers establishing a steric barrier for carbohydrate-active enzymes (CAZymes) that will impact the enzymatic efficiency during the saccharification (Vermaas et al., 2015).

It is known that during the pretreatment process at the pilot plant scale, especially for the steam explosion, some compounds are formed and/or volatilized during the reaction, mainly from hemicellulose fraction. For this reason, as previously reported byAndrade et al. (2017) and Rocha et al. (2012), it is reasoned that the mass balances could not reach 100%. One of the hypotheses is that the sugars react with some of the degrading compounds (HMF, furfural, carboxylic acids, and others) and lignin fragments, leading to humins formation and other compounds which are rarely reported because of its inherent difficulty for analytical detection (Leal Silva et al., 2021).

Despite the inherent challenges for the mass balance of pretreated lignocelluloses, satisfactory data were achieved with above 90% of the cellulose and hemicellulose recovery for the hydrothermally pretreated bagasse (HPTB) and at least 75% for the steam-exploded pretreated bagasse (SEPTB) (Figure 1).

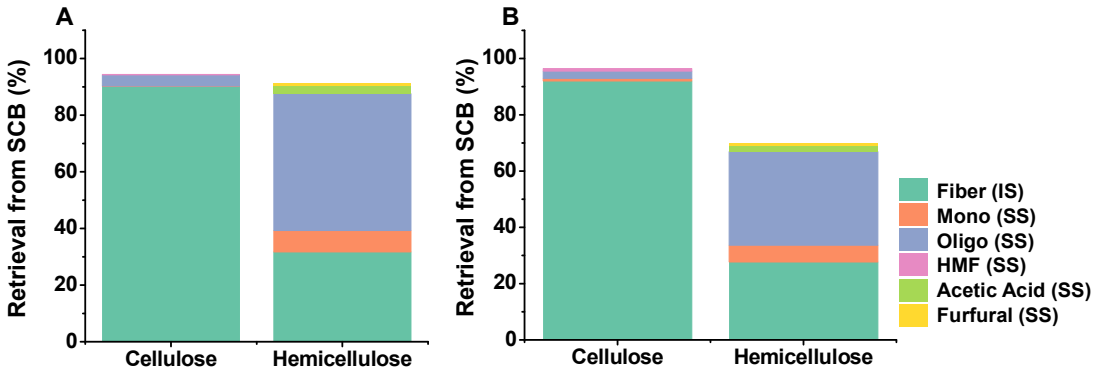


Figure 1: Mass balance after pilot-scale pretreatment based on the insoluble solids (IS) and soluble (SS) content. (A) hydrothermally pretreated bagasse (180 °C for 15 min); (B) steam explosion pretreated bagasse (190 °C for 10 min). Fiber is the solid fraction, mono is the glucose or xylose released, and oligo is the oligomeric fraction of glucose or xylose released.

By the data presented in Figure 1, no significant degradation of the cellulosic fraction was observed. Only traces of HMF were determined, and recovery of this fraction as fibers of approximately 90% for both pretreatments. This is highly desirable, regardless of the biorefinery concept considered, considering that, in the next step, the cellulose will be submitted to enzymatic saccharification to obtain glucose. It is also possible to observe that due to the low severity of the hydrothermal pretreatment, there was practically no release of glucose in the liquid fraction (SS). In contrast, solubilization in a ratio of almost 3:1 (oligo:mono) was observed for the steam explosion (Figure 1B).

Regarding the hemicellulosic fraction, effective 90 and 70% recovery was possible from hydrothermal and steam explosion pretreatments, respectively. In both conditions, nearly 50% of the hemicellulose is available in the liquid fraction, which offers an opportunity for processes in which stream separation is considered, and the solubilized C5 sugars could then be used in an independent fermentation. It is essential to highlight that, for this scenario, optimization still will be necessary, could be in the pretreatment condition and/or a depolymerization step (acid or enzymatic) to obtain monomeric xylose, since for both conditions evaluated in this work, an oligo:mono ratio of 7:1 (HPTB) and 6:1 (SEPTB) were obtained.

By these results, we effectively achieved the expectation of low severity pretreatments, once with severity factors below 4 (3.5 for HPTB and 3.6 for SEPTB), we could solubilize the hemicelluloses with low removal or degradation, following as described by Yuan et al. (2019)

that say it starts to solubilize with minimal changes at a severity of 3, and factors greater than 4.7 imply in reduction of hydrolyzed sugars and high inhibitors formation.

Mechanical refining evaluation

The effect of mechanical refining notably varies depending on biomass type, pretreatment severity, and enzyme formulation and dosage. Previous studies show that refining benefits are limited by biomass recalcitrance and total conversion (Park et al., 2016). It has been reported that high severity pretreatments associated with mechanical refining do not improve enzymatic saccharification (Park et al., 2016). Moreover, high severity pretreatments led to extensive production of harmful inhibitors for the fermentation and might compromise the C5 fraction for biological conversion. Therefore, in this study, we investigated the combination of two types of low severity pretreatments and high-consistency mechanical refining at an industrially relevant environment (pilot plant) to obtain fermentable sugars (glucose) by the enzymatic saccharification of the pretreated sugarcane bagasse.

The optimal conditions for GW and DR were initially investigated using HPTB and the CCRD approach according to the equipment's operational limits. The results presented in Figure 2 highlight that the maximum cellulose conversion into glucose was 50% in 72 h, below conversion levels observed in the literature for pretreatments with higher severity (Andrade et al., 2017; Ramos et al., 2015). Notably, all combinations of mechanical refining with HPTB resulted in higher cellulose conversion levels into glucose. However, the statistical analysis indicates that such values are at the same magnitude as the average of the central point, precluding to infer a clear correlation between saccharification efficiency and gap width or rotation, which was confirmed by the non-significative regression coefficients (R^2) obtained for all the mathematical models in Table 3 and ANOVA. Regardless that no mathematical model could be determined to optimize the mechanical refining process, the positive impact of this additional step on saccharification can be observed, principally after 24 h of reaction (Figure 2), ranging from 5 to 15% of higher conversion rates.

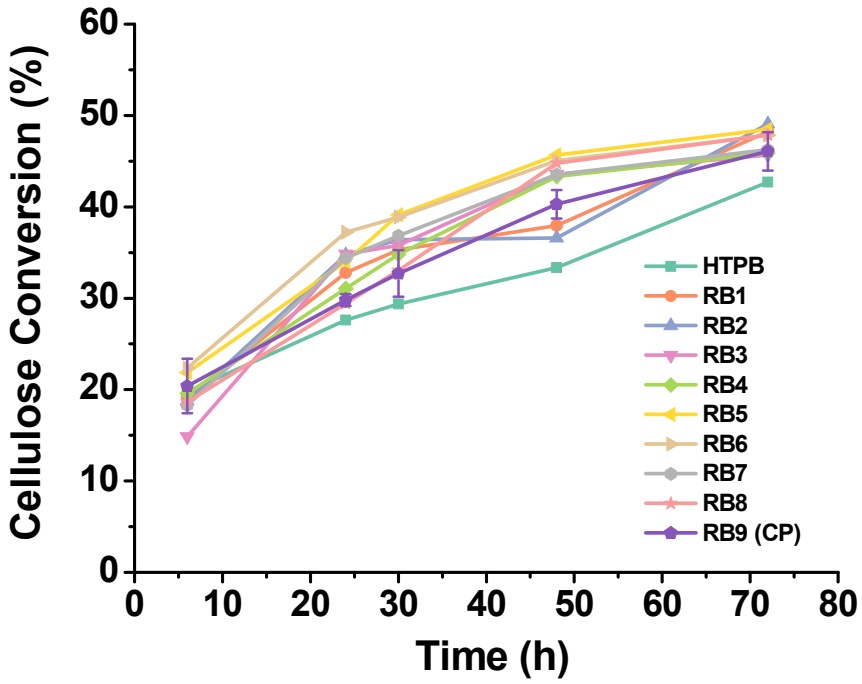


Figure 2: Enzymatic saccharification kinetics for CCRD performed for mechanical refining (RBs) and pretreated bagasse HPTB. Saccharification assays were conducted with 10% (w/w) solid content and an enzyme cocktail load of 10 FPU.gPTB^{-1}. RB conditions are described in Table 1.

Table 3: Mathematical models of mechanical refining CRRD for cellulose conversion response as a function of time.

Time (h)	Mathematical models	R^2	F_{cal}	F_{tab}
6	$Y_1 = 20.40 + 0.63\ x_1 + 0.30\ x_1^2 - 0.37\ x_2 - 1.52\ x_2^2 + 1.27\ x_1\ x_2$	41.26	0.80	5.05
24	$Y_2 = 29.79 + 0.34\ x_1 + 2.81\ x_1^2 - 1.08\ x_2 + 0.96\ x_2^2 - 1.43\ x_1\ x_2$	87.15	8.10	5.05
30	$Y_3 = 32.72 - 0.04\ x_1 + 2.79\ x_1^2 - 0.81\ x_2 + 0.77\ x_2^2 - 0.53\ x_1\ x_2$	68.82	2.60	5.05
48	$Y_4 = 40.29 - 0.31\ x_1 + 1.44\ x_1^2 + 1.77\ x_2 + 0.84\ x_2^2 + 0.28\ x_1\ x_2$	40.63	0.80	5.05
72	$Y_5 = 46.07 + 0.02\ x_1 + 0.95\ x_1^2 - 0.43\ x_2 + 0.40\ x_2^2 - 0.15\ x_1\ x_2$	25.69	0.40	5.05

In addition, we investigated the effect of mechanical refining on another low severity pretreatment by steam explosion, which is currently employed at a commercial scale in lignocellulose biorefineries (Figure 3). Interestingly, the mechanical refining had a more pronounced effect on the saccharification efficiency of steam-exploded pretreated bagasse (SEPTB) compared to HPTB, increasing in 8% its efficiency. It is notable that this gain in the saccharification productivity is observed even at the first hours, and the tendency was maintained throughout the 72 h of reaction, which was not observed for HPTB.

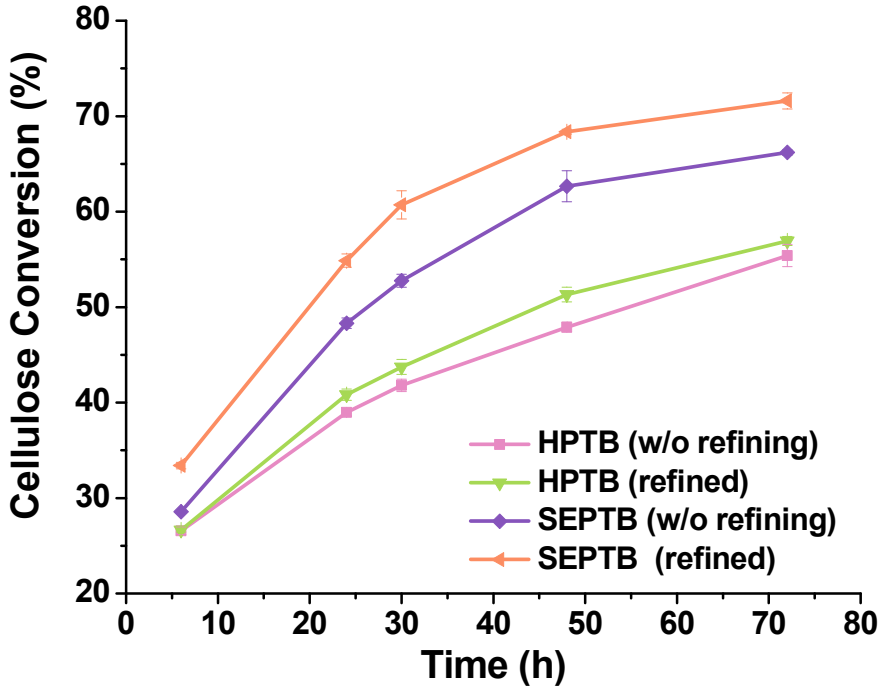


Figure 3: Enzymatic saccharification kinetics for mechanical refining and pretreated bagasse, HPTB or SEPTB. Saccharification assays were performed with 10% (w/w) solid content and an enzyme cocktail load of 10 FPU.gPTB^{-1}.

It indicates that the low severity hydrothermal pretreatment was ineffective in reducing biomass recalcitrance, even though we are working in the range where the benefits of mechanical refining should be significant, as proposed by (Jones et al., 2013) at the laboratory scale. They showed a dependence between the pretreatment severity and enzyme loading. Where the refining was limited by recalcitrance when pretreated biomass achieved a sugar yield of 40% and by total conversion at 80%. Nevertheless, an additional 15% increase in biomass yield was observed when it was 60% before refining. These results suggest the dependence of the refining reactivity on the biomass type and pretreatment while raising the question: does it pays off adding the refining step to the sugar release process?

Most previous works supporting this route at a pilot scale (Corbett et al., 2018; De Assis et al., 2018; Jones et al., 2014) were executed with hardwood and corn stoves, and the equipment used allowed a minimum gap width of 0.05 mm (20 times lower than the applied in this study). At the same time, few works have investigated the refining with pretreated sugarcane bagasse, generally at a lab scale and low consistency (Batalha et al., 2015; Wang et al., 2018b). In the present work, we evaluated the mechanical refining performance at the pilot scale and high consistency associated with the hydrothermal and steam explosion pretreatment. Since the increase in cellulose conversion was higher when the pretreatments were compared,

being 20% instead of the 8% achieved after the refining process with the SEPTB, further research must be conducted to answer the proposed question to determine the relations of refining reactivity to the pretreatment and biomass source.

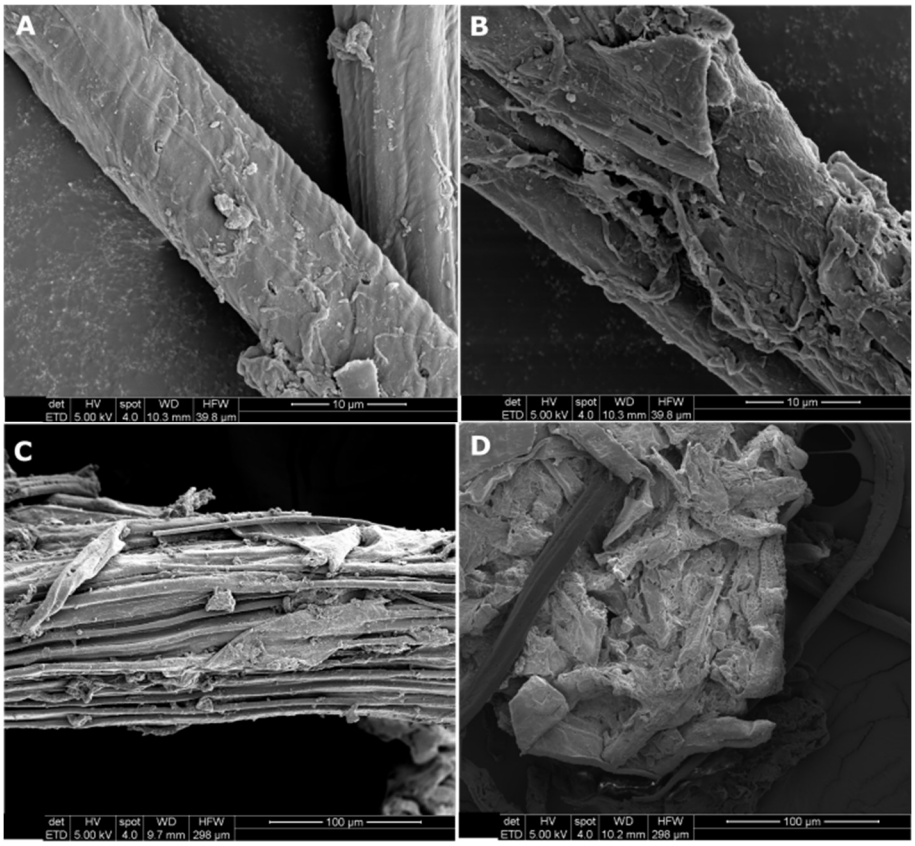


Figure 4: Scanning electron microscopy of unrefined and refined pretreated sugarcane bagasse. (A) HPTB unrefined; (B) HPTB refined (1 mm x 3000 RPM); (C) SEPTB unrefined; (D) SEPTB refined (1 mm x 3000 RPM).

In order to understand the distinct effects of mechanical refining on both pretreated materials, scanning electron microscopy (SEM) was applied to evaluate morphological characteristics. The micrographs show exposed flattened and cylindrical fibers, common to the morphology of sugarcane bagasse for both pretreatments (Figure 4A and C). However, severe fibers opening and delamination are only evident in the steam-exploded material and after its mechanical refining, which also enables access to the fiber tracheid, supportive with saccharification assays (Figure 4C and D). As noticed in Figure 4B, the refined HPTB did not exhibit equivalent morphology, reinforcing the concept that refining reactivity is linked to the pretreatment severity. Such microscopic analysis indicates that the improvement in enzymatic saccharification efficiency by the high-consistency mechanical refining is associated with the exposure of the cell wall fibrils and delamination, likely reflected in higher porosity and swellability of the pretreated biomass.

Conclusion

Aiming at reducing biomass recalcitrance, mechanical refining in high biomass consistency was studied. Improvements in enzymatic digestibility of up to 20% were obtained for SETB compared to HPTB, suggesting that steam explosion pretreatment, associated with mechanical refining, may be an interesting alternative for lignocellulosic biorefineries. SEM micrographs showed that mechanical refining increases fiber delamination and exposure of cell wall fibrils, being the fundamental basis for the increase in enzymatic saccharification. In conclusion, this study advances knowledge about using high-consistency mechanical refining in sugarcane biorefineries, complementing the current data available for hardwoods and corn stover.

References

Andrade, L.P., Crespim, E., de Oliveira, N., de Campos, R.C., Teodoro, J.C., Galvão, C.M.A., Maciel Filho, R., 2017. Influence of sugarcane bagasse variability on sugar recovery for cellulosic ethanol production. Bioresour Technol 241, 75–81. https://doi.org/10.1016/j.biortech.2017.05.081

Arce, C., Kratky, L., 2022. Mechanical pretreatment of lignocellulosic biomass toward enzymatic/fermentative valorization. iScience 25. https://doi.org/https://doi.org/10.1016/j.isci.2022.104610

Batalha, L.A.R., Han, Q., Jameel, H., Chang, H. min, Colodette, J.L., Borges Gomes, F.J., 2015. Production of fermentable sugars from sugarcane bagasse by enzymatic hydrolysis after autohydrolysis and mechanical refining. Bioresour Technol 180, 97–105. https://doi.org/10.1016/j.biortech.2014.12.060

Chen, X., Shekiro, J., Pschorn, T., Sabourin, M., Tao, L., Elander, R., Park, S., Jennings, E., Nelson, R., Trass, O., Flanegan, K., Wang, W., Himmel, M.E., Johnson, D., Tucker, M.P., 2014. A highly efficient dilute alkali deacetylation and mechanical (disc) refining process for the conversion of renewable biomass to lower cost sugars. Biotechnol Biofuels 7. https://doi.org/10.1186/1754-6834-7-98

Corbett, D.B., Knoll, C., Venditti, R., Jameel, H., Park, S., 2020. Fiber fractionation to understand the effect of mechanical refining on fiber structure and resulting enzymatic digestibility of biomass. Biotechnol Bioeng 117, 924–932. https://doi.org/10.1002/bit.27258

Corbett, D.B., Venditti, R., Jameel, H., Park, S., 2018. Effect of Mechanical Refining Energy on the Enzymatic Digestibility of Lignocellulosic Biomass. Ind Eng Chem Res 57, 14648–14655. https://doi.org/10.1021/acs.iecr.8b02932

De Assis, T., Huang, S., Driemeier, C.E., Donohoe, B.S., Kim, C., Kim, S.H., Gonzalez, R., Jameel, H., Park, S., 2018. Toward an understanding of the increase in enzymatic hydrolysis by mechanical refining. Biotechnol Biofuels 11, 1–11. https://doi.org/10.1186/s13068-018-1289-3

Gharehkhani, S., Sadeghinezhad, E., Kazi, S.N., Yarmand, H., Badarudin, A., Safaei, M.R., Zubir, M.N.M., 2015. Basic effects of pulp refining on fiber properties - A review. Carbohydr Polym. https://doi.org/10.1016/j.carbpol.2014.08.047

Ghose, T.K., 1987. Measurement of cellulase activities. Pure and Applied Chemistry 59, 695–702.

Jones, B.W., Venditti, R., Park, S., Jameel, H., 2017. Optimization of pilot scale mechanical disk refining for improvements in enzymatic digestibility of pretreated hardwood lignocellulosics. Bioresources 12, 4567–4593. https://doi.org/10.15376/biores.12.3.4567-4593

Jones, B.W., Venditti, R., Park, S., Jameel, H., Koo, B., 2013. Enhancement in enzymatic hydrolysis by mechanical refining for pretreated hardwood lignocellulosics. Bioresour Technol 147, 353–360. https://doi.org/10.1016/j.biortech.2013.08.030

Koo, B., Park, J., Gonzalez, R., Jameel, H., Park, S., 2019. Two-stage autohydrolysis and mechanical treatment to maximize sugar recovery from sweet sorghum bagasse. Bioresour Technol 276, 140–145. https://doi.org/10.1016/j.biortech.2018.12.112

Leal Silva, J.F., Mariano, A.P., Maciel Filho, R., 2021. Less severe reaction conditions to produce levulinic acid with reduced humins formation at the expense of lower biomass conversion: Is it economically feasible? Fuel Communications 9, 100029. https://doi.org/10.1016/j.jfueco.2021.100029

Olejnik, K., Fabijanska, A., Pelczynski, P., Stanislawska, A., 2019. Controllability of low-consistency chemical pulp refining process. Comput Chem Eng 128, 468–476. https://doi.org/10.1016/j.compchemeng.2019.06.024

Park, J., Jones, B., Koo, B., Chen, X., Tucker, M., Yu, J.H., Pschorn, T., Venditti, R., Park, S., 2016. Use of mechanical refining to improve the production of low-cost sugars from lignocellulosic biomass. Bioresour Technol 199, 59–67. https://doi.org/10.1016/j.biortech.2015.08.059

Ramos, L.P., da Silva, L., Ballem, A.C., Pitarelo, A.P., Chiarello, L.M., Silveira, M.H.L., 2015. Enzymatic hydrolysis of steam-exploded sugarcane bagasse using high total solids and low enzyme loadings. Bioresour Technol 175, 195–202.

Rocha, G.J. de M., Nascimento, V.M., Gonçalves, A.R., Silva, V.F.N., Martín, C., 2015a. Influence of mixed sugarcane bagasse samples evaluated by elemental and physical-chemical composition. Ind Crops Prod 64, 52–58. https://doi.org/10.1016/j.indcrop.2014.11.003

Rocha, G.J.M., Gonçalves, A.R., Nakanishi, S.C., Nascimento, V.M., Silva, V.F.N., 2015b. Pilot scale steam explosion and diluted sulfuric acid pretreatments: Comparative study aiming the sugarcane bagasse saccharification. Ind Crops Prod 74, 810–816.

Rocha, G.J.M., Martín, C., da Silva, V.F.N., Gómez, E.O., Gonçalves, A.R., 2012. Mass balance of pilot-scale pretreatment of sugarcane bagasse by steam explosion followed by alkaline delignification. Bioresour Technol 111, 447–52. https://doi.org/10.1016/j.biortech.2012.02.005

Rodrigues, M.I., Iemma, A.F., 2014. Experimental design and process optimization, Experimental Design and Process Optimization. https://doi.org/10.1201/b17848

Vermaas, J. v., Petridis, L., Qi, X., Schulz, R., Lindner, B., Smith, J.C., 2015. Mechanism of lignin inhibition of enzymatic biomass deconstruction. Biotechnol Biofuels 8, 1–16. https://doi.org/10.1186/s13068-015-0379-8

Wang, Z., Dien, B.S., Rausch, K.D., Tumbleson, M.E., Singh, V., 2018. Fermentation of undetoxified sugarcane bagasse hydrolyzates using a two stage hydrothermal and mechanical refining pretreatment. Bioresour Technol 261, 313–321. https://doi.org/10.1016/j.biortech.2018.04.018

Yuan, H., Song, X., Guan, R., Zhang, L., Li, X., Zuo, X., 2019. Effect of low severity hydrothermal pretreatment on anaerobic digestion performance of corn stover. Bioresour Technol 294. https://doi.org/10.1016/j.biortech.2019.122238

Zou, X., Liang, L., Shen, K., Huang, C., Wu, T., Wu, Y., Fang, G., 2021. Alkali synergetic two-step mechanical refining pretreatment of pond cypress for the fiber with intact 3D structure and ultrahigh cellulose accessibility fabrication. Ind Crops Prod 170, 113741. https://doi.org/10.1016/j.indcrop.2021.113741

Chapter 3

Type of chapter: Research Article
Current status: In preparation

Targeting enzymes to enhance the high solids liquefaction and saccharification efficiency of the engineered *Trichoderma reesei* RUT-C30 secretome

Andrade, L. P.[a,b], Vieira, P. S.[a], Neto, A. A. K. [a], Oliveira, J. V. C [a], Simister, R[c], Gómez , L. D.[c], Lanot, A.[c], McQueen-Mason, S. J.[c], Rocha, G. J. M.[a,*] and Murakami, M. T.[a,*]

[a]Brazilian Biorenewables National Laboratory (LNBR), Brazilian Center for Research in Energy and Materials, Campinas, SP, Brazil
[b]Postgraduate Program in Functional and Molecular Biology (BFM), Institute of Biology, State University of Campinas (UNICAMP), Campinas, SP, Brazil
[c]Centre for Novel Agricultural Products, Department of Biology, University of York, York, United Kingdom

*Correspondence mario.murakami@lnbr.cnpem.br and george.rocha@lnbr.cnpem.br

Abstract

Target enzymes were studied to improve the efficiency of Br_TrR03 (engineered *Trichoderma reesei* RUT-C30 secretome) at high solids saccharification of pretreated sugarcane bagasse (PTB). Using an automated platform, we screened the combinations of endoglucanases (*Bacillus subtilis* GH5R1 and *Humicola insolens* GH45), endoxylanase (*Kalmanozyma brasiliensis* GH11), expansin (*Bacillus subtilis*), and LPMOs (*Kitasatospora papulosa* AA10 and *Termobia domestica* AA15) with the cocktails Br_TrR03 and Cellic® CTec2 at low solids content, and at least one of each enzymatic class enhanced the sugar release of Br_TrR03. At high solids synergism investigation, all evaluated targets have some liquefaction capacity, and the formulation with Br_TrR03+BsGH5R1 presented the most effective saccharification and reduction of the substrate particle size. In agreement with previous work, the endoglucanase activity has shown a significant effect on viscosity even when applied alone to the water-washed, dried, milled, and sieved pretreated sugarcane bagasse (WMPTB). These results highlight relevant activities to improve the *T. reesei* RUT-C30 repertoire for lignocellulose saccharification, providing a rational strategy for biochemical boosting of enzyme cocktails for lignocellulosic biorefineries.

Keywords: Sugarcane bagasse, enzymatic saccharification, enzyme synergism.

Introduction

The increasing demand for energy and the need to reduce carbon emissions have driven the search for sustainable and renewable energy sources. Lignocellulosic biomass is a promising renewable energy source due to its abundance and potential for conversion to biofuels and value-added chemicals (Da Silva et al., 2020). The biochemical route has been considered the most suitable method to convert these polysaccharides into sugar monomers before the bioproduct obtention. Regardless of the application or the methods used for processing biomass into sugar syrups, two fundamental steps are involved in the conversion process. Firstly, the lignocellulosic biomass needs pretreatment to make it more susceptible to enzyme action. The second step involves enzymatic hydrolysis of the pretreated biomass to yield sugar syrups (Da Silva et al., 2020; Lynd et al., 2008; Sun et al., 2023).

Enzymatic conversion of lignocellulosic substrates into sugars involves the action of several enzymes. Cellulases break down the cellulose fibers into glucose mainly by combining the activity of three enzymes; endoglucanases, cellobiohydrolases, and the β-glucosidases. Nevertheless, hemicellulases work in the complex and highly branched xylan structures from hemicellulose by xylanases' cooperation with accessory enzymes such as xyloglucanases, mannanases, arabinofuranosidases, and feruloyl esterases (Gupta et al., 2016).

When targeting, the critical economic distillation concentration of 40 g/L ethanol implies that the enzymatic hydrolysis loading solids must be higher than 15% (Chen and Liu, 2017; Modenbach and Nokes, 2013; Sun et al., 2023). Saccharifications above this content are called high solids, and processing these biomass concentrations can lead to various complications, such as reaction heterogeneity, slow liquefaction, extended hydrolysis times, high enzyme loading, mass transfer issues, and a significant rise in capital investment and energy consumption (Szijártó et al., 2011; van der Zwan et al., 2017). Studies have demonstrated that "liquefaction", i.e., the high solids slurry viscosity reduction, occurs through mechanisms such as material dilution, particle fragmentation, and modification of interparticle interactions during enzymatic hydrolysis (Thygesen et al., 2014b; van der Zwan et al., 2017).

The action of enzymes to enhance high solids biomass transformation has been intensively studied (Cannella and Jørgensen, 2014; Du et al., 2020, 2014; Kristensen et al., 2009; Mukasekuru et al., 2020b; Sant'Ana da Silva et al., 2016; Skovgaard et al., 2014). It has been shown that endoglucanases played a central role in enzyme-mediated liquefaction with viscosity reduction over enzymatic hydrolysis (Gourlay et al., 2018; Skovgaard et al., 2014; Nára Szijártó et al., 2011). Moreover, (Skovgaard et al., 2014) proposed that adding xylanase

facilitated liquefaction directly by solubilizing xylan, thereby diluting the material and indirectly improving the accessibility of the substrate for other enzymes. Alongside, the expansin-like has been considered a significant asset to facilitate the action of hydrolytic enzymes promoting substrate amorphogenesis, loosening the plant cell wall, and providing better access to glycosidic bonds for these enzymes (Ding et al., 2022; Zhang et al., 2021). Literature has also shown that lytic polysaccharide monooxygenases (LPMOs) have been introduced into industrial cocktails due to their capacity to boost the enzymatic conversion by oxidizing the surfaces of insoluble polymeric substrates, leading to improved accessibility of hydrolases to the most recalcitrant parts of the biomass (Cannella and Jørgensen, 2014; Smuts et al., 2023; Velasco et al., 2021). However, most studies evaluating the synergies between enzyme mixtures or the action of a specific enzyme have approached low solids content(Du et al., 2018; Gourlay et al., 2013; Junior et al., 2015; Skovgaard et al., 2014; van der Zwan et al., 2020; Velasco et al., 2021; Zhang et al., 2011). In contrast, the works with high solids generally applied commercial cocktails (Chandrasekar et al., 2021; Chen et al., 2019; Fockink et al., 2017; Lu et al., 2010; Mukasekuru et al., 2020b; Roche et al., 2009).

The balance between slurry fluidity and saccharification is crucial because the challenge of cost-effectively achieving the optimal sugar release lies in the innate recalcitrance of the plant cell wall, even after pretreatment, and finding the better enzyme synergism to achieve it (Sun et al., 2023; Nára Szijártó et al., 2011; van der Zwan et al., 2020). Filamentous fungi are known as excellent producers of enzymes to degrade the biomass in nature, although multiple microorganisms generally accomplish it (Monclaro et al., 2022). The *Trichoderma reesei* was the first one with this ability to be isolated in the Solomon Islands during World War II, and until this date, most strains used by academic and industrial applications originated from him (Bischof et al., 2016; Fonseca et al., 2020). Himmel et al. (2017) light on the discussion related to the scientific reproducibility of cellulase formulations, also pointed out by (Da Silva et al., 2020; Sun et al., 2023) that discussed the difficulty of comparing the results obtained in high-solids saccharifications due to the different experiments designs, biomass source and pretreatments, and enzymatic cocktails.

In this scenario, this present study assesses how selected enzymes act synergically to enhance the secretome of an engineered *Trichoderma reesei* RUT-C30 strain, given that this strain is a well-characterized, reproducible and robust.

Experimental

Pretreatment

The pretreatment was carried out on a pilot scale at the Pilot Process Development Plant (PPDP) from the Brazilian Biorenewables National Laboratory (LNBR), part of the Brazilian Center for Research in Energy and Materials (CNPEM). The sugarcane bagasse (SCB), composed of 42.1% cellulose, 30.5% hemicellulose, 24.4% lignin, 3.5% extractives, and 1.2% ash, was processed in the Dry-Cleaning System from PPDP. In this pneumatic system, the air flows against the SCB, promoting its separation from the non-structural inorganics (stones, pieces of iron and steel, and sand). Steam explosion pretreatment was executed at a feed rate of 10 $kg.h^{-1}$ and a residence time of 10 minutes at 190°C in a stainless-steel continuous reactor from AdvancedBio® (Milford, OH, USA), and the resulting pretreated bagasse (PTB) was homogenized and stored in a cold room. The pretreated bagasse had its chemical characterization determined accordingly to Rocha et al. (2015). The composition of PTB at the start of saccharifications was 59.9% cellulose, 9.2% hemicellulose, 28.3% lignin, and 2.8% ash.

Enzyme targets

The targets were selected from the group library based on factors such as biochemical (optimal pH and temperature close to the ones from the cocktails) and kinetics parameters, expression yield, mechanism of action, CAZy family, diverse organisms' sources, and one as a reference from a previous study (HiGH45). Table 1 describes some biological characteristics of the enzyme targets evaluated to supplement enzymatic cocktails.

Table 1: Targets selected for work execution.

Target Denomination	Source	Activity	CAzy Family	Accession number	PDB
BsGH5-R1	*Bacillus subtilis* 168 (Bacillota)	Endoglucanase	GH5	P10475	3PZV (WT)
HiGH45	*Humicola insolens* (Ascomycota)	Endoglucanase	GH45	P43316.1	2ENG
KbGH11	*Kalmanozyma brasiliensis* (Basidiomycota)	Endoxilanase	GH11	V5EZ11	To be publish
KpAA10	*Kitasatospora papulosa* (Actinomycetota)	LPMO	AA10	UPI0012F47736	6NDQ
TdAA15A	*Thermobia domestica* (Metazoa)	LPMO	AA15	SIW61372.1	5MSZ
BsEXP	*Bacillus subtilis* 168 (Bacillota)	Expansin	n.a.	WP_003231419.1	3D30

Overexpression and purification of the enzymatic targets

Mainly, the clones tested in this work were previously designed and had their conditions optimized. The HiGH45 was provided by Novozymes (Kongens Lyngby, Denmark) as a crude preparation cloned and expressed in *Aspergillus oryzae*. Thermocompetent cells of Escherichia coli strains were transformed with the respective vectors to express the targets described in Table 2. Proteins were purified by two chromatographic steps performed in Äkta Purifier System (GE Healthcare), as detailed in Table 3. Purified samples were analyzed by SDS-PAGE. Protein concentration was estimated (mg/mL) using absorbance spectroscopy at 280 nm in a Nanodrop spectrometer (Thermo Scientific). The molecular mass (kDa) and molar extinction coefficient (ε/1000) were extracted from the sequence using the ExPAsy-ProtParam Tool (https://web.expas y.org/protparam/).

Table 2. Protein expression conditions.

Protein	Strain	Vector	Growth	Expression	Time	Medium	[IPTG]
BsGH5-R1	BL21 (DE3)	pET28a	37 °C – 250 rpm	18°C – 250 rpm	16 h	LB	0.5 mM
KbGH11	ArcticExpress (DE3)	pET28a	37 °C – 250 rpm	16°C – 180 rpm	20 h	LB	0.5 mM
KpAA10	BL21 (DE3) SHuffle	pSUMO	30 °C – 250 rpm	18°C – 180 rpm	16h	ZYM-5052 Auto-induction	n.a.
TdAA15A	Rosetta 2 (DE3)	pET26b	37 °C – 210 rpm	16°C – 180 rpm	16h	M9 with 1% glucose (w/v)	1.0 mM
BsEXP	BL21 (DE3)	pET28a	37 °C – 250 rpm	37 °C – 250 rpm	4h	LB	0.5 mM

Table 3. Protein purification conditions.

Protein	Lysis buffer	1st Purification Step				2nd Purification Step	
		Buffer	Column	Imidazole Gradient	Cu saturation	Buffer	Column
BsGH5-R1	50 mmol.L^{-1} sodium phosphate pH 7, 100 mM NaCl, 20 mmol.L^{-1} imidazole, 0.5 tablets of cOmplete, EDTA-free da Roche®, and 2 mM β-mercaptoethanol	50 mmol.L^{-1} sodium phosphate pH 7, 100 mM NaCl	HisTrap HP (GE Healthcare)	20-500 mmol.L^{-1}	n.a.	50 mmol.L^{-1} sodium phosphate pH 7, 100 mM NaCl, 5% glycerol (v/v)	HiLoad™ 16/60 (GE Healthcare)
HiGH45	n.a.	20 mM triethanolamine pH 7	HiPrep 26/10 (GE Healthcare)	n.a.	n.a.	20 mM triethanolamine pH 7 (Gradient to 1M NaCl)	HiTrap® Q XL (GE Healthcare)
KbGH11	100 mmol.L^{-1} sodium phosphate pH 7.5, 200 mM NaCl, 20 mmol.L^{-1} imidazole, 10% glycerol (v/v), 0.5 tablet of cOmplete, EDTA-free da Roche®, and 2 mM β-mercaptoethanol	100 mmol.L^{-1} sodium phosphate pH 7.5, 200 mM NaCl, 10% glycerol (v/v)	HisTrap HP (GE Healthcare)	20-500 mmol.L^{-1}	n.a.	50mM Sodium acetate pH 4.0	HiLoad™ 16/60 (GE Healthcare)
KpAA10	20 mmol.L^{-1} sodium phosphate pH 7.4, 500 mM NaCl, 20 mmol.L^{-1} imidazole, 0.5 tablets of cOmplete, EDTA-free da Roche®	20 mmol.L^{-1} sodium phosphate pH 7.4, 500 mM NaCl	HisTrap HP (GE Healthcare)	20-500 mmol.L^{-1}	3:1 (Cu:KpAA10), overnight, 4 °C	20 mmol.L^{-1} sodium phosphate pH 7.4, 150 mM NaCl	HiLoad™ 16/60 (GE Healthcare)
TdAA15A	50 mM Tris pH 8, 20 % saccharose, 5 mM MgSO4, 100 µM AEBSF	200 mmol.L^{-1} sodium phosphate pH 7.6, 1.2 M NaCl, 2,5 mM desthiobiotin	StrepTrap HP (GE Healthcare)	n.a.	5:1 (Cu: TdAA15A), 60 minutes, room temperature	10 mmol.L^{-1} sodium phosphate pH 7	HiLoad™ 16/60 (GE Healthcare)
BsEXP	25 mmol.L^{-1} sodium phosphate pH 7.4, 300 mM NaCl, 20 mmol.L^{-1} imidazole, 0.5 tablets of cOmplete, EDTA-free da Roche®, and 2 mM β-mercaptoethanol	25 mmol.L^{-1} sodium phosphate pH 7.4, 300 mM NaCl,	HisTrap HP (GE Healthcare)	20-500 mmol.L^{-1}	n.a.	20 mmol.L^{-1} sodium phosphate pH 7.4, 150 mM NaCl	HiLoad™ 16/60 (GE Healthcare)

Biochemical assays

Novozymes Cellic® CTec2 and a mixture of Celluclast and Novozym 188 in a 5:1 volumetric ratio (CN_5:1); and Br_TrR03 (Fonseca et al., 2020) were used as the enzyme cocktails. Briefly, the proteins from the enzyme cocktails were precipitated with ice-cold acetone and then quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™), based on Lowry's method (Lowry et al., 1951). Bovine Serum Albumin (BSA) was used as a standard. Filter paper activity (FPase) was determined as described by (Ghose, 1987). BsGH5-R1 and KbGH11 activity was confirmed using carboxymethylcellulose (CMC) and beechwood xylan (Megazyme) as substrate. Both reactions were carried out in 50 mM citrate buffer, pH 5.0, at 50 °C for 30 min; it was stopped by adding 1 volume of 3,5-dinitro salicylic acid (DNS) reagent followed by heating at 95 °C for 5 min. Absorbance was measured at 540 nm using an Infinite®200 PRO microplate reader (TECAN Group Ltd., Männedorf, Switzerland). Alternatively, the endo-β-1,4-glucanase and endo-β-1,4-xylanase activity of the targets was verified using Azo-Avicel and Azo-Xylan (Birchwood) (Megazyme) as substrates following the supplier guidelines. Pure glucose or xylose solutions were used as standards.

Activities and protein per mL of cocktail equal 166.1 FPU and 184.9 mg protein for Cellic® CTec2, 47.3 FPU and 90.7 mg protein for CN_5:1, 31.5 FPU and 60.7 mg protein for Br_TrR03.

Preparation of Alcohol insoluble residue (AIR)

Dried at room temperature, grounded, and classified to obtain the fractions retained by the 35- and 8-mesh screens pretreated sugarcane bagasse was AIR-washed to remove soluble sugars by adding 400 ml of water to 10 g of water PTB in a 2 L beaker. After mixing, the suspension was vacuum filtered, and the water was removed and replaced with 400 ml of 96% ethanol, followed by two further ethanol washes before the residue was allowed to dry under ambient conditions.

Screening of enzyme targets using a high throughput platform

The enzyme screening was carried out in an automated platform developed by (Gomez et al., 2010) at the Centre for Novel Agricultural Products (CNAP) from the University of York. AIR-washed PTB had saccharification assays performed with 0.66% of total solids (4mg). The enzyme load of the cocktails was determined by the amount necessary to achieve total reducing

sugars (TRS) lower to 60 μgSugars.mgBiomass^{-1} in 8 hours of reaction. Several loads ranging from 0.5 to 10 mg Protein gCellulose^{-1} were evaluated, and 0.5 mgProtein.gCellulose^{-1} for Cellic® CTec2 and Br_TrR03, and 5 mgProtein.gCellulose^{-1} of CN_5:1 were applied. The cocktails supplementation was assessed employing 5% of each inactive and active enzymatic target at 50°C, pH 5 (25 mM sodium acetate buffer solution) for 8 h. The screening saccharification with inactive targets (Figure 1) was done as a control to guarantee that the effect of the active ones was not due to their adsorption on the lignin fraction of the AIR-washed PTB. TRS was determined with 30 μL of hydrolysate supernatant with 45 μL of 25 mM sodium acetate buffer. Then, 25 μL of 1N NaOH and 50 μL of a solution containing 0.43 mg/mL of 3-methyl-2-benzothiazolone hydrazone (MBTH) and 0.14 mg/mL of dithiothreitol (DDT) were added to the hydrolysate and heated at 60 °C for 20 min. Then, 100 μL of oxidizing reagent [0,2% $FeNH_4$ (SO_4), 0.2% sulfamic acid, and 0.1% HCl] was added for color development. The standard curve for sugar determination had 50, 100, and 150 nmol of glucose. With a total of 250 μL in each well of the plate, reactions were revealed at 620 nm.

Evaluation of the liquefaction of pretreated sugarcane bagasse

To guarantee particles below 2 mm, the PTB was water-washed, dried at room temperature, milled, and sieved for 3 min to obtain the fractions retained by the 100- and 35-mesh screens while eliminating the fines (WMPTB). Each saccharification reaction was carried out independently (n=3) in a 50 mL Falcon tube using a FINEPCR combi-D24 hybridization incubator (Gunpo-si, South Korea) at the maximum rotation (level 9), with a total solids content of 15%, 5 mg protein/g-cellulose of enzyme load (Cellic® CTec2 and Br_TrR03) supplemented with 5% of each active enzymatic target, pH 5 (100 mM citrate buffer solution), and 50 ◦C. For homogeneous reactions, a Falcon tube was used for each experimental point with a total mass of 10 g, and one inox metal sphere (3.5 mm diameter and 1 g each) was inserted. The reference, 0 h, was set up by adding a volume of water instead of the enzyme. The reactions were stopped by heat-inactivation at 95ºC for 45 min in a water bath. For reducing sugars, the samples were diluted by adding 1g of the material to 9 mL of deionized water in a 15 mL Falcon tube; after mixing, it was centrifuged for 10 min at 2880×g. A 1.5 mL supernatant sample was transferred to a 2 ml Eppendorf tube, and then the Falcon tube was stored at -20 °C until analysis. Sugars were quantified by HPLC and particle size by light scattering. One-way analysis of variance (ANOVA) with post hoc Scott-Knott tests was conducted to compare the mean values of TRS.

Analysis of carbohydrate concentrations

HPLC was used to quantify glucose, xylose, arabinose, and cellobiose in the enzymatic hydrolysate samples. The chromatographer was the 1260 Infinity from Agilent, equipped with a refractive index detector and an Aminex HPX 87H column (300 x 7.8 mm) and pre-column (30 x 4.6 mm) at 35ºC with a flow of 0.6 mL.min-1 of 5 mM sulfuric acid solution eluted isocratically. The samples were homogenized and filtered through a Millex syringe filter with a diameter of 13 mm, 0.22 µm. Sample quantification was performed by external calibration.

Diffraction Light Scattering (DLS)

Particle size distributions were evaluated by light scattering using the Beckman Coulter LS13320, ranging from 0.034 to 2000 µm. The instrument was operated with a 780 nm laser light source, 6% obscuration, and a vacuum of 18 in. H_2O, and Fraunhofer light-scattering model. The mathematical treatment of the embedded software assumes spherical particles for converting the 126 photodetector intensities (distributed in scattering angle) to volume percentages (allocated in particle size channels). Particle volume distribution versus equivalent sphere diameter was determined. The assumptions of spherical particle shape might not apply to our substrates' fibrous shape and multimodal distribution. Nonetheless, we have utilized the equivalent sphere diameter and will discuss its implications within these limitations.

Results and Discussion

Screening of enzyme targets by a high throughput platform

The ability of the enzyme targets to act synergically with the cocktails, Cellic® CTec2 and Br_TrR03, to enhance the saccharification efficiency of AIR-washed PTB was screened using an automated platform developed by (Gomez et al., 2010). While mainly the inactive targets presented a negative synergism with the cocktails (Figure 1), a boost effect of the cocktail Br_TrR03 was observed by the supplementation with the LPMO, KpAA10. This result may relate to the reduced non-productive adsorption of the enzymes from the cocktail to the lignin, for example, as had been previously reported with BSA (Bovine Serum Albumin) by Yang and Wyman (2006).

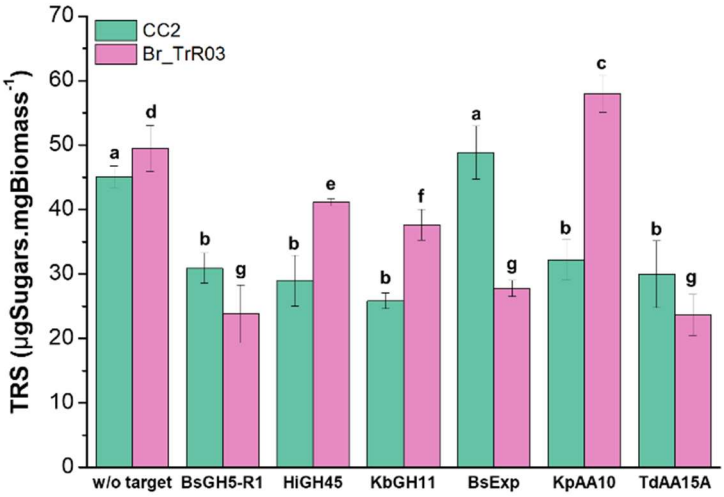


Figure 1: Saccharification efficiency of Br_TrR03 and Cellic® CTec2 enzyme cocktails on post-processed steam-exploded sugarcane bagasse (AIR-washed PTB) supplemented with inactive enzyme targets. Saccharification assays were performed in a high throughput platform with 0.66% (*w/w*) solid content and enzyme cocktail load of 0.5 mgProtein.gCellulose^{-1} and enzyme target load of 0.025 mgProtein.gCellulose^{-1}. Results are expressed as mean ± SD (n = 4, four independent experiments). Scott-Knott post hoc tests ($r < 0.05$) compared Br_TrR03 and Cellic® CTec2 supplementation at 8 hours. Thus, bars sharing the same letter do not present significant differences.

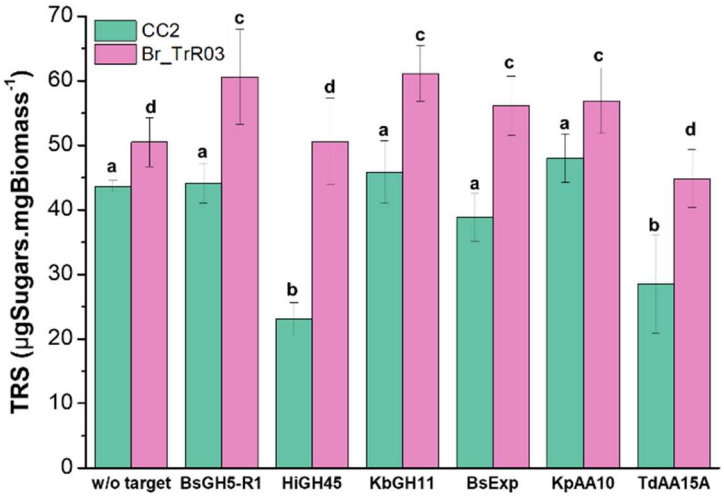


Figure 2: **Saccharification efficiency of Br_TrR03 and Cellic® CTec2 enzyme cocktails on post-processed steam-exploded sugarcane bagasse (AIR-washed PTB) supplemented with active enzyme targets.** Saccharification assays were performed in a high throughput platform with 0.66% (*w/w*) solid content and enzyme cocktail load of 0.5 mgProtein.gCellulose^{-1} and enzyme target load of 0.025 mgProtein.gCellulose^{-1}. Results are expressed as mean ± SD (n = 5, five independent experiments). Scott-Knott post hoc tests ($r < 0.05$) compared Br_TrR03 and Cellic® CTec2 supplementation at 8 hours. Thus, bars sharing the same letter do not present significant differences.

The data from Figure 2 shows a positive influence on sugar release by supplementing the Br_TrR03 cocktail with most of the active targets. The supplementation of the CN_5:1 cocktail (Supplementary Figure 1) revealed the same tendency, however, with a notable increase of TRS by the KbGH11, followed by the BsEXP boost. At the same time, the other targets did not differ from the result obtained without the supplementation. Comparable results were found in previous works (Ding et al., 2022; Junior et al., 2015; Shi et al., 2021; Skovgaard et al., 2014; Nóra Szijártó et al., 2011a; van der Zwan et al., 2020; Velasco et al., 2021) which had evidenced the effects of endoglucanases, endoxylanases, expansins, and LPMOs in enhancing the biomass hydrolysis acting solo or synergically with a mixture of enzymes or commercial cocktails, from low to high solids.

In contrast, none of the targets increased the saccharifications when the Cellic® CTec2 cocktail was supplemented; at least an increment with the xylanase, KbGH11, was expected since the manufacturer recommends the supplementation with Cellic® HTec2 to improve the productivity (Novozymes, 2010). Likewise, supplementation with the targets HiGH45 and TdAA15A did not affect Br_TrR03 and negatively impacted the Cellic® CTec2 yield. These results highlight the complex specificity between the enzyme (target and/or cocktail) and substrate (pretreated biomass). An enzyme could act synergically with a specific cocktail or another enzyme in one circumstance, as the HiGH45 with pretreated aspen in van der Zwan et al. (2020) work and does not with pretreated sugarcane bagasse (present study). Similar behavior is observed for enzymatic cocktails, which could have different results depending on the lignocellulosic biomass's source, recalcitrance, and pretreatment (Baral et al., 2020; Ju et al., 2014).

Besides, we must recognize that these results are from low solids load, and when the feasibility of the process is considered, this is not the desired scenario. Also, different cocktails have different ratios of cellulases and xylanases activities, and the total protein does not properly represent these ratios. Therefore, at low solids, the Br_TrR03 TRS release is better than for Cellic® CTec2, which could be due to the enzyme activity ratio, the substrate physical-chemical characteristics, or yet because the latter is designed to work at high solids concentrations (Novozymes, 2010). These finds corroborate with previous works (Du et al., 2020; Weiss et al., 2019), which have pointed out that the most probable reason is the enzyme-substrate specificity in the enzymatic saccharification reactions. And with van der Zwan et al. (2020) study, recently found that this relation is also highly influenced by biomass concentration and rheology.

Nevertheless, there are infinite targets that could have been evaluated, in the work presented here, we chose six enzymes as possible enhancers. Some have worked synergically with the Br_TrR03 cocktail at low solids load and will continue to be investigated at high solids concentrations. Additionally, Cellic® CTec2 will be used as the commercial positive control.

Evaluating enzyme synergism at high solids concentrations

Several works (Chen and Liu, 2017; Jørgensen et al., 2007; Skovgaard et al., 2014; Nóra Szijártó et al., 2011b) have discussed that the solids content is one of the most critical parameters affecting the enzymatic saccharification process's efficiency. Low biomass loadings are linked to high processing volume and low product concentration, increasing capital and energy costs and requiring work on biomass concentrations of at least 15%. Therefore, we investigate if the enzymes BsGH5-R1, KbGH11, KpAA10, and BsEXP would keep their boost influence in the engineered RUT-C30 secretome at high solids saccharifications designed to evaluate the synergistic effects of the cocktail with target enzymes.

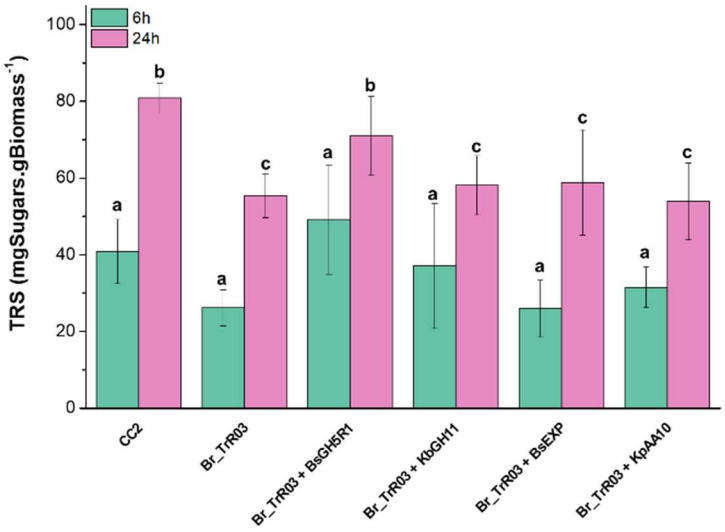


Figure 3: Saccharification efficiency of Br_TrR03 enzyme cocktail on pos-processed steam-exploded sugarcane bagasse (WMPTB) supplemented with active enzyme targets. Saccharification assays were performed with 15% (*w/w*) solid content and enzyme cocktail load of 5 mgProtein.gCellulose^{-1} and enzyme target load of 0.25 mgProtein.gCellulose^{-1}. Results are expressed as mean ± SD (n = 3, three independent experiments). Scott-Knott post hoc tests ($r < 0.05$) compared Br_TrR03 solo and supplemented with Cellic® CTec2 performance at the same dosage over time. Thus, bars sharing the same letter do not present significant differences.

As present in Figure 3, at six hours, all formulations statistically achieved the same sugar release, while at 24 h, the Br_TrR03+BsGH5R1 reached Cellic® CTec2, and both released on average 35 % more sugar than the Br_TrR03 solo or combined with the other targets. Although, in general, the hydrolysis was not complete, with total sugar yields around 15% (Supplementary Figure 2), these results corroborate previous literature findings (Gourlay et al., 2018; Skovgaard et al., 2014) where even low hydrolysis extends result in viscosity alterations. Also, complete saccharification requires a high enzyme load, however, the goal is to find enhancers as candidates for future genetic modifications, which is facilitated by the low load used here. Therefore, these results suggest that automated screening could be a high throughput platform for selecting from a diverse range of enzyme targets.

The low competitiveness in sugar release by the Br_TrR03 enzyme cocktail was only apparent. Once, in a previous study (de Lima et al., 2022), also with the engineered *Trichoderma reesei* RUT-C30 secretome at 15% of solids, showed a significantly higher efficiency when compared to the Cellic® CTec2 applying a load of 10 mgProtein.gDryMass $^{-1}$. The differences observed by the saccharifications of both works were not only because of the enzyme load (four times higher) but can also be credited to the different biomass handling after pretreatment. In contrast to the present study, which had water-washed, dried, milled, sieved, and used a low enzyme load, (de Lima et al., 2022) evaluated the pretreated sugarcane bagasse directly after pretreatment.

As discussed, working with less than 15 % solids content is not technical-economically viable. However, the high recalcitrance from our substrate (PTB) in this concentration led to a rheological behavior beyond rheometer limits (we tried several models (YR-1 Yield Stress, R/S-SST+, and R/S-CPS+ Rheometers, DVIII-Ultra and DV3-TRV viscosimeters from Brookfield®), data not shown), demanding a different analysis method.

Therefore, we evaluate the particle size distributions, since already demonstrated by (Skovgaard et al., 2014), there is an association between lower viscosities and smaller mean particle size. Figure 4 presents a straightforward modification of the WMPTB fibers over time from DLS measurements. We can determine the particle size distribution and transformation over the reaction by assessing the volume of each equivalent diameter and analyzing the differences in peak volumes and how each curve distanced to the left from the determined before reaction (0h). Here we can distinct four peaks ranging from 1 to 200 μm at two and four hours, while after six, remaining just three, with the curve between peaks 2 and 4 getting softener.

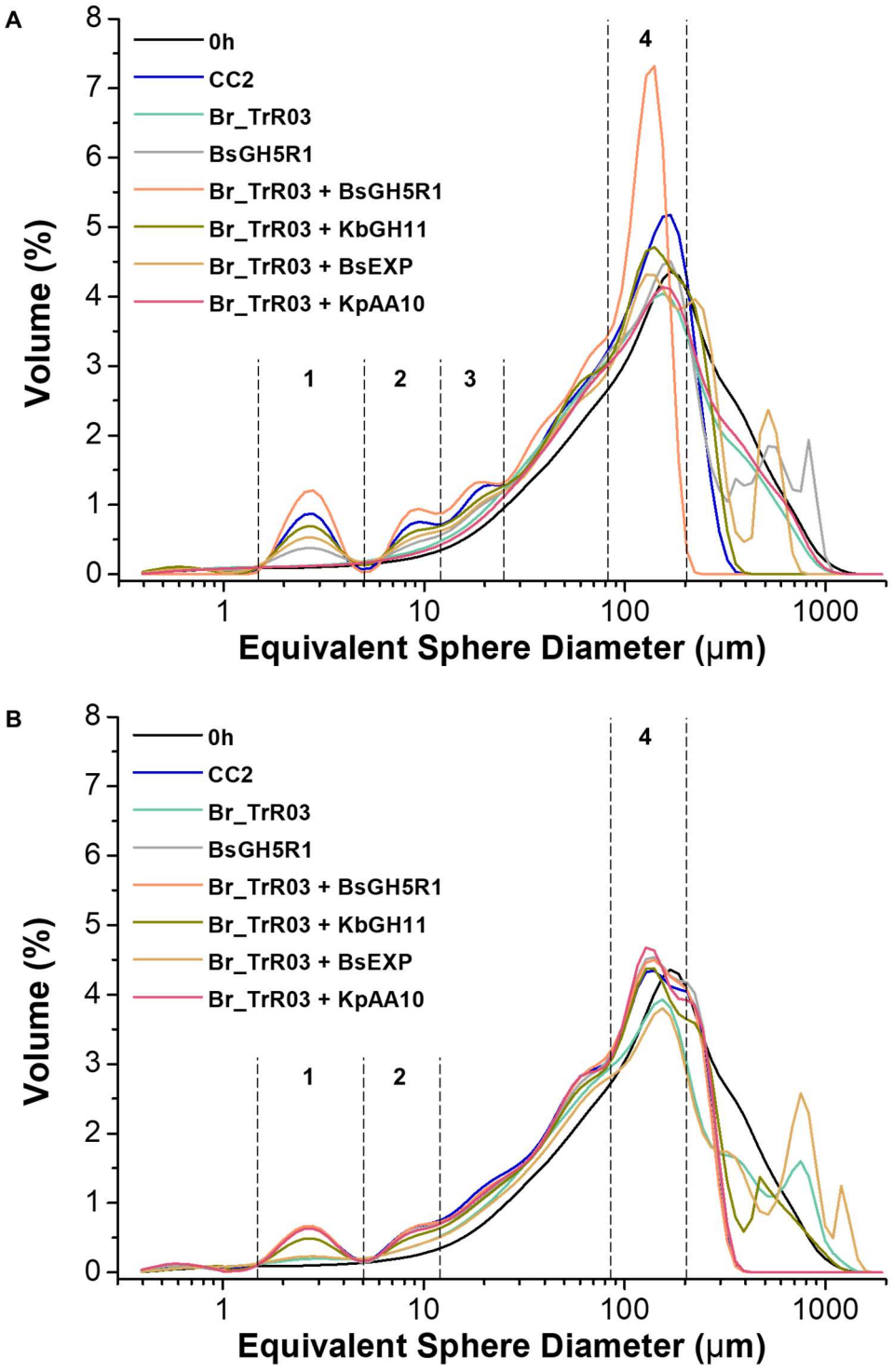


Figure 4: Particle size distributions of enzymatic saccharification of Br_TrR03 enzyme cocktail supplemented with active enzyme targets on pos-processed steam-exploded sugarcane bagasse (WMPTB) at 2 hours (A) and 24 hours (B). Saccharification assays were performed with 15% (*w/w*) solid content and enzyme cocktail load of 5 mgProtein.gCellulose^{-1} and enzyme target load of 0.25 mgProtein.gCellulose^{-1}. Results are expressed as mean (n = 3, three independent experiments).

The formulation, Br_TrR03+BsGH5R1, in which the cocktail was supplemented with the endoglucanase, had its efficiency highlighted from the others. When we increased the reaction complexity (high solids and low load), not only the sugar release was equivalent to the one reached by Cellic® CTec2, but also this blend resulted in the best pattern of reducing the size of the substrate particles over reaction time. In two hours, all the particles in the sample treated with the blend were sizing below 200 μm (half from the obtained with Cellic® CTec2). At peak 4, it reached 7.3% volume, while the other conditions ranged from 4 to 5%. However, after 6 hours (Supplementary Figure 3), more sample show similar volume at peak 4 (Cellic® CTec2 and Br_TrR03+ KpAA10).

At the same time, the supplementation with KbGH11 had its influence reduced over time, probably associated with hemicellulose degradation, even if it does not result in high conversion to monomers. The same behavior can be observed by the combination with BsEXP, which probably had its impact lowered by the reduction of the cellulose polymerization degree after the breakdown of the major chains. Whereas the KpAA10 had an opposite performance from the previous, increasing its effect after four hours by approximating the results of the blends of Br_TrR03+BsGH5R1 and Cellic® CTec2. We could observe this comportment because of the need for a reducing agent and electron donor from the KpAA10, which the amount found in the reaction beginning was insufficient. Still, after a specific reaction time, the released lignin fragments could have assumed this paper, as proposed by (Muraleedharan et al., 2018; Westereng et al., 2015).

Although we observed better results with the endoglucase, BsGH5R1, it contrasts with the ones found by (van der Zwan et al., 2020), in which a more significant viscosity reduction with the cellobiohydrolase, TrGH7, was observed than the one with the endoglucanase, HiGH45, also evaluated in our study and without synergism in our condition. They also tested the mixture, TrGH7/HiGH45, which did not result in viscosity reduction; once the TrGH7 is already expressed by the Br_TrR03 cocktail, a better synergism is likely observed between TrGH7/BsGH5R1. These findings contribute to the discussion that the blend should be tailormade to each biomass because of the high substrate-enzyme specificity (Adsul et al., 2020; Du et al., 2020; Weiss et al., 2019) or that standard substrate and cellulase formulations are necessary for scientific reproducibility assurance (Himmel et al., 2017).

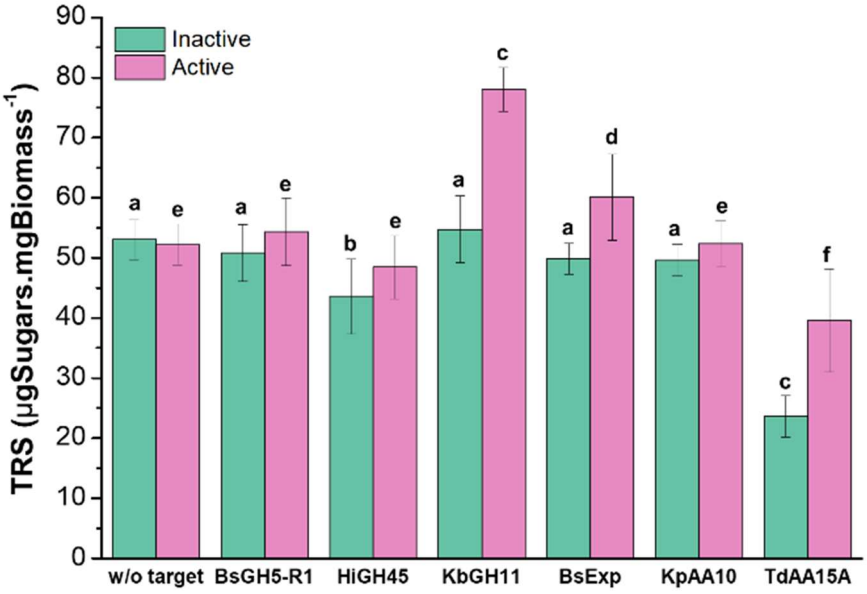
In this sense, additionally, to confirm the effect of the endoglucanase, BsGH5R1, we investigated its action solo in the WMPTB, finding a pronounced effect in reducing the particle size compared to the complex formulations, and after four hours of reaction, it closely followed the results of Br_TrR03+BsGH5R1 and Cellic® CTec2. We contributed to previous studies

demonstrating endoglucanases' individual role in liquefaction (Gourlay et al., 2018; Skovgaard et al., 2014; Nára Szijártó et al., 2011) and in agreement with previous synergic studies (Espinheira et al., 2022; Gourlay et al., 2018). In conclusion, these results indicate space for engineered *Trichoderma reesei* RUT-C30 secretome improvement, BsGH5R1 is the protagonist in WMPTB liquefaction, and the enzymatic armory of *Bacillus subtilis* could be complementary to *Trichoderma reesei*.

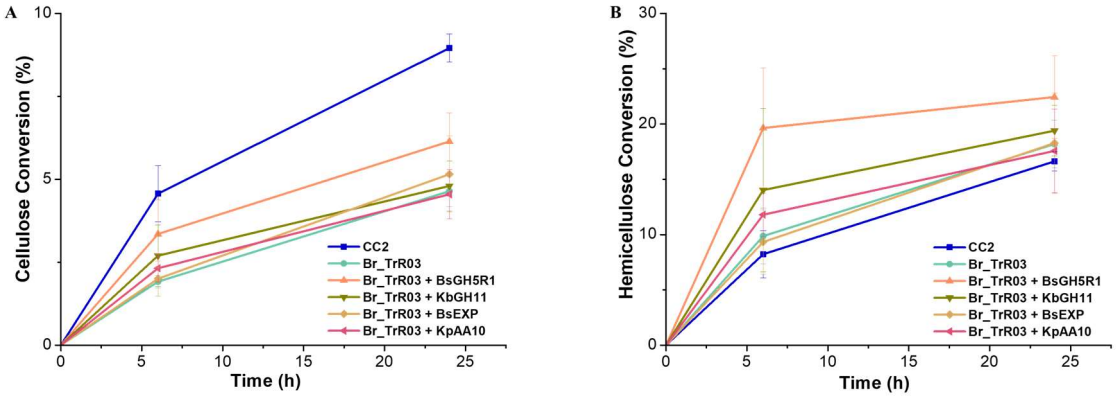
Conclusions

This study compared heterologously expressed enzymes from various organisms to improve the efficiency of engineered *Trichoderma reesei* RUT-C30 secretome in high solids saccharifications of pretreated sugarcane bagasse. The endoglucanase, BsGH5R1 from *Bacillus subtilis*, acts alone or in synergy with the Br_TrR03 cocktail and is the enzyme that increased hydrolysis efficiency the most and reduced the particle size of the substrate faster. Testing enzymes to enhance the enzymatic saccharification and liquefaction efficiency of the engineered *Trichoderma reesei* RUT-C30 secretome enables the identification of targets that displayed synergistic effects and that could be targets for future genetic modifications. The high throughput platform effectively supported the proper selection of the enzymes from diverse targets, eliminating those lacking synergism with the cocktail before evaluation in high solids concentrations. Future research could investigate whether specific enzyme cocktails tailored to the substrate needs could further improve the rate-limiting liquefaction step.

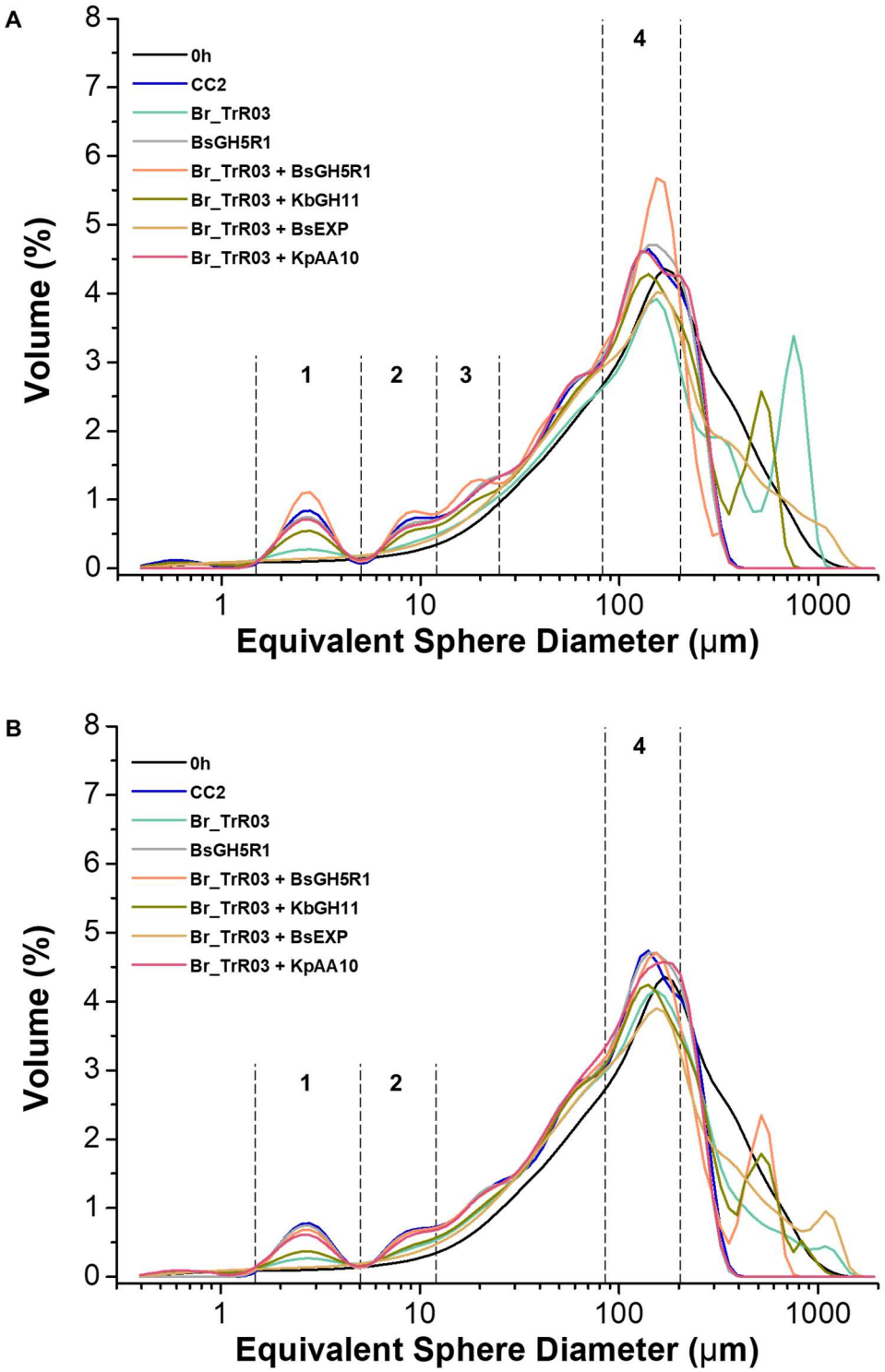
Supplementary data



Supplementary Figure 1: Saccharification efficiency of CN_5:1 enzyme cocktail on pos-processed steam-exploded sugarcane bagasse (AIR-washed PTB) supplemented with inactive and active enzyme targets. Saccharification assays were performed in a high throughput platform with 0.66% (*w/w*) solid content and enzyme cocktail load of 0.5 mgProtein.gCellulose^{-1} and enzyme target load of 0.025 mgProtein.gCellulose^{-1}. Results are expressed as mean ± SD (n = 5, five independent experiments). Scott-Knott post hoc tests ($r < 0.05$) compared Br_TrR03 and Cellic® CTec2 supplementation at 8 hours. Thus, bars sharing the same letter do not present significant differences.



Supplementary Figure 2: Enzymatic saccharification kinetics of Br_TrR03 enzyme cocktail supplemented with active enzyme targets on pos-processed steam-exploded sugarcane bagasse (WMPTB) for cellulose (A) and hemicellulose (B). Saccharification assays were performed with 15% (*w/w*) solid content and enzyme cocktail load of 5 mgProtein.gCellulose^{-1} and enzyme target load of 0.25 mgProtein.gCellulose^{-1}. Results are expressed as mean ± SD (n = 3, three independent experiments).



Supplementary Figure 3: Particle size distributions of enzymatic saccharification of Br_TrR03 enzyme cocktail supplemented with active enzyme targets on pos-processed steam-exploded sugarcane bagasse (WMPTB) at 4 hours (A) and 6 hours (B). Saccharification assays were performed with 15% (*w/w*) solid content and enzyme cocktail load of 5 mgProtein.gCellulose^{-1} and enzyme target load of 0.25 mgProtein.gCellulose^{-1}. Results are expressed as mean (n = 3, three independent experiments).

References

Adsul, M., Sandhu, S.K., Singhania, R.R., Gupta, R., Puri, S.K., Mathur, A., 2020. Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels. Enzyme Microb Technol. https://doi.org/10.1016/j.enzmictec.2019.109442

Baral, P., Jain, L., Kurmi, A.K., Kumar, V., Agrawal, D., 2020. Augmented hydrolysis of acid pretreated sugarcane bagasse by PEG 6000 addition: a case study of Cellic CTec2 with recycling and reuse. Bioprocess Biosyst Eng 43, 473–482. https://doi.org/10.1007/s00449-019-02241-3

Bischof, R.H., Ramoni, J., Seiboth, B., 2016. Cellulases and beyond: The first 70 years of the enzyme producer Trichoderma reesei. Microb Cell Fact. https://doi.org/10.1186/s12934-016-0507-6

Cannella, D., Jørgensen, H., 2014. Do New Cellulolytic Enzyme Preparations Affect the Industrial Strategies for High Solids Lignocellulosic Ethanol Production? Biotechnol. Bioeng 111, 59–68. https://doi.org/10.1002/bit.25098/abstract

Chandrasekar, M., Joshi, L., Krieg, K., Chipkar, S., Burke, E., Debrauske, D.J., Thelen, K.D., Sato, T.K., Ong, R.G., 2021. A high solids field-to-fuel research pipeline to identify interactions between feedstocks and biofuel production. Biotechnol Biofuels 14. https://doi.org/10.1186/s13068-021-02033-6

Chen, H.Z., Liu, Z.H., 2017. Enzymatic hydrolysis of lignocellulosic biomass from low to high solids loading. Eng Life Sci. https://doi.org/10.1002/elsc.201600102

Chen, X., Crawford, N., Wang, W., Kuhn, E., Sievers, D., Tao, L., Tucker, M., 2019. Kinetics and Rheological Behavior of Higher Solid (Solids >20%) Enzymatic Hydrolysis Reactions Using Dilute Acid Pretreated, Deacetylation and Disk Refined, and Deacetylation and Mechanical Refined (DMR) Corn Stover Slurries. ACS Sustain Chem Eng 7, 1633–1641. https://doi.org/10.1021/acssuschemeng.8b05391

Da Silva, A.S.A., Espinheira, R.P., Teixeira, R.S.S., De Souza, M.F., Ferreira-Leitão, V., Bon, E.P.S., 2020. Constraints and advances in high-solids enzymatic hydrolysis of lignocellulosic biomass: A critical review. Biotechnol Biofuels. https://doi.org/10.1186/s13068-020-01697-w

de Lima, E.A., Mandelli, F., Kolling, D., Matsusato Souza, J., de Oliveira Filho, C.A., Ribeiro da Silva, M., Lobo de Mesquita Sampaio, I., Lopes Junqueira, T., Ferreira Chagas, M., Teodoro, J.C., de Morais, E.R., Murakami, M.T., 2022. Development of an economically

competitive Trichoderma-based platform for enzyme production: Bioprocess optimization, pilot plant scale-up, techno-economic analysis and life cycle assessment. Bioresour Technol 364. https://doi.org/10.1016/j.biortech.2022.128019

Ding, S., Liu, X., Hakulinen, N., Taherzadeh, M.J., Wang, Yaru, Wang, Yuan, Qin, X., Wang, X., Yao, B., Luo, H., Tu, T., 2022. Boosting enzymatic degradation of cellulose using a fungal expansin: Structural insight into the pretreatment mechanism. Bioresour Technol 358. https://doi.org/10.1016/j.biortech.2022.127434

Du, J., Liang, J., Gao, X., Liu, G., Qu, Y., 2020. Optimization of an artificial cellulase cocktail for high-solids enzymatic hydrolysis of cellulosic materials with different pretreatment methods. Bioresour Technol 295. https://doi.org/10.1016/j.biortech.2019.122272

Du, J., Zhang, F., Li, Y., Zhang, H., Liang, J., Zheng, H., Huang, H., 2014. Enzymatic liquefaction and saccharification of pretreated corn stover at high-solids concentrations in a horizontal rotating bioreactor. Bioprocess Biosyst Eng 37, 173–181. https://doi.org/10.1007/s00449-013-0983-6

Du, L., Ma, L., Ma, Q., Guo, G., Han, X., Xiao, D., 2018. Hydrolytic boosting of lignocellulosic biomass by a fungal lytic polysaccharide monooxygenase, AnLPMO15g from Aspergillus niger. Ind Crops Prod 126, 309–315. https://doi.org/10.1016/j.indcrop.2018.10.029

Espinheira, R.P., Rocha, V.A.L., Guimarães, T.M., Oliveira, C.A., de Souza, M.F., Domont, G.B., Nogueira, F.C.S., Teixeira, R.S.S., Bon, E.P. da S., Silva, A.S.A. da, 2022. *Aspergillus awamori* endoglucanase-rich supernatant enhances lignocellulosic biomass liquefaction in high-solids enzymatic hydrolysis. Biochem Eng J 183. https://doi.org/10.1016/j.bej.2022.108448

Fockink, D.H., Urio, M.B., Sánchez, J.H., Ramos, L.P., 2017. Enzymatic Hydrolysis of Steam-Treated Sugarcane Bagasse: Effect of Enzyme Loading and Substrate Total Solids on Its Fractal Kinetic Modeling and Rheological Properties. Energy and Fuels 31, 6211–6220. https://doi.org/10.1021/acs.energyfuels.7b00818

Fonseca, L.M., Parreiras, L.S., Murakami, M.T., 2020. Rational engineering of the Trichoderma reesei RUT-C30 strain into an industrially relevant platform for cellulase production. Biotechnol Biofuels 13. https://doi.org/10.1186/s13068-020-01732-w

Ghose, T.K., 1987. Measurement of cellulase activities. Pure and Applied Chemistry 59, 695–702.

Gomez, L.D., Whitehead, C., Barakate, A., Halpin, C., McQueen-Mason, S.J., 2010. Automated saccharification assay for determination of digestibility in plant materials. Biotechnol Biofuels 3, 1–12. https://doi.org/10.1186/1754-6834-3-23

Gourlay, K., Hu, J., Arantes, V., Andberg, M., Saloheimo, M., Penttilä, M., Saddler, J., 2013. Swollenin aids in the amorphogenesis step during the enzymatic hydrolysis of pretreated biomass. Bioresour Technol 142, 498–503. https://doi.org/10.1016/j.biortech.2013.05.053

Gourlay, K., van der Zwan, T., Shourav, M., Saddler, J., 2018. The potential of endoglucanases to rapidly and specifically enhance the rheological properties of micro/nanofibrillated cellulose. Cellulose 25, 977–986. https://doi.org/10.1007/s10570-017-1637-7

Gupta, V.K., Kubicek, C.P., Berrin, J.G., Wilson, D.W., Couturier, M., Berlin, A., Filho, E.X.F., Ezeji, T., 2016. Fungal Enzymes for Bio-Products from Sustainable and Waste Biomass. Trends Biochem Sci 41, 633–645. https://doi.org/10.1016/j.tibs.2016.04.006

Himmel, M.E., Abbas, C.A., Baker, J.O., Bayer, E.A., Bomble, Y.J., Brunecky, R., Chen, X., Felby, C., Jeoh, T., Kumar, R., McCleary, B. V., Pletschke, B.I., Tucker, M.P., Wyman, C.E., Decker, S.R., 2017. Undefined cellulase formulations hinder scientific reproducibility. Biotechnol Biofuels 10. https://doi.org/10.1186/s13068-017-0974-y

Jørgensen, H., Vibe-Pedersen, J., Larsen, J., Felby, C., 2007. Liquefaction of Lignocellulose at High-Solids Concentrations. Biotechnol Bioeng 96, 862–870.

Ju, X., Bowden, M., Engelhard, M., Zhang, X., 2014. Investigating commercial cellulase performances toward specific biomass recalcitrance factors using reference substrates. Appl Microbiol Biotechnol 98, 4409–4420. https://doi.org/10.1007/s00253-013-5450-4

Junior, A.T., Dolce, L.G., de Oliveira Neto, M., Polikarpov, I., 2015. *Xanthomonas campestris* expansin-like X domain is a structurally disordered beta-sheet macromolecule capable of synergistically enhancing enzymatic efficiency of cellulose hydrolysis. Biotechnol Lett 37, 2419–2426. https://doi.org/10.1007/s10529-015-1927-9

Kristensen, J.B., Felby, C., Jørgensen, H., 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol Biofuels 2. https://doi.org/10.1186/1754-6834-2-11

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275. https://doi.org/https://doi.org/10.1016/S0021-9258(19)52451-6

Lu, Y., Wang, Y., Xu, G., Chu, J., Zhuang, Y., Zhang, S., 2010. Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass. Appl Biochem Biotechnol 160, 360–369. https://doi.org/10.1007/s12010-008-8306-0

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E., 2008. How biotech can transform biofuels. Nat Biotechnol 26, 169–72. https://doi.org/10.1038/nbt0208-169

Modenbach, A. a., Nokes, S.E., 2013. Enzymatic hydrolysis of biomass at high-solids loadings – A review. Biomass Bioenergy 56, 526–544. https://doi.org/10.1016/j.biombioe.2013.05.031

Monclaro, A.V., Gorgulho Silva, C. de O., Gomes, H.A.R., Moreira, L.R. de S., Filho, E.X.F., 2022. The enzyme interactome concept in filamentous fungi linked to biomass valorization. Bioresour Technol 344. https://doi.org/10.1016/j.biortech.2021.126200

Mukasekuru, M.R., Kaneza, P., Sun, H., Sun, F.F., He, J., Zheng, P., 2020. Fed-batch high-solids enzymatic saccharification of lignocellulosic substrates with a combination of additives and accessory enzymes. Ind Crops Prod 146. https://doi.org/10.1016/j.indcrop.2020.112156

Muraleedharan, M.N., Zouraris, D., Karantonis, A., Topakas, E., Sandgren, M., Rova, U., Christakopoulos, P., Karnaouri, A., 2018. Effect of lignin fractions isolated from different biomass sources on cellulose oxidation by fungal lytic polysaccharide monooxygenases. Biotechnol Biofuels 11. https://doi.org/10.1186/s13068-018-1294-6

Novozymes, 2010. Fuel ethanol application sheet Cellic® CTec2 and HTec2-Enzymes for hydrolysis of lignocellulosic materials.

Rocha, G.J. de M., Nascimento, V.M., Gonçalves, A.R., Silva, V.F.N., Martín, C., 2015. Influence of mixed sugarcane bagasse samples evaluated by elemental and physical-chemical composition. Ind Crops Prod 64, 52–58. https://doi.org/10.1016/j.indcrop.2014.11.003

Roche, C.M., Dibble, C.J., Stickel, J.J., 2009. Laboratory-scale method for enzymatic saccharification of lignocellulosic biomass at high-solids loadings. Biotechnol Biofuels 2, 1–11. https://doi.org/10.1186/1754-6834-2-28

Sant'Ana da Silva, A., Fernandes de Souza, M., Ballesteros, I., Manzanares, P., Ballesteros, M., P. S. Bon, E., 2016. High-solids content enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse using a laboratory-made enzyme blend and commercial preparations. Process Biochemistry 51, 1561–1567.

Shi, Y., Chen, K., Long, L., Ding, S., 2021. A highly xyloglucan active lytic polysaccharide monooxygenase EpLPMO9A from *Eupenicillium parvum* 4-14 shows boosting effect on hydrolysis of complex lignocellulosic substrates. Int J Biol Macromol 167, 202–213. https://doi.org/10.1016/j.ijbiomac.2020.11.177

Skovgaard, P.A., Thygesen, L.G., Jørgensen, H., Cardona, M., Tozzi, E., Mccarthy, M., Siika-Aho, M., Jeoh, T., 2014. The role of endoglucanase and endoxylanase in liquefaction of hydrothermally pretreated wheat straw. Biotechnol Prog 30, 923–931. https://doi.org/10.1002/btpr.1893

Smuts, I.E., Blakeway, N.J., Rose, S.H., den Haan, R., Viljoen-Bloom, M., van Zyl, W.H., 2023. Supplementation of recombinant cellulases with LPMOs and CDHs improves consolidated bioprocessing of cellulose. Enzyme Microb Technol 164. https://doi.org/10.1016/j.enzmictec.2022.110171

Sun, C., Meng, X., Sun, F., Zhang, J., Tu, M., Chang, J.S., Reungsang, A., Xia, A., Ragauskas, A.J., 2023. Advances and perspectives on mass transfer and enzymatic hydrolysis in the enzyme-mediated lignocellulosic biorefinery: A review. Biotechnol Adv. https://doi.org/10.1016/j.biotechadv.2022.108059

Szijártó, Nára, Horan, E., Zhang, J., Puranen, T., Siika-Aho, M., Viikari, L., 2011a. Thermostable endoglucanases in the liquefaction of hydrothermally pretreated wheat straw. Biotechnol Biofuels 4. https://doi.org/10.1186/1754-6834-4-2

Szijártó, Nóra, Siika-aho, M., Sontag-Strohm, T., Viikari, L., 2011b. Liquefaction of hydrothermally pretreated wheat straw at high-solids content by purified Trichoderma enzymes. Bioresour Technol 102, 1968–1974. https://doi.org/10.1016/j.biortech.2010.09.012

Thygesen, L.G., Thybring, E.E., Johansen, K.S., Felby, C., 2014. The mechanisms of plant cell wall deconstruction during enzymatic hydrolysis. PLoS One 9. https://doi.org/10.1371/journal.pone.0108313

van der Zwan, T., Hu, J., Saddler, J.N., 2017. Mechanistic insights into the liquefaction stage of enzyme-mediated biomass deconstruction. Biotechnol Bioeng 114, 2489–2496. https://doi.org/10.1002/bit.26381

van der Zwan, T., Sigg, A., Hu, J., Chandra, R.P., Saddler, J.N., 2020. Enzyme-Mediated Lignocellulose Liquefaction Is Highly Substrate-Specific and Influenced by the Substrate Concentration or Rheological Regime. Front Bioeng Biotechnol 8. https://doi.org/10.3389/fbioe.2020.00917

Velasco, J., de Oliveira Arnoldi Pellegrini, V., Sepulchro, A.G.V., Kadowaki, M.A.S., Santo, M.C.E., Polikarpov, I., Segato, F., 2021. Comparative analysis of two recombinant LPMOs from *Aspergillus fumigatus* and their effects on sugarcane bagasse saccharification. Enzyme Microb Technol 144. https://doi.org/10.1016/j.enzmictec.2021.109746

Weiss, N.D., Felby, C., Thygesen, L.G., 2019. Enzymatic hydrolysis is limited by biomass-water interactions at high-solids: Improved performance through substrate modifications. Biotechnol Biofuels 12. https://doi.org/10.1186/s13068-018-1339-x

Westereng, B., Cannella, D., Wittrup Agger, J., Jørgensen, H., Larsen Andersen, M., Eijsink, V.G.H., Felby, C., 2015. Enzymatic cellulose oxidation is linked to lignin by long-range electron transfer. Sci Rep 5. https://doi.org/10.1038/srep18561

Yang, B., Wyman, C.E., 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol Bioeng 94, 611–617. https://doi.org/10.1002/bit.20750

Zhang, J., Tuomainen, P., Siika-aho, M., Viikari, L., 2011. Comparison of the synergistic action of two thermostable xylanases from GH families 10 and 11 with thermostable cellulases in lignocellulose hydrolysis. Bioresour Technol 102, 9090–9095. https://doi.org/10.1016/j.biortech.2011.06.085

Zhang, P., Su, R., Duan, Y., Cui, M., Huang, R., Qi, W., He, Z., Thielemans, W., 2021. Synergy between endo/exo-glucanases and expansin enhances enzyme adsorption and cellulose conversion. Carbohydr Polym 253. https://doi.org/10.1016/j.carbpol.2020.117287

Chapter 4

Concluding remarks

There are many challenges involved in establishing a lignocellulosic biorefinery such as high capital and operating costs, technical immaturity, and difficulties in scaling up. This work shed light on some of them, focusing on the development of sugarcane biorefinery through technological and scientific means.

In Chapter 2, we evaluated the transformation promoted in the sugarcane bagasse by high-consistency mechanical refining realized subsequently to low severity pretreatments, both at the pilot scale. When the pretreatments are compared without refining the process, the steam-exploded bagasse (SEPTB) achieves an enzymatic digestibility 20% higher than the hydrothermal one. Still, an additional 8% increase was found when the SEPTB was subjected to mechanical refining. The results highlight the dependence of the refining reactivity on the biomass type and pretreatment and suggest it could be a promising alternative for reducing biomass recalcitrance and enhancing enzymatic digestibility, potentially leading to cost reduction and increased sugar release.

In Chapter 3, through our work, we could identify an endoglucanase from *Bacillus subtilis* BsGH5R1 as a potential candidate for being added to the engineered RUT-C30 strain by genetic modification. It was demonstrated that a high-throughput platform could be effectively used in the enzyme selection from a diverse source of targets, eliminating those lacking synergism.

Overall, this study advances the knowledge concerning technical aspects of pilot-scale high-consistency mechanical refining associated with pretreatment in sugarcane biorefineries, complementing the current data available for hardwoods and corn stover and providing a better understanding of the fiber reactivity modifications. Furthermore, it provides a list of promising candidates to be genetically inserted into the *T. reesei* RUT-C30 to enhance saccharification of pretreated sugarcane bagasse.

Suggested Future Works

The biochemical route is one of the most promising strategies for establishing a lignocellulosic biorefinery. However, to achieve the viable sugar titer of 80g/L, enzymatic hydrolysis must be conducted at high solids (loads >15%). Working under such conditions present several challenges, such as water constraints, mass transfer, long hydrolysis time, slow

liquefaction, and high enzyme loads, to name a few. The findings from this study have contributed to advance in the development of more efficient and sustainable processes for lignocellulosic depolymerization and highlight critical areas of focus that are recommended for future work. These are described as follows:

Techno-economic assessment to evaluate the mechanical refining viability

The results from the present thesis showed that a higher increase in cellulose conversion could be achieved when the pretreatment strategy was altered through mechanical refining. Consequently, further research must be conducted focused on techno-economic analysis to determine in which conditions the mechanical refining is more promising.

Investigation of tailored enzyme cocktails to improve the rate-limiting step: liquefaction

This thesis demonstrated that the enzymatic liquefaction of sugarcane bagasse slurries is highly dependent on the formulation of the applied enzyme cocktail. These results highlight the need of further research to identify or discover enzymes that can effectively catalyze the viscosity reduction at high solids loadings for substrates with different compositions, promoting the increase in sugar release. Considering the substrate-specific nature of enzymatic saccharification, this effort should be tracked in combination and complemented by pretreatment optimization.

Reactors scale down to investigate the performance of enzyme cocktails at high solids load

We could not measure the viscosity nor yield stress from our substrate from the market-available equipment evaluated in this study. This limitation presents an opportunity for future research to determine the minimum reactor scale that could capture industrial conditions, working in scales below than 500 mL. The reactor could be designed using computational fluid dynamics modeling and should integrate sensor technology to provide scale-translatable data and enable isolating or correlating parameters. Results from these scaled-down reactors could provide valuable data for larger-scale reactor optimization.

References

Adsul, M., Sandhu, S.K., Singhania, R.R., Gupta, R., Puri, S.K., Mathur, A., 2020. Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels. Enzyme Microb Technol. https://doi.org/10.1016/j.enzmictec.2019.109442

Andrade, L.P., Crespim, E., de Oliveira, N., de Campos, R.C., Teodoro, J.C., Galvão, C.M.A., Maciel Filho, R., 2017. Influence of sugarcane bagasse variability on sugar recovery for cellulosic ethanol production. Bioresour Technol 241, 75–81. https://doi.org/10.1016/j.biortech.2017.05.081

Arantes, V., Saddler, J.N., 2010. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol Biofuels 3, 4. https://doi.org/10.1186/1754-6834-3-4

Arce, C., Kratky, L., 2022. Mechanical pretreatment of lignocellulosic biomass toward enzymatic/fermentative valorization. iScience 25. https://doi.org/https://doi.org/10.1016/j.isci.2022.104610

Baral, P., Jain, L., Kurmi, A.K., Kumar, V., Agrawal, D., 2020. Augmented hydrolysis of acid pretreated sugarcane bagasse by PEG 6000 addition: a case study of Cellic CTec2 with recycling and reuse. Bioprocess Biosyst Eng 43, 473–482. https://doi.org/10.1007/s00449-019-02241-3

Batalha, L.A.R., Han, Q., Jameel, H., Chang, H. min, Colodette, J.L., Borges Gomes, F.J., 2015. Production of fermentable sugars from sugarcane bagasse by enzymatic hydrolysis after autohydrolysis and mechanical refining. Bioresour Technol 180, 97–105. https://doi.org/10.1016/j.biortech.2014.12.060

Bischof, R.H., Ramoni, J., Seiboth, B., 2016. Cellulases and beyond: The first 70 years of the enzyme producer Trichoderma reesei. Microb Cell Fact. https://doi.org/10.1186/s12934-016-0507-6

Cannella, D., Jørgensen, H., 2014. Do New Cellulolytic Enzyme Preparations Affect the Industrial Strategies for High Solids Lignocellulosic Ethanol Production? Biotechnol. Bioeng 111, 59–68. https://doi.org/10.1002/bit.25098/abstract

Chandrasekar, M., Joshi, L., Krieg, K., Chipkar, S., Burke, E., Debrauske, D.J., Thelen, K.D., Sato, T.K., Ong, R.G., 2021. A high solids field-to-fuel research pipeline to identify interactions between feedstocks and biofuel production. Biotechnol Biofuels 14. https://doi.org/10.1186/s13068-021-02033-6

Chen, H.Z., Liu, Z.H., 2017. Enzymatic hydrolysis of lignocellulosic biomass from low to high solids loading. Eng Life Sci. https://doi.org/10.1002/elsc.201600102

Chen, X., Crawford, N., Wang, W., Kuhn, E., Sievers, D., Tao, L., Tucker, M., 2019. Kinetics and Rheological Behavior of Higher Solid (Solids >20%) Enzymatic Hydrolysis Reactions Using Dilute Acid Pretreated, Deacetylation and Disk Refined, and Deacetylation and Mechanical Refined (DMR) Corn Stover Slurries. ACS Sustain Chem Eng 7, 1633–1641. https://doi.org/10.1021/acssuschemeng.8b05391

Chen, X., Shekiro, J., Pschorn, T., Sabourin, M., Tao, L., Elander, R., Park, S., Jennings, E., Nelson, R., Trass, O., Flanegan, K., Wang, W., Himmel, M.E., Johnson, D., Tucker, M.P., 2014. A highly efficient dilute alkali deacetylation and mechanical (disc) refining process for the conversion of renewable biomass to lower cost sugars. Biotechnol Biofuels 7. https://doi.org/10.1186/1754-6834-7-98

Chylenski, P., Forsberg, Z., Ståhlberg, J., Várnai, A., Lersch, M., Bengtsson, O., Sæbø, S., Horn, S.J., Eijsink, V.G.H., 2017. Development of minimal enzyme cocktails for hydrolysis of sulfite-pulped lignocellulosic biomass. J Biotechnol 246, 16–23. https://doi.org/10.1016/j.jbiotec.2017.02.009

Corbett, D.B., Knoll, C., Venditti, R., Jameel, H., Park, S., 2020. Fiber fractionation to understand the effect of mechanical refining on fiber structure and resulting enzymatic digestibility of biomass. Biotechnol Bioeng 117, 924–932. https://doi.org/10.1002/bit.27258

Corbett, D.B., Venditti, R., Jameel, H., Park, S., 2018. Effect of Mechanical Refining Energy on the Enzymatic Digestibility of Lignocellulosic Biomass. Ind Eng Chem Res 57, 14648–14655. https://doi.org/10.1021/acs.iecr.8b02932

Corrêa, T.L.R., Júnior, A.T., Wolf, L.D., Buckeridge, M.S., Dos Santos, L.V., Murakami, M.T., 2019. An actinobacteria lytic polysaccharide monooxygenase acts on both cellulose and xylan to boost biomass saccharification. Biotechnol Biofuels 12. https://doi.org/10.1186/s13068-019-1449-0

Da Silva, A.S.A., Espinheira, R.P., Teixeira, R.S.S., De Souza, M.F., Ferreira-Leitão, V., Bon, E.P.S., 2020. Constraints and advances in high-solids enzymatic hydrolysis of lignocellulosic biomass: A critical review. Biotechnol Biofuels. https://doi.org/10.1186/s13068-020-01697-w

De Assis, T., Huang, S., Driemeier, C.E., Donohoe, B.S., Kim, C., Kim, S.H., Gonzalez, R., Jameel, H., Park, S., 2018. Toward an understanding of the increase in enzymatic hydrolysis

by mechanical refining. Biotechnol Biofuels 11, 1–11. https://doi.org/10.1186/s13068-018-1289-3

de Lima, E.A., Mandelli, F., Kolling, D., Matsusato Souza, J., de Oliveira Filho, C.A., Ribeiro da Silva, M., Lobo de Mesquita Sampaio, I., Lopes Junqueira, T., Ferreira Chagas, M., Teodoro, J.C., de Morais, E.R., Murakami, M.T., 2022. Development of an economically competitive Trichoderma-based platform for enzyme production: Bioprocess optimization, pilot plant scale-up, techno-economic analysis and life cycle assessment. Bioresour Technol 364. https://doi.org/10.1016/j.biortech.2022.128019

Ding, S., Liu, X., Hakulinen, N., Taherzadeh, M.J., Wang, Yaru, Wang, Yuan, Qin, X., Wang, X., Yao, B., Luo, H., Tu, T., 2022. Boosting enzymatic degradation of cellulose using a fungal expansin: Structural insight into the pretreatment mechanism. Bioresour Technol 358. https://doi.org/10.1016/j.biortech.2022.127434

Du, J., Liang, J., Gao, X., Liu, G., Qu, Y., 2020. Optimization of an artificial cellulase cocktail for high-solids enzymatic hydrolysis of cellulosic materials with different pretreatment methods. Bioresour Technol 295. https://doi.org/10.1016/j.biortech.2019.122272

Du, J., Zhang, F., Li, Y., Zhang, H., Liang, J., Zheng, H., Huang, H., 2014. Enzymatic liquefaction and saccharification of pretreated corn stover at high-solids concentrations in a horizontal rotating bioreactor. Bioprocess Biosyst Eng 37, 173–181. https://doi.org/10.1007/s00449-013-0983-6

Du, L., Ma, L., Ma, Q., Guo, G., Han, X., Xiao, D., 2018. Hydrolytic boosting of lignocellulosic biomass by a fungal lytic polysaccharide monooxygenase, AnLPMO15g from Aspergillus niger. Ind Crops Prod 126, 309–315. https://doi.org/10.1016/j.indcrop.2018.10.029

Espinheira, R.P., Rocha, V.A.L., Guimarães, T.M., Oliveira, C.A., de Souza, M.F., Domont, G.B., Nogueira, F.C.S., Teixeira, R.S.S., Bon, E.P. da S., Silva, A.S.A. da, 2022. *Aspergillus awamori* endoglucanase-rich supernatant enhances lignocellulosic biomass liquefaction in high-solids enzymatic hydrolysis. Biochem Eng J 183. https://doi.org/10.1016/j.bej.2022.108448

Espirito Santo, M.C., Fockink, D.H., Pellegrini, V.O.A., Guimaraes, F.E.G., deAzevedo, E.R., Ramos, L.P., Polikarpov, I., 2020. Physical techniques shed light on the differences in sugarcane bagasse structure subjected to steam explosion pretreatments at equivalent combined severity factors. Ind Crops Prod 158. https://doi.org/10.1016/j.indcrop.2020.113003

Fengel, D., and Wegener, G. (1989). Wood – Chemistry, Ultrastructure, Reactions. Berlin, Germany: De Gruyter.

Fockink, D.H., Urio, M.B., Sánchez, J.H., Ramos, L.P., 2017. Enzymatic Hydrolysis of Steam-Treated Sugarcane Bagasse: Effect of Enzyme Loading and Substrate Total Solids on Its Fractal Kinetic Modeling and Rheological Properties. Energy and Fuels 31, 6211–6220. https://doi.org/10.1021/acs.energyfuels.7b00818

Fonseca, L.M., Parreiras, L.S., Murakami, M.T., 2020. Rational engineering of the Trichoderma reesei RUT-C30 strain into an industrially relevant platform for cellulase production. Biotechnol Biofuels 13. https://doi.org/10.1186/s13068-020-01732-w

García, J.M., Molina, C., Simister, R., Taibo, C.B., Setten, L., Erazzú, L.E., Gómez, L.D., Acevedo, A., 2023. Chemical and histological characterization of internodes of sugarcane and energy-cane hybrids throughout plant development. Ind Crops Prod 199. https://doi.org/10.1016/j.indcrop.2023.116739

Gharehkhani, S., Sadeghinezhad, E., Kazi, S.N., Yarmand, H., Badarudin, A., Safaei, M.R., Zubir, M.N.M., 2015. Basic effects of pulp refining on fiber properties - A review. Carbohydr Polym. https://doi.org/10.1016/j.carbpol.2014.08.047

Ghose, T.K., 1987. Measurement of cellulase activities. Pure and Applied Chemistry 59, 695–702.

Gomez, L.D., Whitehead, C., Barakate, A., Halpin, C., McQueen-Mason, S.J., 2010. Automated saccharification assay for determination of digestibility in plant materials. Biotechnol Biofuels 3, 1–12. https://doi.org/10.1186/1754-6834-3-23

Gourlay, K., Hu, J., Arantes, V., Andberg, M., Saloheimo, M., Penttilä, M., Saddler, J., 2013. Swollenin aids in the amorphogenesis step during the enzymatic hydrolysis of pretreated biomass. Bioresour Technol 142, 498–503. https://doi.org/10.1016/j.biortech.2013.05.053

Gourlay, K., van der Zwan, T., Shourav, M., Saddler, J., 2018. The potential of endoglucanases to rapidly and specifically enhance the rheological properties of micro/nanofibrillated cellulose. Cellulose 25, 977–986. https://doi.org/10.1007/s10570-017-1637-7

Gupta, V.K., Kubicek, C.P., Berrin, J.G., Wilson, D.W., Couturier, M., Berlin, A., Filho, E.X.F., Ezeji, T., 2016. Fungal Enzymes for Bio-Products from Sustainable and Waste Biomass. Trends Biochem Sci 41, 633–645. https://doi.org/10.1016/j.tibs.2016.04.006

Hernández-Beltrán, J.U., Hernández-De Lira, I.O., Cruz-Santos, M.M., Saucedo-Luevanos, A., Hernández-Terán, F., Balagurusamy, N., 2019. Insight into pretreatment methods of lignocellulosic biomass to increase biogas yield: Current state, challenges, and opportunities. Applied Sciences (Switzerland). https://doi.org/10.3390/app9183721

Himmel, M.E., Abbas, C.A., Baker, J.O., Bayer, E.A., Bomble, Y.J., Brunecky, R., Chen, X., Felby, C., Jeoh, T., Kumar, R., McCleary, B. V., Pletschke, B.I., Tucker, M.P., Wyman,

C.E., Decker, S.R., 2017. Undefined cellulase formulations hinder scientific reproducibility. Biotechnol Biofuels 10. https://doi.org/10.1186/s13068-017-0974-y

IPCC Working Group III, 2023. Climate Change 2023: Sixth Assessment Report (AR6), Diriba Korecha Dadi. Panmao Zhai, Interlaken.

Jeoh, T., Santa-Maria, M.C., O'Dell, P.J., 2013. Assessing cellulose microfibrillar structure changes due to cellulose action. Carbohydr Polym 97, 581–586. https://doi.org/10.1016/j.carbpol.2013.05.027

Jones, B.W., Venditti, R., Park, S., Jameel, H., 2017. Optimization of pilot scale mechanical disk refining for improvements in enzymatic digestibility of pretreated hardwood lignocellulosics. Bioresources 12, 4567–4593. https://doi.org/10.15376/biores.12.3.4567-4593

Jones, B.W., Venditti, R., Park, S., Jameel, H., Koo, B., 2013. Enhancement in enzymatic hydrolysis by mechanical refining for pretreated hardwood lignocellulosics. Bioresour Technol 147, 353–360. https://doi.org/10.1016/j.biortech.2013.08.030

Jørgensen, H., Vibe-Pedersen, J., Larsen, J., Felby, C., 2007. Liquefaction of Lignocellulose at High-Solids Concentrations. Biotechnol Bioeng 96, 862–870.

Jørgensen, H., Vibe-Pedersen, J., Larsen, J., Felby, C., 2007. Liquefaction of Lignocellulose at High-Solids Concentrations. Biotechnol Bioeng 96, 862–870.

Ju, X., Bowden, M., Engelhard, M., Zhang, X., 2014. Investigating commercial cellulase performances toward specific biomass recalcitrance factors using reference substrates. Appl Microbiol Biotechnol 98, 4409–4420. https://doi.org/10.1007/s00253-013-5450-4

Junior, A.T., Dolce, L.G., de Oliveira Neto, M., Polikarpov, I., 2015. Xanthomonas campestris expansin-like X domain is a structurally disordered beta-sheet macromolecule capable of synergistically enhancing enzymatic efficiency of cellulose hydrolysis. Biotechnol Lett 37, 2419–2426. https://doi.org/10.1007/s10529-015-1927-9

Khan, M.U., Usman, M., Ashraf, M.A., Dutta, N., Luo, G., Zhang, S., 2022. A review of recent advancements in pretreatment techniques of lignocellulosic materials for biogas production: Opportunities and Limitations. Chemical Engineering Journal Advances. https://doi.org/10.1016/j.ceja.2022.100263

Koo, B., Park, J., Gonzalez, R., Jameel, H., Park, S., 2019. Two-stage autohydrolysis and mechanical treatment to maximize sugar recovery from sweet sorghum bagasse. Bioresour Technol 276, 140–145. https://doi.org/10.1016/j.biortech.2018.12.112

Koo, B.W., Treasure, T.H., Jameel, H., Phillips, R.B., Chang, H.M., Park, S., 2011. Reduction of enzyme dosage by oxygen delignification and mechanical refining for enzymatic

hydrolysis of green liquor-pretreated hardwood. Appl Biochem Biotechnol 165, 832–844. https://doi.org/10.1007/s12010-011-9301-4

Kristensen, J.B., Felby, C., Jørgensen, H., 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol Biofuels 2. https://doi.org/10.1186/1754-6834-2-11

Leal Silva, J.F., Mariano, A.P., Maciel Filho, R., 2021. Less severe reaction conditions to produce levulinic acid with reduced humins formation at the expense of lower biomass conversion: Is it economically feasible? Fuel Communications 9, 100029. https://doi.org/10.1016/j.jfueco.2021.100029

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275. https://doi.org/https://doi.org/10.1016/S0021-9258(19)52451-6

Lu, Y., Wang, Y., Xu, G., Chu, J., Zhuang, Y., Zhang, S., 2010. Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass. Appl Biochem Biotechnol 160, 360–369. https://doi.org/10.1007/s12010-008-8306-0

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E., 2008. How biotech can transform biofuels. Nat Biotechnol 26, 169–72. https://doi.org/10.1038/nbt0208-169

Mamo, G., 2020. Alkaline Active Hemicellulases, in: Advances in Biochemical Engineering/Biotechnology. Springer, pp. 245–291. https://doi.org/10.1007/10_2019_101

Martín, C., Dixit, P., Momayez, F., Jönsson, L.J., 2022. Hydrothermal Pretreatment of Lignocellulosic Feedstocks to Facilitate Biochemical Conversion. Front Bioeng Biotechnol. https://doi.org/10.3389/fbioe.2022.846592

Modenbach, A. a., Nokes, S.E., 2013. Enzymatic hydrolysis of biomass at high-solids loadings – A review. Biomass Bioenergy 56, 526–544. https://doi.org/10.1016/j.biombioe.2013.05.031

Monclaro, A.V., Gorgulho Silva, C. de O., Gomes, H.A.R., Moreira, L.R. de S., Filho, E.X.F., 2022. The enzyme interactome concept in filamentous fungi linked to biomass valorization. Bioresour Technol 344. https://doi.org/10.1016/j.biortech.2021.126200

Mukasekuru, M.R., Kaneza, P., Sun, H., Sun, F.F., He, J., Zheng, P., 2020a. Fed-batch high-solids enzymatic saccharification of lignocellulosic substrates with a combination of additives and accessory enzymes. Ind Crops Prod 146. https://doi.org/10.1016/j.indcrop.2020.112156

Muraleedharan, M.N., Zouraris, D., Karantonis, A., Topakas, E., Sandgren, M., Rova, U., Christakopoulos, P., Karnaouri, A., 2018. Effect of lignin fractions isolated from different biomass sources on cellulose oxidation by fungal lytic polysaccharide monooxygenases. Biotechnol Biofuels 11. https://doi.org/10.1186/s13068-018-1294-6

Nagl, M., Haske-Cornelius, O., Bauer, W., Csarman, F., Ludwig, R., Nyanhongo, G.S., Guebitz, G.M., 2022. Towards a better understanding of synergistic enzyme effects during refining of cellulose fibers. Carbohydrate Polymer Technologies and Applications 4. https://doi.org/10.1016/j.carpta.2022.100223

Nargotra, P., Sharma, V., Lee, Y.C., Tsai, Y.H., Liu, Y.C., Shieh, C.J., Tsai, M.L., Dong, C. Di, Kuo, C.H., 2023. Microbial Lignocellulolytic Enzymes for the Effective Valorization of Lignocellulosic Biomass: A Review. Catalysts. https://doi.org/10.3390/catal13010083

Novozymes, 2010. Fuel ethanol application sheet Cellic® CTec2 and HTec2-Enzymes for hydrolysis of lignocellulosic materials.

Olejnik, K., Fabijanska, A., Pelczynski, P., Stanislawska, A., 2019. Controllability of low-consistency chemical pulp refining process. Comput Chem Eng 128, 468–476. https://doi.org/10.1016/j.compchemeng.2019.06.024

Park, J., Jones, B., Koo, B., Chen, X., Tucker, M., Yu, J.H., Pschorn, T., Venditti, R., Park, S., 2016. Use of mechanical refining to improve the production of low-cost sugars from lignocellulosic biomass. Bioresour Technol 199, 59–67. https://doi.org/10.1016/j.biortech.2015.08.059

Ramos, L.P., da Silva, L., Ballem, A.C., Pitarelo, A.P., Chiarello, L.M., Silveira, M.H.L., 2015. Enzymatic hydrolysis of steam-exploded sugarcane bagasse using high total solids and low enzyme loadings. Bioresour Technol 175, 195–202.

Rocha, G.J. de M., Nascimento, V.M., Gonçalves, A.R., Silva, V.F.N., Martín, C., 2015a. Influence of mixed sugarcane bagasse samples evaluated by elemental and physical-chemical composition. Ind Crops Prod 64, 52–58. https://doi.org/10.1016/j.indcrop.2014.11.003

Rocha, G.J.M., Gonçalves, A.R., Nakanishi, S.C., Nascimento, V.M., Silva, V.F.N., 2015b. Pilot scale steam explosion and diluted sulfuric acid pretreatments: Comparative study aiming the sugarcane bagasse saccharification. Ind Crops Prod 74, 810–816.

Rocha, G.J.M., Martín, C., da Silva, V.F.N., Gómez, E.O., Gonçalves, A.R., 2012. Mass balance of pilot-scale pretreatment of sugarcane bagasse by steam explosion followed by alkaline delignification. Bioresour Technol 111, 447–52. https://doi.org/10.1016/j.biortech.2012.02.005

Roche, C.M., Dibble, C.J., Stickel, J.J., 2009. Laboratory-scale method for enzymatic saccharification of lignocellulosic biomass at high-solids loadings. Biotechnol Biofuels 2, 1–11. https://doi.org/10.1186/1754-6834-2-28

Rodrigues, M.I., Iemma, A.F., 2014. Experimental design and process optimization, Experimental Design and Process Optimization. https://doi.org/10.1201/b17848

Ruiz, H.A., Conrad, M., Sun, S.N., Sanchez, A., Rocha, G.J.M., Romaní, A., Castro, E., Torres, A., Rodríguez-Jasso, R.M., Andrade, L.P., Smirnova, I., Sun, R.C., Meyer, A.S., 2020. Engineering aspects of hydrothermal pretreatment: From batch to continuous operation, scale-up and pilot reactor under biorefinery concept. Bioresour Technol. https://doi.org/10.1016/j.biortech.2019.122685

Ruiz, H.A., Sganzerla, W.G., Larnaudie, V., Veersma, R.J., van Erven, G., Shiva, Ríos-González, L.J., Rodríguez-Jasso, R.M., Rosero-Chasoy, G., Ferrari, M.D., Kabel, M.A., Forster-Carneiro, T., Lareo, C., 2023. Advances in process design, techno-economic assessment and environmental aspects for hydrothermal pretreatment in the fractionation of biomass under biorefinery concept. Bioresour Technol. https://doi.org/10.1016/j.biortech.2022.128469

Saini, J.K., Himanshu, Hemansi, Kaur, A., Mathur, A., 2022. Strategies to enhance enzymatic hydrolysis of lignocellulosic biomass for biorefinery applications: A review. Bioresour Technol 360. https://doi.org/10.1016/j.biortech.2022.127517

Sant'Ana da Silva, A., Fernandes de Souza, M., Ballesteros, I., Manzanares, P., Ballesteros, M., P. S. Bon, E., 2016. High-solids content enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse using a laboratory-made enzyme blend and commercial preparations. Process Biochemistry 51, 1561–1567.

Santucci, B.S., Maziero, P., Rabelo, S.C., Curvelo, A.A.S., Pimenta, M.T.B., 2015. Autohydrolysis of Hemicelluloses from Sugarcane Bagasse During Hydrothermal Pretreatment: a Kinetic Assessment. Bioenergy Res 8, 1778–1787. https://doi.org/10.1007/s12155-015-9632-z

Shi, Y., Chen, K., Long, L., Ding, S., 2021. A highly xyloglucan active lytic polysaccharide monooxygenase EpLPMO9A from Eupenicillium parvum 4-14 shows boosting effect on hydrolysis of complex lignocellulosic substrates. Int J Biol Macromol 167, 202–213. https://doi.org/10.1016/j.ijbiomac.2020.11.177

Shiva, Climent Barba, F., Rodríguez-Jasso, R.M., Sukumaran, R.K., Ruiz, H.A., 2022. High-solids loading processing for an integrated lignocellulosic biorefinery: Effects of transport

phenomena and rheology – A review. Bioresour Technol. https://doi.org/10.1016/j.biortech.2022.127044

Skovgaard, P.A., Thygesen, L.G., Jørgensen, H., Cardona, M., Tozzi, E., Mccarthy, M., Siika-Aho, M., Jeoh, T., 2014. The role of endoglucanase and endoxylanase in liquefaction of hydrothermally pretreated wheat straw. Biotechnol Prog 30, 923–931. https://doi.org/10.1002/btpr.1893

Smuts, I.E., Blakeway, N.J., Rose, S.H., den Haan, R., Viljoen-Bloom, M., van Zyl, W.H., 2023. Supplementation of recombinant cellulases with LPMOs and CDHs improves consolidated bioprocessing of cellulose. Enzyme Microb Technol 164. https://doi.org/10.1016/j.enzmictec.2022.110171

Sun, C., Meng, X., Sun, F., Zhang, J., Tu, M., Chang, J.S., Reungsang, A., Xia, A., Ragauskas, A.J., 2023. Advances and perspectives on mass transfer and enzymatic hydrolysis in the enzyme-mediated lignocellulosic biorefinery: A review. Biotechnol Adv. https://doi.org/10.1016/j.biotechadv.2022.108059

Szijártó, Nára, Horan, E., Zhang, J., Puranen, T., Siika-Aho, M., Viikari, L., 2011a. Thermostable endoglucanases in the liquefaction of hydrothermally pretreated wheat straw. Biotechnol Biofuels 4. https://doi.org/10.1186/1754-6834-4-2

Szijártó, Nóra, Siika-aho, M., Sontag-Strohm, T., Viikari, L., 2011b. Liquefaction of hydrothermally pretreated wheat straw at high-solids content by purified Trichoderma enzymes. Bioresour Technol 102, 1968–1974. https://doi.org/10.1016/j.biortech.2010.09.012

Thygesen, L.G., Thybring, E.E., Johansen, K.S., Felby, C., 2014

. The mechanisms of plant cell wall deconstruction during enzymatic hydrolysis. PLoS One 9. https://doi.org/10.1371/journal.pone.0108313

van der Zwan, T., Hu, J., Saddler, J.N., 2017. Mechanistic insights into the liquefaction stage of enzyme-mediated biomass deconstruction. Biotechnol Bioeng 114, 2489–2496. https://doi.org/10.1002/bit.26381

van der Zwan, T., Sigg, A., Hu, J., Chandra, R.P., Saddler, J.N., 2020. Enzyme-Mediated Lignocellulose Liquefaction Is Highly Substrate-Specific and Influenced by the Substrate Concentration or Rheological Regime. Front Bioeng Biotechnol 8. https://doi.org/10.3389/fbioe.2020.00917

Velasco, J., de Oliveira Arnoldi Pellegrini, V., Sepulchro, A.G.V., Kadowaki, M.A.S., Santo, M.C.E., Polikarpov, I., Segato, F., 2021. Comparative analysis of two recombinant LPMOs

from *Aspergillus fumigatus* and their effects on sugarcane bagasse saccharification. Enzyme Microb Technol 144. https://doi.org/10.1016/j.enzmictec.2021.109746

Vermaas, J. v., Petridis, L., Qi, X., Schulz, R., Lindner, B., Smith, J.C., 2015. Mechanism of lignin inhibition of enzymatic biomass deconstruction. Biotechnol Biofuels 8, 1–16. https://doi.org/10.1186/s13068-015-0379-8

Walker, L.P., Wilson, D.B., Irvin, D.C., McQuire, C., Price, M., 1992. Fragmentation of cellulose by the major Thermomonospora fusca cellulases, Trichoderma reesei CBHI, and their mixtures. Biotechnol Bioeng 40, 1019–1026. https://doi.org/10.1002/bit.260400905

Wang, Z., Dien, B.S., Rausch, K.D., Tumbleson, M.E., Singh, V., 2018. Fermentation of undetoxified sugarcane bagasse hydrolyzates using a two stage hydrothermal and mechanical refining pretreatment. Bioresour Technol 261, 313–321. https://doi.org/10.1016/j.biortech.2018.04.018

Weiss, N.D., Felby, C., Thygesen, L.G., 2019. Enzymatic hydrolysis is limited by biomass-water interactions at high-solids: Improved performance through substrate modifications. Biotechnol Biofuels 12. https://doi.org/10.1186/s13068-018-1339-x

Westereng, B., Cannella, D., Wittrup Agger, J., Jørgensen, H., Larsen Andersen, M., Eijsink, V.G.H., Felby, C., 2015. Enzymatic cellulose oxidation is linked to lignin by long-range electron transfer. Sci Rep 5. https://doi.org/10.1038/srep18561

Yang, B., Wyman, C.E., 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol Bioeng 94, 611–617. https://doi.org/10.1002/bit.20750

Yuan, H., Song, X., Guan, R., Zhang, L., Li, X., Zuo, X., 2019. Effect of low severity hydrothermal pretreatment on anaerobic digestion performance of corn stover. Bioresour Technol 294. https://doi.org/10.1016/j.biortech.2019.122238

Zhang, J., Tuomainen, P., Siika-aho, M., Viikari, L., 2011. Comparison of the synergistic action of two thermostable xylanases from GH families 10 and 11 with thermostable cellulases in lignocellulose hydrolysis. Bioresour Technol 102, 9090–9095. https://doi.org/10.1016/j.biortech.2011.06.085

Zhang, P., Su, R., Duan, Y., Cui, M., Huang, R., Qi, W., He, Z., Thielemans, W., 2021. Synergy between endo/exo-glucanases and expansin enhances enzyme adsorption and cellulose conversion. Carbohydr Polym 253. https://doi.org/10.1016/j.carbpol.2020.117287

Zheng, Y., Zhao, X., Lin, W., Yong, Q., Huang, C., 2023. Revealing the performance of phenoxyethanol-acid pretreatment on the intercellular structure of bamboo and its enzymatic hydrolysis. Ind Crops Prod 199. https://doi.org/10.1016/j.indcrop.2023.116663

Zou, X., Liang, L., Shen, K., Huang, C., Wu, T., Wu, Y., Fang, G., 2021. Alkali synergetic two-step mechanical refining pretreatment of pondcypress for the fiber with intact 3D structure and ultrahigh cellulose accessibility fabrication. Ind Crops Prod 170, 113741. https://doi.org/10.1016/j.indcrop.2021.113741

Anexos

Anexo I. Declaração de Bioética e Biossegurança

COORDENADORIA DE PÓS-GRADUAÇÃO
INSTITUTO DE BIOLOGIA
Universidade Estadual de Campinas
Caixa Postal 6109. 13083-970, Campinas, SP, Brasil
Fone (19) 3521-6378. email: cpgib@unicamp.br

DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "***Alternative strategies to improve the saccharification efficiency of pretreated sugarcane bagasse***", desenvolvida no Programa de Pós-Graduação em Biologia Molecular e Morfofuncional do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

Assinatura: Liliane Pires Andrade
Nome do(a) aluno(a): Liliane Pires Andrade

Assinatura: _____
Nome do(a) orientador(a): Mário Tyago Murakami

Data: 29 de junho de 2023

Anexo II. Declaração de Direitos Autorais

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Alternative strategies to improve the saccharification efficiency of pretreated sugarcane bagasse**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 29 de junho de 2023

Assinatura : Liliane Pires Andrade
Nome do(a) autor(a): **Liliane Pires Andrade**
RG n.° 433.041 SSP/TO

Assinatura : Mário T. Murakami
Nome do(a) orientador(a): **Mário Tyago Murakami**
RG n.° 332736374 SSP/SP