UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Engenharia de Alimentos

Fernando César Barbosa

Optimization of cello-oligosaccharides production by enzymatic hydrolysis of pretreated sugarcane straw using cellulolytic and oxidative enzymes

Otimização da produção de celo-oligossacarídeos por hidrólise enzimática de palha de cana-de-açúcar pré-tratada utilizando enzimas celulolíticas e oxidativas

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For those who think strongly, the impossible is just a question of opinion

I dedicate this Thesis to my family.

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ABSTRACT

Cello-oligosaccharides (COS) are oligomers with 2 to 6 β-1,4-linked glucose units, with potential applications in the food/feed and bioenergy industrial sectors. It has been demonstrated that COS are important functional oligosaccharides because it is able to support the growth of different probiotic strains and, it offers several advantages for fermentative processes when used as substrate in place of glucose to produce bioethanol. Several approaches for COS production optimization have been reported, mainly regarding suitable enzymes and combinations thereof. With this premise, this project has the main goal of optimize the production of COS by enzymatic hydrolysis of pretreated sugarcane straw via heterologous expression of cellulolytic and oxidative enzymes. After the enzymes selection and expression in *Pichia pastoris*, the optimized the combination of the endoglucanases CaCel and CcCel9m, the lytic polysaccharide monooxygenase (LPMO) TrCel61A, the cellobiose dehydrogenase (CDH) NcCDHIIa, with lactose and copper as additives, produced 60.49 mg of COS per g of pretreated sugarcane straw without glucose production. Moreover, under the conditions evaluated in this project, this work also concluded the possible lignin modification/reallocation, the removal of hemicellulose and in consequence its interaction with lignin, and the decrease in crystallinity index appeared to be the most effective characteristic obtained from the steam-explosion for COS production using the selected endoglucanase. Finally, early-stage techno-economic and life cycle assessment for cellopentaose production demonstrated that the upstream sector appeared to be the most relevant sector for both analyses. The assessment demonstrated a reduction between 6.34 and 18.23-fold in the unit production cost in comparison to cellopentaose current market selling price. Moreover, it was possible to observe an overall life cycle impact reduction between 16.2 and 19.9% comparing the baseline with the 3-fold scaled-up + 10% yield scenario. The results demonstrated that the development of a platform for cellopentaose production based on the developed process is feasible.

RESUMO

Os celo-oligossacarídeos (COS) são oligômeros de 2 a 6 unidades de glicose lincadas por ligação β-1,4, com potenciais aplicações nos setores industriais de alimentos/rações e bioenergia. É demonstrado na literatura que os COS são oligossacarídeos funcionais importantes pois são capazes de suportar o crescimento de diferentes cepas probióticas e, além disso, oferecem diversas vantagens para processos fermentativos de produção de bioetanol quando usados como substrato no lugar da glicose. Várias abordagens para otimizar da produção de COS foram descritas, principalmente abordagens relacionadas a escolha das enzimas e as combinações entre elas. Com essa premissa, este projeto teve como objetivo principal otimizar a produção de COS através da hidrólise enzimática de palha de cana-de-açúcar pré-tratada utilizando enzimas celulolíticas e oxidativas expressas de forma heteróloga. Após a seleção e expressão das enzimas em *Pichia pastoris*, a combinação otimizada das enzimas endoglucanases CaCel e CcCel9m, polissacarídeo lítico monooxigenase (LPMO) TrCel61A, celobiose desidrogenase (CDH) NcCDHIIa, com lactose e cobre como aditivos, produziu 60,49 mg de COS por g de palha de cana-de-açúcar pré-tratada sem produção de glicose. Além disso, nas condições avaliadas neste projeto, este trabalho também demonstrou que a possível modificação/realocação da lignina, a remoção da hemicelulose e em consequência sua interação com a lignina, e a diminuição do índice de cristalinidade demonstraram ser as características mais eficazes obtidas a partir do pré-tratamento de explosão vapor para produção de COS usando a endoglucanase selecionada. Por fim, a avaliação inicial técnico-econômica e do ciclo de vida para a produção de celopentaose demonstrou que o setor de upstream foi o setor mais relevante para ambas as análises. A avaliação demonstrou uma redução do custo unitário do produto de 6,34 a 18,23 vezes em comparação ao preço de venda do produto no mercado atual. Além disso, foi possível observar uma redução do impacto de ciclo de vida geral de 16,2 a 19,9% comparando o cenário base com o cenário de rendimento escalonado 3 vezes + 10% de rendimento de hidrólise. Os resultados demonstraram que o desenvolvimento de uma plataforma para produção de celopentaose baseada no processo desenvolvido é viável.

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Chapter 1

1. Introduction

The constant burning of fossil fuel has increased the greenhouse gas emission in the atmosphere, what has contributed to global warming (Ballesteros et al., 2006). Despite concern over dependence on foreign oil imports, national energy security, increase in gas price and environmental impacts (German et al., 2011), the world's energy demand is supplied in supremacy by fossil fuel, a source of energy non-renewable (Twidell and Weir, 2015).

The search for renewable energy source has been constant throughout the world, and alternatives, such as wind, solar, geothermal and biomass has been developed (Farrell et al., 2006). Biomass combustion, for example, accounts for about 95-97% of global bioenergy (Vassilev and Vassileva, 2016) and the ethanol, one of the oldest and most common biofuels in the world, is produced based on biomass feedstocks (Arifin et al., 2014; Devarapalli and Atiyeh, 2015). Ethanol can be considered less toxic compared to fossil fuel, biodegradable and can be produced from a variety of renewable sources (Mansouri, 2016). In addition, United States is considered as the largest ethanol producer in the world (Bertrand et al., 2016; Chiaramonti, 2007), followed by Brazil and Canada (Havlík et al., 2011).

Ethanol production in Brazil represents more than 25% of all world production, and this initiative has begun in 1970's with the Brazilian government "Proálcool" program (Lopes et al., 2016; Soccol et al., 2010), which encouraged the use of sugarcane as the main biomass feedstock material for ethanol production (Madosn and Monceaux, 1995). The use of sugarcane as a source of biofuel is called the first generation of bioethanol (Sims et al., 2008), and in this technique, the glucose, fructose, and sucrose are extracted for subsequent fermentation process (Bai et al.,

2008). However, the appeal for the use of non-food-based feedstocks promoted the development of the second generation of ethanol (Naik et al., 2010).

Beyond the advantage of not competing with food feedstock, the second-generation fuels have the advantages of present higher yields and lower land requirements, promote a less impact on the environment, high availability (Govumoni et al., 2013), and be based mostly on lignocellulosic feedstock, obtained from agriculture residues and industrial organic wastes (Noraini et al., 2014; Ross et al., 2010). Lignocellulosic biomass represents almost 50% of all world biomass produced, reaching an annual production close to 10 - 50 billion tones approximately (Srivastava et al., 2015).

The conversion of lignocellulosic feedstocks in bioethanol can be compromised in three basic steps, named as pretreatment, hydrolysis, and fermentation (Haghighi Mood et al., 2013). The pretreatment, such as biological, chemical, mechanical or physical-chemical (Aditiya et al., 2016), is employed with the objective of disintegrate the cellular biomass wall and then to expose the cellulose and hemicellulose for later breaking down into monomers, such as glucose, by the action of enzymatic or acid hydrolysis (Sun and Cheng, 2002). These monomers will then be able to be converted into alcohols by the action of yeast or bacteria in a fermentation process (Liu et al., 2015a, 2015b; Pessani et al., 2011).

Based on this concept, this project is integrated on the FAPESP Thematic Project “An integrated approach to explore a novel paradigm for biofuel production from lignocellulosic feedstocks” (2015/50612-8t), whose propose is to develop a novel hybrid strategy for second-generation biofuel/chemical production, which should actually reduce fermentation costs compared to current methods; and to develop, improve and design optimal microorganisms and pre-treatment methods able to produce and convert less pre-treated, oligosaccharide rich lignocellulosic feedstocks containing low concentrations of growth inhibitors. The Thematic Project is divided into 5 work packages (WP), and this project, inserted on WP2, was responsible to collect the pretreated sugarcane straw solid fraction (rich in cellulose) from WP1 and produce cello-oligosaccharides for further fermentation and ethanol production by WP3. The representative scheme of the Thematic Project is presented on Figure 1.

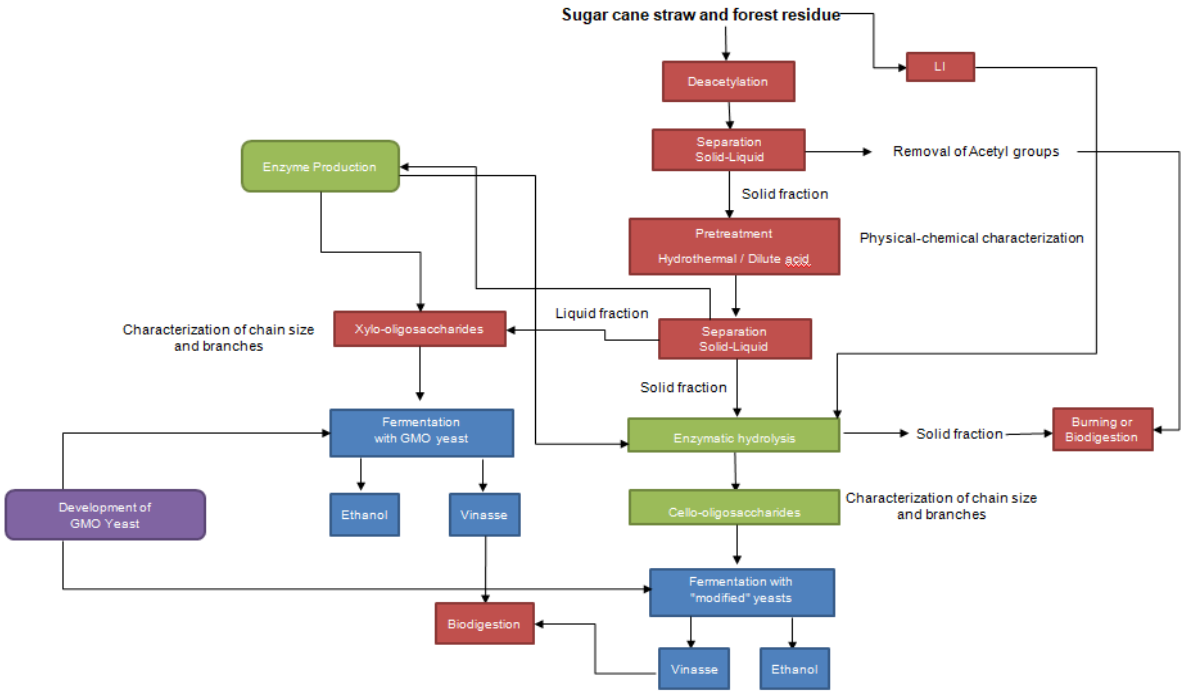

Figure 1. Representative schema of the Thematic Project. Red blocks represent the WP1 (biomass pretreatment); green blocks represent the WP2 (recombinant enzyme production); blue and purple blocks represent the WP3 (strain engineering and fermentation); and the WP4 is responsible for the sustainability and techno-economic analysis.

The sugarcane straw is considered an important and promising source of lignocellulosic biomass (Pratto, 2015). Besides this potential is poorly explored, it represents almost 25% of the total sugarcane vegetal mass, and one ton of sugarcane can generate almost 140 kg of straw (Macedo, 2001; Saad et al., 2008). In Brazil, is estimated that half of this quantity can produce more than 7.5 billion liters of ethanol/ year, which represents 25% of Brazil's annual production (Pratto, 2015). Figure 2 represents an example of sugarcane straw potential for second-generation ethanol production.

The enzymatic conversion of lignocellulose into cello-oligosaccharides (COS), an intermediate bio-product produced during standard enzymatic hydrolysis to produce glucose, seems to be a promising approach for bioethanol production (Chu et al., 2014; Kuba et al., 1990). COS, defined as oligomers of 2 to 6, β-1,4-linked glucose units (Otsuka et al., 2004; Zhao et al., 2009), have been proposed as novel substrates for ethanol fermentation, with potential advantages over glucose including a reduced risk of process contamination, shorter total fermentation times and limited process inhibition by high concentration of glucose (Ahmed et al., 2017; Liang et al., 2013; Mallek-Fakhfakh and Belghith, 2016). Moreover, COS are considered

important functional oligosaccharides (Song et al., 2013) and are significant for the food and feed industrial sectors as a probiotic compound (Uyeno et al., 2013).

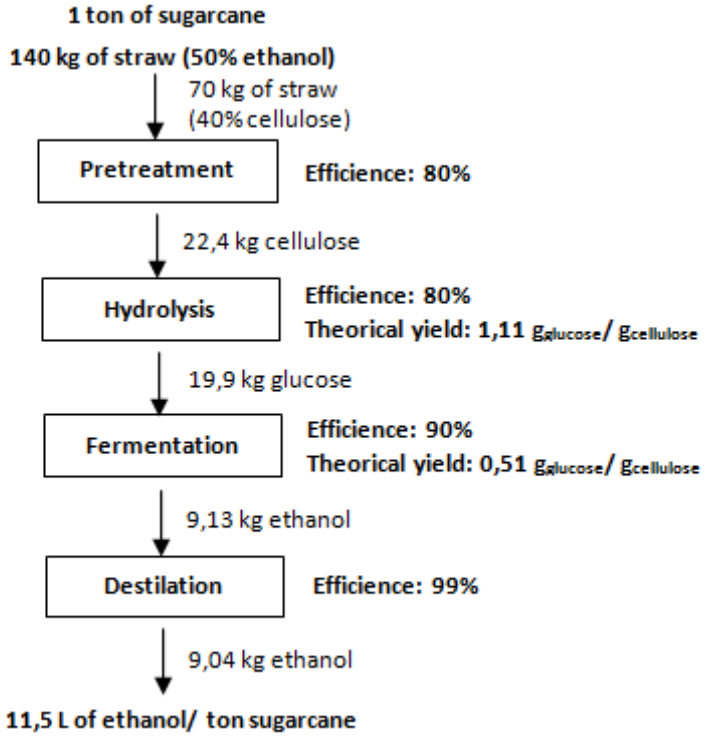

Figure 2. Representative scheme showing the potential of second-generation ethanol production by sugarcane straw.

1.1. Objectives

1.1.1. General Objectives

Considering what was presented here, the general purpose of this work was to optimize the production of cello-oligosaccharides by enzymatic hydrolysis of pretreated sugarcane straw using heterologous expressed cellulolytic and oxidative enzymes.

1.1.2. Specific Objectives

- To generate a database of enzymes (cellulolytic and oxidative) required for degradation of lignocellulosic biomass into COS;
- To select the promising genes from identified enzymes;
- To clone the selected genes in *Pichia pastoris*;

- To produce and purify the super-expressed enzymes in *Pichia pastoris*;
- To evaluate the selected enzymes combination by experimental designs (DoE) to maximize the COS production;
- To understand the correlation between potential endoglucanases, hydrolysis conditions and sugarcane straw morphological and chemical composition for COS production;
- To perform a techno-economic and life cycle analysis of cellopentaose production.

1.2. Thesis Structure

Besides the Introduction (Chapter 1), this Thesis presents other 4 Chapters with results obtained from this project. The Chapter 2 brings a literature review regarding cellulose degradation for bioethanol production. The article, entitled as “Cellulase and oxidative enzymes: new approaches, challenges and perspectives on cellulose degradation for bioethanol production” was published in the Biotechnology Letters scientific journal. The results published in the Biomass and Bioenergy scientific journal is presented in the Chapter 3, with the title “Optimization of cello-oligosaccharides production by enzymatic hydrolysis of hydrothermally pretreated sugarcane straw using cellulolytic and oxidative enzymes”; moreover, the process developed and presented in this article is also under a patent submission process. Chapter 4 represents the article published at Bioresource Technology scientific journal, entitle as “Screening of potential endoglucanases, hydrolysis conditions and different sugarcane straws pretreatments for cello-oligosaccharides production”. Last, Chapter 5 brings the results obtained for the techno-economic and life cycle analysis of cellopentaose production. This work was submitted to Journal of Cleaner Production scientific journal, with the title “Techno-economic and life cycle assessment of cellopentaose production from a bottom-up fermentation approach” and it is under review.

Finally, Chapter 6 promotes a general discussion among the obtained results and suggestions for future development, and Chapter 7 brings the conclusions obtained in this Thesis.

Chapter 2

2. Literature Review

Cellulase and oxidative enzymes: new approaches, challenges, and perspectives on cellulose degradation for bioethanol production

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Cellulase and oxidative enzymes: new approaches, challenges and perspectives on cellulose degradation for bioethanol production

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Abstract

Second-generation bioethanol is a sustainable energy source that can be produced from different renewable materials. However, there is a challenge we must overcome to significantly enhance bioethanol production: the hydrolysis of lignocellulosic biomass to fermentable sugars. Synergistic enzymes, such as endoglucanases, β-glucosidases, cellobiohydrolases, and, more recently, lytic polysaccharide monooxygenases and cellobiose dehydrogenases have been used with great success to hydrolyze pretreated biomass. Further advances in the field of second-generation bioethanol production will likely depend on an increased understanding of the interactions between enzymes and lignocellulosic substrates, the development of enzyme engineering, and the optimization of enzyme mixtures to enhance cellulose hydrolysis.

Keywords: cellobiose dehydrogenase, cellulase, enzymatic hydrolysis, lytic polysaccharide monooxygenase, second-generation bioethanol.

Introduction

Second-generation bioethanol is produced from widely available, cheap lignocellulosic wastes (Álvarez et al., 2016; Rocha-Martín et al., 2017). Sugarcane straw and bagasse, for instance, are abundant agro-industrial wastes composed mainly of cellulose (30–50%), hemicellulose (15–35%), and lignin (10–20%) (Balat, 2011; Mohanty and Abdullahi, 2016). To maximize second-generation bioethanol production from lignocellulosic feedstock, a critical step must be carried out prior to fermentation; the enzymatic hydrolysis of cellulose to fermentable sugars (Soccol et al., 2010).

Enzymatic hydrolysis is a challenging process for bioethanol production and represents an important operational cost (Valdivia et al., 2016). Research has been conducted to reduce the costs of enzyme production, improve hydrolysis performance, and increase the yield of fermentable sugars, thereby leading to productivity gains (Valdivia et al., 2016; Wang et al., 2012).

Cellulases (endo-1,4-β-D-glucanases) are produced by fungi, bacteria, and protozoans. They catalyze the hydrolysis of cellulose to simple reducing sugar products which can then be fermented to bioethanol (Subhedar and Gogate, 2014). Recent discoveries have revealed that accessory enzymes act synergistically with cellulases during cellulose hydrolysis, improving hydrolysis efficiency (Couturier et al., 2016; Hu et al., 2015).

Because of the economic importance of enzymes in second-generation bioethanol production, it is essential to understand how they function and how they can be modified to reduce costs and increase bioethanol yield. In this review, we provide an overview of the structure and catalytic mechanism of cellulases and their auxiliary enzymes in the enzymatic hydrolysis of lignocellulosic biomass for enhanced second-generation bioethanol production as well as the present challenges encountered.

Enzymatic hydrolysis of lignocellulosic biomass for second-generation bioethanol production

Sugarcane tops, trash, and bagasse represent two-thirds of the plant weight and contain about 50% cellulose (Soccol et al., 2010). Bagasse, a fibrous material resulting from juice extraction, is usually treated as waste (Salles Filho et al. 2017),

and sugarcane tops and trash are typically left in the field as crop residue (Sindhu et al., 2016). These lignocellulosic materials are generated—and discarded—in large quantities by the ethanol industry (Zabed et al., 2016). Studies have shown that ethanol yields can be increased by up to 60% in volume by using sugarcane bagasse for second-generation ethanol production (Salles-filho et al., 2017; Soccol et al., 2010).

Cellulose, a linear homopolymer of D-glucose linked by β-1,4 glycosidic bonds, and hemicellulose, a highly branched heteropolymer, are the main constituents of the plant cell wall (Haghighi Mood et al., 2013; Pandey et al., 2000; Sánchez and Cardona, 2008). Intermolecular hydrogen bonds between cellulose units result in a matrix of highly organized crystalline microfibrils and less ordered, amorphous regions (Joshi et al., 2011; Limayem and Ricke, 2012; Zabed et al., 2016). In plants, cellulose and hemicellulose are covalently linked to lignin (Zabed et al., 2016), forming a complex structure that is naturally recalcitrant to depolymerization.

Lignocellulosic biomass can be converted to bioethanol by a biochemical route. This approach requires a pretreatment for degradation of the recalcitrant lignocellulosic structure into lignin, hemicellulose, and cellulose fractions. The pretreatment step is essential to make the cellulose available to the enzymes because it removes lignin and increases the porosity of the substrate, promoting greater exposure of the cellulose surface to the active sites of the cellulases for further fermentation. Different pretreatment methods are compatible with enzymatic hydrolysis and depending on the severity of the treatment, hemicellulases and accessory enzymes are needed for complete degradation. If the pretreatment is chemical, toxic products need to be removed from the medium or inactivated. The success of the hydrolysis step is largely dependent on pretreatment efficiency. After pretreatment, polysaccharides are hydrolyzed to reducing sugars, which can then be converted to ethanol by microbial fermentation, such as yeasts, bacteria and fungi. The microorganisms most commonly employed in lignocellulosic-based bioethanol fermentation are *Saccharomyces cerevisiae* and *Zymomonas mobilis* (Limayem and Ricke, 2012).

Cellulose degradation by enzymes is a complex process. In vitro studies revealed that three processes occur simultaneously during cellulose hydrolysis: (i) physical and chemical changes in solid-phase cellulose, (ii) primary hydrolysis, releasing soluble intermediates from the surface of reactive cellulose molecules, and

(iii) secondary hydrolysis, involving the breakdown of soluble intermediates to lower molecular weight compounds and, ultimately, to glucose (Balat et al., 2008). The hydrolysis rate of lignocellulosic materials decreases rapidly over time. The process is characterized by a fast-initial phase and a slow second phase that can last until complete substrate consumption. This reaction pattern is attributed to the rapid hydrolysis of readily available cellulose fractions combined with end-product inhibition and slow inactivation of enzymes by cellulase adsorption onto lignocellulosic substrates via hydrophobic interactions with lignin (Balat et al., 2008). Substrate concentration, enzyme loading, and temperature are factors that can significantly affect the yield of enzymatic reactions (Kuhad et al., 2016; Saini et al., 2015).

Enzymatic hydrolysis is usually performed using a combination of synergistic enzymes (Sánchez and Cardona, 2008). Compared with acid hydrolysis, the enzymatic route is advantageous because it has lower energy consumption and causes no equipment corrosion problems. Enzymes can hydrolyze cellulose and hemicellulose at mild temperature (45 to 50 C) and pH conditions, which decreases sugar degradation and the formation of reaction inhibitors (Zabed et al., 2017). However, enzymatic reactions are costly: enzymes account for 20 to 30% of the total costs in bioethanol production. Considerable research effort has been directed at optimizing hydrolytic reactions and increasing enzyme activity (Chen and Fu, 2016). New or “improved” enzymes are being developed to enhance cellulose hydrolysis in the presence of hemicellulose and lignin fractions (Sims et al., 2010).

Cellulases

Structure and molecular biology of cellulases

Enzymes are classified into families according to their substrate specificity and amino acid sequence. There is a direct relationship between amino acid sequence and enzyme structure. Cellulases belong to the large family of glycoside hydrolases (GHs). According to the Carbohydrate-Active Enzymes (CAZy) database, endoglucanases are found in the GH families 5–8, 12, 16, 44, 45, 48, 51, 64, 71, 74, 81, 87, 124, and 128, exoglucanases in the GH families 5–7 and 48, and β-glucosidases in the GH families 1, 3, 4, 17, 30, and 116. Recently, auxiliary activity enzymes, such as the oxidoreductases lytic polysaccharide monooxygenases (LPMOs), were added to the GH family (Couturier et al., 2016).

Cellulases are composed of independently folded, structurally and functionally diverse domains or modules (Karmakar and Ray, 2011). Fungal cellulases usually have a simple architecture, containing a catalytic domain at the C-terminus joined by a small poly-linker region to a cellulose-binding domain (CBD) at the N-terminus (Juturu and Wu, 2014). Amino acids with carboxyl groups located within the active site catalyze the reaction by acid-base catalysis through one of two modes, either inversion or retention of the anomeric carbon configuration (Karmakar and Ray, 2011).

Enzymes can be modified or engineered for improved activity and robustness. Directed mutagenesis, random mutagenesis, or a combination of both has been used to obtain improved enzymes for industrial application (Lynd et al., 2002). For instance, Qin et al. (2008) expressed Cel5A, a highly efficient endoglucanase from *Trichoderma reesei*, in *Saccharomyces cerevisiae* using two levels of glycosylation and found that glycosylation increased thermal stability and the optimal pH range without affecting enzymatic activity.

Cellulolytic complexes

Cellulases are a mixture of different cellulolytic enzymes that act synergistically on cellulose, hydrolyzing its bonds (Reczey et al., 1996). Cellulolytic microorganisms produce a complex combination of enzymes that have β-1,4-glycosidic linkage specificity and are highly substrate-specific (CASTRO and PEREIRA, 2010; Mohanty and Abdullahi, 2016; Soccol et al., 2010). A typical cellulase system is composed of at least three enzyme groups: endoglucanases, exoglucanases, and β-glucosidases (Kuhad et al., 2011; Saini et al., 2015; Soccol et al., 2010). Cellulases are classified according to the site of action in the cellulose polymer. Some cellulolytic complexes may also contain exo-1,4-β-D-glucan-4-glucohydrolase (Enzyme Commission number, EC 3.2.1.74) and exo-1,4-β-cellobiosidase (EC 3.2.1.176) (Biswas et al., 2014).

Endoglucanases (EG - EC 3.2.1.4), also known as cellulases (CMCases), have affinity for amorphous cellulose regions and promote a random attack on internal β-glycosidic bonds, releasing oligomers of different lengths, such as cellobiose and cello-oligosaccharides. Their random attacks cause a rapid decrease in chain length, causing viscosity to decrease with the increase in reducing end

groups (Kuhad et al., 2011; Saini et al., 2015; Soccol et al., 2010; Zabed et al., 2017). When endoglucanases act on cellodextrins, the hydrolysis rate increases with polymerization degree, respecting substrate solubility limits. The main end products are cellobiose and cellotriose (Saini et al., 2015).

Exoglucanases (cellobiohydrolases - CBH, avicelase, exo-1,4-β-d-glucanase, EC 3.2.1.91) are processive enzymes with affinity for crystalline cellulose. This group can act on the reducing ends of the molecule (CBH I, exo-1,4-β-cellobiosidase) or on non-reducing ends (CBH II, exo-1,4-β-d-glucanase), releasing β-cellobiose (Juturu and Wu, 2014; Kuhad et al., 2011; Liao et al., 2016; Soccol et al., 2010; Zabed et al., 2017).

β-glucosidases or cellobiases (EC 3.2.1.21) hydrolyze disaccharide cellobiose or cello-oligomers (generated from the action of the two other classes of cellulases) to two glucose molecules (Kuhad et al., 2011; Soccol et al., 2010; Zabed et al., 2017). Cellobiases are essential for the total degradation of cellulose to glucose and play an important role in preventing cellobiose accumulation, a potential cellulase inhibitor (Kuhad et al., 2011).

Cellulase systems are not a mere mixture of representative enzymes but an effective combination of complementary catalysts used for total cellulose degradation (Lynd et al., 2002). Endoglucanases hydrolyze accessible intramolecular β-1,4-glycosidic bonds, producing new reducing ends, which are then cleaved by exoglucanases, releasing cellobiose. Finally, β-glucosidases complete the process by hydrolyzing cellobiose to glucose (Balat et al., 2008). Indeed, the interactions between enzymes and substrate are complexes and the binding affinity also involves changes in the molecular level. Paul et al. (Paul et al., 2020) demonstrated that different microbial cellulases have different main residues involving the cellulose-binding, resulting in different potentials regarding the use of cellulose as a substrate for the high yield of bioethanol.

In addition, in conjunction to the free enzyme system, cellulosomal complexes, a large extracellular complexes composed of multiple enzymes with different binding and substrate specificities, can be combined to increase the cellulose degradation. While the free system operates in a surface limited mechanism, the cellulosomes appear to bind multiple points on the cellulose surface creating large bundles, increasing the surface area for free enzyme penetration (Donohoe and Resch, 2015).

A limitation of using cellulases is that they are inhibited by cellobiose and glucose. Simultaneous saccharification and fermentation (SSF) can be used to overcome this problem. In SSF, cellulose hydrolysis and hydrolysate fermentation occur simultaneously (Balat, 2011). An efficient cellulase cocktail should be able to degrade cellulose in its crystalline form in acidic pH (4 to 5) and under stress conditions (Zabed et al., 2017).

Moreover, the presence of lignin in the substrate results in a decrease of cellulose accessibility by steric hindrance and different enzymes can respond differently to it due to its different adsorption capacity (Donohoe and Resch, 2015). The different levels of cellulose crystallinity also interfere on the cellulase binding capacity and the CGHs are considered to be more important on the crystalline cellulose degradation, while EGs are more important on the amorphous cellulose degradation (Du et al., 2020).

Processive endoglucanases

Endoglucanases are typically non-processive enzymes that attack the cellulose molecule, producing new ends, whereas exoglucanases work processively, releasing cellobiose (Béguin and Aubert, 1994; Teeri and Linder, 1997). However, a few examples of processive endoglucanases, which have a similar mode of action of exo- and endoglucanases, have been reported (Belaich et al., 2016; Irwin et al., 1998; Shoham et al., 2003; Zverlov et al., 2005).

The majority of processive endoglucanases harbor a glycoside hydrolase family 9 (GH9) catalytic domain, while a small part belongs to the glycoside hydrolase family 5 (GH5) family. GH5 processive endoglucanases appear to have a more complex mechanism of action than GH9 endoglucanases; however, their enzymatic routes are still poorly understood (Chang et al., 2018). GH9 processive endoglucanases, in addition to the GH9 catalytic domain, contain an accessory carbohydrate-binding module (CBM), which can significantly influence enzymatic activity (Tomme et al., 1988).

The action of CBM in the first steps of crystalline cellulose degradation was first described in the late 1940s. CBMs are currently classified into more than 43 different families according to their amino acid sequence and binding domains, with over 300 putative sequences identified in over 50 species (Boraston et al., 2004;

Hilden and Johansson, 2004). The presence of CBM adjacent to the GH9 catalytic domain in processive endoglucanases seems to be critical because, without CBM, processive endoglucanases would lose their processivity (Asha et al., 2016; Chiriac et al., 2010; Li et al., 2007; Yennamalli et al., 2014). The CBM ability to assist the cellulases penetration into the bulk of biomass depends on their size, concentration and binding equilibrium (Nimlos et al., 2012).

CBM3 is one of the most versatile and functional families. It is subdivided into four groups, each with a different role. CBM3a and CBM3b increase the binding power of the enzyme to the surface of microcrystalline cellulose, whereas CBM3c is an auxiliary module that feeds a single cellulose chain to the active site, assisting the catalytic module of GH9 enzymes (Jindou et al., 2006; Tormo et al., 1996) and CBM3d is probably evolved from CBM3a but with different appended proteins (Cai et al., 2011). Simultaneous binding of the GH9 catalytic domain and CBM to the cellulose chain also allows processive cleavage of large oligosaccharides from amorphous cellulose (Fig. 1).

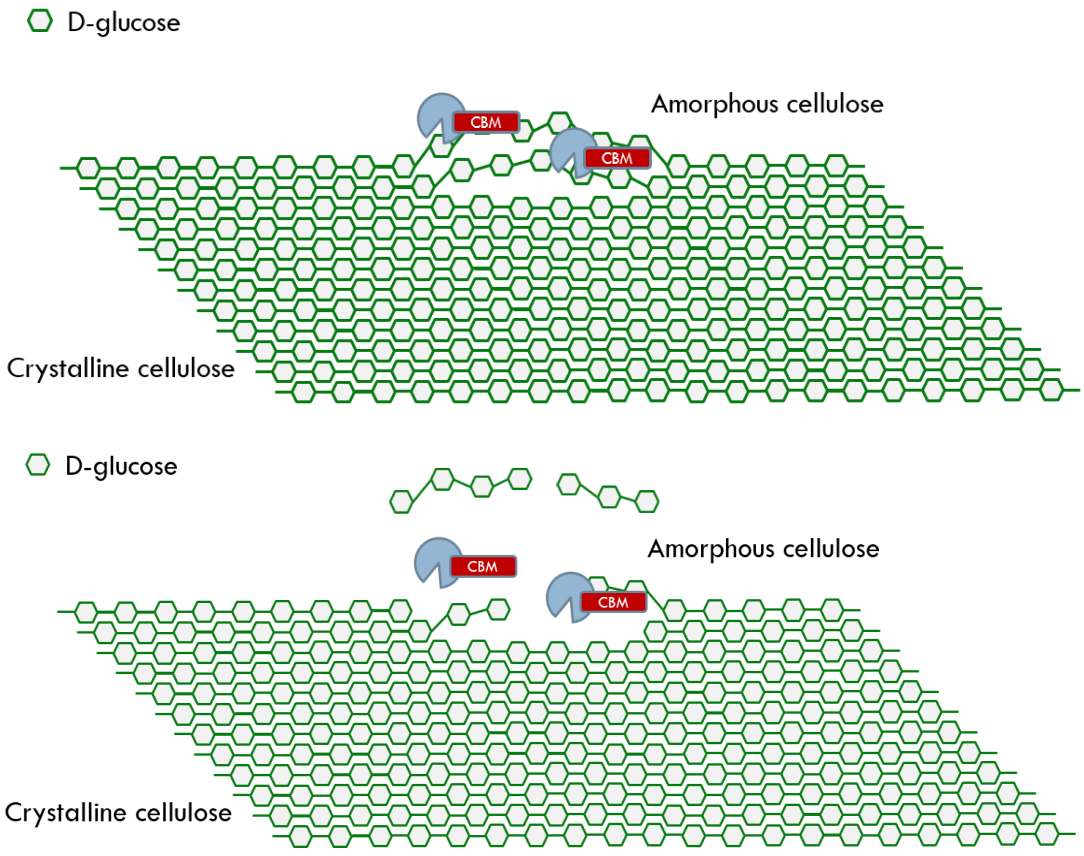

Fig. 1. (A) Simultaneous binding of the catalytic domain and the carbohydrate-binding module (CBM) of processive endoglucanase to the cellulose chain. (B) Cleavage of large oligosaccharides from amorphous cellulose.

Several novel processive endoglucanases have been identified since 2010. Chiriac et al. (Chiriac et al., 2010) showed that Cel9B from *Paenibacillus barcinonensis* has a CBM3c module, a fibronectin type 3 domain (Fn3)-like repeat ($Fn3_{1,2}$), and a CBM3b module adjacent to its GH9 catalytic domain. Zhang et al. (Zhang et al., 2010) showed that Cel9 from *Clostridium phytofermentans* is a processive endoglucanase: the hydrolysis products of regenerated amorphous cellulose were cellotetraose (major product), cellotriose, cellobiose, and glucose, and 71–80% of the reducing sugars produced by Cel9 were soluble. Jeon et al. (Jeon et al., 2012) described EngZ from *Clostridium cellulovorans* as a processive endoglucanase because its capacity to reduce the viscosity of Avicel was intermediate between exo- and endo-type cellulases. Finally, Asha et al. (Asha et al., 2016) demonstrated that the purified enzyme AS-HT-Celuz A from *Aspergillus ochraceus* MTCC 1810 has multiple substrate specificities and acts processively toward both amorphous and crystalline cellulose, indicating the activity of endo- and exoglucanases in different binding sites.

Although efforts have been made to enhance cello-oligosaccharide production by processive endoglucanases, the process is not yet suitable for industrial application. The use of processive endoglucanases alone for bioethanol production has not been explored. Perhaps research on their association with other classes of enzymes can help making this process feasible.

Lytic polysaccharide monooxygenases (LPMOs) and cellobiose dehydrogenases (CDHs): auxiliary enzymes

Until recently, hydrolytic enzymes were considered the only family of enzymes able to degrade recalcitrant cellulose and hemicellulose for subsequent glucose fermentation (Kittl et al., 2012). However, recent studies on LPMO, a copper-dependent enzyme, have modified this view. LPMOs have the potential to increase the efficiency and reduce the costs of fermentation (Rodrigues et al., 2017).

Dimarogona et al. (2012) demonstrated that glycoside hydrolase family 61 (GH-61) from *Sporotrichum thermophile* increased sugar release from pretreated spruce by 20%. Jung et al. (2015) observed a synergistic effect between LPMO (*Gt*GH61) and cellulase (*Gt*Cel5B): the hydrolysis rate of pretreated kenaf and oak increased by 56 and 174%, respectively. The authors confirmed that the presence of cobalt (Co^{2+}) increased the amount of released sugars by 11% in kenaf and 12% in

oak. Song et al. (2018) reported that LPMO (*Tr*AA9A) increased the accessible surface area of bacterial microcrystalline cellulose by separating cellulose ribbons, enhancing hydrolysis yield.

LPMOs were formerly characterized as GHs containing CBM, but, currently, LPMOs are classified as belonging to the auxiliary activity (AA) families AA9, AA10, AA11, AA13, and AA14, according to the CAZy database (Busk and Lange, 2015; Valenzuela et al., 2017). The AA9 family, previously known as GH61, cleaves cellulose chains by oxidation of several carbons (C-1, C-4, and C-6). The AA10 family, the only including bacterial LPMOs, was first described as CBM33; some of its members act on both chitin and cellulose. AA11 family members have the ability to cleave chitin chains by oxidation of C-1. AA13 enzymes cleave starch by oxidizing C-1 at the cleavage site. AA14 enzymes have the potential to cleave xylan via C-1 oxidation (www.cazy.org). CBM is responsible for cleaving crystalline cellulose regions, thereby increasing accessibility to amorphous cellulose and enhancing hydrolysis efficiency (Hu et al., 2014).

AA9 and AA10 have been shown to act synergistically with cellulases during biomass hydrolysis (Bennati-Granier et al., 2015; Ghatge et al., 2015). These LPMOs act through an oxidative mechanism. The copper ion reduces oxygen in the presence of an external electron donor. Then, the reduced oxygen kidnaps a single hydrogen from the substrate, cleaving the β-1,4 glycosidic bond. By oxidizing the glycosidic bond, LPMOs produce an entry point for cellulases, improving biomass degradation (Valenzuela et al., 2017). The active site of LPMO comprises N- and C-terminal histidines complexed with copper. Ascorbic acid and CDH were shown to act as electron donors in the reaction (Courtade et al., 2017). However, Hu et al. (2014) demonstrated that non-cellulosic components of pretreated biomass may act as reducing cofactors for LPMO, obviating the need for external electron donors. Kittl et al. (2012) found that LPMO activity increased in the presence of CDH. Loose et al. (2016) observed that CDH from *Myriococcum thermophilum* acts as an electron donor for AA10 LPMOs.

The recent finding that CDHs may cooperate with LPMOs to enhance cellulose degradation has changed the importance of these enzymes (Ma et al., 2017; Tan et al., 2015). CDHs were known to produce hydrogen peroxide, reduce Fe^{3+} levels, and donate electrons to GH family members (Trimble et al., 2004). CDH are glycosylated extracellular proteins, produced mainly by fungi, formed by a C-

terminal catalytic dehydrogenase domain containing a flavin adenine dinucleotide (FAD) and an N-terminal cytochrome-b-type heme domain (CYT), which transfers electrons from the C-terminal domain to an external electron acceptor (Desriani et al., 2010; Ma et al., 2017). CDHs are divided into class I (produced by basidiomycetes) and class II (produced by ascomycetes). Class IIA CDHs harbor a CBM1 module and class IIB do not (Zamocky et al., 2006).

Although their mechanism of action has not been fully elucidated, it is known that CDHs bind strongly to the cellulose domain and that the dehydrogenase domain oxidizes cellobiose and other cello-oligosaccharides at C-1 to cellobiono-1,5-lactone by reducing FAD. Subsequently, an internal electron transfer from reduced FAD to the CYT domain occurs, followed by electron transfer to an external donor, such as LPMO (Tan et al., 2015). This auxiliary action allows LPMO to perform the redox reactions needed to hydrolyze lignocellulose (Agger et al., 2014).

Current challenges

Many attempts have been made to reduce bioethanol production costs. Enzymatic hydrolysis represents the second largest operational cost (25 to 30%) in bioethanol production, second only to biomass acquisition. In first-generation ethanol production, enzymatic hydrolysis accounts for less than 3% of operational costs. This discrepancy is due to the complex enzymatic cocktail (cellulases, hemicellulases, and accessory enzymes) needed for second-generation bioethanol production. The synergistic action of enzymes is crucial for efficient cellulose degradation.

The major challenge in biomass hydrolysis is the recalcitrant nature of cellulose. New biocatalysts and strategies to improve enzymatic hydrolysis are needed to overcome this issue (Couturier et al., 2016). The complete enzymatic degradation of cellulose into sugar monomers can be achieved, theoretically, by combining enzymes from different families, such as glycosidases, hydrolases, and oxidases. However, experimentally, the combination of such enzymes produced a limited increase in hydrolysis efficiency because of cellulose crystallinity, kinetic complexity, and enzyme inhibition (Couturier et al., 2016).

Enzymes have high costs and are needed in large quantities to obtain significant hydrolysis yields (Bansal et al., 2009). The decrease in hydrolysis rate

over time and the limited knowledge on cellulase kinetics in lignocellulosic substrates are other obstacles to be overcome.

Processive endoglucanases and auxiliary enzymes are potential catalysts for biomass conversion and bioethanol production. However, more research is needed to validate their use in industrial applications. Does the improvement in bioconversion yields obtained with these novel enzymes cover the costs of enzyme production and purification? How much lignin should be removed during pretreatment, considering that lignin can increase the synergy between LPMO and cellulases? Can different LPMOs be selective toward specific reducing agents? What are the optimal proportions of each enzyme class in the enzymatic cocktail? What is the optimal pH and temperature? The potential of enzymatic hydrolysis remains to be fully discovered.

The advantages of enzymatic routes over other hydrolysis methods are a driving force for advances to be made in enzyme-based technologies. Cost-effective processes can be achieved by optimizing pretreatment and reaction parameters but first, researchers should focus on understanding the complex interactions between enzymes and substrates.

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Conflict of Interest

The authors declare that they have no conflicts of interest regarding this publication.

Author contributions

All authors contributed to the study conception and design. Literature search, data analysis and first draft of the manuscript was written by Fernando Cesar Barbosa and Maria Augusta Silvello. Rosana Goldbeck had the idea and critically revised the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Chapter 3

3. Optimization of cello-oligosaccharides production

Optimization of cello-oligosaccharides production by enzymatic hydrolysis of hydrothermally pretreated sugarcane straw using cellulolytic and oxidative enzymes

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Optimization of cello-oligosaccharides production by enzymatic hydrolysis of hydrothermally pretreated sugarcane straw using cellulolytic and oxidative enzymes

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Abstract

Enzymatic hydrolysis of lignocellulosic biomass accounts for 20 to 30% of the total cost of second-generation bioethanol production and many efforts have been made in recent years to overcome the high cost of enzymes. Using cello-oligosaccharides (COS), intermediates in cellulose conversion to glucose, may provide advantages over monomeric glucose fermentation, such as lower risk of growth of process contaminants, shorter fermentation time and limited process inhibition by high concentrations of glucose. In addition, COS are also useful as functional oligosaccharides in the food and feed sectors. This study aimed to optimize COS production for further industrial applications. To the best of our knowledge, this is the first study that has used a design of experiments approach to analyze the synergism between endoglucanases, lytic polysaccharide monooxygenase (LPMO), cellobiose dehydrogenase (CDH) and different additives during the hydrolysis of a pretreated sugarcane straw for COS production. After optimization of enzymatic hydrolysis, a combination of the endoglucanases CaCel and CcCel9m, the LPMO TrCel61A, the CDH NcCDHIIa, with lactose and copper as additives, produced 60.49 mg of COS per g of pretreated sugarcane straw, 1.8-2.7-fold more than the commercial enzyme cocktails Cellic® Ctec2 and Celluclast® 1.5L. The COS/glucose ratio achieved was 298.31, an increase of 3314 and 2294-fold over the commercial enzymatic cocktails, respectively. These results open a new perspective regarding COS production and its industrial application.

Keywords

Cellobiose dehydrogenase; cello-oligosaccharides; endoglucanases; enzymatic hydrolysis; second-generation bioethanol; lytic polysaccharide monooxygenase.

1. Introduction

The implementation of renewable energy sources is becoming increasingly necessary given the negative impacts of indiscriminate use of fossil fuels, such as intensive greenhouse gas emission and global warming (Noraini et al., 2014). Furthermore, the finite nature and price fluctuation of fossil fuels make them an unreliable energy source (Balat, 2011). Biofuels are promising carbon-negative alternatives for the transportation sector that can contribute to the reduction in atmospheric carbon monoxide and dioxide levels (Lynd et al., 2017). In addition, second generation biofuels can be produced from agriculture residues and industrial organic wastes (Noraini et al., 2014; Ross et al., 2010; Song et al., 2013).

The advantages of the use of lignocellulosic biomass for bioenergy production are clear although its enzymatic hydrolysis presents one of the more challenging and expensive steps in second generation bioethanol production, with research into reducing this cost remaining an active field (Valdivia et al., 2016; Wang et al., 2012). The enzymatic conversion of lignocellulose into cello-oligosaccharides (COS), an intermediate bio-product produced during standard enzymatic hydrolysis to produce glucose, seems to be a promising approach for bioethanol production (Chu et al., 2014; Kuba et al., 1990).

COS, defined as oligomers of 2 to 6, β-1,4-linked glucose units (Otsuka et al., 2004; Zhao et al., 2009), have been proposed as novel substrates for ethanol fermentation, with potential advantages over glucose including a reduced risk of process contamination, shorter total process times and limited process inhibition by high concentration of glucose (Ahmed et al., 2017; Liang et al., 2013; Mallek-Fakhfakh and Belghith, 2016; Yang et al., 2015).

Moreover, COS are considered important functional oligosaccharides (Song et al., 2013) and are significant for the food and feed industrial sectors as a probiotic compound (Uyeno et al., 2013); (Karnaouri et al., 2019) confirmed that COS can support the growth of different probiotic strains from *Lactobacilli* and *Bifidobacteria* species. However, there is still limited information regarding large scale production of COS (Chu et al., 2014).

Different approaches for COS production have been investigated including the use of endoglucanases and auxiliary enzymes (AAs), such as lytic polysaccharide mono-oxygenases (LMPOs) and cellobiose dehydrogenase (CDH). A few examples

of processive endoglucanases with both exo and endoglucanase mode of action have been reported (Belaich et al., 2016; Irwin et al., 1998; Shoham et al., 2003; Zverlov et al., 2005); in addition to their recognized glycoside hydrolase family 9 (GH9) catalytic domain, these processive endoglucanases contain an accessory carbohydrate-binding module (CBM) that can significantly influence their enzymatic activity on cellulosic substrate, allowing the processive cleavage of larger oligosaccharides from amorphous cellulose (Hu et al., 2015; Tomme et al., 1988).

LPMOs have a synergistic effect in combination with hydrolases for breaking down cellulosic material through the production of an entry point for the canonical cellulases on the biomass surface (Fushinobu, 2014). Oxidation of glycosidic bonds by LPMOs facilitates cellulose hydrolysis by cellulases and improves bioethanol production from plant biomass (Agger et al., 2014; Borisova et al., 2015; Cannella and Jørgensen, 2014; Liang et al., 2014). Moreover, recent studies support the supposition that LPMOs can create new chain breaks in crystalline substrates, reducing the biomass crystallinity (Hemsworth et al., 2015; Selig et al., 2015; Vermaas et al., 2015; Villares et al., 2017)

CDH is another promising accessory enzyme that acts by oxidizing cellobiose and transferring electrons to the LPMO creating an active form which can reduce molecular oxygen, producing the active intermediate which reacts with cellulose (Henriksson et al., 2000; Hildén et al., 2000; Hilden and Johansson, 2004; Horn et al., 2012).

Due to their value, some methods for producing COS's from cellulosic substrates have previously been evaluated, with different degrees of success. The more successful methods employ hazardous chemicals, such as concentrated hydrochloric and sulphuric acid (Zhang and Lynd, 2003), or extremes in temperature and pressure (Tolonen et al., 2015), limiting their feasibility in different industries. Enzymatic hydrolysis from lignocellulose has also been examined using methods that address a major issue in COS production, namely that COS's exist only as transitionary intermediates during conventional enzymatic hydrolysis of cellulose to glucose by commercial enzyme preparations. Through chromatographic fractionation of a crude enzyme preparation to remove beta-glucosidases, followed by a multi-step hydrolysis process, COS's were obtained as a major product from a lignocellulosic substrate, however a significant glucose fraction remained; furthermore, the majority

of the COS yield consisted of the lowest DP oligomer – cellobiose (Chu et al., 2014). All the methods described above also invariably require additional steps or facilities beyond the single step enzymatic hydrolysis that is conventional in a lignocellulose biorefinery. However, the existence of the aforementioned processive endoglucanases, shown to cleave cellulose into higher DP soluble COS's as a true end product, as well as auxiliary enzymes with synergistic effects on endoglucanase activity suggest that a bottom-up approach to design a cocktail to produce higher DP COS's in a single step reaction is achievable (Belaich et al., 2016; Irwin et al., 1998; Shoham et al., 2003; Zverlov et al., 2005). However, to our knowledge, no empirical study combining the enzymes known from the literature to create a defined, optimized process for COS production from a real lignocellulosic feedstock has been conducted.

In parallel with development of an alternative approach to use COS directly as a substrate for bioethanol production, this study focuses on the optimization of enzymatic the production of COS using this bottom-up approach. To the best of our knowledge, this is the first study that has used design of experiments in order to analyze the synergism of endoglucanases, LPMOs, CDH and different additives for the hydrolysis of hydrothermally pretreated sugarcane straw for COS production, reaching an amount of 60.49 mg/g after optimization. The production of a significant amount of COS, opens a new opportunity for the use of COS in industrial applications, such as bioethanol production or in the food and feed sectors.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals and materials

All chemicals were reagent grade or higher, purchased from Sigma-Aldrich, Merck/Millipore or BD Biosciences. Regenerated amorphous cellulose (PASC) was prepared by the treatment of Avicel with phosphoric acid (Wood and Bhat, 1988). Cellobiose, cellotriaose, cellotetraose, cellopentaose and cellohexaose standards were bought from Megazyme.

2.1.2. Sugarcane straw

Sugarcane straw (dry leaves and green tops) was sampled from a bale at Usina Ferrari (São Paulo, Brazil). The material was air-dried to 10% (w/w) moisture content, determined with an automatic infrared moisture analyzer MA35 (Sartorius Gmbh, Goettingen, Germany) and then hammer-milled to an average size of 0.15 mm - 2.38 mm (-8 / +60 mesh). 30.0 g of sugarcane straw was mixed with NaOH solution and water to give a final concentration of 0.8% NaOH (w/w) in 10% (w/w) of final solid loading in a sealed flask and incubated in a water bath at 60°C for 30 min. The mixture was homogenized and strained through a muslin cloth to recover the solid fraction and rinsed with 1% NaOH solution (w/w) to eliminate residual lignin and sugars followed by water until neutral pH was reached. Then, the solid fraction was submitted to liquid hot water pretreatment in a 316L stainless steel batch reactor with 0.5 L capacity immersed in a glycerin bath. The reactor was filled with 30.0 g of biomass (dry weight) and water to a solid loading of 10% (w/w) and incubated at 190°C for 20 min (residence time). Afterwards, the reactor was immediately cooled in an ice bath and the pretreated sugarcane straw separated by straining through a muslin cloth, rinsed with water until neutralized, dried at room temperature and stored at 4ºC for further use (Brenelli et al., 2020). The chemical composition, determined according to Sluiter et al (Sluiter et al., 2016), was 33.4 ± 0.54% cellulose, 28.31 ± 0.26% hemicellulose, 20.90 ± 0.23% lignin, 8.53 ± 0.25% ash and 11.2 ± 0.52 % extractives for raw sugarcane straw and 52.82 ± 0.81% cellulose, 13.19 ± 0.23% hemicellulose, 22.29 ± 0.16% lignin and 9.55 ± 0.20% ash for two-stage pretreated sugarcane straw.

2.2. Plasmids construction

Synthetic genes encoding the selected endoglucanases, LPMOs and CDH were assembled into the expression vector pPICZ-α B (Thermo Fisher Scientific) overlapping DNA fragments by Gibson assembly (New England BioLabs) (Gibson et al., 2008). LPMOs and CDH genes also encoded for their native secretion signal sequences, while the endoglucanases were cloned downstream of the *S. cerevisiae* alpha factor, both under the control of methanol inducible AOX1 promoter. *E. coli* DH5α (Invitrogen) was used as a host cell for DNA manipulation and *Pichia pastoris* NRRL 11430 (ATCC) was used as the host for recombinant protein production. Luria-

Bertani (LB) medium with 100 µg/mL zeocin (Thermo Fisher Scientific) was used for *E. coli* growth and selection while Yeast Extract-Peptone-Dextrose (YPD) 100 µg/mL zeocin agar plates were used for *P. pastoris* growth and selection.

The plasmid constructs were transformed into *P. pastoris* NRLL 11430 and transformants were analyzed for the correct insertion of genes at the AOX1 locus by PCR with standard primers 5` AOX (GACTGGTTCCAATTGACAAGC) and 3`AOX (GCAAATGGCATTCTGACATCC). A positive clone from each transformation was taken through an expression trial in 50 mL baffled Erlenmeyer flasks for recombinant enzyme production. The cultures were monitored for optical density (OD600), extracellular protein concentration (SDS-PAGE) and enzymatic activity.

2.3. Enzyme expression and purification

The *P. pastoris* strains containing the selected genes were cultivated in 10 mL of Buffered Glycerol-Complex Medium (BMGY) 100 µg/mL zeocin overnight at 30 ºC and 250 rpm. A new culture (OD_{600} 1.0) in 50 mL of Buffered Methanol-Complex Medium (BMMY) was started according to the Pichia Fermentation Process Guide (Thermo Fisher Scientific) at 28 ºC, 200 rpm and 72 hours. 100% methanol was added to the culture every 24 hours to maintain a final concentration of 0.5% (v/v) for induction. At the end of culture, the fermentation broth was centrifuged at 4000 x g for 5 min and the clear culture supernatant was collect for further purification. The selected endoglucanases (Table 1) were purified based on the binding capacity of endoglucanases to the PASC substrate (Zhang et al., 2010). The auxiliary enzymes TrCel61a, NcPMO-02916 and NcCDHIIa (Table 1) were purified following an adaptation of the protocol of (Kittl et al., 2012). After centrifugation, the respective supernatants went through an ammonium sulphate precipitation procedure (20, 15 and 30% respectively) prior to chromatography. The precipitated material was removed by centrifugation and the supernatants were loaded onto 9 mL PHE-Sepharose Fast Flow column (GE Healthcare Biosciences), equilibrated with a 25 mM sodium acetate buffer, pH 5.0, containing their respective saturation amount of ammonium sulphate. Proteins were eluted within 3 column volumes of equilibrated buffer without ammonium sulphate. Fractions containing the respective enzymes were collected and stored at 4 ºC for further activity assays and application.

2.4. Enzymatic activity measurements

Endoglucanase activities were determined according to IUPAC recommendations (Ghose, 1987) using carboxy the conversion of 2 µmol of 2,6-dimethoxyphenol (2,6-DMP) or the formation of 1 µmol of coerulignone (ε469=53,200 M^{-1} cm^{-1}) per minute under reaction conditions - peroxidase activity (Breslmayr et al., 2018). The activity of NcCDHIIa was measured according to the (Harreither et al., 2012) protocol, monitoring the amount of enzyme that oxidizes 1 µmol of the electron acceptor (0.3 mM 2.6-diclorofenolindofenol – ε520=6.8 mM^{-1} cm^{-1}) per minute under the assay conditions.

2.5. Commercial enzymes and determination of enzyme protein concentration

Two commercial cellulase enzyme cocktails, Celluclast® 1.5L (Sigma-Aldrich) and Cellic® CTec2 (Novozymes) were used as positive controls for the enzyme mixtures developed in the experimental designs. Protein concentrations were determined by the Bradford assay (Thermo Fisher Scientific) using bovine serum albumin (BSA) as the calibration standard. The cellulase enzyme cocktail activities were also determined according to IUPAC recommendations (Ghose, 1987) using carboxymethyl cellulose (CMC) as a substrate.

2.6. Enzymatic hydrolysis and experimental designs

Liquid hot water pretreated sugarcane straw hydrolysis by the selected endoglucanases alone was carried out in 50 mL Falcon tubes, at their optimal temperatures and pH, shaken at 150 rpm, with an enzyme loading of 10 U/g of substrate and 1 % (w/v) of substrate for 24, 48 and 72 hours in a shaker incubator (New Brunswick Scientific) according to a protocol adapted from (Goldbeck et al., 2016, 2014). The final liquid volume of each tube was adjusted to 10 mL with sodium phosphate buffer (0.1 M, pH 5.0). Hydrothermally pretreated sugarcane straw hydrolysis by the commercial cocktail enzymes Cellic® Ctec2 and Celluclast® 1.5L was conducted using the same protocol but at 50 °C and pH 5.0. Both hydrolysis reactions were used as controls for further optimized conditions. Endoglucanases, oxidative enzymes and additive (copper, cobalt, lactose and ascorbic acid) concentrations were determined using the design of experiments methodology. Plackett-Burman designs were applied in order to determine the significance effects

of independent variables in two steps: only endoglucanases in a first round, and oxidative enzymes and additives in combination with selected significant endoglucanases in a second round. The designs consisted of 12 experiments and 3 repetitions of the central point, totaling 15 experiments in each round. The dependent variable (COS production) was calculated based on the concentration (mg/g of pretreated sugarcane straw) of cellulose converted into COS (cellobiose to cellohexaose). In the first round of experiments, the coded level +1, -1 and 0 (central point) were defined as 10 U/g, 0 U/g and 5 U/g of substrate; for the second round, the coded level +1, -1 and 0 were defined as 2, 0 and 1 mg of TrCel61a/g of substrate, 1, 0 and 0.5 mg of NcPMO-02916/g of substrate, 1, 0 and 0.5 mg of NcCDHIIa/g of substrate, 2, 0 and 1 mM of ascorbic acid, 1, 0 and 0.5 mM of lactose, 10, 0 and 5 mM of cobalt and copper. Analyses of the effects was conducted with online software Protimiza Experimental Designs® (https://experimental-design.protimiza.com.br). All optimization experiments were performed under these conditions at pH 5.0, 50 °C and 150 rpm for 48 hours.

2.7. Quantification of COS

The content of glucose and COS in the supernatants of the sugarcane straw enzymatic hydrolysis mixtures was determined by High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) using a Dionex ICS-5000 ion chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) according to the method described by (Ávila et al., 2018), with some modifications. Glucose and COS (C2-C6) were separated by gradient elution using ultrapure H_2O (eluent A and D), NaOH 0.25 M (eluent B), NaOAc 1 M/NaOH 0.1 M (eluent C) and) as mobile phases on a Carbopac PA1 column (250 × 4 mm i.d., particle size 10 µm, Thermo Fisher Scientific, Waltham, MA, USA) and CarboPac PA1 guard column (50 × 4 mm i.d., particle size 10 µm, Thermo Fisher Scientific, Waltham, MA, USA). The gradient was performed as follows: 0-36 min, 40% A, 0-42% B and 60-18% C; 36-40 min, 0-3% A, 100-0% B and 0-97% C; 40-42 min, 3-0% A, 0-100% B and 97-0% C; 42-57 min, 40% A and 60% C. The samples were diluted in ultrapure water, filtered through a 0.22 µm PTFE filter and injected into the column using an auto-sampler. The column temperature was maintained at 30 °C, the flowrate was 0.3 mL/min, and the injection volume of the samples was 25 µL. Data

were acquired and processed using Chromeleon software version 7.0. The glucose and COS were identified in samples by comparing the retention times with authentic standards. Calibration curves were constructed using commercial standards to quantify the glucose and COS in the samples. The content of glucose and COS was expressed as mg/L and then converted to mg/g of pretreated sugarcane straw.

2.8. Statistical analysis

The results of the design of experiments (Plackett-Burman) were submitted to a Pareto chart analysis at a 90% confidence level using Protimiza Experimental Design® software and the validation experiments, presented as mean and standard deviation, were performed in duplicate.

3. Results and discussion

3.1. Selection of enzymes

All enzymes employed in this study were selected after an extensive literature review (Table 1). Particularly, endoglucanases were chosen based on their reported capacity to release COS while the oxidative enzymes were chosen based on their characterized enzymatic activity. Data are summarized in Table 1.

Table 1. Selected cellulolytic and oxidative enzymes.

Name	Cazy	NCBI Accession	Organism	Optimal Conditions	References
Processive Endoglucanases					
CaCel9R	GH9 EC3.2.1.4	AJ585346.1	*Clostridium thermocellumF7*	pH 6.0, 78.5°C	[21]
TfCel9A	GH9 EC3.2.1.4	AAB42155.1	*Thermobifida fusca*	pH 5.5, 60°C	[19]
Endoglucanases					
CcCel9M	GH9 EC3.2.1.4	AAG45160.1	*Clostridium cellulolyticum ATCC 35319*	pH 6.5, 37°C	[57]
TrCel45A	GH45 EC3.2.1.4	CAA83846.1	*Trichoderma reesei*	pH 5.0, 60°C	[58]
CaCel	GH45 EC3.2.1.4	ACV50414.1	*Cryptopygus antarcticus*	pH 5.5, 40°C	[59]
Lytic polysaccharide monooxygenases (LPMO)					
TrCel61A	AA9	CAA71999.1	*Trichoderma reesei*	Unknown	[60]
NcPMO-02916	AA9	XP_965598.1	*Neurospora crassa*	Unknown	[43]
Cellobiose dehydrogenase (CDH)					
NcCDHIIa	AA8	EAA273551	*Neurospora crassa OR74A*	Unknown	[43]

3.2. Recombinant production of selected enzymes

Endoglucanase concentrations of nearly 0.1 mg/mL were similar in all cultures after harvesting, with a yield of 10% after purification, lower than the 28% achieved

by (Zhang et al., 2010). Endoglucanase activities were 4-5 U/mg (amount of enzyme capable of releasing 1 µmol of reducing sugars, per minute at 50° C, per gram of substrate) after purification. For the LPMOs, following purification, the concentration achieved for TrCel61A was nearly 2-fold higher than NcPMO-02916: 0.73 and 0.34 mg/mL, respectively. However, a similar activity was found for both enzymes: 0.190 U/mg for TrCel61A and 0.187 U/mg for NcPMO-02916. NcCDHIIa was recovered at a concentration of 0.35 mg/mL after purification and an activity of 12 U/mg in the first batch of production and an activity of 31.9 U/mg in a second batch.

3.3. Pretreated sugarcane straw hydrolysis by selected endoglucanases under their optimal conditions

The glucose and COS production after 48 hours of hydrolysis are summarized in Fig. 1. The COS yield in the commercial enzyme hydrolysates was higher than in the hydrolysates produced from the recombinant enzymes. The total produced COS were: 4.80 (TfCel9A), 7.42 (CtCel9R), 2.82 (CcCel9M), 6.60 (CaCel), 4.05 (TrCel45A), 24.20 (Cellic® CTec2) and 26.54 (Celluclast® 1.5L) mg/g of pretreated sugarcane straw, as shown in Figure 1. However, it is interesting to note that the ratio COS/glucose was much higher using the selected enzymes in comparison to the positive controls, suggesting that these enzymes naturally produced a low amount of glucose (TrCel9A: 14.29, CtCel9R: 16.78, CcCel9M: 9.92, CaCel: 27.12, TrCel45A: 13.51, Cellic® CTec2: 0.089 and Celluclast® 1.5L: 0.123). Therefore, optimization was necessary in order to improve the amount of COS produced while maintaining a high COS/Glucose ratio.

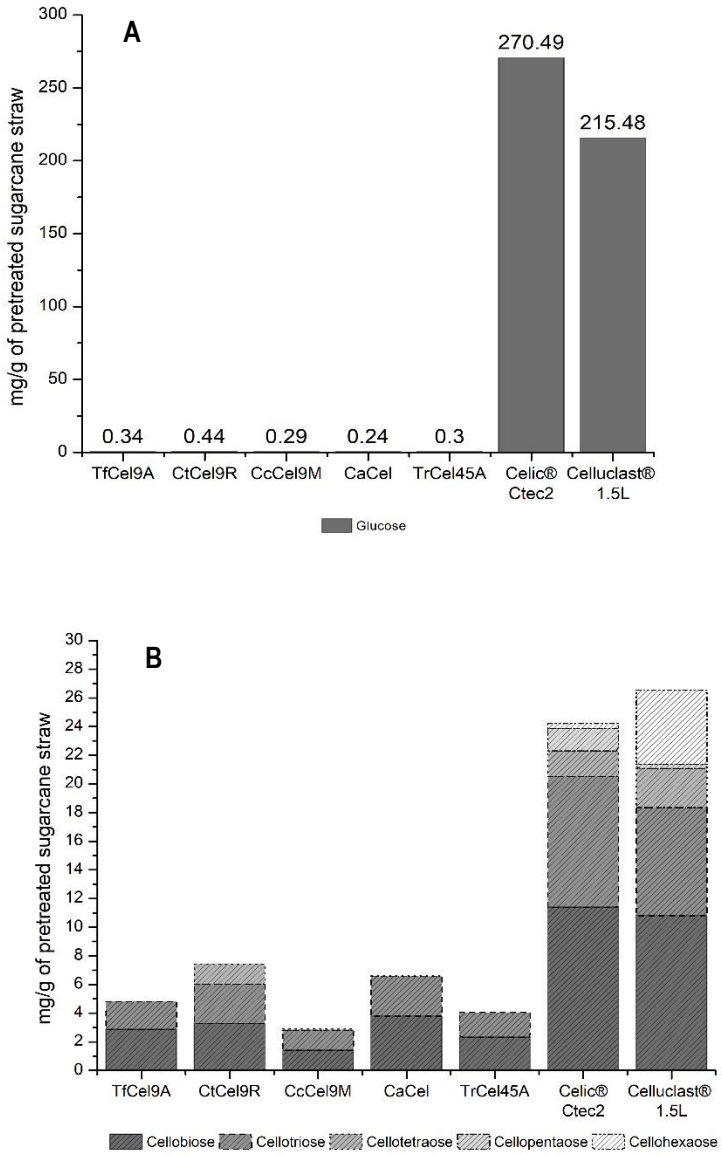

Fig. 1. Glucose and COS production from hydrothermally pretreated sugarcane straw hydrolysis after 48 hours applying selected individual endoglucanase enzymes and enzymatic commercial cocktails under their optimal condition of pH and temperature. A) Glucose production; B) COS production.

3.4. Plackett-Burman (PB) experimental designs

Enzymatic reactions were maintained at pH 5.0 and 50 °C, since mild temperature and pH conditions decrease sugar degradation and the formation of reaction inhibitors and are commonly used in industry (Zabed et al., 2017), using 10 U/g (+1), 0 U/g (-1) and 5 U/g of substrate (0), 1 % (w/v) of pretreated biomass, 150 rpm. Samples were collected after 24, 48 and 72 hours. For the hydrothermally pretreated sugarcane straw, experiment 7 produced the highest amount of COS after

48 hours of hydrolysis, reaching 7.27 mg/g of pretreated sugarcane straw (5.61 and 5.88 mg/g for 24 and 72 hours, respectively), with a COS/Glucose ratio of 25.9 (Table 2). Experiment 7 combined a mixture of CtCel9R, CcCel9M, and CaCel enzymes, and it was similar to the amount found in the CtCel9R hydrolysis alone (7.43 mg/g of pretreated sugarcane straw, Fig. 1 - B). In addition, for all the three sets of samples, the central points were very similar, and no significative COS were found in experiment 12, proving the success and robustness of the design. Finally, the Pareto chart (Fig. 2, α = 10%) for 48 hours hydrolysis showed that CaCel and CcCel9M had significant differences (Table 3; p-value = 0.02 and 0.07, effect = 2.07 and 1.50, respectively), and both enzymes were chosen for further optimizations.

Table 2. Plackett-Burman experimental design codified matrix (P12+3 central points) for the enzymatic hydrolysis of hydrothermally pretreated sugarcane straw (1% w/v) by selected endoglucanases (10 U/g), at pH 5.0 and 50.0 °C, after 48 hours, and the glucose and COS released amount (mg/g of pretreated sugarcane straw). ND = not detected.

#	TfCel9A	CtCel9R	CcCel9M	CaCel	TrCel45A	Glucose (mg/g)	Cellobiose (mg/g)	Cellotriose (mg/g)	Cellotetraose (mg/g)	Cellopentaose (mg/g)	Cellohexaose (mg/g)	COS sum (mg/g)	COS / Glucose
1	1	-1	1	-1	-1	0.22	3.46	1.34	0.34	ND	ND	5.13	23.51
2	1	1	-1	1	-1	0.24	4.84	1.74	ND	ND	ND	6.58	27.04
3	-1	1	1	-1	1	0.23	4.64	1.85	0.04	ND	ND	6.53	28.50
4	1	-1	1	1	-1	0.22	4.66	1.76	ND	ND	ND	6.42	29.07
5	1	1	-1	1	1	0.22	4.14	1.60	ND	ND	ND	5.74	25.98
6	1	1	1	-1	1	0.21	3.96	1.60	ND	ND	ND	5.55	26.82
7	-1	1	1	1	-1	0.28	5.29	1.99	ND	ND	ND	7.27	25.88
8	-1	-1	1	1	1	0.29	4.79	1.87	0.04	ND	ND	6.70	23.27
9	-1	-1	-1	1	1	0.29	4.38	1.78	0.43	ND	ND	6.59	22.38
10	1	-1	-1	-1	1	0.24	2.37	1.17	0.73	ND	ND	4.27	17.93
11	-1	1	-1	-1	-1	0.45	3.01	1.28	0.93	ND	ND	5.23	11.58
12	-1	-1	-1	-1	-1	0.17	0.18	ND	ND	ND	ND	0.18	1.10
13	0	0	0	0	0	0.27	4.61	1.77	ND	ND	ND	6.38	23.83
14	0	0	0	0	0	0.28	4.55	1.79	0.15	ND	ND	6.49	23.36
15	0	0	0	0	0	0.28	4.59	1.83	0.17	ND	ND	6.59	23.86

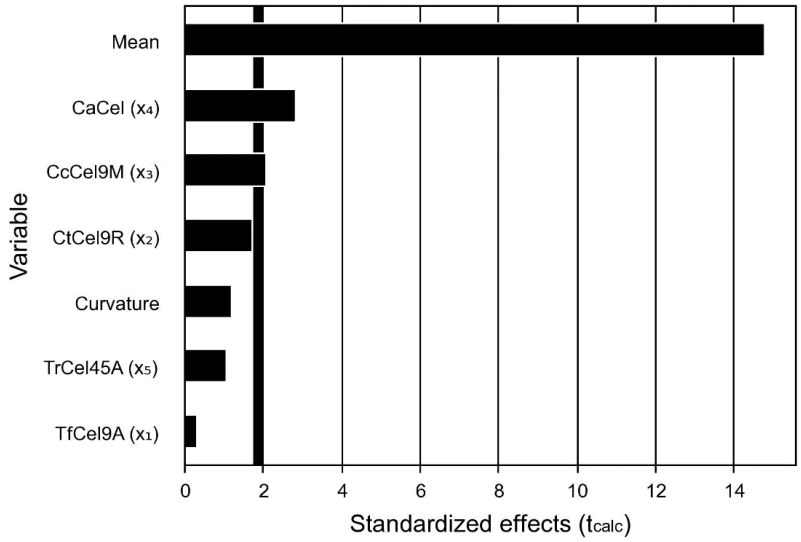

Fig. 2. Pareto chart of standardized effects (p < 0.10) of COS released after enzymatic hydrolysis of hydrothermally pretreated sugarcane straw by a mixture of endoglucanases.

Table 3. Table of effects resulting from the Plackett-Burman experimental design for the evaluation of hydrothermally pretreated sugarcane straw (1% w/v) enzymatic hydrolysis by the selected endoglucanases (10 U/g), at pH 5.0 and 50.0 °C, after 48 hours.

Name	Effect	Standard Error	Calculated t	p-value
Mean	5.52	0.36	15.19	0.00
Curvature	1.94	1.62	1.20	0.27
TfCel9A (x_1)	0.20	0.73	0.27	0.79
CtCel9R (x_2)	1.27	0.73	1.75	0.12
CcCel9M (x_3)	1.50	0.73	2.07	0.07
CaCel (x_4)	2.07	0.73	2.85	0.02
TrCel45A (x_5)	0.76	0.73	1.05	0.32

In the second round of optimization, the action of additives and auxiliary enzymes in COS production was analyzed. To do so, the hydrothermally pretreated sugarcane straw was hydrolyzed with the endoglucanases CaCel and CcCel9M. Experiment 5 (Table 4, TrCel61A +1, NcPMO-02916 +1, Ascorbic Acid -1, NcCDHIIa +1, Lactose +1, Cobalt -1 and Copper +1) resulted in the highest production of COS after 48 hours of hydrolysis, reaching an amount of 25.85 mg of COS per g of pretreated sugarcane straw (Table 4). This represents more than 3.5 times the concentration achieved in previous experiment (PB15 – endoglucanases, 48 hours). The Pareto chart (Fig. 3, α = 10%) for 48 hours hydrolysis shows what variables had a significant effect on COS production. Cobalt negatively influenced COS production (Table 5; p-value = 0.05, effect = -6.95) in contrast to the results of Jung et al., 2015,

in which cobalt in combination with an enzymatic cocktail increased the amount of sugar released from pretreated oak and kenaf by 12 and 11%, respectively. Ascorbic acid also negatively influenced COS production (Table 5; p-value = 0.8, effect = -0.69), suggesting that the NcCDHIIa capacity as an electron donor to LPMOs was satisfactory and no extra electron donor was necessary in the proposed system. The chart also showed that lactose (Table 5; p-value = 0.06, effect = 6.51), an external electron donor for NcCDHIIa, and copper (Table 5; p-value = 0.07, effect = 6.37) positively influenced COS production. Lactose plays a similar electron donation role to cellobiose, suggesting that poor cellobiose availability in the substrate was limiting CDH activity (Kittl et al., 2012). Copper saturation is essential for good LPMOs activity (Horn et al., 2012). Based on these findings, experiment 5 was submitted for validation analysis in order to confirm the optimization of COS production.

The boosting capacity of AAs was also shown by Dimarogona et al: his group obtained a 20% of improvement in sugar yield from pretreated spruce using an AA9 from *Sporotrichum thermophile*, (Dimarogona et al., 2012). The benefits of AAs were also demonstrated in different substrates, such as sugarcane bagasse (Rodríguez-Zúñiga et al., 2015), wheat straw (Cannella and Jørgensen, 2014), corn stover, poplar and lodgepole pine (Hu et al., 2015, 2014).

Table 4. Plackett-Burman experimental design codified matrix (P12+3 central points) for the enzymatic hydrolysis of hydrothermally pretreated sugarcane straw (1% w/v) by selected endoglucanases (10 U/g), oxidative enzymes and additives, at pH 5.0 and 50.0 °C, after 48 hours, together with the amount of glucose and COS released (mg/g of pretreated sugarcane straw). ND = not detected.

#	TrCel61a	NcPMO-02916	Ascorbic acid	NcCDHIIa	Lactose	Cobalt	Copper	Glucose (mg/g)	Cellobiose (mg/g)	Cellotriose (mg/g)	Cellotetraose (mg/g)	Cellopentaose (mg/g)	Cellohexaose (mg/g)	COS sum (mg/g)	COS / Glucose
1	1	-1	1	-1	-1	-1	1	2.92	3.27	1.58	2.61	ND	0.43	7.89	2.70
2	1	1	-1	1	-1	-1	-1	2.30	ND	0.11	0.63	ND	ND	0.74	0.32
3	-1	1	1	-1	1	-1	-1	7.92	1.03	0.08	0.37	0.44	ND	1.92	0.24
4	1	-1	1	1	-1	1	-1	0.16	ND	0.14	ND	0.89	0.03	1.06	6.63
5	1	1	-1	1	1	-1	1	2.39	0.62	ND	0..3	24.93	ND	25.85	10.82
6	1	1	1	-1	1	1	-1	0.19	0.60	0.71	0.63	0.65	ND	2.59	13.63
7	-1	1	1	1	-1	1	1	2.52	ND	0.03	ND	0.12	ND	0.15	0.06
8	-1	-1	1	1	1	-1	1	2.59	0.18	0.09	0.32	13.51	ND	14.10	5.44
9	-1	-1	-1	1	1	1	-1	0.18	ND	ND	ND	4.08	0.16	4.24	23.56
10	-1	-1	-1	-1	1	1	1	ND	0.03	0.23	0.34	ND	ND	0.60	-
11	-1	1	-1	-1	-1	1	1	0.40	0.06	ND	ND	0.24	ND	0.30	0.75
12	-1	-1	-1	-1	-1	-1	-1	0.17	0.12	ND	ND	ND	ND	0.12	0.71
13	0	0	0	0	0	0	0	ND	ND	0.05	ND	1.21	ND	1.26	-
14	0	0	0	0	0	0	0	ND	ND	0.05	ND	0.93	ND	0.98	-
15	0	0	0	0	0	0	0	ND	ND	0.05	ND	1.24	ND	1.29	-

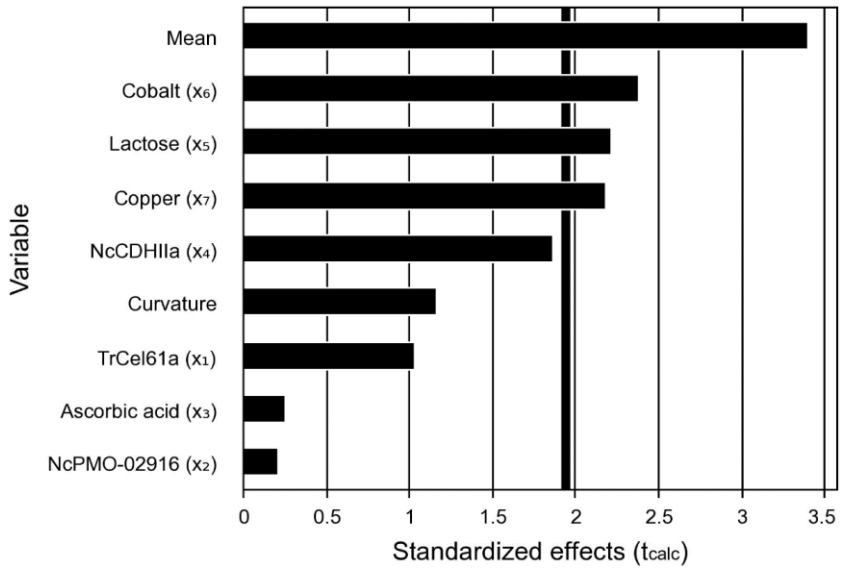

Fig. 3. Pareto chart of standardized effects ($p < 0.10$) of COS released after enzymatic hydrolysis of hydrothermally pretreated sugarcane straw by the mixture of oxidative enzymes and additives.

Table 5 Table of effects resulting from the Plackett-Burman experimental design for the enzymatic hydrolysis of hydrothermally pretreated sugarcane straw (1% w/v) by the selected endoglucanases (10 U/g), oxidative enzymes and additives, at pH 5.0 and 50.0 °C, after 48 hours.

Name	Effect	Standard Error	Calculated t	p-value
Mean	4.96	1.45	3.41	0.01
Curvature	-7.57	6.51	-1.16	0.29
TrCel61a (x_1)	2.98	2.91	1.03	0.34
NcPMO-02916 (x_2)	0.59	2.91	0.20	0.85
Ascorbic acid (x_3)	-0.69	2.91	-0.24	0.82
NcCDHIIa (x_4)	5.45	2.91	1.87	0.11
Lactose (x_5)	6.51	2.91	2.24	0.07
Cobalt (x_6)	-6.95	2.91	-2.39	0.05
Copper (x_7)	6.37	2.91	2.19	0.07

3.5. Experimental design validation

The optimized conditions for hydrolysis of hydrothermally pretreated sugarcane straw as well as comparison to commercial enzyme cocktails (Cellic® CTec2 and Celluclast® 1.5L) were validated (Table 6, Fig. 4). This produced a COS concentration of 60.49 ± 0.82 mg/L. This was higher than expected from the PB15 - auxiliary enzymes and additives; however, it may be explained by the higher activity of the NcCDHIIa enzyme used in the validation experiments. On a normalized scale,

it produced 2.15 mg of COS/g of pretreated sugarcane straw per 1 U of NcCDHIIa/mg of pretreated sugarcane straw in the PB15 experiment, and in the validation test, 1.90 mg of COS/g of pretreated sugarcane straw was produced per 1 U of NcCDHIIa/mg of pretreated sugarcane straw. This observation highlights the importance of CDH in COS production. It is also evident that the optimized conditions produced between 1.8 and 2.7-fold more COS than the commercial enzyme cocktails. Regarding the COS/Glucose ratio, the optimized condition generated a ratio of 298.31 against 0.09 and 0.13 for Cellic® Ctec2 and Celluclast® 1.5L, an increase of 3314.55 and 2294.69-fold, respectively.

It is interesting to note that mainly cellopentaose was produced under the optimized conditions while no cellopentaose or cellohexaose were detected in the endoglucanase PB experiment, the reason for which has yet to be determined. Depending on the substrate/product equilibrium, the synthesis of higher DP (degree of polymerization) COS during enzymatic hydrolysis by retaining endoglucanases is possible due to their ability to catalyze the reverse transglycosylation reaction, in which the enzyme-COS intermediate reacts with a second oligomer as opposed to water, resulting in their linking via a new glycosidic bond (Claeyssens et al., 1990; Harjunpää et al., 1999). However, all the endoglucanases used in this study belong to glycoside hydrolase families containing cellulases with inverting mechanisms of hydrolysis, meaning that transglycosylation is unlikely to be the mechanism for cellopentaose formation under the optimized conditions. Moreover, the enzymatic commercial cocktail converted an average of 283.11 mg/g of cellulose into sugars (glucose and oligomers), while the optimized conditions using the constructed cocktail converted only 60.7 mg/g of cellulose into sugars, indicating that further optimization is required and possible in order to increase COS production (Table 6, sum of produces sugars).

Table 6 Validation experiment of enzymatic hydrolysis of hydrothermally pretreated sugarcane straw (1%, w/v), after 48 hours, at pH 5.0 and 50.0 °C, under optimized conditions and comparison to enzymatic commercial cocktails (mg/g of pretreated sugarcane straw).

#	Glucose (mg/g)	Cellobiose (mg/g)	Cellotriose (mg/g)	Cellotetraose (mg/g)	Cellopentaose (mg/g)	Cellohexaose (mg/g)	COS sum (mg/g)	COS / Glucose
Optimized Condition	0.20 ± 0.01	1.67 ± 0.03	0.86 ± 0.01	1.89 ± 0.02	52.66 ± 1.03	3.42 ± 0.15	60.49 ± 0.82	298.31 ± 2.02
Cellic® Ctec2	255.47 ± 56.55	7.20 ± 5.10	4.70 ± 4.02	5.53 ± 3.97	0.66 ± 0.53	4.15 ± 1.47	22.24 ± 6.09	0.09 ± 0.00
Celluclast® 1.5L	254.97 ± 21.95	21.03 ± 13.64	6.74 ± 3.37	4.32 ± 3.58	0.77 ± 1.09	0.68 ± 0.45	33.54 ± 13.21	0.13 ± 0.01

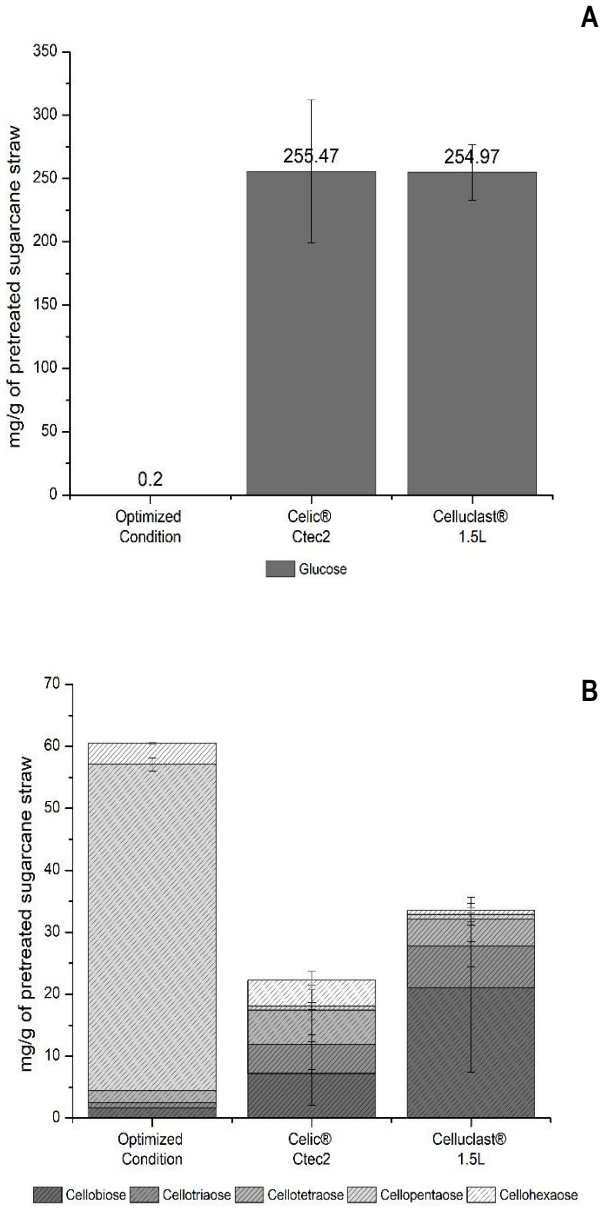

Fig. 4. Glucose and COS production from hydrothermally pretreated sugarcane straw hydrolysis after 48 hours of hydrolysis under optimized conditions and comparison to commercial cocktails. A) Glucose production; B) COS production.

In a further set of experiments, the potential benefit of using two LPMOs and the importance of the NcCDHIIa in the hydrolysis were evaluated (Table 7). To do so, each LPMO enzyme was evaluated individually in the presence and absence of NcCDHIIa under the same optimized conditions and experimental parameters. The results showed that the presence of NcPMO-02916 was not essential for COS production since the production was similar to the amount produced in the validation

experiments (61.72 ± 1.26 mg/g of pretreated sugarcane straw), and that the majority of observed LPMO activity could be attributed to the presence of TrCel61a. However, the absence of NcCDHIIa decreased COS production to 8.90 ± 0.25 mg/g of pretreated sugarcane straw, a similar amount found in the experiments without auxiliary enzymes and additives (7.27 mg/g). These results demonstrated the synergism between LPMO and CDH enzymes, where the CDH acts by donating electrons to the LPMO, which subsequently reduces electron acceptors such as molecular oxygen. The reduction of oxygen forms the reactive species necessary to attack the surface of cellulose (Hildén et al., 2000; Horn et al., 2012).

Table 7. Evaluation of the importance of NcCDHIIa and NcPMO-02916 under validation experimental condition for the hydrolysis of hydrothermally pretreated sugarcane straw (1% w/v), after 48 hours, at pH 5.0 and 50.0 °C (mg/g of pretreated sugarcane straw). ND = not detected.

Optimized Condition	Glucose (mg/g)	Cellobiose (mg/g)	Cellotriose (mg/g)	Cellotetraose (mg/g)	Cellopentaose (mg/g)	Cellohexaose (mg/g)	COS sum (mg/g)	COS/ Glucose
Without NcPMO-02916	ND	1.84 ± 0.08	1.10 ± 0.09	2.23 ± 0.17	53.96 ± 1.05	2.59 ± 0.14	61.72 ± 1.26	-
Without NcPMO-02916 and NcCDHIIa	0.34 ± 0.02	2.32 ± 0.12	1.81 ± 0.00	2.50 ± 0.15	2.27 ± 0.22	ND	8.90 ± 0.25	26.17 ± 0.26

4. Conclusion

The optimized conditions produced 60.49 mg of COS per g of hydrothermally pretreated sugarcane straw after 48-hour hydrolysis. This amount represents an increase of 1.8 - 2.7-fold compared to the commercial enzymatic cocktails with a COS/Glucose ratio 3314.55 and 2294.69-fold higher than the Cellic® Ctec2 and Celluclast® 1.5L's ratio, respectively. Further improvement in yield may be possible by improving the accessibility to cellulose by modifying the pretreatment. To the best of our knowledge, this is the first study that has used a design of experiments approach to analyze the synergism of endoglucanases, LPMOs, CDH and different additives to hydrolyze pretreated sugarcane straw/lignocellulose into cello-oligosaccharides. These results open a new perspective for COS production and its possible application.

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Chapter 4

4. Endoglucanases, hydrolysis conditions and pretreatments for COS production

Screening of potential endoglucanases, hydrolysis conditions and different sugarcane straws pretreatments for cello-oligosaccharides production

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Screening of potential endoglucanases, hydrolysis conditions and different sugarcane straws pretreatments for cello-oligosaccharides production

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Abstract

Cello-oligosaccharides (COS) are oligomers with 2 to 6 β-1,4-linked glucose units, with potential applications in the food/feed and bioenergy industrial sectors. In this study, the combination of five heterologous expressed endoglucanases varying the temperature and pH conditions were evaluated by design of experiments for COS production. Afterwards, the best combination was tested to produce COS from different pretreated sugarcane straws: ionic liquid, diluted acid, hydrothermal and steam-explosion. The results showed that steam explosion pretreated sugarcane straw treated with CtCel9R enzyme at 50°C and pH 5.0 yielded 13.4 mg COS g biomass^{-1}, 5-18-fold higher compared to the other pretreated straws. Under the conditions evaluated, the removal of hemicellulose and decrease in the cellulose crystallinity can benefits the enzymatic hydrolysis. This is the first study that combined the evaluation of different enzymes, conditions, and sugarcane straw pretreatments to optimize COS production in a single step without glucose formation.

Keywords

cello-oligosaccharides; endoglucanases; sugarcane straw; pretreatment; hydrolysis.

1. Introduction

A path toward lignocellulosic biomass for advanced biofuels and bioproducts within the biorefinery concept ensures future energy security and sustainability of cellulosic and sugar-based industries (Mahmood et al., 2019). Among the available feedstock candidates, sugarcane straw is gaining attention to produce bioethanol and value-added products, in addition to heat and electrical power generation (Brenelli et al., 2020; Ferrari et al., 2019). The bioproducts portfolio which can be obtained through chemical and biochemical conversion technologies includes xylitol, acetic acid, furfural, antioxidants, xylo-oligosaccharides (XOS) and cello-oligosaccharides (COS) (Brienzo et al., 2017; Kaschuk and Frollini, 2018; Kruyeniski et al., 2019; Zhai et al., 2018).

COS are biologically important molecules and it has been related to food, feed, and bioenergy industrial sectors (Barbosa et al., 2020b). COS are defined as linear oligomers saccharides consisting of 2 to 6, β-1,4-linked glucose units (Otsuka et al., 2004; Zhao et al., 2009), and important functional molecules because it was able to support the growth of different probiotic strains (Karnaouri et al., 2019). Moreover, when combined with fermentative microorganisms capable of consuming oligomeric sugars, COS can be used for bioethanol production in place of glucose once it can give some fermentation advantages, such as shorter fermentation time, fewer process contaminants and limited process inhibition by high concentration of glucose (Ahmed et al., 2017; Mallek-Fakhfakh and Belghith, 2016; Yang et al., 2015).

COS can be derived from the biomass cellulose fraction through different vias, such as acid hydrolysis, hydrolysis over carbon catalysts, mild thermal conversion, and controlled enzymatic hydrolysis. The latter is considered selective and greener compared to the others approaches with the advantage that can be designed according to the degree of polymerization desired and minimal production of monomers (Brienzo et al., 2017; Kaschuk and Frollini, 2018; Kruyeniski et al., 2019; Zhai et al., 2018).

A typical enzymatic hydrolysis cocktail contains exoglucanases and endoglucanases. Endoglucanases have affinity for amorphous cellulose regions and promote a random attack on internal β-glycosidic bonds, releasing oligomers of different lengths, mainly cellobiose and cellotriose. Among the endoglucanases, processive endoglucanases have been reported as the mainly class of enzymes able

to release oligosaccharides with a broad range of degree of polymerization (DP) from amorphous cellulose. The majority of processive endoglucanases harbor an accessory carbohydrate-binding module (CBM) in addition to the traditional glycoside hydrolase family 9 (GH9) catalytic domain, allowing them to have both exo- and endoglucanase mode of action (Barbosa et al., 2020; Hu et al., 2015; Karnaouri et al., 2019).

Several approaches for COS production optimization have been developed with the use of regular endoglucanases, processive endoglucanases and auxiliary enzymes (AAs), such as lytic polysaccharide mono-oxygenases (LMPOs) and cellobiose dehydrogenase (CDH) (Barbosa et al., 2020). Recently, this group reached a COS production of approximately 60 mg/g of pretreated sugarcane straw using a combination of heterologous expressed processive endoglucanases (10 U/g), LPMO (2 mg/g) and CDH (1 mg/g) (Barbosa et al., 2020b).

There are several factors that can affect the enzymatic hydrolysis conversion yield and they can be related both to enzymes and biomass properties resulted from the pretreatments (Amit1 et al., 2018; Lin et al., 2020). Regarding the enzymes, parameters such as loading, synergy, temperature, pH and agitation must be considered (Kumar and Pruthi, 2014; Sarkar et al., 2012; Van Dyk and Pletschke, 2012). The choice of the right pretreatment for a particular feedstock is essential to reduce the biomass recalcitrance, hence increasing the accessibility of cellulose for COS production via enzymatic hydrolysis (Leu and Zhu, 2013; Várnai et al., 2010).

In this study, we evaluated five different processive endoglucanases of bacterial and fungal origin, belonging to GH9 and GH45 families for their ability to release COS from pretreated-sugarcane straw. First, we analyzed the optimal combination of endoglucanases, pH, and temperature by design of experiments (DoE) to produce COS from hydrothermal-pretreated straw (HyD). Afterwards, enzymatic hydrolysis at the selected condition after the DoE study was carried out with ionic liquid (IL), diluted acid (DA) and steam-explosion (SE)-pretreated sugarcane straw and the COS yields evaluated.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were analytical grade, purchased from Sigma-Aldrich, Merck/Millipore, or BD Biosciences. Regenerated amorphous cellulose (PASC) was

prepared by the treatment of Avicel with phosphoric acid (Wood and Bhat, 1988). Cellobiose, cellotriose, cellotetraose, cellopentaose and cellohexaose standards were bought from Megazyme (> 95% purity).

Sugarcane straw, composed by green tops and dry leaves were air-dried until ~10% (w/w) moisture content, hammer-milled to obtain an average size of 0.15 mm - 2.38 mm (-8 /+60 mesh) and partially de-ashed using a Disintegrator DM 540 (IRBI, Araçatuba, São Paulo, Brazil). The recovered sugarcane straw, containing approximately 7% (w/w) moisture content, was stored at room temperature before use. Chemical characterization regarding cellulose, hemicellulose, lignin and ash content of all pretreated sugarcane straws were determined according to Justin B. Sluiter et al., 2016.

2.1.1. Hydrothermal-pretreated sugarcane straw

The Hyd-pretreated sugarcane (two-stage pretreatment) was obtained according described previously (Brenelli et al., 2020). Briefly, the reactions were conducted in a 350 L capacity reactor, built out of 276 Hastelloy steel, designed by the Brazilian Biorenewables National Laboratory (LNBR) and Pope Scientific INC. The reactor was loaded with 20 kg of sugarcane straw (dry basis) and NaOH solution (approximately 200 L, 0.8%, w/w), achieving a solid concentration of 9% (w/w). The reaction mixture was heated at 60°C for 30 min. Then, the reactor was discharged, and the solid fraction was separated from the black liquor by filtration in a Nutsche filter with a 100 L capacity (Pope Scientific INC- 276 Hastelloy steel) and thoroughly washed until pH 7. The mild alkaline-pretreated sugarcane straw and water were added in the reactor achieving a solid concentration of 9% (w/w) and the reaction mixture was heated at 190°C for 20 min. Afterwards, the reactor was discharged and the solid fraction (Hyd-pretreated sugarcane straw) was separated from the soluble fraction by filtration in a Nutsche filter with a 100 L capacity, thoroughly washed until neutral pH was reached and air-dried at room temperature (25 ± 2°C) until moisture content below 10% (w/w).

2.1.2. Ionic liquid pretreated sugarcane straw

The IL-pretreated sugarcane straw was obtained according described previously (Ferrari et al., 2020). Briefly, sugarcane straw was milled, passed through a set of sieves and the material retained between 16- and 24-mesh sieves (0.7−1.19

mm in diameter) was used for the experiments. Pretreatment was carried out in a stainless-steel reactor consisting of a jacketed vessel with a maximum operating pressure of 10 bar and an anchor impeller. A mixture of 60 % (w/w) H_2O, 30 % (w/w) 2-hydroxyethylammonium acetate ([Mea][Ac]) and 10 %(w/w) 2-hydroxyethylammonium hexanoate ([Mea][Hex]) was used to pretreat 37.24 g of the biomass, 8.7% (w/w) moisture content, at 20 %(w/w) of solid loading and 130 °C (Ferrari et al., 2019). After 4 h of pretreatment, 300 g of water was added to the samples, and solid and liquid fractions were separated using a 125 µm nylon filter. Then, solid fractions were washed twice with 300 g of water and dried overnight at 105 °C.

2.1.3. Diluted acid pretreated sugarcane straw

The diluted acid pretreatment was carried out in 316L stainless steel batch reactor of 0.5 L capacity. The reactor was filled with 20.0 g of sugarcane straw (dry basis), deionized water and sulfuric acid 72% (w/w) to 0.5% v/v final concentration, and solid concentration of 9% (w/w). The reactor was heated in a glycerin bath calibrated to perform reaction at 140°C. After 15 min of effective treatment time (defined as residence time at target temperature plus corrective time due to heating ramp), the reactor was immediately cooled in an ice bath. The reaction products were separated by straining using a 125 µm nylon filter. The solid fraction (DA-pretreated sugarcane straw) was thoroughly washed until neutral pH was reached and air dried at room temperature (25 ± 2°C) until moisture content below 10% (w/w).

2.1.4. Steam-explosion pretreated sugarcane straw

The steam-explosion pretreatment was carried out in 20 L reactor built out of 276 Hastelloy steel designed by LNBR and Pope Scientific INC. The reactor was loaded with 1.5 kg of sugarcane straw (dry basis), hermetically closed and steam was injected until a pressure of approximately 1.2 MPa (equivalent to 195 °C) was achieved. After 10 min of pressurization, the reactor was suddenly depressurized, the steam-exploded slurry, referred as SE-pretreated sugarcane straw (~70% moisture content) was discharged, collected and dried at room temperature (20 ± 2°C) until 10% (w/w) moisture content.

2.2. X-Ray diffraction (XRD), crystallinity index (CI) and Infrared spectroscopy (FTIR)

Approximately 3.0 g of each pretreated sugarcane straw sample was mixed with analytical grade isopropanol, to ensure fixation, and pressed against a support of polymethyl methacrylate (PMMA) in disc form with 2 mm depth and 25 mm diameter. A Rigaku X-ray diffractometer Rint 2100 VPC/N (Software PDXL2) equipped with Cu radiation source with wavelength 1.542 Å (voltage of 40 kV and current of 30 mA) and 0.2 mm slits was used. The θ-2θ scans were performed in the range of 5–40° with 0.02° steps and 2 s per step. The time for obtaining the XRD diffractogram was approximately 20 min per sample (Caliari et al., 2017).

Crystallinity values were determined according to the method of Segal et al., 1959, according to the following equation.

$$CI\ (\%) = \left(\frac{I002 - Iam}{I002}\right) * 100$$

where CI (%) is the crystallinity index calculated as a percentage, I002 is the diffraction intensity associated with crystalline cellulose (maximum diffraction between 20° < 2θ < 25°), and Iam is the intensity associated to amorphous cellulose (minimum diffraction between 15° < 2θ < 20°). The band at 1514 cm^{-1} was chosen as an internal standard.

FTIR were obtained on a JASCO FT/IR-4100 (Tokyo, Japan) spectrophotometer, using a KBr disk containing finely ground samples. Thirty-two scans were taken of each sample recorded from 4000 to 500 cm^{-1} at a resolution of 1 cm^{-1} (Oliveira Moutta et al., 2013).

2.3. Enzymes selection and Plasmids construction

The five endoglucanases used in this study were chosen based on their capacity to release COS from lignocellulosic materials. The literature has already reported that CtCel9R, CcCel9M, TfCel9A and TrCel45a enzymes release mainly cellotetraose, whereas the enzyme CaCel releases mainly cellobiose and cellotriaose. The Table 1 summarizes the enzymes name, Cazy number, NCBI accession, organism origin, and the pH and temperature optimal conditions described in the literature.

Table 1. Selected cellulolytic enzymes.

Name	Cazy	NCBI Accession	Organism	Optimal Conditions	References
Processive Endoglucanases					
CtCel9R	GH9 EC3.2.1.4	AJ585346.1	*Clostridium thermocellumF7*	pH 6.0, 78.5°C	(Zverlov et al., 2005)
TfCel9A	GH9 EC3.2.1.4	AAB42155.1	*Thermobifida fusca*	pH 5.5, 60°C	(Irwin et al., 1998)
Endoglucanases					
CcCel9M	GH9 EC3.2.1.4	AAG45160.1	*Clostridium cellulolyticum ATCC 35319*	pH 6.5, 37°C	(Belaich et al., 2002)
TrCel45A	GH45 EC3.2.1.4	CAA83846.1	*Trichoderma reesei*	pH 5.0, 60°C	(Karlsson et al., 2002)
CaCel	GH45 EC3.2.1.4	ACV50414.1	*Cryptopygus antarcticus*	pH 5.5, 40°C	(Song et al., 2017)

Synthetic genes encoding the selected endoglucanases were assembled into the pPICZ-α B vector (Thermo Fisher Scientific) by Gibson assembly (New England BioLabs). *E. coli* DH5α (Invitrogen) was used as a host cell for DNA manipulation and *Pichia pastoris* NRRL 11430 (ATCC) was used as the host for recombinant protein production. Luria-Bertani (LB) medium with 100 µg/mL zeocin (ThermoFisher Scientific) was used for *E. coli* growth and selection while Yeast Extract-Peptone-Dextrose (YPD) 100 µg/mL zeocin agar plates were used for *P. pastoris* growth and selection (Barbosa et al., 2020b).

The selected genes were cloned into *P. pastoris* expression vector pPICZα and transformed into *P. pastoris* NRLL 11430. Transformants were checked for correct insertion of genes by PCR with standard primers 5` AOX (GACTGGTTCCAATTGACAAGC) and 3`AOX (GCAAATGGCATTCTGACATCC). A positive clone of each transformation was taken through an expression trial in 50 mL baffled Erlenmeyer for recombinant enzyme production. The cultures were monitored for optical density (OD), extracellular protein concentration (SDS-PAGE) and enzymatic activity.

2.4. Enzymes expression, purification, and activity

According to Barbosa et al., 2020b, the *P. pastoris* strains containing the selected genes were cultivated in 10 mL of Buffered Glycerol-Complex Medium (BMGY) 100 µg/mL zeocin overnight at 30 ºC and 250 rpm. A new culture (OD_{600} 1.0) in 50 mL of Buffered Methanol-Complex Medium (BMMY) was started according to the Pichia Fermentation Process Guide at 28 ºC, 200 rpm and 72 hours. Pure methanol solution was added to the culture every 24 hours to maintain a final concentration of 0.5% (v/v) for induction. At the end of culture, the fermentation broth was centrifuged at 4000 x g for 5 min and the clear culture supernatant was collect

for further purification. The endoglucanases were purified based on the binding capacity of endoglucanases to the PASC substrate (Zhang et al., 2010). Briefly, 200 mg of each enzyme was mixed with 1 g of PASC for 15 minutes and released using pure ethylene glycol solution. Their activities were determined according to IUPAC recommendations (Ghose, 1987) using carboxymethyl cellulose (CMC) as a substrate. Endoglucanase concentrations were similar in all cultures after harvesting, nearly to 0.1 mg/mL with a yield of 10% after purification, lower than the 28% achieved by (Zhang et al., 2010). Endoglucanase activities were 4-5 U/mg (defined as the amount of enzyme capable of releasing 1 µmol of reducing sugars, per minute at 50° C, per gram of substrate) after purification.

2.5. Commercial enzymes and determination of enzyme protein concentration

Two commercial cellulase enzyme cocktails, Celluclast® 1.5L (Sigma-Aldrich) and Cellic® CTec2 (Novozymes) were used for comparative purposes with the enzyme mixtures developed in the experimental designs. Protein concentrations were determined by Bradford using a prefabricated assay (Thermo Fisher Scientific) and bovine serum albumin (BSA) as the calibration standard.

2.6. Enzymatic composition and process conditions

A Design of Experiments (DoE) was applied by a Plackett-Burman design in order to analyze the effects of the factors (independent variables) in the response (dependent variable) (Rodrigues and Iemma, 2014). The five selected endoglucanases, pH and temperature were simultaneously analyzed. HyD-pretreated sugarcane straw was used in this experiment, which it was carried out in 50 mL Falcon tubes, at 150 rpm, 1 % (w/v) of substrate for 48 hours in a shaker incubator (New Brunswick Scientific) according to an adapted protocol from (Goldbeck et al., 2016). The final volume of each experiment was adjusted to 10 mL with sodium phosphate buffer (0.1 M, pH 5.0). The designs consisted of 12 experiments and 3 repetition of the central point, totaling 15 experiments. The dependent variable (COS production) was calculated based on the concentration (mg/g of pretreated sugarcane straw) of cellulose converted into COS (cellobiose to cellohexaose The real and coded levels -1, 0 (central point) and +1 for the factors endoglucanases (from 0 to 10 U/g substrate), pH (from 5.0 to 6.5) and temperature (from 40 to 60 ºC) of each experiment are presented with the response values in the results section.

Analyses of the effects was conducted with online software Protimiza Experimental Designs®.

2.7. Enzymatic hydrolysis

Based on the statistical analysis obtained in the DoE experiment regarding enzyme, temperature, and pH, the hydrolysis assays of the different pretreated sugarcane straws (HyD, IL, DA, SE-pretreated sugarcane straw) were performed. The hydrolyses were carried out again in 50 mL Falcon tubes, with 10 U/g of pretreated sugarcane straw, at 150 rpm, 1 % (w/v) of substrate for 48 hours in a shaker incubator (New Brunswick Scientific). The final volume of each experiment was adjusted to 10 mL with sodium phosphate buffer (0.1 M, pH 5.0). The different pretreated sugarcane straws were also hydrolyzed by the commercial cocktail enzymes Cellic® Ctec2 and Celluclast® 1.5L under the same protocol conditions and at manufacturer recommended temperature and pH (50 °C and pH 5.0).

2.8. Quantification of COS

The content of glucose and COS in the supernatants of the enzymatic hydrolysis assays was performed by High Performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) using an ion chromatographer Dionex ICS-5000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the method described by (Ávila et al., 2018), with some modifications. Glucose and COS (C2-C6) were separated by gradient elution using ultrapure H_2O (eluent A), NaOH 0.25 M (eluent B), NaOAc 1 M/NaOH 0.1 M (eluent C) and ultrapure H_2O (eluent A) as mobile phases on a Carbopac PA1 column (250 × 4 mm i.d., particle size 10 µm, Thermo Fisher Scientific, Waltham, MA, USA) and CarboPac PA1 guard column (50 × 4 mm i.d., particle size 10 µm, Thermo Fisher Scientific, Waltham, MA, USA). The gradient was performed as follows: 0-36 min, 40% A, 0-42% B and 60-18% C; 36-40 min, 0-3% A, 100-0% B and 0-97% C; 40-42 min, 3-0% A, 0-100% B and 97-0% C; 42-57 min, 40% A and 60% C. The samples were diluted in ultrapure water, filtered through a 0.22 µm PTFE filter, and injected into the column using an auto-sampler. The column temperature was maintained at 30 °C, the flowrate was 0.3 mL/min, and the injection volume of the samples was 25 µL. Data were acquired and processed using Chromeleon software version 7.0. The glucose and COS were identified in samples by comparing the retention times of

authentic standards and the samples. Calibration curves were constructed with commercial standards to quantify the glucose and COS in the samples. The content of glucose and COS was expressed as mg/L and then converted to mg/g of pretreated sugarcane straw (Barbosa et al., 2020b).

2.9. Statistical analysis

The pretreated sugarcane straw compositions and crystallinities were analyzed at 95% of confidence using Origin® software (ANOVA and Tukey). The results of the design experiments (Plackett-Burman) were submitted to a Pareto chart analysis at a 90% confidence level using Protimiza Experimental Design® software and the validation experiments, presented as mean and standard deviation, were performed in duplicate.

3. Results and discussion

3.1. Plackett-Burman (PB) experimental design

The enzymatic reactions were performed according the conditions described in Table 2 using purified enzymes. As can be seen, the experiment 11 (containing only CtCel9R at 60°C and pH 6.5) resulted in the highest amount of COS after 48 hours of hydrolysis, reaching 9.01 mg/g pretreated HyD-pretreated sugarcane straw and the highest COS/Glucose ratio (2.58). In opposite, experiment 1 (containing TfCel9A and CcCel9M at 60°C and pH 5.0) produced the lowest amount of COS among the experiments, 4.25-fold lower than the experiment 11. The yields achieved at the central points were very similar for all the three sets of samples (8.19, 7.67 and 7.57 mg of COS/g of HyD-pretreated sugarcane straw) and, as expected, COS were rarely found in samples from experiment 12 (no enzymes), proving the success and robustness of the design.

Table 2. Plackett-Burman experimental design codified matrix (P12+3 central points) for the evaluation of hydrothermally pretreated sugarcane straw (1% w/v) enzymatic hydrolysis by the selected endoglucanases (10 U/g), varying the pH and temperature, after 48 hours; and its glucose and COS released amount (mg/g of pretreated sugarcane straw). ND = not detected.

#	TfCel9A (U/g)	CtCel9R (U/g)	CcCel9M (U/g)	CaCel (U/g)	TrCel45A (U/g)	pH	Temperature (°C)	Glucose (mg/g)	Cellobiose (mg/g)	Cellotriose (mg/g)	Cellotetraose (mg/g)	Cellopentaose (mg/g)	Cellohexaose (mg/g)	COS sum (mg/g)	COS / Glucose
1	10	0	10	0	0	5.0	60	0.24	1.30	0.57	0.25	ND	ND	2.12	0.87
2	10	10	0	10	0	5.0	40	0.62	6.25	0.54	ND	ND	0.75	7.54	1.22
3	0	10	10	0	10	5.0	40	0.60	5.83	0.43	ND	ND	0.87	7.13	1.18
4	10	0	10	10	0	6.5	40	0.19	3.21	1.15	ND	0.13	0.19	4.68	2.53
5	10	10	0	10	10	5.0	60	0.35	4.69	1.12	ND	0.13	0.56	6.49	1.85
6	10	10	10	0	10	6.5	40	0.24	3.33	0.76	ND	0.31	0.43	4.82	2.03
7	0	10	10	10	0	6.5	60	0.35	5.96	1.67	ND	ND	0.50	8.13	2.33
8	0	0	10	10	10	5.0	60	0.28	3.47	1.26	ND	ND	ND	4.73	1.68
9	0	0	0	10	10	6.5	40	0.22	3.62	1.19	0.08	ND	ND	4.90	2.23
10	10	0	0	0	10	6.5	60	0.24	3.28	1.19	0.00	ND	ND	4.47	1.89
11	0	10	0	0	0	6.5	60	0.35	7.43	1.19	0.10	ND	0.29	9.01	2.58
12	0	0	0	0	0	5.0	40	0.13	0.15	ND	ND	ND	ND	0.15	0.12
13	5	5	5	5	5	5.75	50	0.48	6.54	0.92	ND	ND	0.73	8.19	1.72
14	5	5	5	5	5	5.75	50	0.49	6.11	0.86	ND	ND	0.70	7.67	1.57
15	5	5	5	5	5	5.75	50	0.47	6.10	0.82	ND	ND	0.64	7.57	1.61

Several studies have shown that processive endoglucanases are able to generate COS from amorphous cellulose (Asha et al., 2016; Jeon et al., 2012; Zhang et al., 2018). Since regular and processive endoglucanase have different mode of action (Barbosa et al., 2020), in this study we evaluated the synergism between three regular endoglucases (two from family GH45 and one from family GH9) and two processive endoglucanase from family GH9 of bacterial and fungi origin, all described in the literature with the capacity to release COS (Table 1). However, the Pareto chart (α = 10%) for 48 hours of hydrolysis showed that only CtCel9R had significant differences (Fig. 1, Table 3; p-value = 0.006, effect = 3.68). All other enzymes demonstrated non-significant effects on COS production. Moreover, the factors pH and temperature did not demonstrate to be significative on COS production (Fig. 1, Table 3; p-value = 0.187 and 0.318, respectively). The PB experiment was performed to investigate the possible synergism among the endoglucanases, but the table of effects resulting from the experimental design demonstrated that the enzymes TfCel9A and CcCel9M had an antagonist effect in combination with other enzymes for COS production (Table 3). The reason for this finding is unclear, however, we assume that the presence of some specific enzyme or some inhibitors generated during the HyD- pretreatment associated to the different optimal pH and temperature of each enzyme can negatively have influenced the enzymes' action. In addition, a higher substrate specificity can have caused the positive effect found to the CtCel9R enzyme. For this reason, the enzyme CtCel9R was chosen to hydrolyze the different pretreated sugarcane straws.

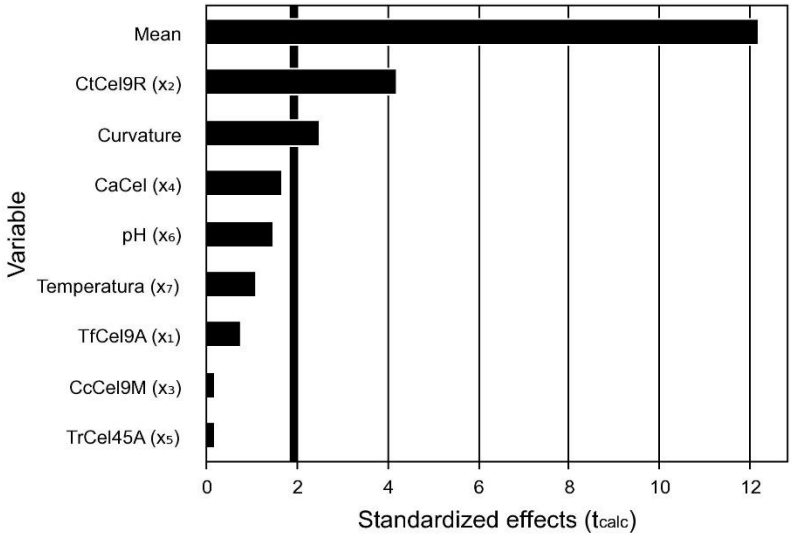


Fig. 1. Pareto chart of standardized effects ($p < 0.10$) of COS released after enzymatic hydrolysis of hydrothermally pretreated sugarcane straw by a mixture of endoglucanases, pH and temperature.

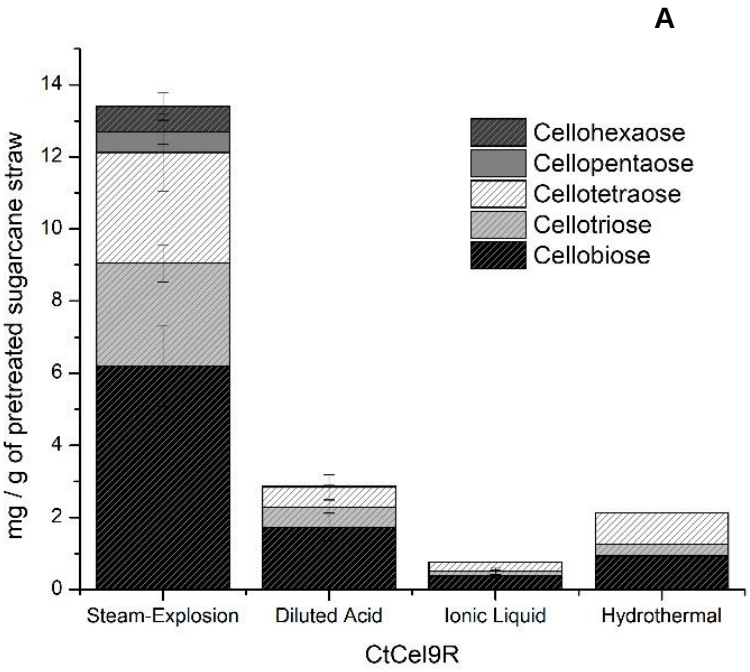
Table 3. Table of effects resulting from the Plackett-Burman experimental design for the evaluation of hydrothermally pretreated sugarcane straw (1% w/v) enzymatic hydrolysis, after 48 hours.

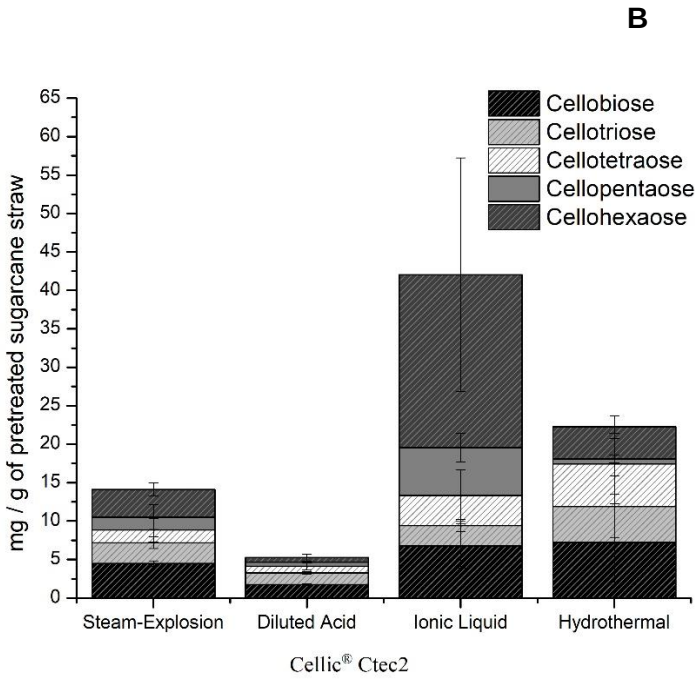
Name	Effect	Standard Error	Calculated t	p-value
Mean	5.35	0.44	12.18	0.000
Curvature	4.93	1.96	2.51	0.046
TfCel9A (x_1)	-0.66	0.88	-0.75	0.484
CtCel9R (x_2)	3.68	0.88	4.19	0.006
CcCel9M (x_3)	-0.16	0.88	-0.18	0.863
CaCel (x_4)	1.46	0.88	1.67	0.147
TrCel45A (x_5)	0.15	0.88	0.17	0.869
pH (x_6)	1.31	0.88	1.49	0.187
Temperature (x_7)	0.95	0.88	1.09	0.318

3.2. Hydrolysis of different pretreated sugarcane straws

In this study, sugarcane straw was submitted to four different pretreatments in order to promote morphological and chemical changes in biomass and consequently increase cellulose digestibility to produce COS employing the selected processive endoglucanase CtCel9R. The hydrolysis of Hyd-, IL-, DA-, SE- pretreated sugarcane straw using the enzyme CtCel9R as well as its comparison with commercial enzyme cocktails (Cellic® CTec2 and Celluclast® 1.5L) were performed at pH 5.0 and 50 °C, since no significative difference was found in the PB experiment and mild temperature and pH conditions are commonly used in the industry (Zabed et al., 2017).

The Figures 2 the production of COS after the hydrolysis of the four pretreated sugarcane straws with CtCel9R, Cellic® CTec2 and Celluclast® 1.5L after the 48 hours, respectively. CtCel9R was able to produce 13.40 mg of COS/g of SE-pretreated sugarcane straw, 4.7, 6.3 and 17.6-fold higher than the amount produced using the DA-, HyD- and IL- pretreated sugarcane straw, respectively (Fig. 2A).





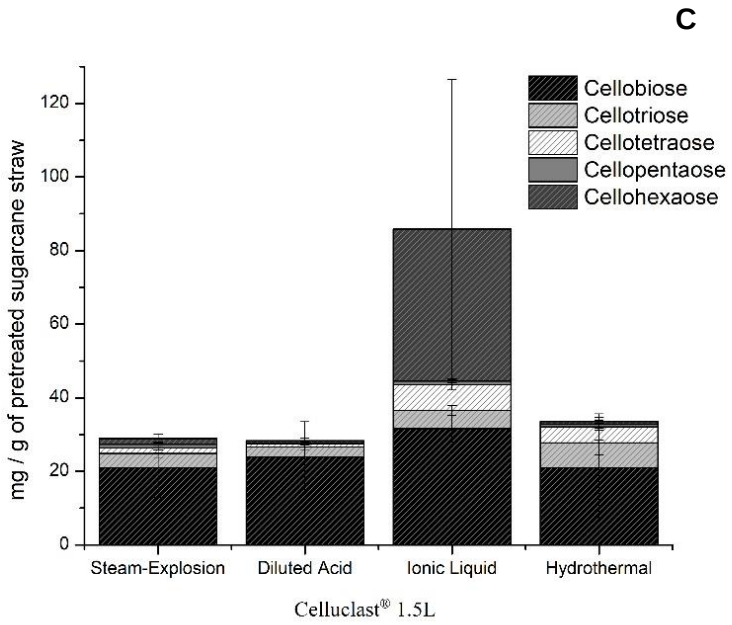


Fig. 2. COS production by CtCel9R (A), Cellic® Ctec2 (B) and Celluclast® 1.5L (C) from steam-explosion, diluted acid, ionic liquid, and hydrothermally pretreated sugarcane straws hydrolysis after 48 hours.

It is interesting to note that the IL-pretreated sugarcane straw was the only one that increased the biomass digestibility for COS production for both cocktails, reaching an amount of 42.04 mg of COS/g (2.5, 8.0 and 2.0-times higher than the amount produced using the steam-explosion, diluted acid and hydrothermal pretreatments) and 85.85 mg of COS/g (2.8, 2.8 and 3.0-times higher than the amount produced using the steam-explosion, diluted acid and hydrothermal pretreatments), respectively (Fig. 2B and C). Despite both cocktails were not designed for COS production, Cellic® CTec2 and Celluclast® 1.5L produced 3.14 and 6.40-fold higher the amount reached by the CtCel9R enzyme, respectively.

Regarding glucose production, the Figure 3 shows that the enzyme CtCel9R produced a small amount glucose only in SE-pretreated material (15.6 mg/g of pretreated sugarcane straw), whereas a high amount of glucose was detected in all other pretreated straws with both Cellic® CTec2 (54.6 – 255.4 mg/g of pretreated sugarcane straw) and Celluclast® 1.5L (38.7 – 254.9 mg/g of pretreated sugarcane straw) . The amount of glucose produced by both cocktails were similar with a slight increase of production in the hydrolysis with Cellic® CTec2, which was already expected because these cocktails possess β-glucosidase (Rodrigues et al., 2015). In

terms of digestibility of biomass under the tested conditions, the SE- pretreatment generated the highest digestible biomass for the CtCel9R enzyme, reaching a digestibility of 2.9% (against 0.21, 0.10 and 0.31 % for HyD-, IL- and DA- pretreated straws, respectively). Regarding the hydrolysis with Cellic® CTec2, the maximum digestibility obtained was for the HyD-pretreated sugarcane straw (27.77%), followed by SE- (24.36%), DA- (18.17%) and IL- (9.67%). The HyD- pretreatment was also able to generate the highest digestible biomass for the Celluclast® 1.5L cocktail (28.85%), followed by the DA- pretreatment (17.34%), LI- pretreatment (12.46%) and SE- pretreatment (12.07%).

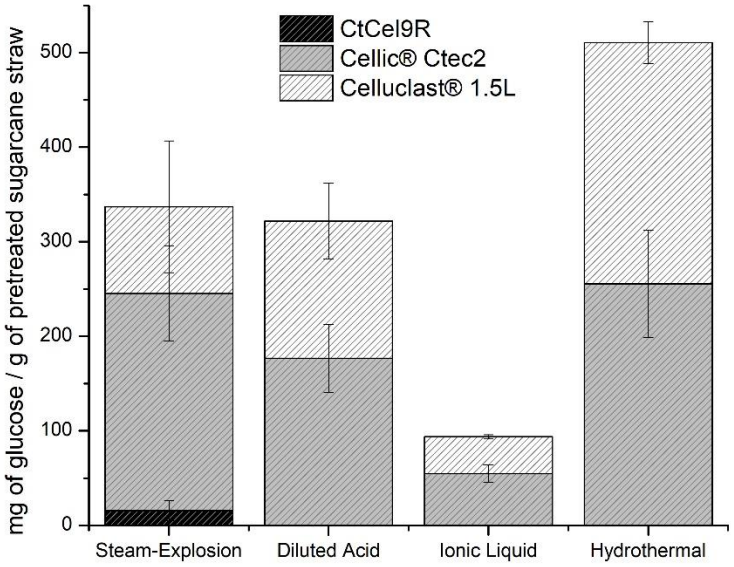


Fig. 3. Glucose production by CtCel9R, Cellic® Ctec2 and Celluclast® 1.5L from steam-explosion, diluted acid, ionic liquid, and hydrothermally pretreated sugarcane straws hydrolysis after 48 hours.

3.3. Chemical and morphological analysis of the pretreated sugarcane

Chemical and morphological changes that occurred in sugarcane straw after being submitted to different pretreatments to decrease recalcitrance are important to understand the relations between the performance of the selected endoglucanase and COS yields. For this reason, the cellulose crystallinity along with FTIR analysis and chemical composition were performed for the four sugarcane straw materials in order to investigate the influence of pretreatments on crystallinity and cellulose

enzymatic hydrolysis into COS.

The compositions of the different pretreated sugarcane straws used to produce COS are presented in Table 4. HyD- and DA-pretreated sugarcane straw had the highest content of cellulose and similar hemicellulose content. As expected, steam explosion pretreatment efficiently solubilized hemicellulose while ionic liquid preserved it, resulting in materials with low and high content of hemicellulose, respectively. However, ionic liquid pretreatment has removed mainly lignin from the biomass, achieving values comparable to the hydrothermal pretreatment. Nevertheless, the later was combined with a mild alkaline pretreatment prior the hydrothermal as an additional strategy for xylo-oligosaccharides recovery into the solubilized fraction (Brenelli et al., 2020), what explains the extent of lignin removal compared to the steam explosion pre-treatment.

Table 4. Sugarcane straws chemical composition and crystallinity index after the four different pretreatments. NM = not measured; "a, b, c" = ANOVA analysis at 5% of confidence for each substrate component.

Component	Pretreatments			
	Ionic Liquid	**Diluted Acid**	**Steam-Explosion**	**Hydrothermal**
Cellulose	45.80 ± 1.90^{a}	52.71 ± 0.21^{b}	43.16 ± 0.79^{a}	52.82 ± 0.81^{b}
Hemicellulose	28.30 ± 0.70^{a}	12.54 ± 0.10^{b}	9.66 ± 0.84^{c}	13.19 ± 0.23^{b}
Lignin	21.30 ± 1.40^{a}	31.61 ± 0.44^{b}	34.99 ± 0.59^{c}	22.29 ± 0.16^{a}
Ashes	NM	2.9 ± 0.02^{a}	12.18 ± 0.48^{b}	9.55 ± 0.20^{c}
Total	95.4 ± 0.85	99.76 ± 0.20	101.35 ± 0.75	97.85 ± 0.99
Crystallinity Index	42.3 %	40.0 %	18.0 %	39.3 %

The profile of FTIR spectrum of the different pretreated sugarcane straws demonstrated a shoulder at 2850 cm^{-1}, assigned to the vibration of the OCH_3 groups that are present in lignin (Nada et al., 1998), lower intense in the steam explosion pretreated straw in comparison to the other materials, which may suggest that this pretreatment caused a lignin modification/reallocation (Brienzo et al., 2017). The band at 1424 cm^{-1}, related to the acetyl groups between lignin and hemicellulose, exhibited lower intensity in the SE-pretreated sample, which can indicate that the pretreatment efficiency promoted breakage of ester group and significant exposure to the hemicellulose (Moutta et al., 2013; Selvaraj and Gobikrishnan, 2020). The bands 1051 cm^{-1} and 1163 cm^{-1}, representing the primary and secondary OH groups, and 2912 cm^{-1}, that can be attributed to C-H aliphatic axial deformation in the CH_2 and

CH_3 groups from cellulose, lignin and hemicellulose, demonstrated a higher intensity in HyD-, DA- and IL- pretreated straws, what can be related to a higher crystallinity degree when compared to the material obtained from the SE- pretreatment (Moutta et al., 2013; Shao et al., 2008). Last, the XRD showed that all the samples exhibited typical cellulose diffraction peaks, excepted by SE-pretreated straw (2θ=22.5° and 2θ=16.0°), indicating a possible loss of the ordered structure of the cellulose chains that interconnect by the fibril. Many studies have already indicated that there is an increase in CI when biomass is subjected this type of pretreatment (Moutta et al., 2013; Shao et al., 2008).

Regarding the crystallinity index, SE-pretreated sugarcane straw exhibited the lowest value, CI=18% followed by HyD- (CI = 39.26%), DA- (CI = 40%) and IL- (42.33%) pretreated sugarcane straw (Table 4), indicating a possible correlation between CI and COS production (Fig. 4). SE-pretreatment is one of the most successful and widely used methods for enhance biomass enzymatic digestibility: in the process, steam condensates and permeates the biomass, initiating an autohydrolysis reaction resulting in the cleavage of glycosidic bonds; and when the pressure is relieved, the biomass fibers evaporates causing the mechanical disruption of the lignocellulosic matrix and reduction of the biomass particle size (Duque et al., 2016).

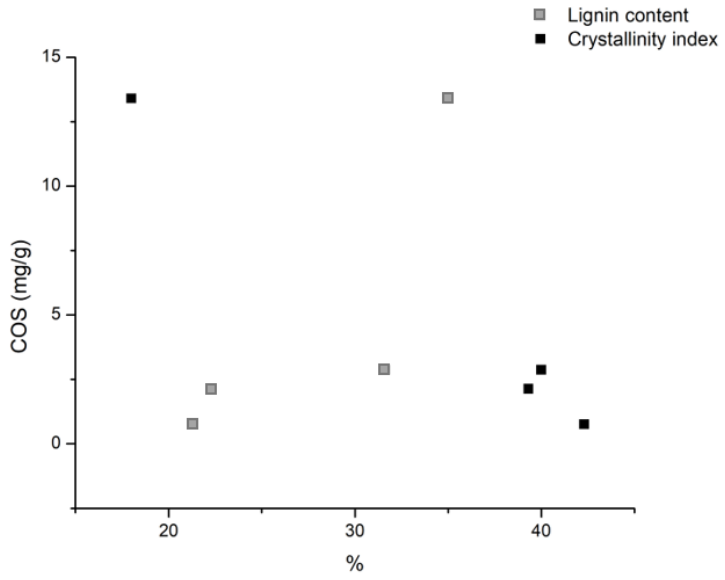


Fig. 4. Relation between COS production and pretreated sugarcane straw lignin content and crystallinity index.

Residual lignins in the pretreated straw can prevented the action of the processive endoglucanase through physical hindrance or unproductive enzyme binding, as already demonstrated for other pretreated lignocellulose and cellulases (He et al., 2018; Huang et al., 2019). Apparently, there is no correlation between lignin content present in the pretreated straw and cellulose crystallinity indices or lignin content and COS yield, as showed in Figure 4. Thus, the lignin content did not adequately describe possible favorable changes in cellulose, such as high surface area and porosity, low hydrophobicity which might have led better CtCel9R enzyme performance to produce COS. Based on that is only suggested that the SE- pretreatment may have caused a lignin modification/reallocation, which may have improved CtCel9R accessibility of sugarcane straw.

The chemical and morphological changes that occurred during sugarcane straw pretreatments were important to understand the relations between the selected endoglucanase performance and COS production. The comparison between COS yields from the different pretreated sugarcane straws by the processive endoglucanase CtCel9R indicates that a possible lignin modification/reallocation, the removal of hemicellulose and decrease in the cellulose crystallinity can benefits the enzymatic hydrolysis, hence increasing COS production. However, a deeper investigation has to be carried out to fully understand the relation between biomass composition and processive endoglucanases for COS production, since depending on the pretreatment severity and biomass, undesired phenomena such as lignin condensation and aggregation, cellulose co-crystallization may occur and influence the enzymatic hydrolysis.

4. Conclusion

The SE- pretreatment demonstrated to be the most effective treatment for COS production in this study. CtCel9R enzyme produced 13.40 mg of COS/g of SE-pretreated sugarcane straw, an increase of 4.7, 6.3 and 17.6-fold in relation to the DA-, HyD- and IL- pretreatments, respectively. The possible lignin modification/reallocation, the removal of hemicellulose and in consequence its interaction with lignin, and the decrease in crystallinity index appeared to be the most effective characteristic obtained from the SE- pretreatment for COS production. The results presented in this work bring important elucidations for further studies regarding large-scale COS production for industrial applications.

Supplementary data for this work can be found after the references of this Chapter.

Acknowledgments

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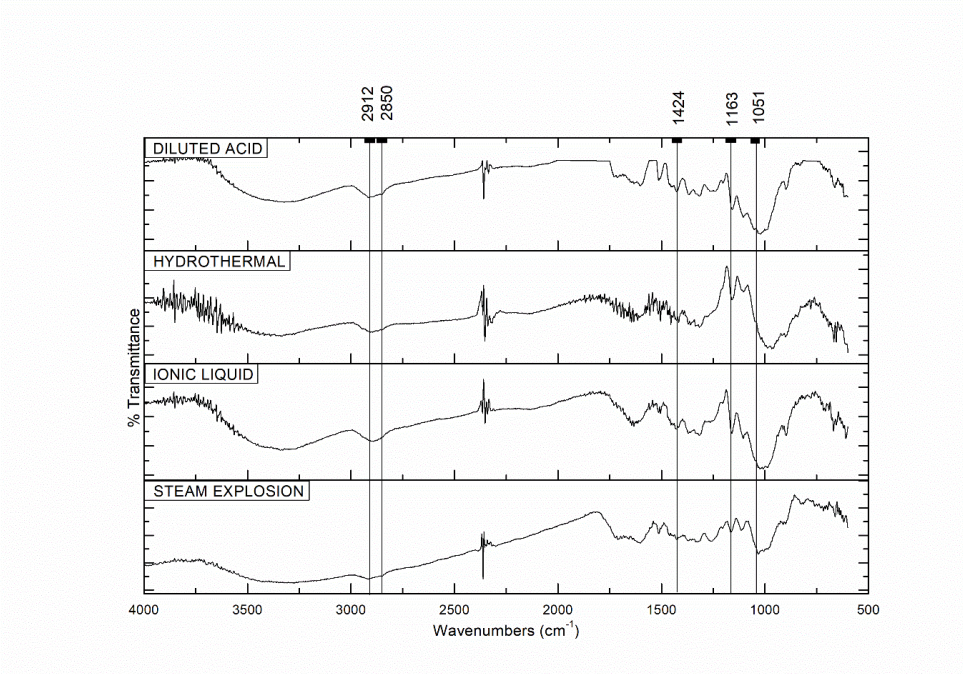
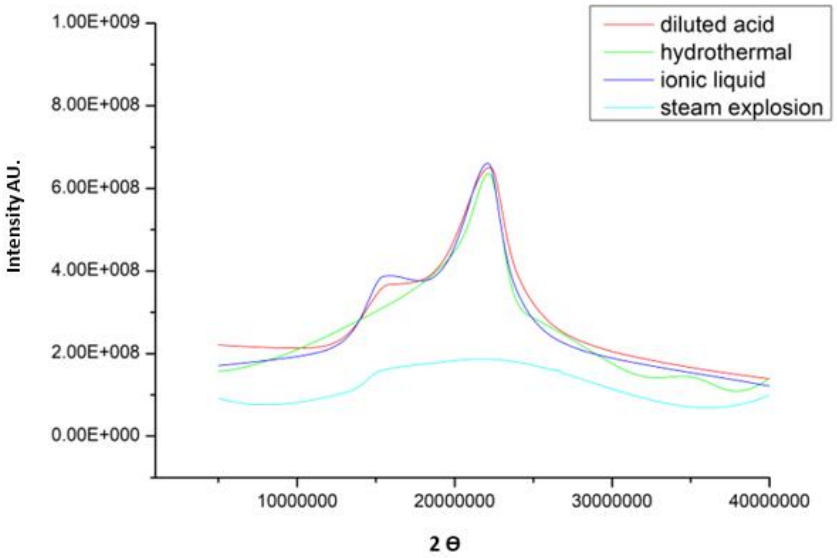


Supplementary material 1. Normalized FT-IR spectra obtained for the four different pretreated sugarcane straws.



Supplementary material 2. X-Ray diffractograms obtained for the four different pretreated sugarcane straws.

Chapter 5

5. Techno-economic and life cycle assessment

Techno-economic and life cycle assessment of cellopentaose production from a bottom-up fermentation approach

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Techno-economic and life cycle assessment of cellopentaose production from a bottom-up fermentation approach

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Abstract

Cello-oligosaccharides (COS) have been proposed as novel substrates for ethanol production and as functional oligosaccharides for the food and feed industrial sectors. With a high market added value, some methods for producing COS from cellulosic substrates have previously been evaluated, however, there is still limited information regarding large scale production of COS. Previously, this group published a work in which 60.49 mg of COS/g of pretreated sugarcane straw was produced without glucose formation, and 87% of produced COS being cellopentaose. Since COS/cellopentaose process analysis are rarely described in the literature, the primary aim of this study was to estimate the cellopentaose production cost using the developed process. Moreover, this study also investigated the life cycle assessment (LCA) of the developed process to identify potential environmental impact. The results demonstrated that was possible to obtain a cellopentaose unit production cost varying between USD 1.15 to 0.40/mg depending on the adopted scenario, a reduction of 6.34 and 18.23-fold in comparison to cellopentaose current market selling price. Also, an optimization in the upstream sector (significant in both assessments) can reduce both the equipment cost (the most significant “total plant direct cost”) and the overall life cycle impact (LCIA). Last, the results suggested that a better understanding of the hydrolysis solid/liquid proportion is determinant to reduce the bulk material cost, and it was possible to observe a LCIA reduction between 16.2 and 19.9% comparing the baseline with the most promising scenario. These results open a new perspective regarding COS/cellopentaose production and its industrial application.

Keywords

Cello-oligosaccharides, cellopentaose, techno-economic, life cycle, process analysis, production cost.

1. Introduction

Cello-oligosaccharides (COS) are defined as oligomers of 2 to 6, β-1,4-linked glucose units (Otsuka et al., 2004; Zhao et al., 2009) which have been proposed as novel substrates for ethanol production in addition to their established applications as functional oligosaccharides for the food and feed industrial sectors (Ahmed et al., 2017; Song et al., 2013; Uyeno et al., 2013). Regarding ethanol production, COS have some advantages over glucose, including a reduced risk of process contamination and limited process inhibition by high glucose concentration (Ahmed et al., 2017; Liang et al., 2013; Mallek-Fakhfakh and Belghith, 2016; Yang et al., 2015). As a probiotic compound, the Karnaouri group (Karnaouri et al., 2019) confirmed that COS can support the growth of different probiotic strains, such as *Lactobacilli* and *Bifidobacteria* species.

With a high market aggregate value, some methods for producing COS from cellulosic substrates have previously been evaluated, but with different degrees of success. The more successful methods employ hazardous chemicals, such as concentrated hydrochloric and sulfuric acid (Zhang and Lynd, 2003), or extremes in temperature and pressure (Tolonen et al., 2015), limiting their feasibility in different industries. Enzymatic hydrolysis from lignocellulose has also been examined using methods that address a major issue in COS production, namely that COS's exist only as transitionary intermediates during conventional enzymatic hydrolysis of cellulose to glucose by commercial enzyme preparations. Through chromatographic fractionation of a crude enzyme preparation to remove beta-glucosidases, followed by a multi-step hydrolysis process, COS's were obtained as a major product from a lignocellulosic substrate, however a significant glucose fraction remained; furthermore, the majority of the COS yield consisted of the lowest DP oligomer – cellobiose (Chu et al., 2014). All the methods described above also invariably require additional steps or facilities beyond the single step enzymatic hydrolysis that is conventional in a lignocellulose biorefinery. However, the existence of specific endoglucanases with synergistic effects with auxiliary enzymes, shown to cleave cellulose into higher DP soluble COS's as a true end product, suggesting that a bottom-up approach to design a cocktail to produce higher DP COS's in a single step reaction is achievable (Belaich et al., 2016; Irwin et al., 1998; Shoham et al., 2003; Zverlov et al., 2005).

Previously, this group published the first study using design of experiments in order to analyze the synergism of endoglucanases, LPMOs, CDH and different additives for the hydrolysis of pretreated sugarcane straw for COS production through a bottom-up approach, reaching 60.49 mg of COS/g after optimization without glucose formation and, curiously, 87% of produced COS was cellopentaose (Barbosa et al., 2020). However, there is still limited information regarding large scale production of COS (Chu et al., 2014), and, indeed, there is even less information in the literature regarding the production of cellopentaose, a COS with 5 β-1,4-linked glucose.

Due to the fact that COS/cellopentaose production and process analysis are rarely reported, the primary aim of this study was not to investigate the feasibility of the individual unit operations or a specific market demand, but to estimate the cellopentaose production cost using the developed process and to analyze the potential environmental impact through a life cycle assessment of the developed process.

The Life Cycle Assessment (LCA) tool is widely used to analyze products and processes from a holistic perspective. By its means, it is possible to identify hotspots in a given production chain and investigate potential environmental damage, such as climate change and impacts in human health. As such the LCA tool has supported the pursuit of truly sustainable solutions, resulting in its ongoing adoption as a key implementation in early-stage process development (McManus and Taylor, 2015; Wiloso et al., 2012).

Based on these assumptions, the industrial platform reproduced in this work is assumed to be constructed in Brazil since the country is the major sugarcane producer in the world, generating more than 65M tons of sugarcane straw per year. In addition, the investment and operational costs are also lower in Brazil than in Europe or the USA. It is expected that this process analysis will open a new perspective for the use of COS in industrial applications.

2. Materials and methods

2.1. Design Basis

Despite there being no specific market data for cello-oligosaccharides or cellopentaose, the company Market Study Report established that the global oligosaccharide market size is expected to grow within the forecast period of 2020 to

2025 with a CAGR of 6.0%, projected to reach USD 1912.4 million by 2025 from USD 1513.1 million in 2019 (Market Study Report, 2020). To explore the different scale-up scenarios without saturating the market and exceeding demand, it was assumed that COS marketing represents a quarter of this amount (with xylo-oligosaccharides, fructo-oligosaccharides, and galacto-oligosaccharides) and that cellopentaose represents 100% of COS market, we presume that cellopentaose moved USD 378.25 million in 2019. Based on the cellopentaose selling price found at Sigma-Aldrich (USD 34.72/mg, > 80% purity) and Megazyme (USD 7.29/mg, > 95% purity) websites, we assume that cellopentaose moved close to 51 kg of material in 2019.

Therefore, a baseline scenario was established to produce approximately 18 kg of cellopentaose per year (35% of market share) based on the method previously described by this group (Barbosa et al., 2020). Moreover, 5 other scenarios were evaluated in order to reach the assumed total cellopentaose market share: baseline + 10% of hydrolysis yield, 2-fold scaled-up process, 2-fold scaled-up process + 10% of hydrolysis yield, 3-fold scaled-up process and 3-fold scaled-up process + 10% of hydrolysis yield. The main process sections – sugarcane straw pretreatment, xylo-oligosaccharide (XOS) production, enzyme production and purification, and COS production – are indicated by different colors in Fig. 1.

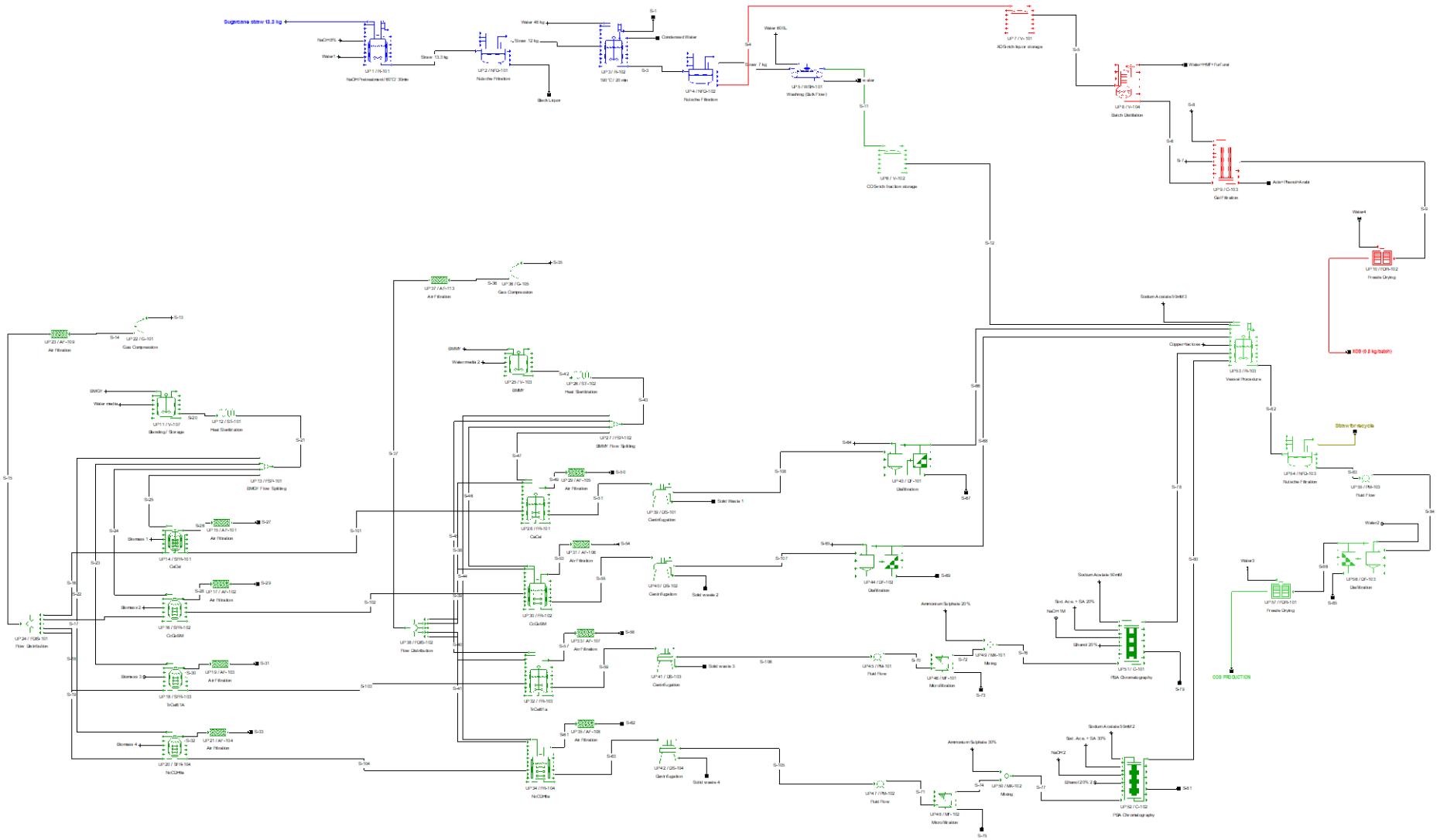

Fig. 1. Flowsheet of developed cellopentaose production process. Blue equipment represents the sugarcane straw pretreatment; red equipment represents XOS production; green equipment represents the enzymes production and purification for COS production, and lime equipment represents sugarcane straw hydrolysis and cellopentaose purification.

2.2. Modeling and simulation software

2.2.1. Economic assessment

The economic assessment of all scenarios was performed using SuperPro Designer. The international cost of the used equipment, the annual production, lowest selling price and the life cycle input data were also provided by SuperPro Designer.

2.2.2. Life cycle assessment (LCA)

The 2 co-products from the biorefinery (cellopentaose and xylooligosaccharides - XOS) were analyzed by the means of LCA. This study adopted a cradle-to-gate approach, performing a mass allocation between the cellopentaose and the XOS mixture. The Life Cycle Inventories (LCI) were built on SimaPro v9, with background data sourced from EcoInvent 3.5. The APOS (allocation at point-of-substitution) were the datasets of choice, with the “market for” inventories being used to better represent the market share for different manufacturers and processes with the same output.

When available, inventories regionalized for Brazil (BR) were preferred, but otherwise, “Rest of the World” (RoW) and “Global” (GLO) datasets were used. Foreground data was adapted from the six simulation scenarios’ energy and mass balances, accounting for inputs, utilities and emissions. A new dataset was built for sugarcane straw, using published inventories adapted for the Brazilian context (Cavalett et al., 2012).

A few adaptations, however, were necessary to include all the inputs accounted in the SuperPro simulations. Sodium acetate was considered as the output from reacting sodium hydroxide and acetic acid. Each kg of lactose was assumed to correspond to 30.77 kg of whey. Yeast extract, peptone, biotin and casamino acids were accounted as fodder yeast from breweries. The Brazilian electricity production mix present in EcoInvent 3.5 was updated to 2020 figures (EPE, 2020). Cooling energy and steam were considered to be generated with renewable energy, from biogas, with off-site production.

Recipe (2016) was selected as the Life Cycle Impact Assessment (LCIA) method, due to its normalization inventory being relevant in the global context. From the available 18 impact categories, four were chosen for further analysis: Global

Warming Potential (GWP), Marine Ecotoxicity (M. ecotoxicity), Human Carcinogenic Toxicity (Human CT) and Human Non-Carcinogenic Toxicity (Human nCT). This selection was made considering the normalized LCIA results.

2.2.3. Sugarcane straw pretreatment section

The sugarcane straw pretreatment applied was established by Brenelli group (Brenelli et al., 2020). Briefly, the pretreatment consisted of a two-step process: a mild alkaline followed by hydrothermal pretreatment to maximize the production of XOS from the hemicellulose fraction (liquid fraction) and generate a cellulose-rich fraction to be converted into cello-oligomers (solid fraction).

In the baseline scenario 13.3 kg of sugarcane straw was used per batch. In the first stage of the treatment the sugarcane straw was mixed with NaOH to a final concentration of 0.8% (w/v) and 115 kg of water in a 300L reactor for 30 minutes and 60 °C. After that, the material went through a filtration procedure and the retained material, containing 12 kg of sugarcane straw, went through the second treatment stage in a 150L reactor. 48L of water was added to the reactor, and the reaction occurred under 190 °C for 20 minutes. After that, the material went through a new Nutsche filtration module in which the XOS and COS streams were separated. The reaction produced ~70 kg of XOS stream containing 5 kg of xylo-oligomers rich liquid, and ~30 kg of COS stream containing 7 kg of cellulose-rich sugarcane straw. The XOS stream was stored in a 200L storage tank prior to the XOS purification procedure, and the cello-oligosaccharides stream were cleaned with water prior to the storage in a 100L storage tank and subsequent enzymatic hydrolysis.

2.2.4. Xylo-oligosaccharides production section

The purification of the XOS stream was based on Ho et al. (2014) with some modifications. In short, the ~70 kg of XOS stream went through a distillation (89.9L capacity) process to eliminate the furfural and hydroxymethylfurfural. After that, the material went through a gel filtration procedure to eliminate the remaining formic acid, phenols, and arabinose. Finally, the material went through a freeze-drying procedure to produce a powder rich in xylo-oligosaccharides. Based on the market price, it was established that the XOS could also be sold as a secondary product at a price of USD 50/kg.

2.2.5. Cello-oligosaccharides production section

The COS production section was based on the previous work published by this group (Barbosa et al., 2020). Briefly, an optimal condition for COS production was established involving the synergism of the endoglucanases CcCel9M (GH9 from *Clostridium cellulolyticum* ATCC 35319) and CaCel (GH45 from *Cryptopygus antarcticus*) at 10 U/g of pretreated sugarcane straw; the lytic polysaccharide monooxygenase (LPMO) TrCel61A (AA9 from *Trichoderma reesei*) at 2 mg/g of pretreated sugarcane straw; the cellobiose dehydrogenase (CDH) NcCDHIIa (AA8 from *Neurospora crassa OR74A*) at 1 mg/g of pretreated sugarcane straw; and the additives copper (10 mM) and lactose (1 mM).

All enzymes were heterologously expressed in *Pichia pastoris* under the vector pPICZ-α B (Thermo Fisher Scientific). LPMOs and CDH genes were encoded with their native secretion signal sequences, while the endoglucanases were cloned downstream of the *S. cerevisiae* alpha factor, both under the control of methanol inducible *AOX1* promoter. The enzyme production consisted in two steps: cell growth and enzyme production. For cell growth, the *P. pastoris* strains containing the selected genes were cultivated in Buffered Glycerol-Complex Medium (BMGY) for 48 hours at 30 ºC and 250 rpm to achieve a target cell density of 38 g/L. In the second step, the cultures were maintained at 28 ºC and 200 rpm for 72 hours for enzyme production. At the end of the culture period, fermentation broths were centrifuged for biomass and cell debris removal. The endoglucanases were purified using tangential flow filtration equipment (concentration followed by a diafiltration) for the removal of impurities and buffer exchange prior to the hydrolysis step. The LPMO and CDH went through a microfiltration procedure prior to chromatography. Then the materials were loaded onto PHE-Sepharose Fast Flow column (GE Healthcare Biosciences), equilibrated with a 25 mM sodium acetate buffer, pH 5.0 containing their respective saturation amount of ammonium sulphate. Proteins were eluted within 3 column volumes of equilibrated buffer without ammonium sulphate. Fractions containing the respective enzymes were collected for the hydrolysis step.

Based on the results published previously (Barbosa et al., 2020) , a final concentration of ~0.1 mg/mL were obtained for the 2 endoglucanase cultures after harvesting, with a yield of 10% after purification and an activity of 4-5 U/mg. For TrCel61A, the concentration achieved was 0.73 mg/mL with an activity of 0.190 U/mg following purification. Lastly, NcCDHIIa was recovered at a concentration of 0.35

mg/mL after purification and an activity of 31.9 U/mg. In the baseline scenario, 2 seed fermenters with 200L capacity were used to grow the *P. pastoris* cells containing the endoglucanase genes, and 2 seed fermenters with 50L capacity were used to grow the *P. pastoris* cells containing the LPMO and CDH genes; 2 other fermenters with a 2000L capacity were used to produce the endoglucanases and 2 fermenters with a 500L capacity were used to produce the LPMO and CDH enzymes.

The pretreated sugarcane straw hydrolysis condition was established at 50°C, pH 5.0, 150 rpm for 48 hours and 1% w/v. According to the results published previously, the hydrolysis achieved a 6.049% conversion of sugarcane straw into sugars (or 11.45% conversion of cellulose into sugars). The hydrolysis produced 1.67 ± 0.03 mg/g of cellobiose, 0.86 ± 0.01 mg/g of cellotriose, 1.89 ± 0.02 mg/g of cellotetraose, 52.66 ± 1.03 mg/g of cellopentaose and 3.42 ± 0.15 mg/g of cellohexaose. In the baseline scenario, the hydrolysis was conducted in an 800L capacity reactor and, after hydrolysis, the material went through a filtration step for the removal of unconverted pretreated sugarcane straw that could also be reused in the process. After that, the material went through a tangential flow filtration process (concentration followed by a diafiltration) to remove impurities, such as copper, lactose, culture media and enzymes, finishing in a freeze-drying procedure to produce a powder rich in cellopentaose (> 80% purity). The 6.049% conversion yield of sugarcane straw into sugars obtained is lower compared to the average conversion yield found in the literature for biomass into sugars (Brienzo et al., 2017; Jin et al., 2020; Karnaouri et al., 2019; Kruyeniski et al., 2019), therefore, we also analyzed the proposed scenarios (scaled-up) with a 10% conversion yield since the hydrolysis can still be optimized.

3. Results and discussion

3.1. Economic assessment

In the baseline scenario, the xylo-oligomers rich liquid produced 0.8 kg of XOS/batch and the cellulose-rich sugarcane straw produced 0.45 kg of cellopentaose/batch. Focusing in the cellopentaose production, SuperPro Designer suggested a maximum of 41 batches per year, resulting in a cellopentaose yield of 18.61 kg/year (35.85% of the market share), with a total investment of USD 91.805.162, an annual operating cost of USD 21.469.832 and a unit production cost of only USD 1.15/mg. In the baseline scenario, the unit production cost obtained was

6.34-fold lower than the conventional market price. In the baseline + 10% conversion yield scenario, with a similar total investment and annual operating cost, a total quantity of 29.78 kg of cellopentaose/year could be produced and 57.39% of the market share could be reached, with a production cost of USD 0.72/mg, at ~10-fold lower than the conventional market price.

In the 2-fold scaled-up scenario, a maximum of 40 batches per year could occur, resulting in a cellopentaose yield of 36.33 kg/year. This amount represents 70% of the market share. Thereby, a total investment of USD 108.358.024 and an annual operating cost of USD 27.192.032 would be necessary. The unit production cost obtained for the scenario was USD 0.75/mg, similar to the unit cost obtained in the baseline + 10% conversion yield but with an additional 6kg of cellopentaose produced per year. In the 2-fold scaled-up + 10% conversion yield scenario, a total of 58.21kg of cellopentaose/year could be produced with an over market size production of 6kg. Interestingly, the unit production cost was projected to decrease to USD 0.47/mg, more than 15-fold lower than the conventional market price with a similar total investment and annual operating cost.

Lastly, the 3-fold scaled-up scenario can produce a total of 47.61 kg of cellopentaose/year, reaching almost 100% of the market share with a maximum of 35 batches per year. The unit production cost obtained was USD 0.64/mg, 11.39-fold lower than the current market price. To do so, an investment of USD 120.350.326 with an annual operating cost of USD 30.405.885 would be required. In the last and most optimistic scenario, 3-fold scaled-up + 10% conversion yield, 76.62 kg of cellopentaose could be produced per year with a similar total investment and annual operating cost, with a unit product cost of only USD 0.40/mg, presenting a price reduction of ~18-fold.

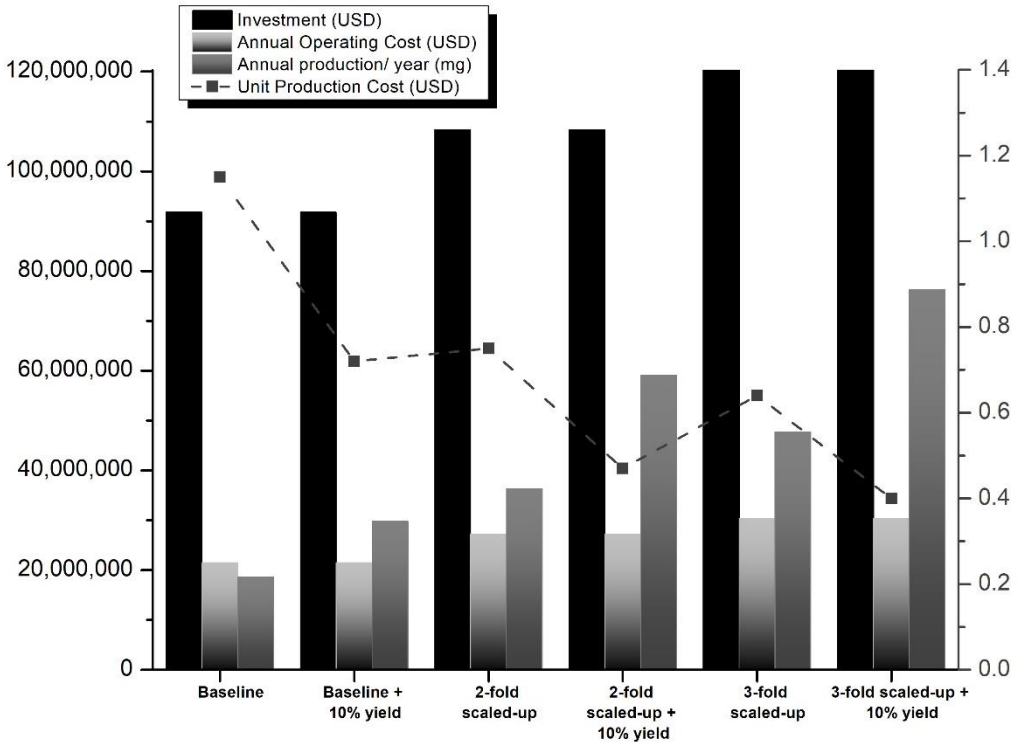

Fig. 2. Comparative overview of the required investment, annual operating cost, annual production per year and unit production cost for each scenario analyzed.

Figure 2 summarizes the economic assessment data. It is interesting to note that while there is an increase in 31.08% in the cost investment and 41.61% in annual operating cost, there is a reduction of 65.22% in unit production cost from the baseline to the 3-fold scaled-up + 10% yield scenario. In addition, there is an increase of 309.69% of annual production capacity per year between both mentioned scenarios. Lastly, the figure also shows that the unit production cost for a 2-fold scaled-up scenario is similar to the baseline + 10% yield scenario, since the increment in annual production nullifies the increment in investment and annual operating costs. This finding is not reproducible for the 3-fold scaled-up scenario in comparison to 2-fold scaled-up + 10% yield because the annual production per year in the latter is higher than the annual production per year of the 3-fold scaled-up scenario.

3.2. Cost composition

The Direct Fixed Capital (DFC) represented the largest component of the required investment in all scenarios, reaching ~94% of the total value, followed by the

startup cost (~5%) and the working capital (~1%). In addition, the DFC can be divided into 3 subcategories: Contractor's Fees & Contingency, Total Plant Indirect Cost (TPIC) and Total Plant Direct Cost (TPDC). For all the scenarios analyzed in this study, the contribution of each category was proportional and did not change: ~13%, ~32% and ~54%, respectively.

Within the category of Contractor's Fees & Contingency, the contractor's fee represented ~66% of the total investment, with contingency accounting for the remaining ~34%. This pattern was observed in all scenarios. In a similar way, the same representative amount was found for the subitems engineering (~41%) and construction (~59%) within the category TPIC. Last, the TDPC was composed by equipment cost (~31%), installation (~10%), process pricing (~11%), instrumentation (~12%), insulation (~1%), electrical (~3%), buildings (~14%), yard improvement (~5%) and auxiliary facilities (~13%) in all scenarios.

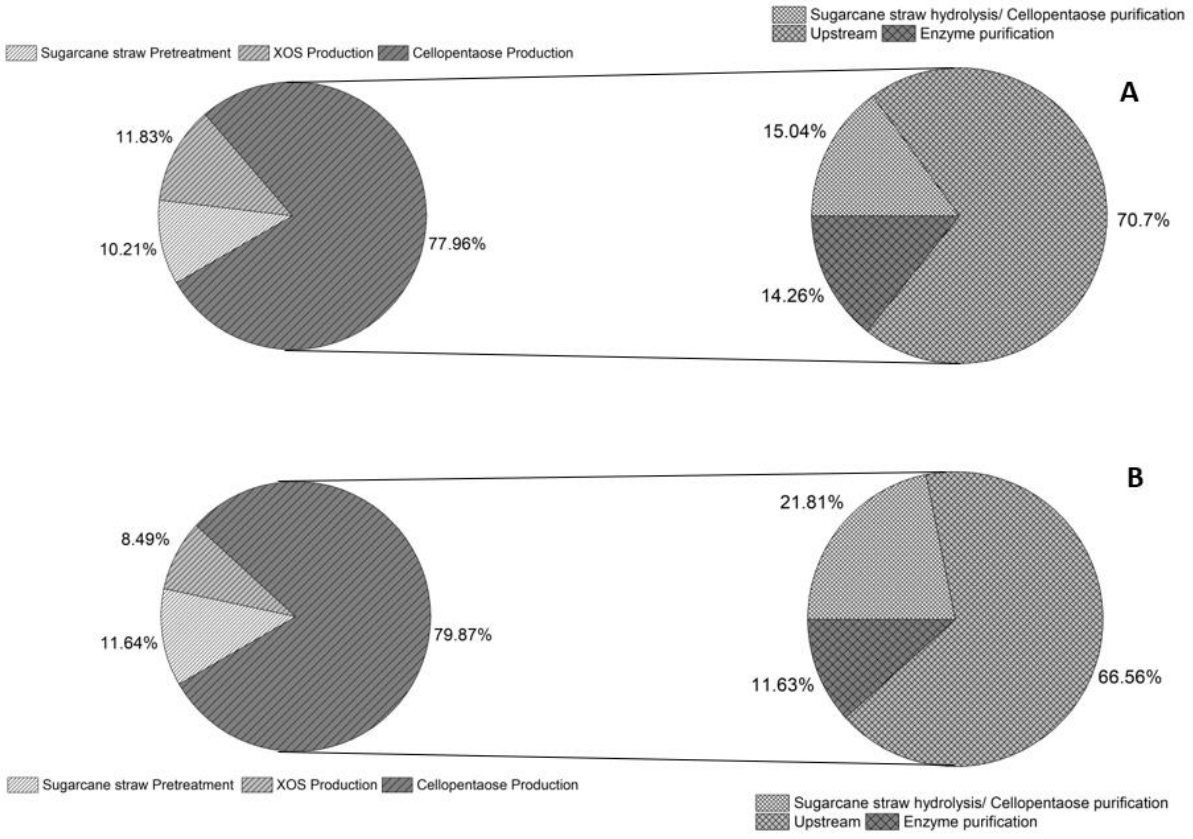

Fig. 3. Cost composition. A: general process equipment cost composition and cellopentaose production cost composition for the baseline scenario. B: general process equipment cost composition and cellopentaose production cost composition for the 3-fold scaled-up + 10% yield scenario.

57 items of equipment were necessary for the developed process. In the baseline scenario, cellopentaose production-related equipment costs represented ~80% of the total equipment cost, followed by sugarcane pretreatment-related equipment (~12%) and XOS production-related equipment. However, in the most optimistic scenario, 3-fold scaled-up + 10% yield, cellopentaose production-related equipment cost (~78%) was followed by XOS production-related equipment cost (12%) and sugarcane pretreatment-related equipment cost (~10%), indicating that the costs related to XOS purification tend to be higher than the costs related to sugarcane straw pretreatment in higher scales (Fig. 3).

Moreover, for the cellopentaose production-related equipment cost, the upstream-related equipment represented ~71% of the total cost in the baseline scenario and ~66% of the total cost in the 3-fold scaled-up + 10% yield scenario, indicating that a decrease in upstream-related equipment cost can be expected through an increase in scale, and also that an optimization in enzyme production can significantly reduce the equipment investment. In addition, the equipment costs related to sugarcane straw hydrolysis/cellopentaose purification and enzymes purification were similar in the baseline scenario: ~15% and 14%, respectively. However, the equipment costs related to sugarcane straw hydrolysis/cellopentaose purification increased to ~22% while the enzyme purification-related equipment cost decreased to ~12% in the 3-fold scaled-up + 10% yield (Fig. 3). This finding can be justified by the 2-fold increment in the freeze-dryer and dialfilter equipment costs in the 3-fold scaled-up + 10% yield scenario.

Regarding the input material costs, the sodium acetate 50 mM solution used as a buffer in the sugarcane straw hydrolysis with a total solid loading of 1% w/v represented ~84% of the total bulk material cost. This finding, in addition to the increase in the sugarcane straw hydrolysis/cellopentaose purification-related equipment costs, suggests that a future optimization strategies would require determining experimentally whether simultaneously increasing biomass and enzyme loading in the reaction will result in a linear increase in COS production, thereby permitting to decrease reaction volumes and minimizing materials/ operation costs. A 1% solids loading for saccharification is low compared to industrial standards so this could be increased in the future, and that this, therefore, represents a sort of worst-case scenario which is, nevertheless still profitable.

The cost related to waste treatment was similar in all scenarios, in which the

aqueous waste treatment represented ~94% of the total waste treatment costs mainly due to the treatment cost of the “acid+phenol+arabinose” stream from XOS purification (representing ~24% of the total liquid waste treatment cost).

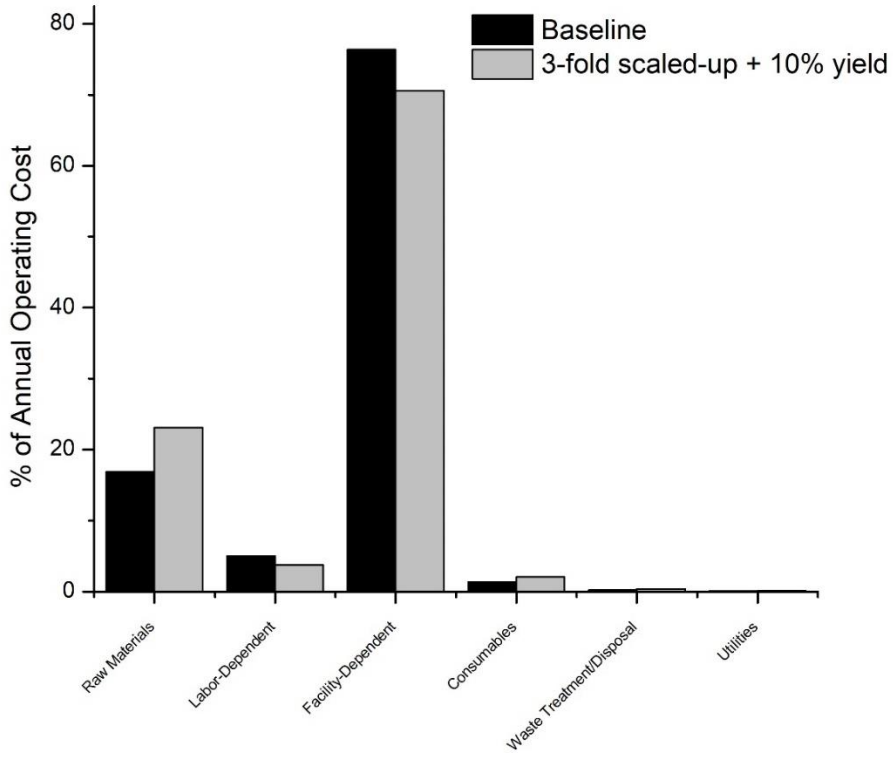

Fig. 4. Comparative overview of the annual operating cost for each analyzed scenario.

Lastly, Figure 3 represents the annual operating cost for the baseline and 3-fold scaled-up + 10% yield scenarios. The data indicates that the majority is related to facility-dependent costs (~70-75%), followed by raw material (~17-23%), labor-dependent (~ 4-5%), consumables (~ 1-2%), waste treatment/disposal (~ 0.3%) and utilities (~0.1%), indicating that an optimization on specific process steps, as already discussed, can lead a drop on equipment/facility dependent costs, causing a possible reduction in the annual operating and unit production costs.

3.3. Project Indices

Gross margin (difference between revenue and cost of products sold, divided by revenue), return of investment (ROI - relationship between the amount of money earned as a result of an investment and the amount of money invested) and payback time (an indicator of the time to return on an investment) projected indices were also

calculated by SuperPro Designer for the baseline and 3-fold scaled-up + 10% yield scenarios. For each scenario, two different cellopentaose selling prices were applied: USD 7.29/mg (the lowest market price) and the unit production cost obtained in each scenario + 70% of profit (USD 1.96/mg and USD 0.68/mg, respectively).

In the baseline scenario, the projected gross margin was 84.18% when cellopentaose was sold at USD 7.29/mg and 41.05% when it was sold at USD 1.96/mg. A ROI of 83.66% with a payback time of 1.20 years was projected when cellopentaose was sold at USD 7.29/mg, while a ROI of 18.76% with a payback time of 5.33 years was projected when cellopentaose was sold at USD 1.96/mg. In the 3-fold scaled-up + 10% yield scenario, a gross margin of 94.53%, a ROI of 271.04% and a payback time of 0.37 years were projected when cellopentaose was sold at USD 7.29/mg; while a gross margin, ROI and payback time of 41.46%, of 19.72% and 5.07 years were projected when cellopentaose was sold at USD 0.68/mg. These results indicate that the developed process for cellopentaose production appears to be profitable, and the product selling price has a significant impact on the economic performance of the plant. It is important to highlight that these indices were calculated using the SuperPro Designer software and may eventually vary if other factors are chosen related to the local economy, for example.

Lastly, the point where the company's revenues equal its costs - break-even point - was also evaluated based on the data generated by SuperPro Designer. Table 1 shows that the break-even occurs in less than a year when cellopentaose is sold at USD 7.29/mg, and in more than five years when the cellopentaose is sold at unit production cost + 70% of profit in both baseline and 3-fold scaled-up + 10% yield scenarios.

Table 1. Comparative break-even points for baseline and 3-fold scaled-up + 10% yield scenarios.

Scenarios	Fixed Investment Cost	Unit Sales Price	Unit Production Cost	Break-Even (kg)	Annual Production	Break-Even time (years)
Baseline	$ 87,017,000.00	$ 7.29	$ 1.15	14.17	18.61	0.76
Baseline (70% Profit)	$ 87,017,000.00	$ 1.96	$ 1.15	107.43	18.61	5.77
3-fold scaled-up + 10% yield	$113,866,000.00	$ 7.29	$ 0.40	16.53	76.26	0.22
3-fold scaled-up + 10% yield (70% Profit)	$113,866,000.00	$ 0.68	$ 0.40	406.66	76.26	5.33

3.4. Life cycle assessment (LCA)

The following LCA results focused on cellopentaose as the main product. All the subsequent observations and conclusions are also applicable to the XOS

coproduct since no disaggregation was done to the biorefinery process data and mass allocation was performed.

In order to identify the process' environmental bottlenecks, each identified burden in the LCIA was attributed to their correspondent process unit. In the Baseline scenario (Fig. 5), the upstream sector was found to be the largest contributor to the overall impact in all four categories, particularly in global warming, with 81%. This is particularly due to the usage of utilities, representing the unit with the largest consumption of: low pressure steam (83%), cooling water (>99%), glycol (93%) and electricity (80%). However other inputs in this sector are also substantial contributors, such as anhydrous ethanol, acetic acid, and ammonium sulfate.

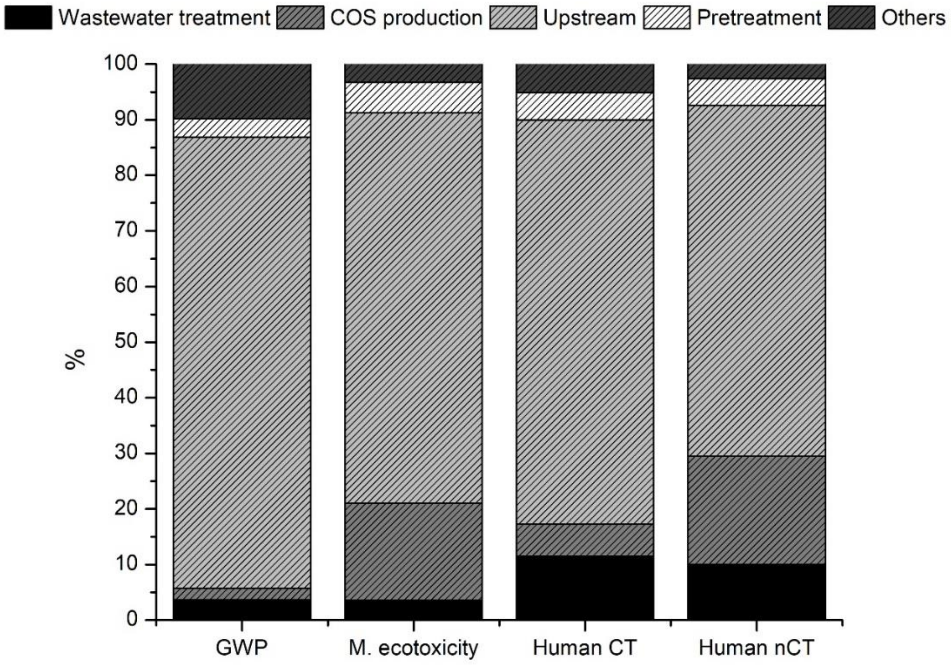

Fig. 5. LCIA for 1 kg of cellopentaose production in baseline scenario.

While less prominent than the upstream sector, a significant impact share can be assigned to COS production which, for the purpose of this analysis, comprises both sugarcane straw hydrolysis and cellopentaose purification. Within these processes, the use of copper sulfate was indicated in M. ecotoxicity, and the consumption of electricity and steam, for Human nCT. Wastewater treatment, on the other hand, showed a stronger significance for both Human Toxicities. The impact from sugarcane straw pretreatment is mostly associated with the consumption of

steam and sodium hydroxide. Other process units and inputs, such as sugarcane straw, XOS purification, and process activity are accounted for in “others”. However, the impact reported for this category can be largely attributed to the extra 25% of electricity consumption assigned by SuperPro Designer for unlisted equipment and system general load.

Comparing the general LCIA results amongst the six scenarios (Fig**.** 6), the effect of scale-up in the overall impact becomes clear. By increasing batch size, input and utility consumption becomes more efficient, leading to a dilution effect in the LCIA, and the same occurred for the “+10%” scenarios, potentialized by the extra yield in the hydrolysis. In general, it was possible to observe a reduction in the indicators between 16.2 and 19.9%, comparing the baseline with the 3-fold scaled-up + 10% yield scenarios, among all impact categories. The impact distribution among units also remained consistent among the different scenarios, with the upstream sector consistently identified as the main hotspot.

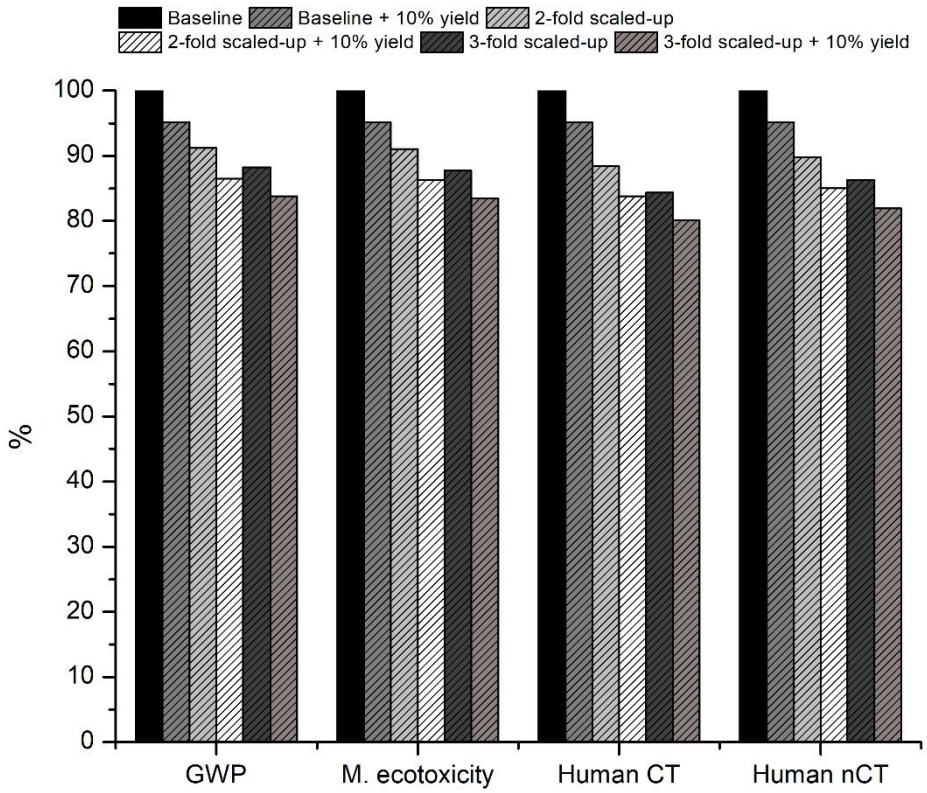

Fig. 6. Comparative general LCIA for the six scenarios (FU: 1 kg of cellopentaose).

While the proposed bioprocess is a novel approach for the production of COS, there are LCA studies available in the literature that explored the production of other kinds of oligosaccharides, such as XOS (Lopes et al., 2019) and pectin-derived oligosaccharides (POS) (Gonzalez-Garcia et al., 2018). Regarding the GWP, XOS' GWP ranged from 3.8 to 5.5 $kgCO_{2eq}.kg_{XOS}^{-1}$ in the first, and, in the second, POS were obtained with a GWP from 50 to 56 $kgCO_{2eq}.kg_{POS}^{-1}$ - compared to this work, the GWPs reported in the literature are, at least, 65% smaller compared to 3-fold scaled-up + 10% yield scenario (Table 1). However, it is important to remark that both references did not include on-site enzyme production (upstream), the most notable hotspot found in this work. By factoring out this section, the GWP for the proposed scenarios would range from 23.9 to 30.8 $kgCO_{2eq}.kg_{COS}^{-1}$.

Moreover, human carcinogenic toxicity and marine ecotoxicity were also explored for POS production, and ranged between 9.1 to 10.9 $kg_{1,4\text{-}DCB}.kg_{POS}^{-1}$ and 53 to 204 $kg_{1,4\text{-}DCB}.kg_{POS}^{-1}$, respectively. Compared to this work, results for human carcinogenic toxicity are within range, and for marine ecotoxicity this work presents lower marks of one order of magnitude (Table 2).

Table 2. Comparative LCIA results for the six scenarios.

Impact category	Unit	Scenarios					
		Baseline	Baseline + 10% yield	2-fold scaled-up	2-fold scaled-up + 10% yield	3-fold scaled-up	3-fold scaled-up + 10% yield
Global warming	kg CO2 eq	164	142	156	145	150	137
Marine ecotoxicity	kg 1,4-DCB	14.2	12.2	13.5	12.5	12.9	11.8
Human carcinogenic toxicity	kg 1,4-DCB	9.51	7.97	9.05	8.02	8.41	7.62
Human non-carcinogenic toxicity	kg 1,4-DCB	294	250	280	254	264	241

4. Conclusion

This article successfully performed the techno-economic and life cycle assessment of cellopentaose production. Regarding the cellopentaose production cost estimation using the developed process, it was possible to obtain a unit production cost varying between USD 1.15 to 0.40/mg for the baseline and the 3-fold scaled-up + 10% yield scenario, a reduction of 6.34 and 18.23-fold in comparison to the cellopentaose current market selling price. Regarding equipment costs, the upstream-related equipment was the most significant, therefore improvements in enzyme production will have the greatest potential for reducing equipment investment. Moreover, the techno-economic analysis suggested that a better understanding of the hydrolysis solid/liquid proportion is determinant to reduce the

bulk material cost. In the LCA, the upstream sector was also found to be the largest contributor to the overall impact in all 4 assessed categories. It was also observed that scale-up and higher reaction yields had a positive effect in the LCIA indicators, with a potential reduction between 16.2 and 19.9% when comparing the baseline with the 3-fold scaled-up + 10% yield scenarios.

Acknowledgments

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Chapter 6

6. General discussion

In the last 2 decades, the society began to recognize the opportunities offered by the bioeconomy through the biorefinery concept. The possibility to produce energy, chemicals, and materials from plant-based and sustainable raw materials has promoted investments in R&D activities around the world (Cherubini, 2010). Besides ethanol, producers are now looking for high-value co-products in order to increase the value chain and production flexibility, such as butanol, lactic acid, coniferol, ethylene, furfural, cellulose microfibers, polyhydroxyalkanoates (PHA), phenolic compounds (ferulic acid), xylo-oligosaccharides and cello-oligosaccharides (Rosales-Calderon and Arantes, 2019). This project first intended to produce cello-oligosaccharides for bioethanol production, however, based on the cellopentaose production predominance and on the biorefinery concept, the capacity to produce high-value cellopentaose in a commercial scale was evaluated.

Chapter 2 brought an extensive literature review regarding lignocellulosic material and its degradation based on cellulase and oxidative enzymes. In this Chapter was discussed the synergism among enzymes (endoglucanases, β-glucosidases, cellobiohydrolases, lytic polysaccharide monooxygenases, and cellobiose dehydrogenases) to successfully hydrolyze the pretreated biomass, and the need for a better understanding of the interactions between enzymes and lignocellulosic substrates, the development of enzyme engineering, and the optimization of enzyme mixtures to enhance cellulose hydrolysis.

Based on this searching, Chapter 3 elucidated an efficient approach for COS production without glucose formation. After the optimization strategy developed, a combination of the endoglucanases CaCel and CcCel9m, the LPMO TrCel61A, the

CDH NcCDHIIa, with lactose and copper as additives, produced 60.49 mg of COS per g of hydrothermally pretreated sugarcane straw with a 298.31 COS/glucose ratio. Interesting, more than 85% of produced COS was cellopentaose, suggesting that a bottom-up approach to design a cocktail to produce higher degree of polymerization (DP) COS's in a single step reaction is achievable. The process developed is under patent submission.

In order to deeper understand the relation between substrate chemical/morphological composition and enzymes for COS production, Chapter 4 was addressed. Sugarcane straws with four different pretreatments were tested, and under the evaluated conditions, the possible lignin modification/reallocation, the removal of hemicellulose and in consequence its interaction with lignin, and the decrease in crystallinity index appeared to be the most effective characteristic obtained from the steam explosion (SE-) pretreatment for COS production. Intriguingly, it was expected to obtain a higher amount of COS using the SE-pretreated straw under the optimized production method developed, however, almost the same amount of COS was obtained with hydrothermally (6.05% substrate to COS conversion yield), ionic liquid (5.62% substrate to COS conversion yield), or SE- (6.40% substrate to COS conversion yield) pretreated sugarcane straws (Table 1). These results indicate a possible conversion yield limitation on the biomass to high DP COS's, suggesting that a further process optimization, mainly regarding the activity of CDH enzyme, may still be possible. Interesting to note that the cellopentaose production predominance is also real for the other pretreated sugarcane straws: among all COS produced, the cellopentaose amount was 94.7% using ionic liquid material and 79.2% using steam-explosion substrate.

Table 1. COS comparative production using different pretreated sugarcane straws. ND = not detected.

#	Glucose (mg/g)	Cellobiose (mg/g)	Cellotriose (mg/g)	Cellotetraose (mg/g)	Cellopentaose (mg/g)	Cellohexaose (mg/g)	COS sum (mg/g)	COS / Glucose
Hydrothermal	0.20 ± 0.01	1.67 ± 0.03	0.86 ± 0.01	1.89 ± 0.02	52.66 ± 1.03	3.42 ± 0.15	60.49 ± 0.82	298.86 ± 2.02
Ionic Liquid	0.85 ± 0.23	0.76 ± 0.06	ND	0.25 ± 0.00	54.07 ± 0.61	1.20 ± 0.19	56.21 ± 0.95	68.34 ± 1.94
Steam Explosion	7.09 ± 0.10	1.93 ± 0.13	0.82 ± 0.09	0.34 ± 0.07	53.44 ± 2.69	3.86 ± 0.10	60.40 ± 2.30	8.52 ± 0.04

Finally, Chapter 5 presented the results obtained for early-stage techno-economic and life cycle assessment for cellopentaose production. The assessment demonstrated that it was possible to obtain a unit production cost varying between USD 1.15 to 0.40/mg for the baseline and the 3-fold scaled-up + 10% yield scenario,

a reduction of 6.34 and 18.23-fold in comparison to cellopentaose current market selling price. The upstream sector appeared to be the most relevant sector for both analyses. Regarding equipment costs into the total plant direct cost section, the upstream-related equipment cost was the most significant, therefore, an optimization in enzyme production can significantly reduce the equipment investment. Moreover, the techno-economic analysis suggested that a better understanding of the hydrolysis solid/liquid proportion is determinant to reduce the bulk material cost. In the LCA, the upstream sector was also found to be the largest contributor to the overall impact in the four selected categories, and in general, it was possible to observe an overall impact reduction between 16.2 and 19.9% comparing the baseline with the 3-fold scaled-up + 10% yield scenario. The results demonstrated that the development of a platform for cellopentaose production based on the developed process is feasible.

Chapter 7

7. General conclusions

- The main goal of producing cello-oligosaccharides with no or minimal glucose formation was achieved;

- The combination of the endoglucanases CaCel (10 U/g) and CcCel9m (10 U/g), the LPMO TrCel61A (2 mg/g), the CDH NcCDHIIa (1 mg/g), with lactose (1 mM) and copper (10 mM) most produced COS;

- The optimized condition produced 60.49 mg of COS per g of hydrothermally pretreated sugarcane straw after 48-hour hydrolysis (pH 5.0, 50 °C and 150 rpm), an increase of 1.8 - 2.7-fold compared to the commercial enzymatic cocktails Cellic® Ctec2 and Celluclast® 1.5L;

- The developed approach produced COS with a 298 COS/glucose ratio, 3314.55 and 2294.69-fold higher than the Cellic® Ctec2 and Celluclast® 1.5L's ratio;

- Using only endoglucanases in the hydrolysis, it was found a poor correlation between lignin content and crystallinity index, or lignin content and COS yield among the different pretreated sugarcane straws tested;

- Using only endoglucanases in the hydrolysis, the possible lignin modification/reallocation, the removal of hemicellulose and in consequence

its interaction with lignin, and the decrease in crystallinity index appeared to be the most effective characteristic obtained from the steam explosion pretreatment for COS production;

- Despite the steam explosion pretreatment appears to be the most effective for COS production, a similar amount of COS was obtained with sugarcane straws with different pretreatments (hydrothermal, ionic liquid and steam explosion) through the optimized approach;

- Using the optimized approach, the major COS produced was cellopentaose (≥ 80%) in all sugarcane straws analyzed;

- Techno-economic assessment demonstrated that it was possible to obtain a unit production cost varying between USD 1.15 to 0.40/mg for the baseline and the 3-fold scaled-up + 10% yield scenario, a reduction of 6.34 and 18.23-fold in comparison to cellopentaose current market selling price;

- The upstream sector appeared to be the most relevant sector for techno-economic and life cycle assessment;

- An optimization in enzyme production (upstream) can significantly reduce the equipment investment (total plant direct cost);

- A better understanding of the hydrolysis solid/liquid proportion is determinant to reduce the bulk material cost;

- It was possible to observe an overall impact reduction between 16.2 and 19.9% comparing the baseline with the 3-fold scaled-up + 10% yield scenario;

- The results demonstrated that the development of a platform for cellopentaose production based on the developed process is feasible.

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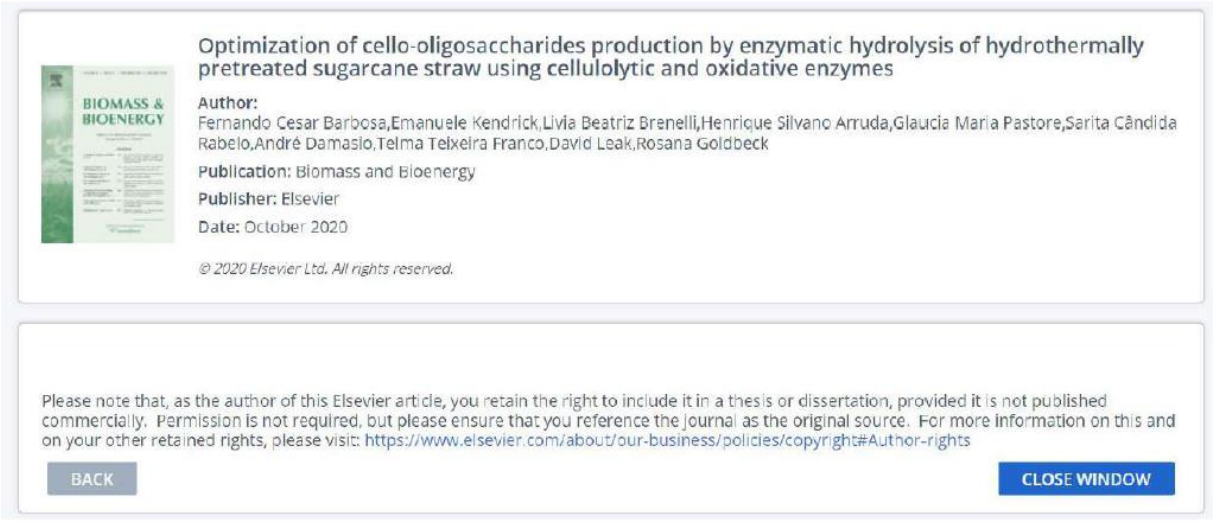

Optimization of cello-oligosaccharides production by enzymatic hydrolysis of hydrothermally pretreated sugarcane straw using cellulolytic and oxidative enzymes

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