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PROGRAMA INTEGRADO (UNESP, USP E UNICAMP) DE PÓS-GRADUAÇÃO EM BIOENERGIA

CELLOOLIGOSACCHARIDES AND XYLOOLIGOSACCHARIDES PRODUCTION FROM SUGARCANE BAGASSE AND COTTON RESIDUES USING CHEMICAL, PHYSICAL AND BIOLOGICAL APPROACHES

JEFFERSON POLES FELIPUCI

Tese apresentada ao Instituto de Pesquisa em Bioenergia de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Bioenergia.

Orientador(a): Prof. Dr. Michel Brienzo

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ABSTRACT

Cellooligosaccharides (COS) and xylooligosaccharides (XOS) are oligomers with interest in several areas, such as agriculture and food industry. The production of COS and XOS depends on the pretreatment involved in the biomass. This study was dedicated to COS and XOS production from two different biomasses: sugarcane bagasse and cotton residue. The methods involved chemical, physical and biological pretreatment, and a combination of them. Also, for both studies, x-ray, FTIR-ATR and scanning electron microscope (SEM) analysis were performed. For sugarcane bagasse, the objective was the production of COS and XOS by a combination of physical and biological pretreatments, while for cotton residue the objective was the production of COS using chemical, physical and biological pretreatments, individually. The sugarcane bagasse was biologically pretreated for 5 months with *Coniophora puteana* (CBMAI 0870), *Gloeophyllum trabeum* (CBMAI 0872), and *Pleurotus ostreatus* (CCIBt 2338). A part of the biomass was ball-milled, and the other part was knife-milled, then the enzymatic hydrolysis with cellulase and xylanase was applied in different enzyme loads, ranging from 20 to 50 IU/g. After five months of pretreatment with *C. puteana*, followed by enzymatic hydrolysis using cellulase at 50 IU/g in combination with knife milling, the yield of COS reached 26.23%. In contrast, when the same pretreatment was applied but with ball milling instead, the yield of COS increased to 36.65%. The best XOS result was 78.12% yield after the biological pretreatment with *G trabeum* after 1 month of pretreatment. For the cotton residue, NaOH was used with different concentrations and temperature, ball milling with three different times of reactions and biological pretreatment using *Gloeophyllum trabeum* (CBMAI 0872) growth for 15 days in liquid medium. The COS yield reached 8.68% from untreated cotton using 100 IU/g of enzyme, against 11.61% of COS which was alkali pretreated using 50% NaOH (w/w) at 100 ºC. The biological pretreatment reached 9.13% of COS yield using the same enzyme load. The ball milling method obtained the best COS yield, reaching 30.11% after 6 h of milling. For both studies, the x-ray, FTIR-ATR and SEM analysis showed differences in the biomasses in comparison to the untreated ones, mainly after the ball milling pretreatment, decreasing drastically the crystallinity index, from 65.38% to 47.85% after 3 h of ball milling. This study examines the effects of chemical, physical, and biological pretreatments on sugarcane bagasse and cotton in the production of COS and XOS.

Keywords: oligosaccharides, biomass, sugarcane bagasse, cotton, enzymatic hydrolysis.

RESUMO

Celo-oligosacarídeos (COS) e xilo-ogosacarídeos (XOS) são oligômeros com interesse em diversas áreas, como a agricultura e a indústria alimentícia. A produção de COS e XOS depende do pré-tratamento envolvido na biomassa. Este estudo foi dedicado à produção de COS e XOS a partir de duas biomassas diferentes: bagaço de cana-de-açúcar e resíduo de algodão. Os métodos envolveram pré-tratamento químico, físico e biológico, e uma combinação deles. Também, para ambos os estudos, foram realizadas análises por raios-x, FTIR-ATR e microscópio eletrônico de varredura (MEV). Para o bagaço de cana, o objetivo foi a produção de COS e XOS por combinação de pré-tratamentos físicos e biológicos, enquanto que para o resíduo de algodão o objetivo foi a produção de COS utilizando pré-tratamentos químicos, físicos e biológicos, individualmente. O bagaço de cana foi pré-tratado biologicamente por 5 meses com *Coniophora puteana* (CBMAI 0870), *Gloeophyllum trabeum* (CBMAI 0872) e *Pleurotus ostreatus* (CCIBt 2338). Uma parte da biomassa foi moída em esferas e a outra parte foi moída com faca, então a hidrólise enzimática com celulase e xilanase foi aplicada em diferentes cargas de enzimas. Após cinco meses de pré-tratamento com *C. puteana*, seguido de hidrólise enzimática utilizando celulase a 50 UI/g em combinação com moagem com faca, o rendimento de COS atingiu 26,23%. Em contraste, quando o mesmo pré-tratamento foi aplicado, mas com moagem de esferas, o rendimento de COS aumentou para 36,65%. O melhor resultado do XOS foi 78,12% de rendimento após o pré-tratamento biológico com *G. trabeum* após 1 mês de pré-tratamento. Para o resíduo de algodão, foi utilizado NaOH com diferentes concentrações e temperaturas, moagem em esferas com três tempos diferentes de reações e pré-tratamento biológico utilizando crescimento de *Gloeophyllum trabeum* (CBMAI 0872) por 15 dias em meio líquido. O rendimento de COS atingiu 8,68% a partir do algodão não tratado com 100 UI/g de enzima, contra 11,61% de COS que foi alcalino pré-tratado com 50% de NaOH (m/m) a 100 ºC. O pré-tratamento biológico atingiu 9,13% do rendimento de COS utilizando a mesma quantidade de enzima. Além disso, o método de moagem por esferas obteve o melhor rendimento de COS, atingindo 30,11% após 6 h de moagem. Para ambos os estudos, as análises de raios-x, FTIR-ATR e MEV mostraram diferenças nas biomassas em comparação com as não tratadas, principalmente após o pré-tratamento da moagem de esferas, diminuindo drasticamente o índice de cristalinidade, de 65,38% do índice de cristalinidade da amostra não tratada para 47,85% após 3 h de moagem de esferas. Este estudo examina os efeitos de pré-tratamentos químicos, físicos e biológicos em bagaço de cana-de-açúcar e algodão na produção de COS e XOS.

Palavras-chave: oligossacarídeos, biomassa, bagaço de cana-de-açúcar, algodão, hidrólise enzimática

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THESIS INTRODUCTION

Biomass is a biological material that can be converted into energy or high added value products. Sugarcane bagasse and cotton residues are examples of biomasses that can be used to produce cellooligosaccharides and xylooligosaccharides, or second generation ethanol, in the sugarcane bagasse case. The potential of biomasses can be an ideal substitute for fossil fuels in energy industries, once the origin of the biomasses are natural resources. Bio-based products have been used over the years as a replacer or a diminisher for oil-based products, they are present in products such as engines, packaging, medicines, and others. The bioproducts can be originated from vegetal biomass like crops, vegetable oils, forests, and waste from agriculture, cities and industry (SCAPINI, *et al*., 2021; QASEEM, SHAHEEN, WU, 2021). Before being turned into a bioproduct, the biomass needs to pass through a process called pretreatment, which consists of modifying the lignocellulosic structure aiming to expose the macromolecules in it or reduce the recalcitrance. Pretreatments can be separated into chemical, physical and biological pretreatment, and a combination of them (FELIPUCI, *et al*., 2020).

Chemical pretreatments consist of the use of acid or alkali reagents to modify the biomass structure: acid conditions solubilize hemicellulose, while alkali conditions change the lignin structure, reduce the cellulose crystallinity and partially solubilize the hemicellulose and lignin. Physical pretreatments consist of use of a physique condition, such as milling, reducing the lignocellulosic particle size and crystallinity. Usually, chemical and physical pretreatments are combined to reach a better result. However, chemical pretreatment usually generates undesirable compounds and demands much energy (FELIPUCI, *et al*., 2020; 2021). There are also the physic-chemic pretreatments, such as steam explosion, consisting in a saturated steam under a controlled pressure and temperature. Biological pretreatment is the use of microorganisms to degrade or modify vegetal biomass structure employing their special enzymatic complexes (IOELOVICH, 2016). Biological pretreatment is already known as a sustainable option to conventional methods used in industries due to does not generate inhibitory and toxic compounds and is a cheaper method compared to chemical and physical pretreatments. Biological pretreatment can be combined with chemical and physical methods to reach better results using less chemicals and energy, making this method an economically and an eco-friendly feasible process

(FELIPUCI, *et al*., 2020; IOELOVICH, 2016; AGBOR, *et al*., 2011). Physical pretreatments consist in use techniques to reduce the size of the biomass particles, such as knife milling or ball milling, however the physical pretreatment is known to demand more energy in the process, but is faster and don't generate undesirable compounds (FELIPUCI *et al*, 2020 and 2021; RASTOGI, SHRIVASTAVA, 2017).

The lignocellulosic material is mainly composed of cellulose, hemicellulose and lignin, which are strictly connected, and this connection makes the material resistant to pretreatments. This is responsible for the high recalcitrance of the material. Microorganisms have the capacity to produce specific enzymes that break the lignocellulosic structure and decrease the recalcitrance. Combining methods focusing on decreasing the material recalcitrance and breaking the lignocellulosic structure, then applying enzymatic hydrolysis (carbohydrate hydrolysis), can generate oligomers from cellulose and xylan with more value-added, such as cellooligosaccharides (COS) and xylooligosaccharides (XOS), respectively (FELIPUCI, *et al*., 2021; SINDHU, BINOD, PANDEY, 2016; RASTOGI, SHRIVASTAVA, 2017).

COS are oligomers formed by β-1,4 linked D-glucose units with a few numbers of glucose. COS are not digestible and can show prebiotic effect, stimulating *in vitro* growth of healthy microorganisms, such as *Lactobacillus* and *Bifidobacterium* strains (ADEBOLA, *et al*., 2014; VÁSQUEZ, *et al*., 2000; CAO, GUO, HUA, 2020; PALANIAPPAN, 2021). XOS are sugar oligomers made up of xylose units, they are also considered healthy due to their capacity to prevent disorders in human nutrition, such as gastrointestinal disorders and infection, obesity control and better nutrient absorption (KUMAR, *et al*., 2021; MANO, 2017). This thesis aimed to debate the methods involved in COS and XOS production, testing chemicals, physical and biological pretreatments, and the combination of them, considering the biotechnological and economics aspects.

OBJECTIVES

The objective of this study was to produce COS and XOS using sugarcane bagasse and cotton waste via biological and physical pretreatment, and via chemical, physical and biological pretreatment, respectively. A combination of pretreatment was evaluated and the pretreated material was characterized.

Specific Objectives

- Evaluate the structural changes of sugarcane bagasse due to biological pretreatment with white-rot fungi;
- Evaluate the physical pretreatment effects in the sugarcane bagasse after the biological pretreatment;
- Evaluate the production of COS and XOS via enzymatic hydrolysis of a combination of pretreated sugarcane bagasse;
- Evaluate the structural changes in cotton biomass after physical, chemical and biological pretreatment and a combination of them;
- Evaluate the production of COS via enzymatic hydrolysis using cotton residue after ball milling, alkaline and biological pretreatment.

Thesis format and organization

This thesis was organized into chapters, which were designed considering independent publications such as book chapter and papers. The organization was as follows: General introduction and objectives; Chapter 1 – book chapter: Biotechnological Aspects of Microbial Pretreatment of Lignocellulosic Biomass (DOI: https://doi.org/10.1007/978-981-15-9593-6_6), published in Biorefineries: A Step Towards Renewable and Clean Energy, published in Springer, reuse authorization license number 5947030195987 by Spring Nature. This chapter presents a bibliography review about the biological pretreatment in lignocellulosic biomasses. In the chapter was discussed the biotechnological aspects of this technique and presented the microorganisms involved in the process, as well as the enzymes and possibilities of the biological pretreatment in the industry; Chapter 2 – article: Efficient production of cellooligosaccharides and xylooligosaccharides by combined biological pretreatment and enzymatic hydrolysis process (DOI: https://doi.org/10.1007/s13399-024-05703-1), published in Biomass Conversion and Biorefinery, from Springer, reuse authorization license number 5945500157455 by Spring Nature. Chapter 2 is an article about biological pretreatment, involving the production of COS and XOS using two kinds of physical pretreatment, such as knife mill and ball mill, evaluating different charges of enzymes. Also, were evaluated the changes in the lignocellulosic biomass structure after biological pretreatment and the crystallinity index after knife and ball

milling. Chapter 3 - article: Cellooligosaccharides production from cotton residue using biological pretreatment, alkali and milling approaches. The third chapter is an article about COS production from cotton residue involving chemical, physical and biological pretreatments. With the focus on the COS production, a waste with high cellulose content was chosen. The cotton residue was also analyzed by x-ray, FTIR-ATR and scanning electron microscope to evaluate the modifications in the biomass before and after the pretreatments; General conclusions.

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CHAPTER I: BIOTECHNOLOGICAL ASPECTS OF MICROBIAL PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

ABSTRACT

In several areas, products are obtained from lignocellulosic biomass, such as bioethanol and personal items. Notwithstanding, it features high recalcitrance, hence its use often demands pretreatment and hydrolysis stages to reach bio-based final products. Industrially, the most common method is the chemical pretreatment which, as the name implies, involves chemical components with potential environmental risks. This procedure is responsible to increase biomass accessibility and to enhance polysaccharides achieving in subsequent stages. Biological pretreatment presents a new perspective to replace or cooperate with its chemical counterpart, once microorganisms can modify the lignocellulosic structure and facilitate accessibility to macromolecules of interest. According to the above, this chapter covers the potential of biological pretreatment as well as the mechanisms of microbial degradation, their enzymes, and the impacts on the economy worldwide.

Keywords: sugarcane bagasse; fungus; microorganism; biological pretreatment; degradation

1.1 INTRODUCTION

Recent technological, social and environmental changes have brought new needs in both science and industry for developing alternative technologies that make it possible to achieve similar products, than those obtained from petroleum sources (RUAN *et al*. 2019). Since the last years of the 19th century, the world energy matrix has been based on fossil fuels (British Petroleum 2019). Among the possibilities to replace oil, biomass has become the most important resource, able to generate several products by different routes, with the great advantage of being environmentally friendly (GUEDES *et al*. 2019). In this perspective, bio-based products are currently part of everyday life, with applications in sectors such as engines, packaging, medicines, and many others. With or without slight treatment/modifications, vegetal biomass like crops, vegetable oils, forest, agricultural waste, and also the municipal and industrial ones are used to produce bioproducts (SOROKINA *et al*. 2017; ROSALES-CALDERON and ARANTES 2019). However, turning vegetal biomass into bioproducts may become a challenge, since the raw material needs to be undergone to different types of stages during the conversion process until reaching suitable yields (HOLWERDA *et al*. 2019). Pretreatment has a huge importance in the steps of value-added products generated from biomass systems, where complex structure presented in plants must be conditioned for subsequent stages (ANTUNES *et al*. 2019).

The most used methods of biomass pretreatments, such as chemical and physical procedures, have in common the demand for plenty of chemical reagents and/or energy inputs in its process. Such chemicals are widely used in industries to separate biomass components in order to manufacture all kinds of (bio-) products, but in consequence, those reagents are found polluters for the environment. Nowadays, facing an economic and global warm crisis, it is essential and recommended looking for alternatives to low-cost, less oil-dependent, and non-polluting manufacturing methods.

Biological pretreatment of biomass is already known as an option to conventional methods used in industries. This method does not generate toxic and inhibitory compounds and need low quantity of chemical and energy input, which makes it an economically and eco-friendly feasible process. Biological pretreatment also can be used before a chemical or physical pretreatment: the biological stage can provide a better decrease of the recalcitrance while the chemical stage provides the

separation of the macromolecules. This combination can reduce the costs and chemicals in the whole process (SHINDU *et al*. 2016; SINGH *et al*. 2018; AGBOR *et al*. 2011, FELIPUCI 2020).

In this chapter will be discussed how biological pretreatment works, including the enzymes and microorganisms involved in the biomass structure modification. Moreover, the benefits and disadvantages of this method are discussed, as well as value-added and commodity products, mainly on large scale.

1.2 BIOLOGICAL PRETREATMENT

Biological pretreatment of lignocellulosic biomass became a fundamental research topic since it is clear that a near-term economy will depend on the supply of biomass to produce bioproducts and bioenergy. It is related to the use of microorganisms, aiming to degrade or modify vegetal biomass structure employing their special enzymatic complexes (AGBOR 2011; SINDHU *et al*. 2016). Among the vast variety of species in the world, fungi and bacteria are well known to produce specific enzymes for lignocellulose deconstruction, called cellulases, hemicellulases, and ligninases. These enzymes are capable to degrade natural macromolecules found in the plant cell wall, such as cellulose, hemicelluloses, and lignin. Cellulose and hemicelluloses, for instance, are hydrolyzed into smaller molecules (the monomeric sugars) (SHARMA *et al*. 2019).

Among the numerous enzymes produced by fungi that degrade cellulose, hemicellulose and lignin, the most studied are: endo-glucanases, exo-glucanases and β-glucosidases that hydrolyze cellulose; endo-xylanases, β-xylosidases, acetyl xylan esterases and others that degrade xylan and laccases, manganese-peroxidases and lignin-peroxidases that degrade lignin (PAMIDIPATI and AHMED 2019; GAUTAM *et al*. 2019; MALGAS *et al*. 2019).

The species of fungi that degrade lignin are known as white rot. The ones that depolymerize cellulose and hemicelluloses are named brown rot because the wood degraded takes a brownish appearance, due to the loss of polysaccharides (cellulose and hemicellulose) remaining high amounts of lignin (HATAKKA and HAMMEL 2011). Biological pretreatment does not generate toxic compound (degradation products, inhibitors) during its process and it is ecologically promising, which is an advantage comparing to other usual methods. Moreover, results can be optimized when the

strains are pre-selected (SINDHU *et al*. 2016; VAN KUIJK *et al*. 2015). In the biodegradation, variable microbial communities is important to the quality of the final results due to its vast amount of enzymes. However, in addition to the microorganism itself, biomass composition, temperature, humidity, pH, aeration rate, incubation time and biomass particle size are elements that can also affect the result and the quality of the pretreatment (SINDHU *et al*. 2016; FANG *et al*. 2012; LI *et al*. 2012; IQBAL *et al*. 2013; FATOKUN *et al*. 2016).

Usually, biological pretreatment needs long-time requirements (10-14 days), space, and careful growth conditions to work. In industrial scale it may be less attractive but the biological pretreatment can be used together with chemicals and physical pretreatment. The potential of delignification by microorganisms combining with chemical and physical methods is inviting because of the complete degradation of lignocellulosic biomass components, mainly lignin, that can take a long time to reach significant results (AGBOR *et al*. 2011; HATAKKA 1994; HATAKKA *et al*. 1993).
Recalcitrance is the capacity of a biomass resist to a pretreatment or to enzyme action. The quantity and organization of the components into the cell wall such as cellulose crystallinity are factors that may change the recalcitrance level of biomass (NAIDU *et al*. 2018; MELATI *et al*., 2019; PARK *et al*. 2010). Lignin is suggested to has a crucial hole in the recalcitrance due to its resistance against pathogens and insects, and its removal influences the access to the polysaccharides (SHIMIZU *et al*., 2020; SCHMATZ *et al*., 2020; ZHAO *et al*. 2012; PHITSUWAN *et al*. 2013)

High recalcitrance is a challenge in the search for better methods of macromolecules isolation from biomass. Accordingly, different pretreatment methods have been developed, aspiring to circumvent this problem in order to separate its components. One method to work around the recalcitrance problem is to select varieties with low lignin content (BRIENZO *et al*., 2015), or delignify biomass decreasing lignin content, considering that lignin is a barrier in carbohydrate extraction (SHIMIZU *et al*., 2020; BRIENZO *et al*., 2017). An usual pretreatment focuses on improving the formation or capability to form fermentable sugars by hydrolysis; avoid by-product formation that may prevent subsequent processes and be a good cost-benefit ratio method (MELATI *et al*., 2019). Thus, biological pretreatment is an option to replace or co-work with other methods of pretreatment by attending such ideal requirements.

Other way to degrade lignocellulosic biomass is using co-culture, which use more than one microorganism. This method is based in to use fungus or/and bacteria to degrade the lignocellulosic biomass. However, competition between microorganisms for the substrate is not recommended, and it can be used one after other. This technique is useful due to both microorganisms encompass large quantities of enzymes, which can complete degrade the lignocellulosic material. This process can be used in different areas such as agronomy (degrade pesticides) and industry (carpet decolorization) (YOON *et al*. 2014; SARIWATI *et al*. 2017; WANG *et al*. 2017; SARIWATI *et al*. 2017; KUMARI and NARAIAN 2016).

1.2.1 Lignocellulosic Biomass structure

Lignocellulosic biomass englobes all organic matter directly from plant sources. It is the largest source of carbohydrates in nature, with a great variety, abundance and availability, involving wood, agro-industrial waste, municipal waste, and plants. What draws attention to these materials is that they are renewable resources with energy potential. This presents them as possible substitutes for fossil fuels, generating sustainable energy through bioethanol and co-generation of electric energy (by a burning process) (NANDA *et al*. 2015). Consequently, interest in research, both in scientific and industrial fields, grows constantly (BILGILI *et al*. 2017; MAO *et al*. 2015; ASLAN 2016; TOKLU 2017; SHARMA *et al*. 2019).

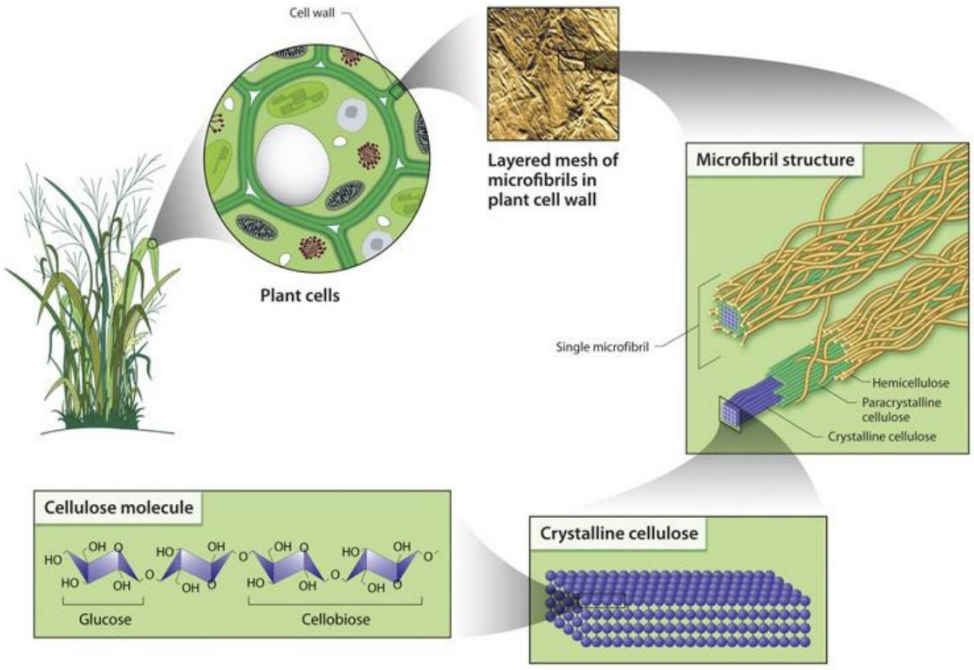
One of the most used lignocellulosic biomass is the sugarcane bagasse (*Saccharum* spp) Currently, the bagasse is used in the production of electrical and thermal energy through its combustion in high-pressure boilers in plants (FERNANDES *et al*. 2018). Another application aims to obtain second-generation ethanol (cellulosic ethanol), serving as an alternative to replace fossil fuels.

Lignocellulosic biomass is also used in the production of clothing, artificial skin, paper, and other products in common use (MIZUHASHI *et al*. 2015; KIM *et al*. 2014). More specifically, in biotechnology and biomass conversion, it is possible to produce briquettes, carbon adsorbents, and biofilms. The production of these items depends on the treatment that those biomasses will be undergone. For separation of each macromolecule, there is one or a series of treatments to be based on biological routes. The main characteristic of vegetal biomass is its lignocellulosic structure existing into the cell wall, presented in all plant forms. Its composition is mainly cellulose,

hemicelluloses, and lignin, with less quantities pectins, proteins, and extractives (NAIDU *et al*. 2018). Quantities of each component change according to biomass and soil types, geographic localization, and other factors (De VASCONCELOS, 2015). The three main components (cellulose, hemicelluloses, and lignin) in the cell wall are organized in a way that recalcitrance is increased, making its separation harder in biotechnological processes. Cellulose and hemicelluloses are strongly connected by hydrogen bonds. Hemicelluloses can be located between cellulose fibers, while lignin is connected to the carbohydrates forming a complex interaction network (SCHMATZ *et al*. 2020; BUSSE-WICHER 2016).

Cellulose is the major macromolecule in the plant cell wall (Fig 1). The quantity varies according to biomass type: cotton presented around 95% and sugarcane bagasse showed 25 to 45% (NAIDU *et al*. 2018). It is also considered most abundant organic polymer found on the planet Cellulose is an arrangement constituted by cellobiose unities (glucose dimers) joined by β-1,4 glycosidic chains. In the cellulose structure, there are amorphous regions which are organized regions demined crystalline and non-crystalline zones (IOELOVICH 2016). Cellulose is widely sought in the industry as raw material for common use products, such as varnish, films, paper, among others. Due to several industrial interests, cellulose isolation from biomass is widely studied. Cellulose can be separated from other carbohydrates by alkaline treatment, or broken by acid treatment. In the case of alkaline treatment, ester linkages break down, resulting in structural modification of the cell wall and facilitating separation from hemicelluloses (GALLETTI and ANTONETTI 2012).

Figure 1.1 - Schematic representation of lignocellulosic biomass emphasizing the cellulose macromolecule



Source: JASMANIA and THIELEMANS 2018

Hemicelluloses, different from cellulose, are composed of more than one monosaccharide: pentoses, hexoses and uronic acids. In pentoses group is found xylose and arabinose; in hexoses group is found mannose, glucose and galactose and in uronic acids is found glucuronic and galacturonic acids. Those monosaccharides can also be subdivided into three main groups: xyloglucans, xylans, and mannans, that are formed by subunits of mannose. Many of the OH groups at C2 and C3 of xylose units are substituted with O-acetyl groups. The monosaccharides are connected by β and α glycosidic bonds and can have between 80-200 units. Hemicelluloses have amorphous characteristics and a lower degree of polymerization than cellulose. It makes up 15 to 35% of lignocellulosic biomass and it is associated to the integrity of the plant cell wall, having great importance in its shape and resistance. Hemicelluloses correspond to one-third of all renewable carbon on the planet. Hemicellulose has been studied for several applications, with a feature for oligomers such as xylooligosaccharides and manooligosaccharides (De FREITAS *et al*., 2019; CHIYANZU *et al*., 2014).

Lignin is a biomass macromolecule composed of phenylpropane units of p-hydroxyphenyl (H), syringyl (S) and guaiacyl (G). This polyphenolic structure is

organized irregularly and has an amorphous structure. Depending on species, lignin comprehends between 10 to 20% of lignocellulosic biomass, being the third most abundant macromolecule in the plant cell wall. For plants, lignin helps in protection against insects and fungi and also contributes to growth development and mechanical strength. This protection is one of the reasons to the biosynthesis, once infections, metabolic stress and disturbances in cell wall structure are starters to the plant initiate the process (VANHOLME *et al*. 2010). It is arranged mainly on the secondary wall, making it rigid and waterproof. The most common linkages in lignin macromolecule is the β-O-4, however there are others kind of linkages more resistant to chemical degradation, such as β-5, β- β, 5-5, 5-O-4 and β-1 linkages. Lignin organization is linked with hemicelluloses, together with its irregular structure and a gigantic number of possibilities for connections between its forming units, which suggests that there is a low chance of existing two similar lignin molecules (RALPH *et al*. 2004). This favors the increasing recalcitrance of its biomass (SCHMATZ *et al*., 2020). Lignin is an obstacle for a process dedicated to macromolecule separations as it remains as residual content/contaminant (FELIPUCI, 2020).

1.2.2 Microorganisms in Biological pretreatments

Microorganisms are considered of key function in biological pretreatments of lignocellulosic biomass. Degradation capacity of microorganisms is widely known, mainly because of the degradative potential of its enzymes, which are produced during its growth. Biological pretreatment technology has generated results in several areas involving biotechnology, bioremediation, biopulping among others.

The most common microorganisms applied in biological pretreatment are white-rot, brown-rot, and soft-rot fungi, besides bacteria. These microorganisms are capable to consume all components in lignocellulosic biomass, mainly lignin, and the capacity to mineralize lignin into carbon dioxide and water. Brown-rot fungi are known to degrade polysaccharides more efficiently, and only slightly modifies the lignin, while white-rot fungi can degrade lignin with more facility (KIRK and MOORE 1972; KIRK and HIGHLEY 1973). Holocelluloses/lignin ratio presented in biomass after degradation can be used to measure the fungal effect on the biomass decomposition. The effect on the biomass components can be classified at different ratios: Class 1 (corresponds to brown decomposition agents): ratio less than one; Class 2: whose process has a low

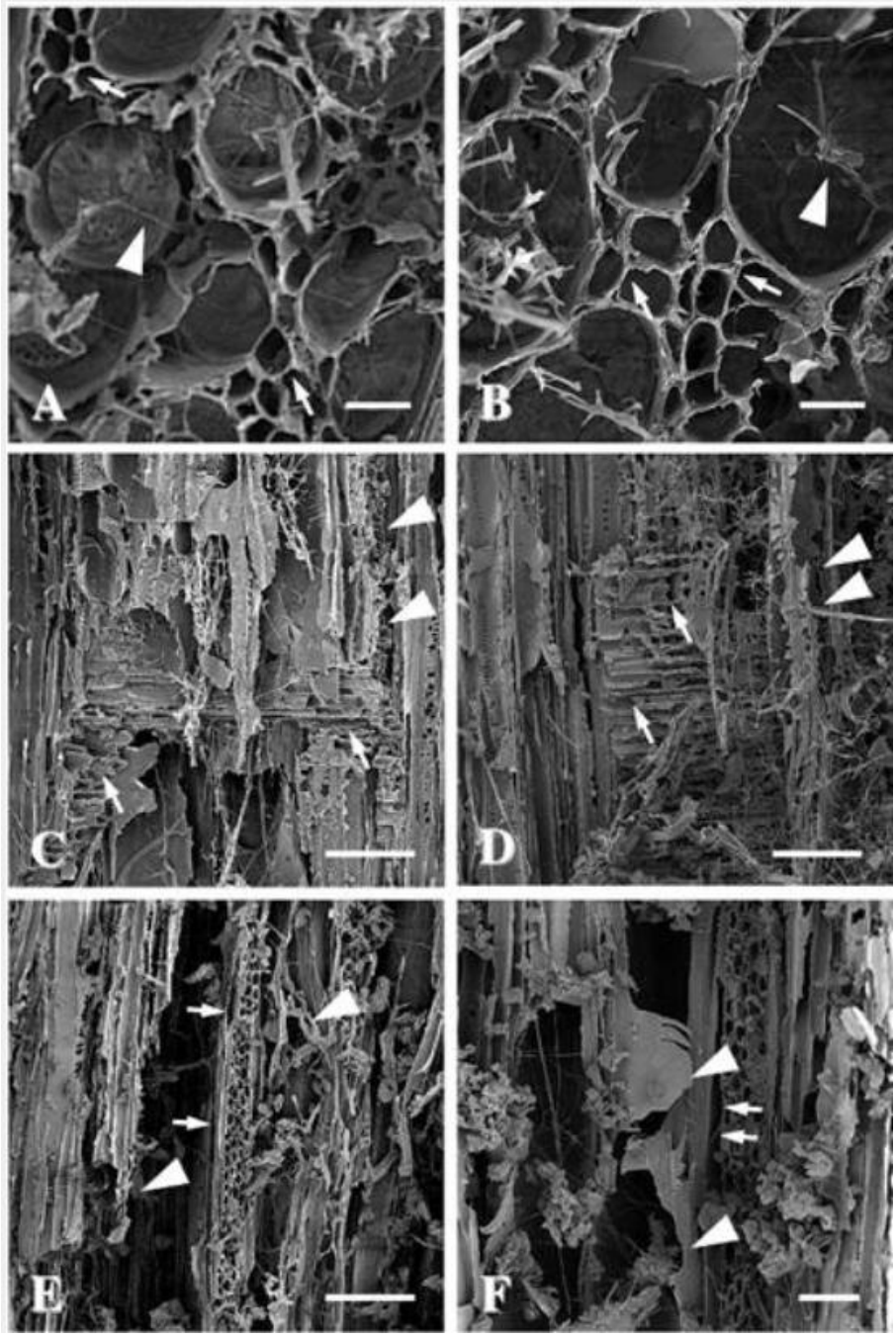
amount of residual lignin; Class 3: holocelluloses content is two to five times higher than lignin content; Both classes 2 and 3 correspond to white decomposition agents (TROJANOWSKI 2001).

1.2.2.1 White-Rot Fungi

Industrially white-rot fungi are well known as lignin consumers, found in Basidiomycota phylum. Those comprehend over than 90 % of all Basidiomycetes that rot woods. (RILEY *et al* 2014). This phylum has been studied in several areas, including medicine (MADHANRAJ *et al*. 2019), agriculture (DUPLESSIS *et al*. 2011), and forestry (MARTIN *et al*. 2008). This phylum also includes mushrooms (MORIN *et al*. 2012), and pathogens of plants, animals, and other fungi (DUPLESSIS *et al*. 2011; DAWSON 2007).

White-rot fungi have great potential to degrade lignocellulosic biomass (Fig 2). Although those fungi also can degrade polysaccharides, they are known as a well specific lignin degrader (RUDAKIYA and GUPTE 2017). Syringil (S) units of lignin usually are preferred instead of guaiacyl (G) units, due to its less resistance to degradation. In certain conditions, white-rot fungi are lignin-selective depending on several factors, like cultivation time, temperature, wood species, and other variables (HATAKKA and HAMMEL 2011; HAKALA *et al*. 2004). The degradation ability of these fungi has been quite studied not only in lignocellulosic biomass researches, but also in other areas, such as bioremediation, food, pharma, and other industries. These abilities allows the fungi grow in restrictive conditions, such as lignocellulosic wastes In the last decade, several studies focused on these group showed results to degrade pesticides (KAUR *et al*. 2016; GOUMA *et al*. 2019), to increase productivity, efficiency, and quality of several products (KUSHWAHA *et al*. 2018) and applied in pulp and paper industry (SINGH 2018).

Figure 1.2 **-** Scanning electron micrographs of beech wood degradation by white-rot fungi after 120 days



Source: BARI *et al,* 2018. A, C, and E: *Pleurotus ostreatus*; B, D, and F *Trametes versicolor*. A and B shows cross-sections (bar 20 µm): the arrows point cell walls already degraded and arrowheads point colonization of hyphae in the cell lumina; C and D show radial sections (bar 100 µm): the arrows point a entire decomposition of ray parenchyma and arrowheads point deconstruction of cell walls and vessels; E and F show tangential sections (bar 100 µm): the arrows point the separation of ray wall with vessels lumina, while arrowhead point disintegration of woody structure.

1.2.2.2. Brown-Rot Fungi

Brown-rot fungi are also found in the Basidiomycota group, representing nearly 7 % of this phylum (HATAKKA and HAMMEL 2011; GOODELI 2003). An examples of families that concern to brown-rot fungi are: Adustoporiaceae, Postiaceae Auriporiaceae and others (LIU *et al*, 2022) Evolutionarily, most of this group are derived from white-rot fungi, probably by losing of decay capability and biodegradative mechanisms (HIBBETT and THORN 2001). Otherwise, white-rot and brown-rot classification are discussed, since new genetic studies suggest continuum rather than a dichotomy between these two groups. In this case, authors suggest that the “white-rot fungi” term would be restricted to fungi that consume all the cell wall macromolecules through activity of lignin-degrading peroxidases (RILEY *et al*. 2014).

The brown color of brown-rot fungi is due to residual lignin left after degradation. It is caused by fungi enzymatic arsenal that degrades polysaccharides. Hemicellulose degradation is faster and polysaccharide depolymerization involves oxidative components and hydrolytic enzymes (HATAKKA and HAMMEl 2011).

Degradation capacity is widely known in the bio-pulping area. Bio-pulping is a process where wood chips are treated by microorganisms to improve quality and make stronger paper produced. This method reduces toxicity and pitch content (GUPTA 2019). Using some species of brown-rot fungi with worms to degrade paper mill sludge is a useful strategy to enhance cellulose decomposition (NEGI and SUTHAR 2018).

1.2.2.3. Bacteria

Bacteria are known to produce cellulolytic, hemicellulolytic, and ligninolytic enzymes that can also be used in biological pretreatment (SHARMA *et al*. 2019). An advantage in comparison to fungal pretreatment is that some bacteria can grow faster than fungi besides degrade lignin into small particles. (HATAKKA 2005; KURAKAKE *et al*. 2007). Examples of wood degrading bacteria can be: Betaproteobacteria, Gammaproteobacteria, and Acidobacteria (JOHNTON *et al*, 2016).

Although bacteria can properly degrade lignocellulosic biomass, its sole use as biological pretreatment has not proved efficient. However, it can improve the enzymatic digestion of lignocellulose after applying another pretreatment, such as physicochemical method (ZHUO *et al*. 2018). Co-culture using bacteria and/or fungi can degrade lignocellulosic biomass almost completely due to high enzymatic activity.

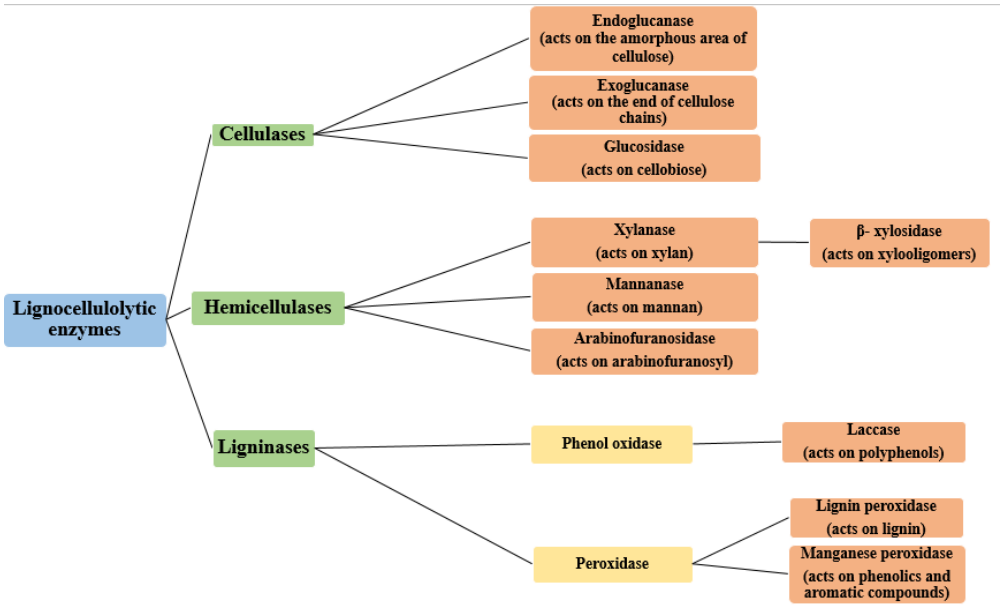
Selecting the best strains, that can produce necessary enzymes is essential for an efficient biological pretreatment in order to produce biofuels and bioproducts (SHARMA *et al*. 2019).

1.2.3 Enzymes Involved in Biological Pretreatment

The effectiveness of a biological pretreatment depends on enzymes ability to address biochemical and physical barriers to hydrolysis. Therefore, a mix of enzymes can co-work to increase biomass access by expanding small pores and open the cell wall matrix (AMIN *et al*. 2017).

Lignocellulose degradation by microorganisms is mainly accomplished by a system of extracellular enzymes that hydrolyze and oxidize the biomass component (Fig 3). Hydrolases (cellulases and hemicellulases) are produced by hydrolytic system to degrade polysaccharides and oxidative catalytic system to degrades lignin by the production of ligninases (SAJITH *et al*. 2016).

Figure 1.3 - Simplified representation of lignocellulolytic enzymes and their action mode



Source: SAJITH *et al*. 2016.

1.2.3.1 Cellulases

Cellulases are glycosyl hydrolases (GHs) produced by microorganisms while they grow on lignocellulosic materials. They hydrolyze cellulose into shorter chain

polysaccharides by breaking down β-1,4-glycosidic bonds. In their structure, they usually have a catalytic domain at the N- terminal and a carbohydrate-binding module at the C- terminal. The first one cleaves the glycosidic linkage and the second one destiny the catalytic domain to the polysaccharide substrate (JAYASEKARA and RATNAYAKE 2019; OBENG *et al*. 2017).

Three main enzymes comprise cellulases enzyme system, endoglucanases (endo-β-1,4-D-glucanases; EC 3.2.1.4), exoglucanases (exo-β-1,4-D-glucanases; EC 3.2.1.91) and glucosidases (β-D-glucoside glucanhydrolases, EC 3.2.1.21). These enzymes are categorized as per their structure and function, however their collaborative work is essential for complete hydrolysis of the complex cellulose fibers (SAJITH *et al*. 2016).

Endoglucanases generate oligosaccharides with free chain ends by hydrolyzing internal β-1,4-glycosidic bonds and acting randomly on amorphous areas of cellulose. These enzymes can convert cellodextrin (intermediate product of cellulose hydrolysis) into cellobiose and glucose (SINGH *et al*. 2016). Endoglucanases has rapid dissociation, can reduce chain length and viscosity by acting on cellulose but exhibit no activity against crystalline cellulose such as avicel (AKAMINE *et al*. 2018; OBENG *et al*. 2017; SAJITH *et al*. 2016).

Exoglucanases acts on the crystalline region of cellulose and release cellobiose as product from reducing (EC 3.2.1.91) or non-reducing ends (EC 3.2.1.176). The oligosaccharide chain portion that each enzyme attacks are related to its classification. However the actions of the enzymes are unidirectional in a long-chain oligomer (OBENG *et al*. 2017; SINGH *et al*. 2016). These enzymes are more active against crystalline cellulose substrates such as avicel and cellooligosaccharides but do not hydrolyze soluble resultants of cellulose like carboxymethyl cellulose (JAYASEKARA and RATNAYAKE 2019; SAJITH *et al*. 2016).

β-glucosidases present rigid structure with an active site that favors disaccharides entry, however, they also can hydrolyze low degree of polymerization soluble cellodextrins. These enzymes act on cellobiose to complete the hydrolysis process of cellulose. As result, glucose with a free hydroxyl group at C^4 from the non-reducing end of oligosaccharides are released (OBENG *et al*. 2017; SAJITH *et al*. 2016).

Retention and reversion are catalytic mechanisms that lead to successful cellulose hydrolysis. This is performed by two catalytic amino acid residues of the

enzymes, a proton donor and a nucleophile. Both of them stereochemicaly modifies the anomeric carbon configuration, facilitating enzymatic cleavage of the glycosidic bonds (GARVEY *et al*. 2013).

Cellulolytic enzyme multisystem can suffer inhibition by its products. For this reason, β-glucosidases and exoglucanases are essential to alleviate exo- and endoglucanases, respectively, from feedback inhibition. In the same way, β-glucosidase is also inhibited by glucose, therefore is necessary a search for glucose tolerant β-glucosidases (OBENG *et al*. 2017). Complementary action of these cellulases is crucial for efficient hydrolysis in order to obtain glucose residues, which can be used for several applications such as the production of biofuel and chemicals. Among microorganisms, fungi are responsible for approximately 80% of cellulose hydrolysis and therefore, considered great cellulase producers (SINGH *et al*. 2016).

1.2.3.2 Hemicellulases

Efficient hemicellulose hydrolysis of lignocellulosic biomass improves hydrolysis yield and consequently reduces enzyme costs and dosages, which makes crucial the use of hemicellulases. They are most often glycoside hydrolases and are usually produced by microorganisms together with cellulases. The hemicellulose backbone of a lignocellulosic biomass can be composed by different polysaccharides, depending on the source (SINDHU *et al*. 2016; SINGH *et al*. 2016).

Mannan and xylan are the most common hemicelluloses found in nature. Xylan is the main hemicellulose in lignocellulosic biomass from agriculture residues, comprised of xylose units in the backbone chain that are usually linked to acetyl and ferulic groups, arabinofuranosyl or glucuronic acid residues. Therefore, multiple enzymes are necessary to decompose xylan, including endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37) that acht on the main chain of xylan. The enzymes that work on the pending grups are α-arabinofuranosidase (EC 3.2.1.55), and α-glucoronidases (EC 3.2.1.139) (ÁBREGO, CHEN & WAN, 2017). In addition, acetyl xylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73), and p-coumaric acid esterases (EC 3.1.1.x) are also requested for the complete deconstruction of xylan (CHADHA *et al*. 2019). Hemicellulases structures are consisted by a catalytic domain to perform enzyme functions. They can be glycosyl hydrolases that cleaves glycosidic bonds or

can be carbohydrate esterases that hydrolyze ester bonds, between xylan and acetic acid or ferulic acid substitutions (JUTURU and WU, 2013).

Xylanases hydrolyze β-1,4 linkages in xylan backbone chain, producing xylooligosaccharides. Most of them belong to glycoside hydrolase (GH) families 10 and 11, however enzymes that are exclusively active on D-xylose-containing substrates, known as “true xylanases”, are only on family 11 (TYAGI, PATIL and GUPTA, 2019). β-xylosidases hydrolyze a low degree of polymerization xylooligomers, produced by xylan hydrolysis, into xylose. Xylanases action is inhibited by xylooligomers produced in the hydrolysis, therefore β-xylosidases action removes end-product inhibition increasing the efficiency of xylanases (CHADHA, RAI and MAHAJAN, 2019).

β-mannanases hydrolyze mannan-based hemicelluloses. As result, short β-1,4-mannooligomers are released that can be hydrolyzed into mannose by β-mannosidases. Arabinofuranosidases catalyze the removal of arabinosyl substituents and facilitate an increase in access points of xylanase to xylan Both β-mannanases and arabinofuranosidases are required for mannan or arabinofuranosyl containing hemicelluloses (TERRONE *et al*. 2020). The α-1,2-glycosidic bond can be broken down by α-D-glucuronidases releasing glucuronic acid from the xylan chain (CHADHA, RAI and MAHAJAN, 2019; SINGH *et al*. 2016).

Acetyl xylan esterases are enzymes responsible to remove acetyl groups linked to β-D-xylopyranosyl residues by hydrolyzing the ester bonds. The accessibility of enzymes that break the backbone by steric hindrance can be interfered by acetyl side-groups, therefore their removal makes the xylanases action easier. Ferulic acid esterases and p-coumaric acid esterases also catalyze ester bonds on xylan. The first enzymes are recognized to break down ester linkages between ferulic acid and arabinose substitutions on xylan, and the second acts on the bond between arabinose and p-coumaric acid (CHADHA, RAI and MAHAJAN 2019; BAJPAI 2014).

Due to the complex chemical structure of hemicelluloses, its hydrolysis into its constituent monomers requires catalytic action of versatile enzymes that work synergistically. Hemicellulolytic enzymes can be produced by different fungi and bacteria, however, the source of most commercially important hemicellulases are fungi (MANJU and CHADHA 2011). They have biotechnological potential and several industrial applications, like hemicelluloses hydrolysis of lignocellulosic biomass, improving cellulose saccharification (CHADHA, RAI and MAHAJAN 2019).

1.2.3.3 Ligninases

Lignin is one of the main responsible for recalcitrance in lignocellulosic biomass because its complex structure, protecting polysaccharides (SCHMATZ *et al*. 2020). To break down the lignin structure, microorganisms developed some specific extracellular enzymes based on oxidative reactions. In nature, lignin degradation is important to the biogeochemical carbon cycle (RUIZ-DUEÑAS and MARTÍNES 2009). Those enzymes are also used in the bioremediation process and its action is an important step for lignin removal in industries that work with cellulosic biomass (JHA 2019).

Ligninases are, generally, separated in two different types: phenol oxidases and peroxidases. Laccases are an example of phenol oxidases. Lignin degradation by laccases (EC 1.10.3.2) is normally by oxidation of phenolic compounds, yielding quinines and phenoxy radicals. Peroxidases make part of oxidoreductases family. This group of enzymes catalyzes lignin depolymerization utilizing H_2O_2 (SAJITH *et al*. 2016).

Laccase enzymes are observed in plants, insects, bacteria, and fungi, mainly in the white-rot group. In fungi, these enzymes are involved not just in lignin degradation but also in sporulation, pigmentation of the fungus, detoxification, and fruiting body (CLUTTERBUCK 1990; THURSTON 1994). The molecular weight of laccase is around 50 to 100 kDa and they are classified as multicopper oxidases, which can be monomeric, dimeric, or tetrameric. Laccase use molecular oxygen to oxidize phenolic rings to phenolic radicals. Laccase can cleave Cα–Cβ cleavage, aryl-alkyl cleavage, and Cα-oxidation. Products may be submitted through non-enzymatic reaction, like polymerization, hydration, or dismutation, or a second enzyme-catalyzed oxidation (MADHAVI and LELE 2009; SAJITH *et al*. 2016). With a redox mediators present, laccases can also catalyze the breakdown of non-phenolic lignin structures, and cleave β-O-4 linkages.

Lignin peroxidase (EC 1.11.10.14) is considered one of the key enzymes in plant cell wall degradation due to its ability to oxidize non-phenol lignin structures. This reaction can cleavage Cα–Cβ bonds, mediating ring-opening reactions. Lignin peroxidases are oxidized by hydrogen peroxide, and, this catalysis results in the creation of intermediate radicals such as phenoxy and veratryl alcohol (WONG 2009; RUIZ-DUEÑAS and MARTÍNES 2009). Lignin peroxidase and laccase are considered

“partners” enzymes in certain conditions, due to substrate provided by lignin peroxidase after lignin degradation (BOOMINATHAN and REDDY 1992).

Manganese peroxidase (EC 1.11.1.13) attacks both phenolic and non-phenolic lignin units. This enzyme works as a mediator in enzymatic activity, once it is converted from Mn^{2+} into Mn^{3+}. Several monomeric phenols are oxidized by Mn^{3+} cation, including dyes and phenolic lignin model compounds (DATTA *et al*. 2017).

1.2.4. Enzymatic Hydrolysis of Biological Pretreated Material

In a biorefinery system, lignocellulosic biomass hydrolysis is an essential phase in the whole process, since through hydrolysis intermediate products are obtained by breaking up of macromolecules existent in pretreated biomass (BICHOT *et al*. 2018; POCAN *et al*. 2018). The intermediate denomination is because these products will be used at a subsequent stage of conversion, the main intermediate products are monomers such as hexoses and pentoses coming from cellulose and hemicelluloses (LOOW *et al*. 2016). Hydrolysis or saccharification can be performed by acid, enzymatic or combined procedures, among the aforementioned, the biological process is possibly the most researched in the last years (POCAN *et al*. 2018). Hydrolysis by biological routes shows benefits associated to mild temperature in operation, high ratio (quantitative) between obtained product and precursors (monomers), minimal corrosion problems and in enzymatic hydrolysis does not produce inhibitory chemicals that can modify enzymes activities (AMEZCUA-ALLIERI *et al*. 2017; JAHNAVI *et al*. 2017).

The key to the biological hydrolysis of pretreated lignocellulosic biomass is the hydrolytic enzymes; cellulose saccharification happens by deed of cellulolytic enzymes (cellulases), and hemicelluloses splitting befalls by action of hemicellulolytic enzymes (hemicellulases) (BHARDWAJ *et al*. 2019; BARBOSA *et al*. 2020). These enzymes can be synthesized mainly by fungi, bacteria, yeast, or algae through its controlled growth in solid or submersed fermentations (DOTSENKO *et al*. 2018; ARUWAJOYE *et al*. 2020). Instead of producing hydrolytic enzymes, there is the alternative to purchase commercial enzymes prepared by different industries dedicated to synthesize and purify enzymatic cocktails that act according to specific conditions in hydrolysis (FLORES-GÓMEZ *et al*. 2018). Table 1.1 shows a summary of some

characteristics related to hydrolytic enzymes, their mode of action, product formation, and inhibitory aspects.

Table 1.1 - Properties of cellulases and hemicellulases action on lignocellulosic biomass

Macromolecule	Enzyme	EC / Synonym	Act on	Product	Compounds that cause inhibition	References
Cellulose	Endoglycanases	3.2.1.4 / Carboxylmethylcellulases (CMCases)	β-(1→4) bonds at non-crystalline sections	Reducing and non-reducing new parts	Cellobiose	(MURPHY *et al*. 2013; PARK *et al*. 2019)
	Exoglycanases	3.2.1.91 / Cellobiohydrolases (CBHs) or Avicelases	β-(1→4) bonds at cellulose ends and new reducing parts and non-reducing parts	Cellobiose and other glycooligomers	Glycose	(FRY 2003; VIANNA BERNARDi *et al*. 2019)
	β-glycosidases	3.2.1.21 / Cellobiases	β-(1→4) bonds at cellobiose	Glycose	Glycose, mannose and galactose	(TETER *et al*. 2014; HSIEH *et al*. 2014)
Xylan	Endoxylanases	3.2.1.8	β-(1→4) bonds at xylan backbone	Xylobiose and other xylooligomers	Xylobiose and Xylotriose	(PUCHARt *et al*. 2018; FU *et al*. 2019)
	β-xylosidases	3.2.1.37	β-(1→4) bonds at xylobiose	Xylose	Xylose	(YEOMAN *et al*. 2010; BOSETTO *et al*. 2016)
	α-arabinofuranosidases	3.2.1.55	α-(1→2), α-(1→3) and α-(1→5) bonds at xylose-arabinose linkages	Xylose and Arabinose	Glycose and Galactose	(NUMAN and BHOSLE 2006; YEOMAN *et al*. 2010; MALGAS *et al*. 2019)
	α-glucuronidases	3.2.1.139	α-(1→2) bonds at glucuronic acid-xylose linkages	Methyl glucuronic acid and xylose	--	(DASHNYAM *et al*. 2018; MALGAS *et al*. 2019)
	β-galactosidases	3.2.1.23 / Lactases	β-(1→4) bonds at galactose-xylose linkages	Galactose and Xylose	Galactose	(HUSAIN 2010; KHOSRAVI *et al*. 2015; MALGAS *et al*. 2019)
	acetyl xylan esterases	3.1.1.6	Acetyl groups located at xylan branches	Acetyl groups	*Organophosphate compounds	(MONTORO-GARCÍA *et al*. 2011; PAWAR *et al*. 2016;

							RAZEQ *et al*. 2018)
	ferulic acid esterases	3.1.1.73	Ester bonds in arabinose-ferulic acid linkages	Arabinose, Ferulic acid and p-coumaric acid (phenolic acids**)		--	(SZWAJGiER *et al*. 2010; LOPES *et al*. 2018)
	p-coumaric acid esterases	3.1.1.73	Ester bonds in arabinose-p-coumaric acid linkages	Arabinose and p-coumaric acid (phenolic acid**)		--	(LOPES *et al*. 2018)
Linear mannan	Endomannanases	3.2.178	β-(1→4) bonds at mannan backbone	Non-reducing new parts and mannobiose and mannotriose	Galactose	Glycose and	(VRIES *et al*. 2005; DA CRUZ 2013; LOPES *et al*. 2018)
	β-mannosidases	3.2.1.25	Non-reducing new parts and other mannosaccharides	Mannose		Sucrose	(MCCABE *et al*. 1990; DA CRUZ 2013; LOPES *et al*. 2018)
	α-galactosidases	3.2.1.22	α-(1→6) bonds at galactose-mannose linkages	Galactose and Mannose		*Ag^{2+} and Hg^{+}	(Da CRUZ 2013; SIRISHA *et al*. 2015; LOPES *et al*. 2018)
Galactan	Endogalactanases	3.2.1.89	α-(1→3) bonds at galactanan backbone	Galacto-disaccharide Galacto-trisaccharide and Galcto-tetrasaccharide		--	(GONZÁLEZ-AYÓN *et al*. 2019)
	β-galactosidases	3.2.1.23 / Lactases	β-(1→4) bonds at galactose-xylose linkages	Galactose and Xylose		Galactose	(HUSAIN 2010; KHOSRAVI *et al*. 2015; MALGAS *et al*. 2019)

Source: Authors *Product did not generate in pretreatment and/or hydrolysis of lignocellulosic biomass; ** Cause inhibition in most of hydrolytic enzymes

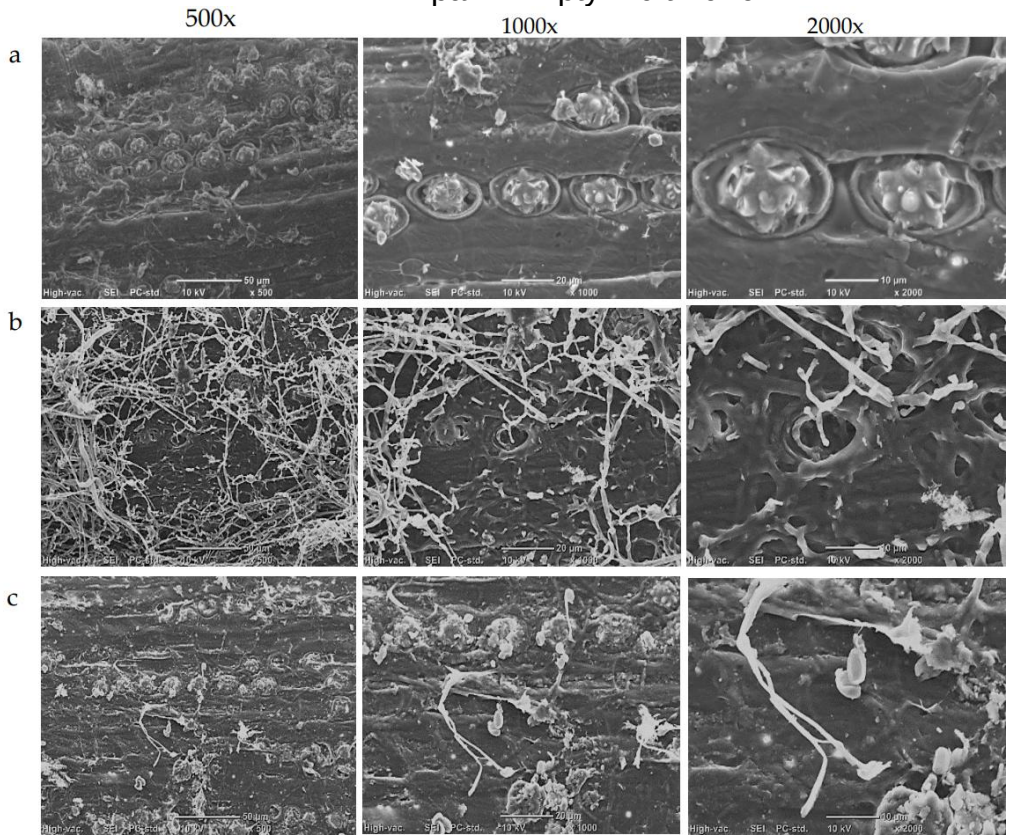
Finally, it should be taken into account that hydrolytic enzymes can suffer deactivation by temperature, pH, reaction time, stirring intensity, enzymatic loads, and mixing modes (BALAN 2014; HU *et al*. 2016; SINGHVI and GOKHALE 2019). Substrate characteristics and modifications over the enzymatic hydrolysis can increase the material recalcitrance (WALLACE *et al*. 2016). Therefore, it is recommended to develop new researches with new conditions that exploit novel tolerance levels for increasing pretreatment and hydrolysis yields.

1.2.5 Mechanisms of Cell Wall Degradation by Microorganisms

During periods of fungal growth, cell wall undergoes structural modifications that allow access to inside components (RILEY *et al*. 2014). Although degrading enzymes are known and studied, degradation can occur in a different manner according to situations: chemical structure and composition of the cell wall are different among woody materials (or non-wood) and enzymatic arsenals of microorganisms are different among them (Fig 4). These factors determine the degradation level of the material and make it difficult to fully understand how biomass is consumed and how the degradation process occurs. Thus enzymes involved in the degradation process must be suitable to each substrate. Furthermore, it is important to evaluate which microorganism and its respective strain are most adequate for each kind of substrate.

Degradation efficiency by microorganisms depends, in many cases, on the chemical structure of molecules and on the presence of efficient enzymes in degrading compounds, which are specific for most substrates (PEREIRA and FREITAS 2012). Biomass chemical structure can influence the metabolism of the microorganisms, especially regarding rates and extent of biodegradation. In the case of catabolic enzymes that have low specificity for its substrate, xenobiotics with a chemical structure similar to natural compounds can be recognized by an active enzyme system and, consequently, used by microorganisms as a source of nutrients and energy (PEREIRA and FREITAS 2012).

Figure 1.4 - Scanning electron microscope images on the surface of the Oil palm Empty Fruit Bunch



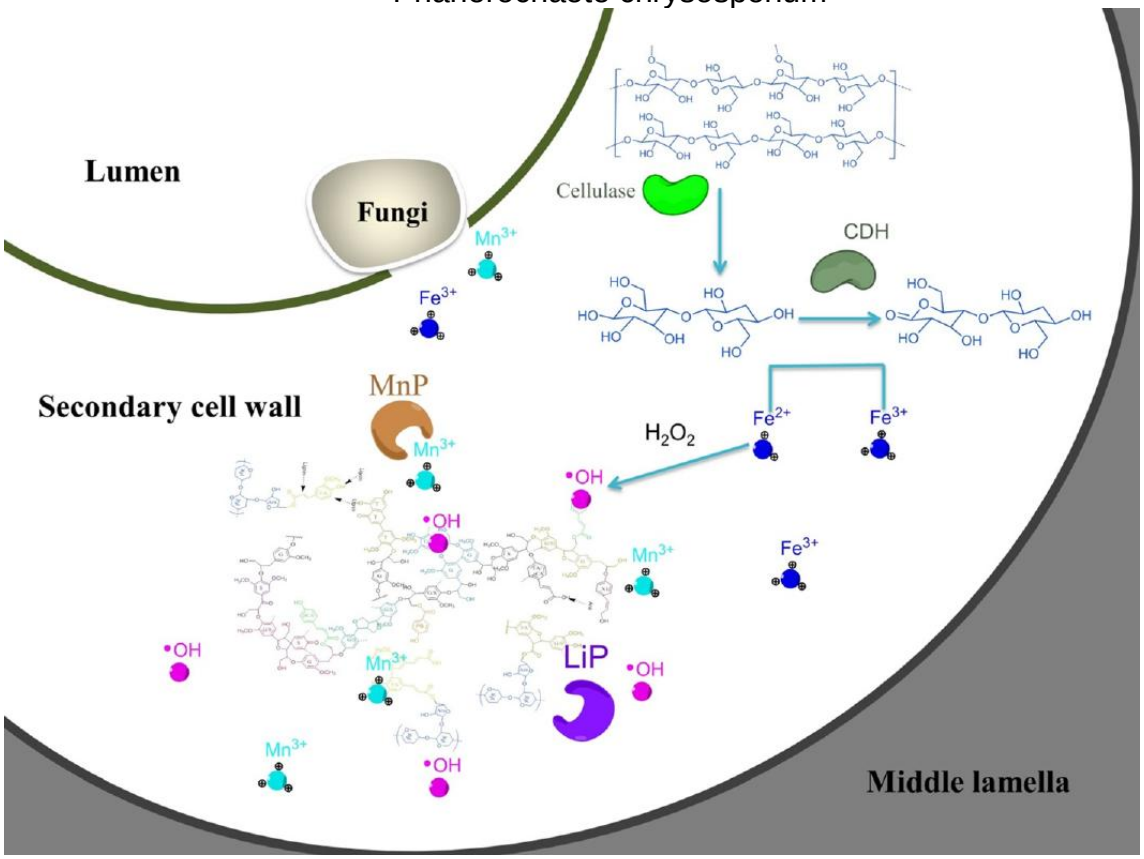
Source: ARBAAIN *et al* 2019. (a) Untreated; (b) biologicaly pretreated using *Schizophyllum commune* (ENN1); (c) biologicaly pretreated using *Phanerochaete chrysosporium*.

Carbon sources can influence fungi growth, which can affect growing patterns (MANNAA and KIM 2017). Hyphae development allows better colonization of lignocellulosic material and also penetrate easily to plant cell walls than bacteria, reaching macromolecules unavailable for those microorganisms (PEREIRA and FREITAS 2012). Enzymes are a crucial tool for the degradation of lignocellulosic biomass. Microorganisms release those enzymes which work in a synergistic and independently action, such as peroxidases, laccases, xylanases, and the other enzymes.

An example of cell wall degradation is proposed in figure 5 (ZENG *et al*. 2014). In this degradation proposal, the plant cell wall is degraded by *Phanerochaete chrysosporium*, which is capable to degrade all components of the lignocellulosic biomass. Fungal hyphae attach inside the cell wall, secreting enzymes. Manganese peroxidases (MnP) oxidize Mn2+ to Mn3+ and break the phenolic and non-phenolic lignin units (DATTA *et al*. 2017; WONG 2009). Lignin peroxidases (LiP) oxidize non-phenolic structures to mineralized lignin, cleaving Cα–Cβ bonds, mediating ring-

opening reactions (WONG 2009; RUIZ-DUEÑAS and MARTÍNES 2009). This process occurs in the secondary cell wall, in which are located structural carbohydrates as well as aromatic backbone. Cellulases hydrolyze β-1,4-glycosidic bonds and act on the microcrystalline region in cellulose chain to break the cellulose into monomers of cellobiose and D-glucose. Cellobiose dehydrogenases co-work with cellulases to break cellulose chains into small saccharides, generating hydroxyl radicals, H_2O_2, and Fe^{3+}.

Figure 1.5 - Proposed process of degradation of the wheat straw cell wall by Phanerochaete chrysosporium



Source: ZENG *et al*. 2014

Although the process of degradation could be different from all microorganisms, the enzymes work similarly but secreted at a different amount, and one characteristic that can be noticed is the variety of the lignocellulosic structure/composition. In wheat lignin degradation using analytical pyrolysis was revealed that Cα–Cβ bonds and free phenolic units are preferred than non-phenolic units by *Pleurotus eryngii* and *Phanerochaete chrysosporium*. This preferential is due to the redox potential, that is lower in comparison with the etherified ones, permitting easier oxidation by ligninolytic

peroxidases and laccases produced by the fungi. *In vitro*, applying enzyme in lignocellulosic biomass, *P. eryngii* is capable to reduce the phenolic content of lignin, evidencing its capacity of modifying a lignocellulosic materials (MARTÍNEZ *et al*. 2001; CAMARERO *et al*. 2001). Another example of lignocellulosic biomass deconstruction is with the brown-rot fungi *Penicillium echinulatum*. In this case, using different carbon sources, was grown wild-type (2HH) and a mutant strain (S1M29). It was realized that the mutant was more capable to produce cellulases and hemicellulases, showing that the variety of microorganisms can differentiate by the quantity of enzymes produced (SCHNEIDER *et al*. 2016).

1.3. ECONOMIC IMPACTS AND CHALLENGES ON INDUSTRIAL SCALE INVOLVING BIOLOGICAL PRETREATMENT

Studies involving biological pretreatments are needed today for several reasons, including environmental friendly process, chemical reduction, and energy savings. There is a growing number of items produced from fossil derivatives such as plastics and tires that are not renewable, in addition to remaining in nature indefinitely. Nevertheless, it is important to mention that a biotechnological route should concern about energy and chemical reagents applied, aiming to be more advantageous than traditional processes.

For biofuels, specifically, greenhouse gases bring concern and it is on the part of governments. Gas derived from fossil is already being replaced by biofuels, which draws attention to new processes of production and ways to reduce costs. The type of biomass, process complexity, and value of by-product influence the choice of pretreatment (BAJPAI 2016). Despite chemical pretreatments holding the main focus on these procedures, biological pretreatments are able to optimize those processes in several levels, for instance: reduce the water, chemicals, and energy spent, generate less inhibitor and toxic compounds, reduce the costs and improve performance and yield.

In the food industry, one of the most worrying problems is waste since all economic classes in society have a certain degree of waste generation (MCCARTHY and LIU 2017). This food that is not used can be turned into energy by the biological or thermochemical process. Biological pretreatment in food waste has advantages in comparison with conventional methods of pretreatment such as low cost and simplicity

(PHAM *et al* 2015). Lignocellulosic biomass products can be a source of material and energy in order to support a more sustainable society. Products of direct consumption or second value-added are already present in human life such as paper, fibers and textiles, nanocellulose, organic acids, furfural, and others (ZAMANI 2015). Food and biofuels are examples where biological pretreatment can be used to improve the productivity and reduce costs. Moreover, the high quantity of products produced from biological pretreatment in lignocellulosic biomass makes them more accessible and, consequently, cheapest.

Biological pretreatment can be economical, however, it is hard to speculate how much can impact and change the human lifestyle. The extensive number of products that can be produced with lignocellulosic biomass after a biological pretreatment makes harder this count, considering the production cost and sell value of each one. Products individually can be speculated is considering the cost. An example, the xylan extraction using biological pretreatment before chemical (H_2O_2) pretreatment reduced the need for the chemical reagent to reach the same results, which means less cost in the process (FELIPUCI 2020). On the other hand, the production of fermentable sugar by biological pretreatment of corn stover using posterior enzymatic hydrolysis showed to be more expensive (1.41 $/kg) than steam explosion (0.43 $/kg), dilute sulfuric acid (0.42 $/kg) and ammonia fiber explosion (0.65 $/kg) methods, anyway, the prices of all pretreatments depends from several factors (BARAI and SHAH 2017). In this case, there was no need of detoxification using biological pretreatment. However, this method investigated required reactors, mainly due to long pretreatment time. Biological pretreatment could considerer an option of process outside not using any reactor, but face other problems such as contamination.

Although the advantages of an experimental scale, the use of biological pretreatment in the industry is still a challenge. Recent studies showed the potential of microorganisms in biofuels productions using biological pretreatment (YAHMED *et al* 2017; ZABED *et al* 2019). However, it is a common view of all the difficulties involved in biological pretreatment on a large scale. Microorganism utilization in biotechnological processes requires certain precautions, which needs to add one or more steps in the process: contamination and sterilization of growth site are some examples. Furthermore, microorganism growth is slow, while sugars are fundamental as an energy source (VASCO-CORREA *et al*. 2016; UMMALYMA 2019). An option to

improve the process and pass through those problems is the genetic engineering as well as co-culture of suitable microbial consortium (SHARMA *et al*, 2019).

1.4. CONCLUDING REMARKS

Biological pretreatment has several advantages over traditional biomass separation methods. Application of microorganisms and their enzymes, in addition to enhancing the breakdown of lignocellulosic structure, makes the process cheaper and less aggressive to nature. An important advantage is no by-products generation, improving the fermentable sugars production by enzymatic hydrolysis of cellulose, with appreciable cost-benefit, among other benefits.

Microorganisms present great potential for industrial use. Employment of microorganisms in pretreatments, or just their enzymes, can provide a reduction of energy and chemical reagents consumption in the separation process of lignocellulosic biomass macromolecules. Microorganism co-cultivation is a valid technique option with biotechnological potential, once the enzymes produced by the microorganisms can complement each other, achieving a greater degree of degradation. Mechanism degradation of plant cell wall depends on the microorganism in question and, mainly, on its enzyme production and action on lignocellulosic biomass. Even though the use of the micro in the industrial-scale requires greater cultivation assistance, it still offers important advantages: there are cost reduction and yield improvement for the biorefinery area and also less chemical residues in the environment.

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CHAPTER II: EFFICIENT PRODUCTION OF CELLOOLIGOSACCHARIDES AND XYLOOLIGOSACCHARIDES BY COMBINED BIOLOGICAL PRETREATMENT AND ENZYMATIC HYDROLYSIS PROCESS

ABSTRACT

Xylooligosaccharide and cellooligosaccharide production has been improved over the years using different kinds of methods. Chemical and physical methods are the most used due to their facility in reducing the biomass recalcitrance; however, such methods require a great deal of energy and generate undesirable compounds. On the other hand, biological pretreatment is an option to overcome the high energy demand and pollution issues and optimize production. In this work, sugarcane bagasse was biologically pretreated with *Coniophora puteana* (CBMAI 0870), *Gloeophyllum trabeum* (CBMAI 0872), and *Pleurotus ostreatus* (CCIBt 2338) for 5 months. After the biological pretreatment, part of the material was milled in a knife mill (20-mesh) and another part was milled in a ball mill (powder aspect). The material was enzymatic hydrolyzed with cellulase (20, 50, and 100 IU/g) and xylanase (50 IU/g) combined with the milling techniques to produce cellooligosaccharides and xylooligosaccharides. Xylooligosaccharides and cellooligosaccharides were characterized by ATR-FTIR analysis, scanning electron microscopy images and X-ray to evaluate the modifications in the lignocellulosic structure. The enzymatic hydrolysis using cellulase (50 IU/g) combined with knife milling resulted in 26.23% of cellooligosaccharide conversion after 5 months of cultivation with *C. puteana*, while cellulase with the same enzymatic charge combined with ball milling resulted in 36.65% of cellooligosaccharide conversion using the same fungus and the same time of cultivation. Xylooligosaccharide conversion also reached better results when compared to untreated material: the best result was 78.12% of xylooligosaccharide conversion after the biological pretreatment with *G. trabeum*. Finally, scanning electron microscope images made it possible to observe gaps formed by the fungi growth in the biomass in comparison to untreated material; and x-ray analysis showed evidence of the effect of ball milling pretreatment in the biomass, reducing its crystallinity.

Keywords: Ball mill · Biomass recalcitrance · Non-digestible oligomers · Prebiotic compounds · Soluble fiber · White-rot fungi

2.1 INTRODUCTION

In the literature, the process of converting lignocellulosic biomass into value-added products is commonly referred to as lignocellulosic biorefinery. This approach expands potential applications of lignocellulosic materials beyond generating heat energy and ethanol to include the production of by-products and value-added molecules (SRIARIYANUN and DHARMALINGAM 2023; RUENSODSAI and SRIARIYANUN 2022; FORSAN *et al* 2021; SCAPINI *et al* 2021; QUASEEM, SHAHEEN and WU 2021)

Two value-added molecules are xylooligosaccharides (XOS) and cellooligosaccharides (COS). These molecules comprise xylose and glucose, respectively, and are known to be interesting to human health. XOS is considered healthy due to its capacity to prevent disorders in human nutrition, such as gastrointestinal disorders and infection, obesity control, and better nutrient absorption (ADEBOLA, CORCORAN AND MORGAN 2014; VÁZQUEZ *et al* 2000; CAO *et al* 2020). XOS also presents various degrees of polymerization, from 2 to 10 xylose units, and the wide range of pH and thermostability makes this sugar a good prebiotic molecule (KUMAR *et al* 2021; MANO *et al* 2018). On the other hand, COS are oligomers formed by β-1,4 linked D-glucose units, with few numbers of glucose units, starting with 2 units, called cellobiose. A characteristic of COS is that the shorter COS (from 3 to 6 glucose units) are water soluble, while longer COS tend to be insoluble. The soluble COS is not digestible by humans and can be prebiotic. It stimulates the growth of healthy bacteria in vitro, such as *Lactobacillus* and *Bifidobacterium* (ÁVILA *et al* 2021; FUJII *et al* 2016; KARNAOURI *et al* 2019; SANZ, GIBSON and RASTALL 2005). To reach the desirable material, such as COS and XOS, lignocellulosic biomass is submitted to a pretreatment and enzymatic hydrolysis. The pretreatment is important to reduce the recalcitrance of the lignocellulosic material, while the enzymatic hydrolysis will break down the sugars into small units, forming COS and XOS.

Choosing the correct pretreatment is essential to modify the lignocellulosic material structure, as well as the correct enzyme for the enzymatic hydrolysis step. The main pretreatments to overcome the material recalcitrance are chemical, physical, and biological, or a combination of them (FELIPUCI *et al* 2020), and each one has its limits and advantages. Physical and chemical pretreatments are faster; however, they demand more energy and generate undesirable compounds, respectively. An example of chemical pretreatment is the acid and alkali pretreatment. A characteristic of an acid

pretreatment is to solubilize hemicellulose and lignin, while the alkali pretreatment changes the lignin structure, reducing cellulose crystallinity and partially solubilizing the hemicellulose. An example of physical pretreatment can be steam explosions, which are used to auto-hydrolyze hemicellulose, or ball milling, reducing the size of the lignocellulosic material particles. On the other hand, biological pretreatment is cheaper and does not generate undesirable compounds. However, it requires time and proper isolation without contaminants to the process and depends on the microorganism involved in the process. White rot fungi, for example, are known to degrade lignin, while brown rot fungi degrade cellulose and hemicellulose (FELIPUCI *et al* 2020 and 2021; RASTOGI and SHRIVASTAVA 2017).

The tendency to overcome the limitations of the pretreatments is to use a combination of different pretreatments (FELIPUCI *et al* 2020 and 2021). Various methods could be used to achieve a good COS and XOS yield (KUMAR *et al* 2021, SANTANA *et al* 2023; FANG *et al* 2022; MENSAH *et al* 2024), according to each pretreatment characteristic. The inspiration to use a biological pretreatment before enzymatic hydrolysis came from the fungi's capacity of changing the lignocellulosic structure, decreasing the biomass recalcitrance and facilitating the enzymatic hydrolysis (FELIPUCI *et al* 2021). Moreover, using different milling methods is a good alternative to decrease biomass recalcitrance even more (GOMIDE *et al* 2019).

Production of value-added products has been improved over the last decade and different technologies are important to find sustainable ways to produce more with less environmental impact. Experiments using biologically pretreated material followed by enzymatic hydrolysis have a huge potential for eco-friendly production. Regarding this, the objective of this study is to optimize the COS and XOS production using a combination of biological pretreatment combined with physical pretreatments before the enzymatic hydrolysis step, based on the hypothesis that both pretreatments can decrease the sugarcane bagasse recalcitrance. Moreover, an analysis with ATR-FTIR spectra, scanning electron microscope, and an X-ray technique were applied to verify the structural changes in the biomass (FELIPUCI *et al* 2021).

2.2 MATERIALS AND METHODS

2.2.1 Material and bagasse preparation

The sugarcane bagasse was supplied by a local factory (Usina Sao Joao, Araras, SP, Brazil). The industrial bagasse was humidified to 75% and transferred to polypropylene bags. A falcon tube was placed into the central part of each bag as an opening for introducing the fungal inoculum after the set was twice autoclaved at 121 °C for 1 h. The control sample was prepared the same way, however no fungi was inoculated. A total of 15 bags were prepared/fungal strain.

2.2.2 Biological pretreatment

The microorganisms used in this study were *Coniophora puteana* (CBMAI 0870) and *Gloeophyllum trabeum* (CBMAI 0872) (Brazilian Collection of Environment and Industry Microorganisms, Campinas State University-Unicamp, Campinas, Sao Paulo, Brazil), and *Pleurotus ostreatus* (CCIBt 2338) (from the Sao Paulo Botanic Institute).

The microorganisms were cultivated for 7 to 15 days at 25 °C in Potato Dextrose Agar (PDA) (KASVI Ltda). To prepare the PDA medium, 39 g of PDA was added to 1 L of distilled water; then, it was autoclaved in 200-mL portions. The liquid was poured into Petri dishes (20 mL each); then, it was exposed to UV light. After completing the PDA preparation, the microorganisms were reactivated and put in the Petri dishes (CASTELLANI 1963). One-fifth of the PDA from Petri dishes was inoculated in the polypropylene bag. Inside the polypropylene bag, each culture was left to grow for 1 to 5 months at 25 °C. Every month, one bag was taken as a sample and the biologically pretreated bagasse was dried at room temperature, milled at 20-mesh, and oven-dried at 60 °C for 24 h. A fraction of the biological pretreated sugarcane bagasse was milled in a ball milling for 1 h for later experiments. The samples were separated according to the fungus applied in the pretreatment.

2.2.3 Chemical characterization

After the biological pretreatment, and also for the control sample, the sugarcane bagasse chemical characterization (CC) was carried out as follows: 0.3 g of bagasse (dry basis) was added with 3 mL of 72% sulfuric acid (Sigma-Aldrich 320,501-1L) to a 100 mL Schott bottle and allowed to react in a water bath at 45 °C for 7 min. After the reaction time, 84 mL of distilled water was added and then autoclaved for 30 min at 121 °C. Then, a filtration step, using a crucible (16 μm), separated the liquid and the

solid fractions. The liquid fraction was used to determine the soluble lignin and the sugars and acetic acid in HPLC, using a Bio-Rad Aminex HPX-87H (300 . 7.8 mm) column in 45 °C, a refraction index detector Waters 2414, H2SO4 50 mM of mobile phase, a flow of 0.6 mL/min, and a sample volume of 20 µL. The solid fraction was dried for 24 h in an oven at 105 °C to determine the insoluble lignin. The soluble lignin was measured using a spectrophotometer at 215 and 280 nm adopting a standard method (GOUVEIA 2009; NREL protocol). The total lignin was reported, meaning the sum of soluble and insoluble lignin.

2.2.4 Enzymatic hydrolysis

In the present study, two enzymes were used: cellulase cocktail (Cellic Ctec2–Novozymes, with 1455 IU/mL of endoglucanase) and xylanase. The xylanase was produced from *Aspergillus versicolor* (isolated from Brazilian soil) at our lab (DE FREITAS *et al* 2021). *A. versicolor* was grown on oat wheat and the xylanase was purified using chromatography on DEAESephadex A-50 column (20 . 1.8 cm) equilibrated with the
dialysis buffer and Sephadex G-75 column (70 . 1.8 cm) equilibrated with the same buffer (CARMONA *et al* 1998). The purified endoxylanase showed activity of 414.4 IU/mL.

For all experiments, the enzymatic hydrolysis was performed with 1 g of material, in 5 mL of sodium acetate buffer solution in pH 5.2. The buffer solution was prepared with sodium acetate (Dinamica–Quimica Contemporanea Ltda, COD: 1015) and acetic acid (Dinamica–Quimica Contemporanea Ltda, COD: P.10.0021.000.00.81). It was performed for 24 h at 50 °C (for cellulase) or 55 °C (for xylanase) in a shaker at 150 rpm. After hydrolysis, the samples were boiled for 7 min, filtered in 0.44 µm and 0.22 µm filters, and then went through HPLC to determine the sugars. The enzymatic hydrolysis was made in three different steps, differing in the milling method, enzyme, and enzymatic charge. The first experiment was enzymatic hydrolysis with cellulase using material milled in a knife mill (20-mesh) and three different charges: 20 IU/g, 50 IU/g, and 100 IU/g. The second one used cellulase cocktail after milling the material in a ball mill, using an intermediary enzyme charge (50 IU/g). In this experiment, the samples cultivated for 1 and 5 months were

hydrolyzed. The ball milling was performed in a 150 mL reactor using a 110 g metallic ball with 617 blows per minute, in a ball mill model MA 350 (MARCONI Equipamentos para Laboratorios Ltda). In the third experiment, the samples were milled using a knife mill, and the hydrolysis was performed using xylanase with an intermediary enzyme charge (50 IU/g).

2.2.5 Analysis of ATR-FTIR

Knife-milled untreated and biologically pretreated bagasse before and after enzymatic hydrolysis with cellulase with 50 IU/g were analyzed by infrared (FTIR) attenuated total reflectance (ATR), using an FTIR-IRAffinity-1S Shimadzu spectrophotometer. The samples analyzed by FTIR-ATR were only from the first and last months of cultivation as the objective of this experiment was to study the difference caused by the biological pretreatment and enzymatic hydrolysis in the lignocellulosic structure.

2.2.6 Biomass images from electron microscope

The 20-mesh milled untreated and biologically pretreated samples of three consecutive months were measured using scanning electron microscopy (SEM, EVO-LS-15, Carl Zeiss), operated at 15 kV with a spot size of 3.0–4.0 mm and a working distance (WD) of 9.5 mm.

2.2.7 X-ray analysis

The analyzed samples were from the first and last months of cultivation for each microorganism and also with untreated material. All samples were compared after grinding in the ball mill and knife mill with a particle size of 20 mesh. A Shimadzu XRD-6000 model diffractometer equipped with a Cu anode (λ = 1.54059 A) was used. Measurements were taken at 40 kV and 30 mA voltages in θ–2θ configuration from 2θ = 13 to 25°, with a step size of 0.02° and a scan speed of 2°/min. The proportion of crystalline regions in bioplastics was determined from the crystallinity index (CrI), from Eq. 1:

$$CrI = \left[\frac{I20 - Iam}{I20}\right] * 100$$

Equation 1: Crystallinity index formula where I20 is the intensity from plane diffraction at 2θ = 20°, and Iam represents the background scattering intensity (MACEDO *et al* 2023).

2.2.8 Statistical analysis

The statistical analysis was made in DX3Trial-expert software (DesignExpert, Stat-Ease company, version DX 6.0.6). ANOVA was used to compare statistical differences between untreated material with the biologically pretreated material. Different letters mean statistical differences between the samples.

2.3 RESULTS AND DISCUSSION

2.3.1 Chemical characterization

The sugarcane bagasse was submitted to a chemical characterization to determine the percentage of cellulose, hemicellulose, and lignin (Table 2.1). Corroborating previous literature (FELIPUCI *et al* 2021), the changes in the lignocellulosic components can be observed. It is important to note that, in the chemical composition table, the ANOVA test was made comparing only the untreated samples to the samples corresponding to each microorganism, not comparing the microorganisms themselves (Supplementary Table 1).

A consequence of biological pretreatment is the consumption of cell wall components. The implication of consuming the cellulose and hemicellulose was the lignin content increase after the biological pretreatment. In this case, the lignin content of the samples pretreated with *G. trabeum* and *C. puteana* reached 60.49% after 1 month of cultivation with *G. trabeum*, while the untreated samples reached 21.55% of lignin content. In this case, both fungi statistically changed the lignin content of the biomass compared to untreated samples. However, the lignin content of untreated samples was statistically similar for *P. ostreatus*, except for the fourth month, reaching 34.60% of lignin content. The increase in lignin is related to the lignocellulosic structure

modifications as fungi tend to first consume more readily available sugars instead of lignin (KEREM, JENSEN and HAMMEL 1999).

For cellulose content, almost all samples were statistically similar to the untreated sample, except for the one that was biologically pretreated with *C. puteana* after 5 months. The higher cellulose content was observed after 2 months of biological pretreatment with *G. trabeum*, reaching 45.40%, while the lower was in the last month of cultivation with *C. puteana* 30.69%. For total hemicelluloses, a decrease for *G. trabeum* and *C. puteana* was observed, from 27.66% for untreated samples to 16.7% after 1 month of cultivation with *G. trabeum*, and 15.17% for *C. puteana* after 4 months of biological pretreatment. However, the hemicelluloses percentage increased after 5 months of cultivation with *P. ostreatus*, reaching 38.86%. Generally, the hemicellulose content changed statistically in almost all samples, except those from 1-month culture with *C. puteana* and *P. ostreatus*. The same pattern for cellulose and hemicellulose content after biological pretreatment with these fungi was reported in the literature (FELIPUCI *et al* 2021).

The preference for sugars or lignin is related to the microorganism enzymatic arsenal. White-rot fungi prefer to degrade lignin, while brown-rot fungi degrade other polysaccharides. However, regardless of which macromolecule was consumed, a consequence of degradation is the change in the lignocellulosic structure and its recalcitrance (ZENG *et al* 2014). Once the recalcitrance decreases, the enzymatic hydrolysis may be more efficient in COS and XOS production, which is the main objective of this work, leading this characteristic to have a major impact on the final results.

Table 2.1 - Chemical composition of untreated and biologically pretreated sugarcane bagasse material.

Microorganism	Growth Time (months)	Cellulose (%)	Hemicellulose (%)	Total lignin (%)
Untreated	**0**	40.53 ± 5.41^{a,b}	27.66 ± 3.15 ^{a}	21.55 ± 0.20^{a}
***Gloeophyllum trabeum* (CBMAI 0872)**	**1**	37.60 ± 1.63^{a}	16.7 ± 1.74^{b}	60.49 ± 1.70^{b}
	2	45.40 ± 0.96^{b}	20.08 ± 2.09^{b}	53.06 ± 4.62^{b}
	3	37.14 ± 1.07^{a}	17.57 ± 4.72^{b}	33.57 ± 1.65^{a,d}
	4	42.42 ± 2.94^{a,b}	16.06 ± 1.75^{b}	57.70 ± 1.41^{b}
	5	43.30 ± 1.16^{a,b}	15.66 ± 0.25^{b}	34.95 ± 3.91^{d}
***Coniophora puteana* (CBMAI 0870)**	**1**	41.55 ± 0^{a}	22.12 ± 5.44^{a,c}	25.24 ± 0.38^{a,c}
	2	43.27 ± 0.06^{a}	16.22 ± 0.08^{b,c}	41.58 ± 0.77^{b}
	3	41.21 ± 1.53^{a}	17.98 ± 0.46^{b,c}	38.09 ± 7.91^{b,c}
	4	32.81 ± 1.72^{c}	15.17 ± 0.11^{b}	42.75 ± 2.74^{b}
	5	30.69 ± 0.00^{c}	15.24 ± 0.19^{b}	36.63 ± 1.86^{b,c}
***Pleurotus ostreatus* (CCIBt 2338)**	**1**	35.84 ± 0.54^{a}	32.33 ± 3.58^{a,c}	26,43 ± 0^{a,b}
	2	40.12 ± 0.06^{a}	35.79 ± 3.85^{b,c}	29.72 ± 4.86^{a,b}
	3	39.51 ± 0.36^{a}	38.35 ± 1.74^{b,c}	26.07 ± 2.19^{a,b}
	4	41.29 ± 0.92^{a}	38.34 ± 0.63^{b,c}	34.60 ± 3.17^{b}
	5	40.28 ± 1.00^{a}	38.86 ± 0.80^{b}	26.47 ± 0.52^{a,b}

Source: Author. Significant statistical differences (p-value less than 0.05) were symbolized by different letters in the same column for each microorganism.

2.3.2 Enzymatic hydrolysis

The enzymatic hydrolysis occurred both in untreated and biologically pretreated sugarcane bagasse samples using three different enzymatic charges, including ball and knife mills. The purpose of differing the enzymatic charges was to compare the effectiveness of enzyme charge over the 20-mesh milled bagasse, after the biological pretreatment. Besides that, a comparison using the material after ball milling was made. The statistical analysis compared the untreated samples with each microorganism, and then, the enzymatic charges for each line of the table.

Table 2.2 presents the bagasse milled at 20-mesh, hydrolyzed by cellulase at 20, 50, and 100 IU/g. The table that represents the quantity of glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose, and larger than cellohexaose, individually, can be found in Supplementary Table 2.2, in the Supplementary material. For most samples, the COS yield was better using 100 IU/g of cellulase. However, the

most evident value was 26.39% of COS yield in the fifth month of biological pretreatment with *Coniophora puteana* using 50 IU/g of enzyme charge. Comparing the data from the COS yield, it was observed by using the ANOVA test that most of the results between 50 and 100 IU/g were not significantly different.

The material treated with *G. trabeum* and enzymatic hydrolysis produced more COS than the untreated one; however, most samples were statistically similar, except those cultivated for 5 months (20 IU/g), reaching 14.47% of COS; 1, 3, and 5 months (50 IU/g), reaching 12.37%, 12.76%, and 11.38% of COS, respectively; and 1 month (100 IU/g), reaching 16.91% of COS. *C. puteana* followed the same pattern as *G. trabeum*. In this case, the samples that are different were cultivated for 5 months (20 IU/g and 100 IU/g) and 4 and 5 months (50 IU/g). A different pattern was shown by *P. ostreatus* when enzymatic hydrolyzed with cellulose 50 and 100 IU/g were used: most of the results presented less COS yield compared to the untreated biomass, reaching only 3.52% of COS yield using 50 IU/g of cellulase in the material cultivated for two months.

Considering the comparison of COS yield among the fungi, it is important to highlight that some samples showed significant differences throughout all the months, such as the fifth month for *G. trabeum* and the third month for *P. ostreatus* when using 20 IU/g; and the fifth month for *C. puteana* using 50 and 100 IU/g of cellulase. All ANOVA tests for enzymatic hydrolysis are shown in the Supplementary material (Tables 2.3, 2.4 and 2.5).

Table 2.2 - COS conversion (%) after enzymatic hydrolysis of biologically pretreated sugarcane bagasse using three different enzymatic charges of cellulase.

Samples (20-mesh milled)	Cultivation time (months)	Enzymatic Charge		
		20 IU/g	50 IU/g	100 IU/g
Untreated	-	2.42 ± 2.57^{aA}	5.65 ± 1.56^{aA}	9.39 ± 4.15^{aA}
Gloeophyllum trabeum (CBMAI 0872)	1	4.61 ± 0.64^{aA}	$12.37 \pm 0.04^{bA,B}$	16.91 ± 5.33^{bB}
	2	4.10 ± 2.51^{aA}	7.04 ± 2.12^{aA}	$10.11 \pm 0.83^{a,bA}$
	3	6.52 ± 2.50^{aA}	12.76 ± 2.10^{bB}	$14.97 \pm 0.08^{a,bB}$
	4	5.96 ± 1.55^{aA}	$8.08 \pm 2.11^{a,cA}$	9.17 ± 0.66^{aA}
	5	14.47 ± 1.63^{bA}	$11.38 \pm 0.62^{b,cA}$	$11.51 \pm 3.98^{a,bA}$
Coniophora puteana (CBMAI 0870)	1	2.30 ± 0^{aA}	4.87 ± 0.96^{aA}	6.58 ± 2.69^{aA}
	2	4.70 ± 0^{aA}	$9.77 \pm 0.88^{a,cA}$	10.86 ± 3.58^{aA}
	3	5.79 ± 1.58^{aA}	$9.01 \pm 2.98^{a,cA,B}$	13.22 ± 0^{aB}
	4	10.07 ± 2.08^{aA}	$12.46 \pm 0.81^{b,cA}$	11.78 ± 1.96^{aA}
	5	21.79 ± 7.09^{bA}	26.39 ± 3.29^{dA}	25.22 ± 7.29^{bA}
Pleurotus ostreatus (CCIBt 2338)	1	$5.24 \pm 0^{a,bA}$	5.93 ± 0.13^{aA}	$6.21 \pm 1.30^{a,bA}$
	2	$5.61 \pm 0^{a,bA}$	3.52 ± 1.39^{aA}	$5.60 \pm 0.75^{a,bA}$
	3	7.80 ± 1.14^{bA}	4.69 ± 1.14^{aB}	$4.77 \pm 0^{bA,B}$
	4	4.09 ± 1.29^{aA}	6.40 ± 1.72^{aA}	$6.32 \pm 0.62^{a,bA}$
	5	$5.24 \pm 0.71^{a,bA}$	4.68 ± 1.03^{aA}	4.53 ± 1.10^{bA}

Source: Author. Significant statistical differences (p-value lesser than 0.05) were symbolized by different letters in the same column and line for each microorganism.

Table 2.3 shows the COS yield (%) of cellulase applied to biologically pretreated sugarcane bagasse after ball milling, including the quantities of glucose, celobiose, cellotriose, celotetraose, cellopentaose, cellohexaose, and sugars larger than cellohexaose, individually, in the same conditions. In this experiment, only 2 months of cultivation were selected.

The untreated sample showed the lowest COS yield (8.87%). For the biologically pretreated material with *G. trabeum,* the best result was 22.58%, cultivated for 1 month. For *Coniophora puteana* and *Pleurotus ostreatus*, the best result was in the fifth (36.65%) and fourth month (16.11%) of cultivation, respectively. The value of 36.65% of COS yield for *C. puteana* was the best result for this experiment: more than twice the COS conversion compared to the sample corresponding to the first month. The ANOVA analysis showed the statistical comparison between the untreated sample and each microorganism. The statistical similarity appeared only for the samples

represented by month 5 for *G. trabeum* and month 1 for *P. ostreatus*. All samples compared to each other showed significant differences (Supplementary material).

The result of COS yield (Table 2.3), where all samples were milled with ball milling, was greater than any other sample milled to the 20-mesh, including those that used 100 IU/g of cellulase in the enzymatic hydrolysis. Ball milling was already used as an enhancer for enzymatic hydrolysis of cellulosic materials. Mais *et al*. (2002) (MAIS *et al* 2002) used ball milling to increase the cellulose hydrolysis in *Pseudotsuga menziesii* (Pinophyta, Plantae) both at room temperature (25 °C) and at 45 °C. In this case, pre- and post-treatments in the lignin removal were reported. Wu *et al*. (2021) used ball milling to enhance enzymatic hydrolysis in *Populus tremuloides* (Magnoliophyta, Plantae) and reported 66.5% of glucose at a low enzyme dose (10 FPU/g). The difference in this work came from the milling step: the samples were intermittently milled to optimize the enzymatic hydrolysis, while in the present work, the milling and hydrolyzation were carried out in different steps. In addition, the ball milling pretreatment modified the microcrystalline cellulose properties, managing to increase the capacity enzymatic hydrolysis (GOMIDE *et al* 2019). Although those experiments did not aim to produce COS, the effect of ball milling used together with enzymatic hydrolysis was clear: the ball milling method enhanced the capacity of enzymatic hydrolysis. In fact, the combination of ball milling with other methods to enhance enzymatic processes or production of some biomass-based products are common in the literature (MA *et al* 2022; KIM *et al* 2018; ZHANG *et al* 2024; ZAKARIA *et al* 2014; LIN *et al* 2010). However, there is a lack of literature on experiments using ball milling and biological pretreatment to enhance enzymatic hydrolysis for COS production. The results of the present study show the great potential of this technique, and ball milling after biological pretreatment can be used together for other purposes.

Table 2.3 - COS conversion (%) after enzymatic hydrolysis of ball-milled biologically pretreated sugarcane bagasse using cellulase.

Samples (milled with ball-mill)	Cultivation time	Enzymatic charge						
		50 IU/g						
Fungi	Month	Glucose	C2	C3	C4	C5	C6	COS
Untreated		33.24	7.21	0	0.1	0	1.58	8.87± 0.28[a]
G. trabeum	1	46.26	15.28	1.29	0.1	1.48	4.44	22.58 ± 1.64[b]
	5	30.28	5.24	0	0.44	0.39	3.50	9.56 ± 0.36[a]
C. puteana	2	32.55	10.12	0.98	0.09	1.11	3.35	15.64 ± 1.34[b]
	5	64.13	22.83	1.97	0.13	1.6	10.12	36.65 ± 2.52[c]
P. ostreatus	1	38.83	6.66	0	0.11	0	2.10	8.86 ± 0.87[a]
	4	48.58	11.39	0.69	0.25	0.72	3.08	16.11 ± 1.11[b]

Source: Author. Significant statistical differences (p-value lesser than 0.05) were symbolized by different letters in the same column for each microorganism. *C1* glucose; *C2* celobiose; *C3* celotriose; *C4* celotetraose; *C5* celopentaose; *C6* celohexaose; > C6 chains larger than celohexaose. *COS* gluco-oligosaccharides = C2 + C3 + C4 + C5 + C6 + > C6.

XOS production was evaluated using xylanase (50 IU/g) applied to 20-mesh untreated and biologically pretreated material (Table 2.4). The untreated sample reached 2.95% of XOS yield. For *G. trabeum* and *C. puteana*, almost all samples converted more than 40% of XOS, reaching 78.56% in the sample pretreated with *G. trabeum* after one month of cultivation. For the samples pretreated with *C. puteana*, the best result was achieved after 5 months of cultivation, reaching 66.26% of XOS yield. The samples that were biologically pretreated with *P. ostreatus* had the lowest XOS conversion of XOS in the first month of cultivation, 9.17%. For xylanase production, the statistical comparison revealed significant differences between all samples compared to the untreated one.

The XOS production occurred through the breakdown of the glycosidic linkage in the xylan chain. For XOS production, there are a few studies in the literature using a milling technique and a biological pretreatment together (ZHANG *et al* 2021). These are usually performed using chemical, physical–chemical pretreatments, or enzymatic hydrolysis, aiming at the food, medical and pharmaceutical industries (AMORIM *et al* 2019). Experiments producing XOS reached 67.43% of XOS conversion using *Aspergillus versicolor* endoxylanase with a 65 IU/g of enzymatic load for 24 h at 55 °C in sugarcane bagasse and leaves (FORSAN *et al* 2023), while this experiment reached

improved results (78.12% after 1 month of cultivation with *Gloeophyllum trabeum*) using less enzyme. However, in this case, the enzyme was used directly in the xylan, which can optimize the results. It was observed that the quantity of xylose varied according to the microorganism and time of cultivation. It is known that the reaction time, temperature, quantity and type of enzyme and even the rotation/shaking (rpm) during enzymatic hydrolysis can change the xylose and XOS yield (GAUTÉRIO *et al* 2022).

Table 2.4 - XOS production after enzymatic hydrolysis of untreated and biologically pretreated sugarcane bagasse.

Samples (20-mesh)	Cultivation time	Enzymatic charge 50 IU/g						
		XOS (%)						
Fungi	Month	X2	X3	X4	X5	X6	>X6	XOS
Untreated		0	0	0.06	0	0	2.90	2.95 ± 0.10[a]
G. trabeum	1	21.41	9.68	0.12	0	0	47.35	78.56 ± 0.03[b]
	2	16.39	6.55	0.08	0	0	31.74	54.75 ± 0.43[c]
	3	0	3.63	0.08	0.07	0	38.22	41.99 ± 5.92[d]
	4	0	3.56	0.11	0	0	3845	42.12 ± 6.18[d]
	5	0	10.96	0.11	0	0	39.02	50.08 ± 5.61[c,d]
C. puteana	1	0	0	0.08	0	4.86	10.37	15.31 ± 0.01[b]
	2	0	9.39	1.19	1.34	0	39.13	51.05 ± 1.02[c]
	3	0	6.80	1.72	0	0	36.63	45.15 ± 1.20[d]
	4	0	6.71	0.50	0	0	41.58	48.78 ± 1.82[d]
	5	0	4.43	0.11	0	0	61.73	66.26 ± 10.41[c]
P. ostreatus	1	0	0	0.05	0	0	9.13	9.17 ± 0.001[b]
	2	0	0	0.04	0	0	7.40	7.44 ± 0.03[c]
	3	0	0	0.04	0	0.92	7.77	8.72 ± 0.44[b]
	4	0	0	0.04	0	0	9.05	9.09 ± 0.46[b]
	5	0	0	0.04	0	0.76	6.94	7.73 ± 0.44[c]

Source: Author. Significant statistical differences (p-value lesser than 0.05) were symbolized by different letters in the same column for each microorganism. *X1 xyl*ose; *x2* xylobiose; *X3* xylotriose; *X4* xylotetraose; *X5* xylopentaose; *X6* xylohexaose; > X6 chains larger than xylohexaose. *XOS* Xylo-oligosaccharides = X2 + X3 + X4 + X5 + X6 + > X6

2.3.3 FTIR-ATR analysis

FTIR-ATR was applied to untreated and biologically pretreated material in different cultivation times and before and after enzymatic hydrolysis using cellulase with 50 IU/g of enzymatic charge (Fig. 2.1). All samples were milled in a 20-mesh.

Table 2.5 presents wavebands corresponding to the functional groups in sugarcane bagasse.

Table 2.5 - ATR absorbance wavebands in lignocellulosic biomass.

Wavenumber	Functional group	Polymer	Reference
1735	C = O stretching	Hemicellulose	CHEN *et al* 2016
	Carbonyl-ester bond stretching of the p-coumaric acid unit	Lignin	NAZIR *et al* 2023
1600	Aromatic ring vibration	Lignin	GARCÍA *et al* 2012
1510	C=C in aromatic ring	Lignin	GARCÍA *et al* 2012
1245	C–O of the acetyl group	Hemicellulose	LI *et al* 2012; VART *et al* 2009
1161	C–O–C vibration pyranose ring	Cellulose, hemicellulose	OUYANG *et al* 2015; NAKASONE *et al* 2016
1050	C–O–C vibrations in the anomeric region of the xylans	Hemicellulose	JU *et al* 2011; HARIPRIYA *et al* 2014
897	C–O–C stretch of β-glycosidic bonds	Cellulose, hemicellulose	XIAO *et al* 2014; ZHANG *et al* 2010; LE TROEDEC *et al* 2008
833	C–H out-of-plane in p-hydroxyphenyl units	Lignin	HOAREAU *et al* 2004

Source: Author

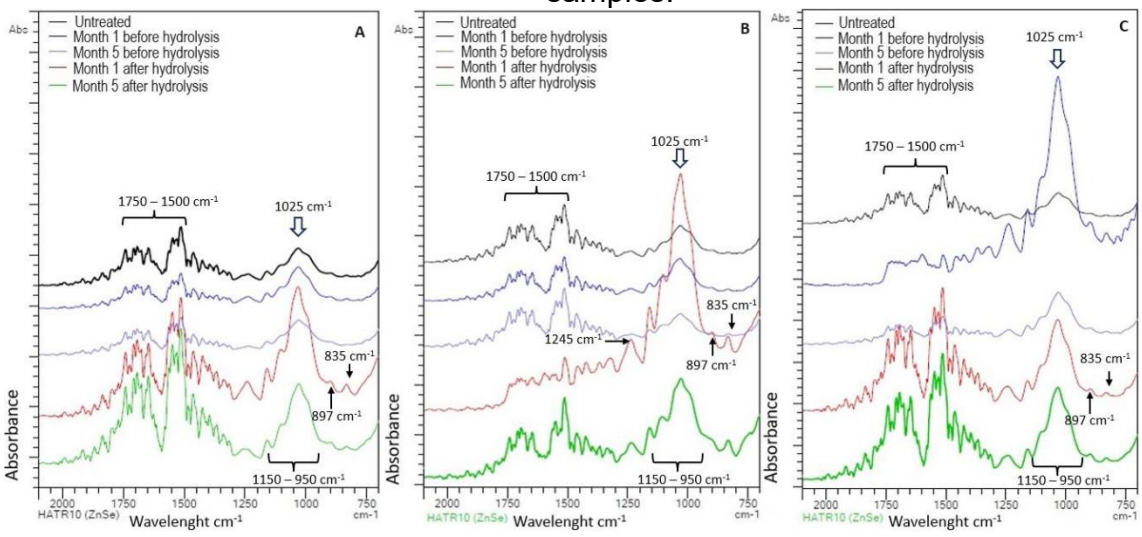
Both samples of *C. puteana* and *P. ostreatus* showed bands at 1750–1500 cm^{-1} region, after enzymatic hydrolysis. It is known that this region corresponds to thenon-conjugating C = O stretching functional group from hemicellulose and C = C stretching from the aromatic ring vibration from lignin, respectively (XU *et al* 2013). The bands around 1510 cm^{-1} are evident in all samples after enzymatic hydrolysis, showing a modification in the lignin structure after the enzymatic hydrolysis with cellulase, once lignin and the sugars are strictly connected in the plant cell wall. The band 835 cm^{-1} also is atributed to lignin (C-H out of plane in *p*-hydroxyphenyl units) and it is evident in all samples after enzymatic hydrolysis (HOAREAU *et al* 2004; XU *et al* 2013; FAIX 1991).

All enzymatically hydrolyzed samples also showed a band at the 1025 cm^{-1} wavelength. This band is between a region (897 and 1159 cm^{-1}) that indicates the presence of hemicellulose and cellulose in the material, specifically the C–O–C β-glycosidic linkages between the sugar units in cellulose and hemicellulose (XIAO *et al* 2014; ZHANG *et al* 2010; LE TROEDEC *et al* 2008; HOAREAU *et al* 2004; XU *et al* 2013; FAIX 1991; NIKONENKO *et al* 2000). This showed a change in the polysaccharide structure, specifically a glycosidic linkage formation (RILEY *et al* 2014).

For the *C. puteana* and *G. trabeum* samples, in the first month after enzymatic hydrolysis, the clearest peaks in this region were observed, probably due to the effects from the enzyme in the sugars. However, these bands are less evident in the fifth month possibly due to less quantity of sugars at this time of cultivation. This argument can be supported by the quantity of hemicellulose remaining after biological pretreatment for these two fungi (Table 2.1). This region also covers the 897 cm^{-1} band, which is attributed to vibrations belonging to polysaccharides, which is also evident in all samples after enzymatic hydrolysis.

All samples not enzymatically hydrolyzed showed the same patterns as the untreated sample, except the 1025 cm^{-1} band in the *P. ostreatus*. In this case, the structure of polysaccharides may experience modifications by the microorganism itself. The similarity of the material before enzymatic hydrolysis with untreated material demonstrates that the main modification in the lignocellulosic structure was made only after the enzymatic hydrolysis.

Figure 2.1 - A: ATR spectra of untreated and biologically pretreated with *C. puteana* (CBMAI 0870) bagasse samples; B: ATR spectra of untreated and biologically pretreated with *G. trabeum* (CBMAI 0872) bagasse samples. C: ATR spectra of untreated and biologically pretreated with *P. ostreatus* (CCIBt 2338) bagasse samples.



Source: Author

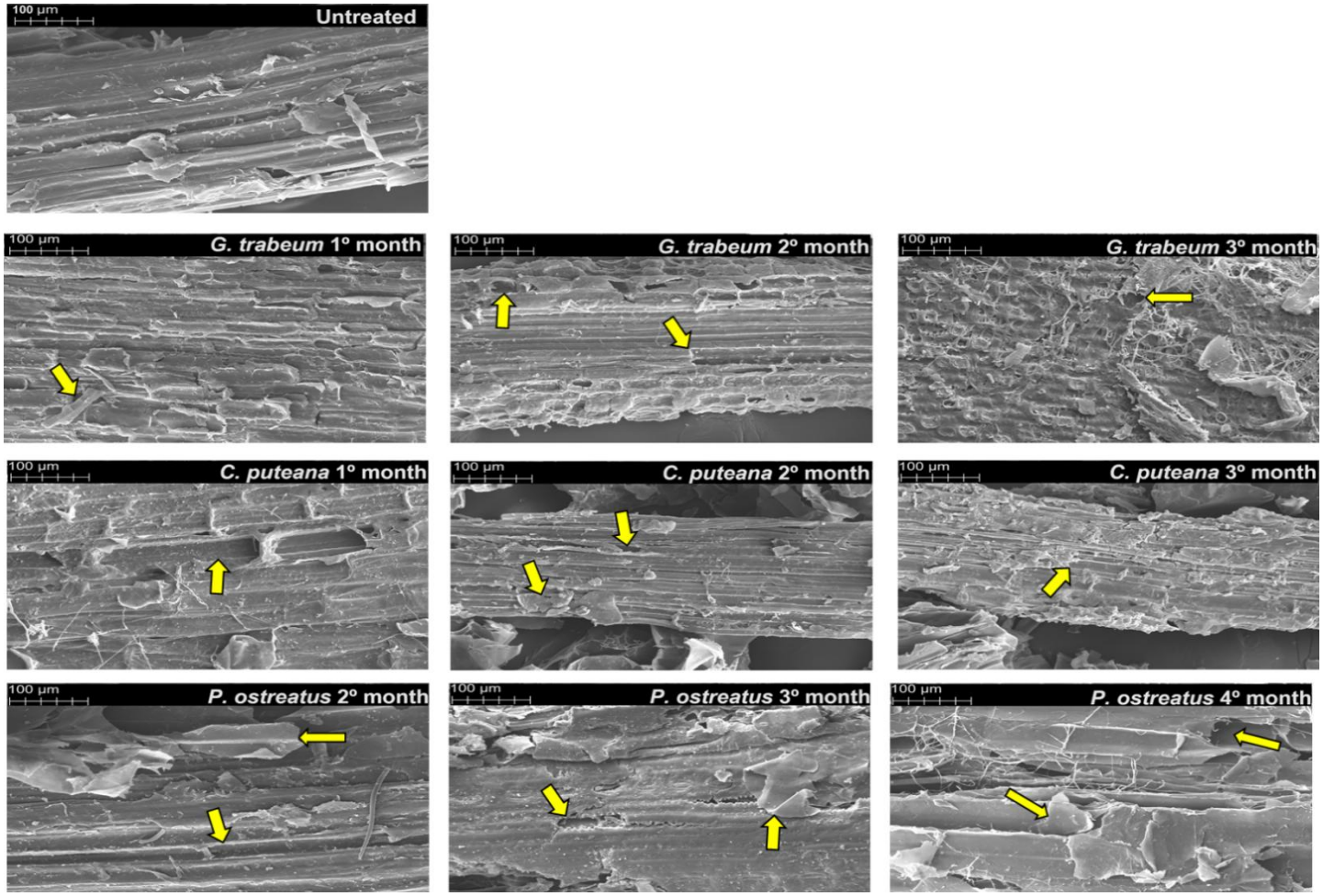
2.3.4 Scanning electron microscope images

Scanning electron microscopy was applied to compare the degree of physical modification between untreated and biologically pretreated sugarcane bagasse (Fig. 2.2). Then, 3 months were analyzed in sequence compared to the untreated biomass to show the continuous modification caused by the fungus action. The arrows show gaps formed by the fungi growing, in addition to the continuous degradation during the experiment.

The untreated sugarcane bagasse showed no gaps and a preserved structure. It was observed that the biomass degradation by the fungi started in the cell wall, where it experienced structural modification, changing its recalcitrance and chemical composition (RILEY *et al* 2014). In the cell wall, the components (lignin, cellulose, and hemicellulose) are degraded by specific enzymes produced by the fungus, which can be more or less specific for each macromolecule. Each microorganism has its own enzymatic arsenal, with particular ways of degrading biomass. Some white rot fungi do not completely degrade the lignin complex to reach the polysaccharides, as only a partial structural modification in the cell wall may allow access of larger molecular size enzymes to the polysaccharides (ZENG *et al* 2014).

The biomass degradation by *G. trabeum*, *C. puteana*, and *P. ostreatus* can be seen in Fig. 2, mainly when comparing the beginning and the end of the fungal growth. These images clearly show the way microorganisms modify the lignocellulosic structure to access the cellulose and hemicellulose during the degradation process. The arrows show evidence of the structural modification in the cell wall, while the untreated biomass remains intact.

Figure 2.2 - Scanning electron microscope image from the untreated and biologically pretreated sugarcane bagasse. The yellow arrows show the gaps formed by the fungi growth (*G. trabeum* 2nd month, *G. trabeum* 3rd month, *C. puteana* 1st month, *C. puteana* 2nd month, *P. ostreatus* 2nd month, *P. ostreatus* 3rd month and *P. ostreatus* 4th month) and the structural modifications in the lignocellulosic biomass (*G. trabeum* 1st month, *C. puteana* 2nd month, *C. puteana* 3rd month, *P. ostreatus* 2nd month, *P. ostreatus* 3rd month and *P. ostreatus* 4th month).



Source: Author

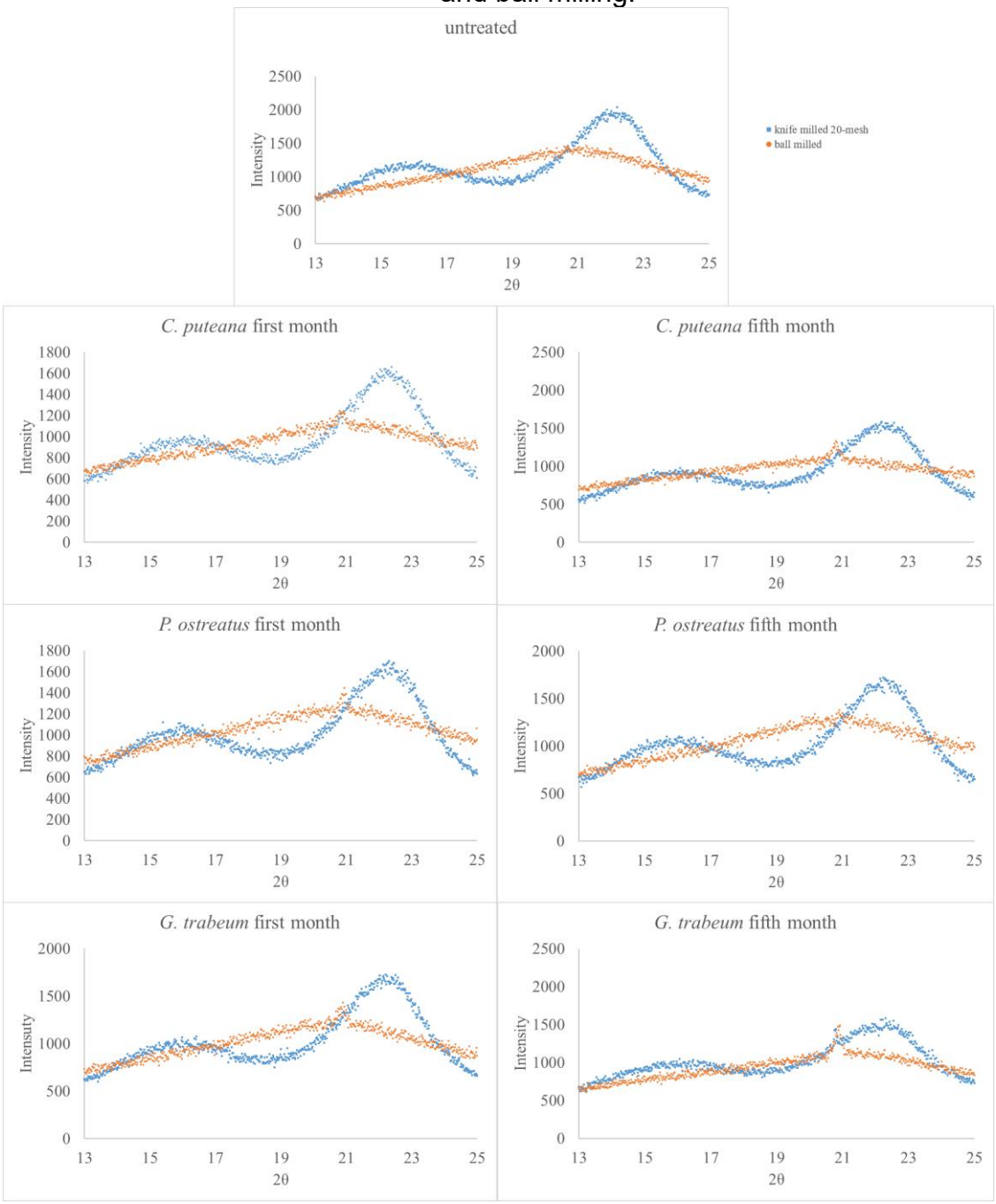
2.3.5 X-ray analysis

X-ray diffraction was applied to observe the crystallinity in the lignocellulosic structure, comparing the knife and ball milling (Fig. 2.3). The X-ray analysis diffraction reveals the peak, meaning the samples have a crystallinity index (MACEDO *et al* 2022).

Two peaks were observed in all samples using knife milling: the first one around $2\theta = 16°$ and the second one around $2\theta = 22°$. Seiwert *et al*. (2021) reported that the diffraction pattern in 18.2° is related to the organized xylan, which may be the case at the peak at $2\theta = 16°$. The diffraction pattern at 21–22° is related to the cellulose organized structure at ~ 22.8° (KAFLE *et al* 2015). Comparing the curve related to the ball-milled samples, it was not possible to determine the crystallinity, including the untreated ones. In this case, the peak was no longer in $2\theta = 22°$. This characteristic of the curve means low crystallinity of the cellulose chain, meaning that the ball milling method was, in fact, efficient in deconstructing the lignocellulosic structure. This pattern was shown by all samples.

When comparing the results between the crystallinity index and COS production (Table 2.3), both after ball milling, it can be assumed that this pretreatment is related to the recalcitrance reduction of the biomass. The effect of ball milling was already analyzed in other biomasses for different reasons (WANG, ZHU and DEUSS 2021; SITOTAW *et al* 2023); however, all the conclusions led to the crystallinity decrease after ball milling.

Figure 2.3 - X-ray diffraction from sugarcane bagasse after biological pretreatment and ball milling.



Source: Author

2.4 CONCLUSIONS

This work was based on the hypothesis that biological pretreatment combined with physical methods could modify the lignocellulosic structure. These modifications may reduce the biomass recalcitrance, facilitating the enzymatic hydrolysis step. The biological pretreatment effect was perceptible by the scanning electron images, and the chemical characterization analysis verified that the polysaccharides were partially

consumed. However, the COS and XOS yield was better than the untreated biomass, resulting in 26.39% of COS for *C. puteana* after 5 months of cultivation. This occurred due to the effects of fungi growth in the sugarcane bagasse, reducing its recalcitrance. The cellulose crystallinity was reduced after ball milling, as indicated by x-ray analysis. The quantity of COS yield after this pretreatment was higher than any samples that were only milled with a knife mill, reaching 36.65% of COS yield. In fact, after ball milling, only the intermediary enzyme charge (50 IU/g) was sufficient to achieve all values for the COS yield concerning the samples that were knife milled, proving that the ball mill method also reduces the biomass recalcitrance. The higher values for XOS yield were achieved for the samples biologically pretreated with *G. trabeum* and *C. puteana*, reaching 78.56% of XOS for the first one after one month of cultivation. It is important to note that these samples were only milled with the knife mill and the enzyme charge was 50 IU/g. These results can be optimized by increasing the quantity of enzymes or by milling the material in the ball mill. The most evident limitation of the biological pretreatment is the time of cultivation. However, the present study showed potential for combined pretreatments, mainly the biological pretreatments and ball mill method. Future experiments can be conducted using chemical pretreatments or different kinds of milling techniques together with biological pretreatments.

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CHAPTER III: CELLOOLIGOSACCHARIDES PRODUCTION FROM COTTON RESIDUE APPLYING BIOLOGICAL, CHEMICAL, AND PHYSICAL PRETREATMENTS

ABSTRACT

Cotton residues are left behind in the harvesting process and are usually not used for industry. However, this lignocellulosic material has great potential to be used as feedstock for added-value products such as cellooligosaccharides (COS). The process of producing COS is dependent on the pretreatment used. This study evaluated chemical, physical and biological pretreatments to produce COS and evaluated the efficiency of these pretreatments for this biomass. The alkali pretreatment was used NaOH 10, 25 and 50% (w/w), at 60 and 100 ºC; the physical pretreatment consisted of ball milling the cotton residue for 1, 3 and 6 h; and the biological pretreatment cultivated fungus *G. trabeum* (CBMAI 0872) for 15 days in liquid medium. After all pretreatments, the biomass was enzymatically hydrolyzed with cellulase using 20, 50 and 100 IU/g of enzyme. Also, the untreated and pretreated biomass were analyzed by X-ray, FTIR-ATR and scanning electron microscope techniques to evaluate the modifications in the cotton residue. The cotton residue showed modifications in the chemical composition after the alkali and biological pretreatments, mainly in the hemicellulose content. The COS yield reached 8.68% from untreated cotton using 100 IU/g of enzyme, against 11.61% of COS which was alkali pretreated using 50% NaOH (w/w) at 100 ºC. The biological pretreatment reached 9.13% of COS using the same enzyme charge. The ball milling method obtained the best values for COS yield, reaching 30.11% after 6 h of milling. X-ray analysis showed a decrease in cotton crystallinity after the physical pretreatment, mainly after 6 h, when the cotton lost completely its crystallinity. FTIR-ATR showed small differences for all pretreatment in comparison to untreated cotton, and scanning electron microscopy showed visual differences only in ball-milled samples, indicating a recalcitrance decrease, corroborating the values of COS yield. This study demonstrates the capacity of the pretreatments to change the lignocellulosic structure, mainly after ball milling, facilitating the enzymatic hydrolysis and improving the COS yield.

3.1 INTRODUCTION

Cotton textiles are widely used for multiple products, mainly for clothes, shoes, and accessories manufacturing. According to the Agricultural Economy Institute (IEA), the world supply of cotton plumes production reached 44.48 million tons in the 21/22 crop year. For Brazil, the production was approximately 2.83 million tons for the 2022/23 crop, while in China and USA the production was 5.99 and 3.38 million tons in this same period, respectively (ZEFERRINO, RAMOS, 2022). These figures highlight the high importance of cotton in our society, mainly in developed countries. However, the production of cotton requires a considerable amount of water, energy and chemicals in the production line, mainly if the final product is a high-added value product. The residues generated cannot be reused in the fabrics due to hygiene risks but can be used in composting or for generating energy by gasification, pyrolysis, and anaerobic digestion methods (SCHMIDT, 2016; TAUSIF *et al*, 2018; HAMAWAND *et al*, 2016).

In recent years, increasing attention has been directed toward the valorization of lignocellulosic residues, including cotton waste, as feedstocks for high-value bioproducts such as cellooligosaccharides (COS). Pretreatments play a crucial role in this process, as they are designed to alter the lignocellulosic structure of biomass, reducing its recalcitrance and enhancing the efficiency of subsequent steps such as enzymatic hydrolysis. Proper pretreatment can significantly reduce the energy and chemical requirements of the overall process, thereby lowering production costs. Numerous studies have explored a variety of pretreatment strategies, including their combinations, to optimize COS yields (SANTANA, *et al*., 2023; FANG *et al*., 2022; MENSAH *et al*., 2024). The most common pretreatments for COS production from lignocellulosic biomass are chemical, physical, and biological pretreatments. Chemical pretreatments are separated into acid and alkali types: acid pretreatment primarily hydrolyzes hemicellulose, while the alkali pretreatment reduces cellulose crystallinity, partially solubilizes hemicellulose and lignin. Ball milling is a physical pretreatment capable of reducing biomass crystallinity efficiently, and biological pretreatment involves a microorganism such as bacteria or fungus to degrade the biomass through an enzymatic process (FELIPUCI, 2020).

Cotton is characterized by its high cellulose content. During cotton fiber development, particularly in the synthesis of the secondary cell wall, over 90% of the dry weight is composed of cellulose (HAIGLER *et al*., 2012; LEE, KAFLE, PARK,

2015). Cellulose structure is composed of glucose dimers (cellobiose) linked by β-1,4 glycosidic chains. A characteristic of cellulose organization is the appearance of more organized regions named crystalline zones, while there are regions that are non-organized, named amorphous zones (IOELOVICH 2016; FOCKINK, *et al* 2020). The mechanical resistance of cellulose is related to its degree of crystallinity (DADI, SCHALL, VARANASI, 2007). Cellulose is a raw material for several uses in industries such as paper, film, and others. As a result, cellulose isolation and separation are widely studied.

After pretreatment, the lignocellulosic structure decreases its recalcitrance and configuration, exposing the cellulose chains and becoming more susceptible to enzymatic hydrolysis. Some options for cellulose conversion into COS are by enzymatic hydrolysis, which produces a short glucose chain with a few numbers of units. COS are formed by 2 glucose (cellobiose) until 6 glucose units (cellohexaose) that are water-soluble, while longer chains tend to be insoluble. Soluble COS exhibit prebiotic properties, promoting the *in vitro* growth of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium* strains (FUJII *et al*. 2016; KARNAOURI *et al*. 2019b; SANZ *et al*. 2005). In addition, COS have potential applications in food, pharmaceutical, animal and cosmetic industries (KENDRICK *et al*., 2022; ÁVILA *et al*., 2021, FORSAN, 2024).

In the present study, cotton residue was pretreated using chemical, physical and biological approaches to optimize COS yield following enzymatic hydrolysis with three different enzyme loadings. The underlying hypothesis was that the physical pretreatment would reduce the recalcitrance of the lignocellulosic structure, and the chemical and biological pretreatments would alter the cell wall components. With these modifications, the enzyme was expected to access and hydrolyze the cellulose more effectively, thereby enhancing COS production. The study focused on COS generation from cotton residues through three independent pretreatment strategies applied prior to enzymatic hydrolysis: biological pretreatment, alkali pretreatment and ball-milling. X-ray, scanning electron microscope and ATR-FTIR analysis were applied in all samples to evaluate crystallinity and physical modifications.

3.2 MATERIAL AND METHODS

3.2.1 Material and cotton preparation

Cotton residue was supplied by EMBRAPA. It came with humidity at 9,89% and was milled at 20-mesh using a knife mill. All the subsequent experiments were conducted using the 20-mesh milled cotton and the mass measurements were performed on a dry basis (FERNANDES *et al*, 2020).

3.2.2 Pretreatments

The samples were milled in a ball mill model MA 350 (MARCONI Equipamentos para Laboratorios Ltda). This equipment has a 150 mL reactor with a 110 g metallic ball and blows 617 times per minute. In this pretreatment, the samples were milled for 1, 3 and 6 h.

For the alkaline pretreatment, 20-mesh milled cotton was treated with three different NaOH concentrations: 10%, 25%, and 50% (w/w). The experiment was performed using 1g of biomass in a proportion of 1:10 biomass/solution. The reaction was performed in a water bath for 30 min at two temperatures: 60 ºC and 100 ºC. After the reaction time, the samples were filtered and the solid fraction was washed with diluted water to neutralize the pH using 5 mol/L HCl. The solid fraction was dried in an oven at 50 ºC and stocked for further analysis.

3.2.3 Biological pretreatment

This pretreatment was performed using 5 g of cotton in a 100 mL Bushnell Haas (BH) liquid medium, and the fungus used was *Gloeophyllum trabeum* (CBMAI 0872) (Brazilian Collection of Environment and Industry Microorganisms, Campinas State University-Unicamp, Campinas, Sao Paulo, Brazil). Before the liquid medium preparation, the fungus was pre-cultivated in Petri dishes with Potato Dextrose Agar (PDA) for 7 days at 28 ºC. After the growth period, the PDA cultures were transferred to a 250 mL Erlenmeyer flask containing 100 mL of BH liquid medium. The experiment was performed for 15 days in a shaker (Tecnal – TE-420) at 25 ºC and 150 rpm. After incubation, the liquid medium with biomass and PDA was filtered, and the solid fraction was separated, dried at 60 °C and stored for further analysis (BRANDÃO, *et al*. 2019).

3.2.4 Chemical characterization

Untreated, alkali and biologically pretreated cottons were chemically characterized. A total of 0.3 g of cotton was added to a test tube with 3 mL of 72% sulfuric acid (Sigma-Aldrich 320,501-1L) and reacted in a water bath at 30 °C for 1 h. Subsequently, 84 mL of distilled water was added to dilute the acid and the mixture was autoclaved for 1 h at 121 ºC. After the autoclave step, the sample was filtered through a crucible (16 µm), separating the liquid and solid fractions. The liquid fraction was used to determine the sugars by HPLC method, while the solid fraction was dried in an oven at 105 °C for 24 hours to determine the insoluble lignin. For the sugars, the cellulose content was estimated based on glucose, and hemicellulose was estimated using xylose, arabinose, and acetyl content. The soluble lignin was measured using a spectrophotometer at 215 and 280 nm based on a standard method (GOUVEIA *et al*., 2009). The HPLC analysis was carried out using a Bio-Rad Aminex HPX-87H (300 × 7.8 mm) column at 45 °C, a Waters 2414 refractive index detector, 50 mmol/L H_2SO_4 as the mobile phase, a flow rate of 0.6 mL/min, and a sample volume of 20 µL.

3.2.5 Enzymatic hydrolysis

The enzymatic hydrolysis used endoglucanase (Celluclast 1.5 L, Novozymes, CCN03196) with three different loads (20, 50 and 100 IU/g). The cellulase activity was 951 IU/mL, based on the carboxymethyl cellulase (CMC) assay for endo-β-1,4-glucanase method (GHOSE, 1987). For the cellulase activity measurement, the enzyme was diluted in five different dilutions. Then, for the reaction, was used 0.5 mL of CMC 2% and the diluted enzyme in bath water at 50 ºC for 30 min, then added 3 mL of DNS and boiled for 5 min. After the boiling, the solution was diluted by adding 20 mL of distilled water and measured in a spectrophotometer at 540 nm.

The enzymatic hydrolysis experiment was performed using 0.1 g of cotton in a falcon tube containing 5 mL of sodium acetate buffer (pH 5.2) and enzyme solution. The buffer was prepared with sodium acetate and acetic acid. The reaction was performed for 24 h in a shaker at 150 rpm and 50 ºC. After the hydrolysis, the samples were boiled for 5 min, centrifuged at 5000 rpm for 15 min and filtered through a 0.44 µm and Sep-Pak filters, prior to HPLC analysis.

3.2.6 Crystallinity index (X-ray)

The x-ray analysis used a SmatLab SE operating in from 2*θ of* 13 to 25°, with a step size of 0.02° and a scan speed of 5°/min. All samples were analyzed to determine the crystallinity index after the pretreatments, comparing to the untreated sample. The crystallinity index (CrI) was determined by the Eq. 1:

$$CrI = \left[\frac{I20 - Iam}{I20}\right] * 100$$

Equation 1: Crystallinity index formula where I20 is the intensity from plane diffraction at 2θ = 22°, and Iam represents the background scattering intensity (MACEDO *et al*., 2022).

3.2.7 FTIR-ATR analysis

FTIR-ATR analysis was performed in a equipment model FT-IR ALPHA Platinum-ATR from Bruker brand and the software used was OPUS 7.5. The resolution used was 4 cm^{-1}, the sample scan time and background scan time were 32 scans and wavenamber used was 700-2100 cm^{-1}.

3.2.8 Scanning electron microscope images

The scanning electron images were performed in an equipment model JSM-IT500HR from JEOL brand. The samples were gold metalized and the magnitude of the images was 1000 and 2000 times. All the samples were analyzed to evaluate the physical modifications after the pretreatments.

3.2.9 Statistical analysis

The software DX3Trial-expert software (DesignExpert, Stat-Ease company, version DX 6.0.6) was used to do the ANOVA statistical analysis for chemical characterization and enzymatic hydrolysis. All pretreatments were compared to untreated samples, and the differences between samples were noted by different letters in the tables with a confidence level of 95% (*p*-value less than 0.05). The statistical results and tables were in the supplementary material section.

3.3 RESULTS AND DISCUSSION

3.3.1 Chemical characterization

The cotton residue was chemically characterized to determine the percentage of cellulose, hemicellulose, and lignin (Table 3.1), and an ANOVA test was performed to compare the values from the pretreatments with the untreated control. All samples were analyzed, except those subjected to ball milling, as the significant reduction in particle size rendered them incompatible with the chemical characterization method. The untreated cotton presented 51.81% of cellulose, 9.29% of hemicellulose, and 33.16% of lignin. It is important to note that commercially used cotton, such as that found in everyday products, is basically pure cellulose, presenting a white color and free of lignin. In contrast, industrial cotton residue is unprocessed and impure.. The material used in the present study was brown and collected from early stage of the harvesting process. High quantities of lignin were reported in the literature for cotton residues (WANG *et al*., 2016; MOU *et al*., 2024), while pure cotton fiber has no lignin in the composition (CHOKSHI *et al*., 2022). Reddy (2009) compared the composition between cotton and cotton stalks and found a difference in the lignin content: 13.7% of lignin for cotton stalk fiber against 0.7% for cotton. This difference may be due to the variety of cotton stalks and the extraction and characterization methods used.

The cellulose percentage for all pretreatments significantly changed after the alkali and biological pretreatments. For alkali pretreatment, all percentages of cellulose increased, reaching 70.82% for 25% (w/w) NaOH at 100 ºC, while the lowest value for cellulose was 59.48% using 10% (w/w) NaOH at 60 ºC. For the biological pretreatment, the cellulose percentage decreased to 43.59%. However, despite the cellulose percentage increase, the cellulose (content) was removed after both alkali and biological pretreatment. For alkali pretreatment, it was observed an increment of cellulose remotion according to the intensity of NaOH: 9.73 g/100 g of cellulose was removed after pretreating using 10% NaOH (w/w) at 60 ºC, while 17.37 g/100 g of cellulose was removed using 50% NaOH (w/w) at 100 ºC. After the biological pretreatment, 25 g/100 g of cellulose was removed/consumed.

The hemicellulose percentage decreased in all pretreatments: for the alkali pretreatment the lowest value was 0.85% for 50% (w/w) NaOH at 60 ºC, and the highest value was 1.74% for 10% (w/w) NaOH at 100 ºC, while the hemicellulose content in the biological pretreatment was 2.88%. This data from cellulose and hemicellulose indicates a clear characteristic of these pretreatments: the alkali pretreatment capacity of solubilization of hemicellulose and lignin, consequently an

increase of cellulose percentage (FORSAN, DE REITAS, BRIENZO, 2024; SHIMIZU *et al*., 2018); and a capacity of sugars solubilization from the *G. trabeum* (FELIPUCI *et al*., 2021).

The lignin content decreased for all samples that were alkali pretreated, with a maximum value of 27.10% for 50% (w/w) NaOH at 100 ºC, and a minimum value of 22.64% for 25% (w/w) NaOH at 100 ºC. These values were consistent with the literature, since NaOH is known to solubilize lignin and hemicellulose. Meanwhile, lignin percentage in the biologically pretreated sample increased to 40.02%, due to the removal of other components like hemicellulose (MELATI *et al*., 2019). This increase was related to the chemical composition after the biological pretreatment, however, it was important to note that the lignin was also partially degraded by the fungus: a total of 8.55 g/100 g of lignin was consumed after the biological pretreatment. This compositional patternafter biological pretreatment is known in the literature for various biomasses studies (KEREM, JENSEN, HAMMEL, 1999; VASCO-CORREA, GE, LI, 2016; FELIPUCI *et al*., 2024)

Table 3.1 - Chemical composition of untreated and pretreated cotton residue

Cotton samples			Compostioin (%, dry basis)				Component removal (g/100g)			
			Cellulose	Hemicellulose	Lignin	E*	MR (%)	Cellulose	Hemicellulose	Lignin
Untreated			51.81 ± 0.90^{a}	9.29 ± 0.51^{a}	33.16 ± 1.14^{a}	17.76 ± 0.44	-	-	-	-
Alkaline pretreatment	60 ºC	10% (w/w)	59.48 ± 2.36^{b}	1.57 ± 1.13^{b}	26.80 ± 0.87^{b}	-	70.74	9.73	8.18	14.21
		25% (w/w)	65.82 ± 0.78^{c,d,e}	1.26 ± 0.58^{b}	25.91 ± 0.91^{b,c}	-	65.40	8.76	8.47	16.22
		50% (w/w)	64.66 ± 6.56^{b,d}	0.85 ± 0.22^{b}	25.75 ± 1.51^{b,c}	-	62.28	11.53	8.76	17.13
	100 ºC	10% (w/w)	63.00 ± 1.74^{b,d}	1.74 ± 0.04^{b}	23.51 ± 3.08^{c,d}	-	61.07	13.33	8.23	18.81
		25% (w/w)	70.82 ± 1.61^{e}	1.15 ± 0.22^{b}	22.64 ± 1.40^{d}	-	54.72	13.06	8.66	20.78
		50% (w/w)	67.42 ± 2.74^{d,e}	1.48 ± 0.24^{b}	27.10 ± 0.38^{b}	-	51.08	17.37	8.54	19.32
Biological pretreatment – *Gloeophyllum trabeum*			43.59 ± 5.15^{f}	2.88 ± 4.23^{b}	40.02 ± 1.20^{e}	-	61.50	25.00	7.52	8.55

* Extractive; MR: mass recuperation. Significant statistical differences (*p*-value less than 0.05) were symbolized by different letters in the same column for each microorganism.

3.3.2 COS yield after enzymatic hydrolysis

Enzymatic hydrolysis was performed using cellulase (endoglucanase) with three different enzyme charges: 20, 50, and 100 IU/g of material. These three values

were chosen to represent low, intermediate, and high enzyme concentrations, and compare the COS yield after the hydrolysis (FORSAN, 2024; FELIPUCI, 2021; FELIPUCI, 2024). Table 2 shows the glucose, C2 (cellobiose) and >C3 (cellotriose to hexose) yields from hydrolysis using 20 IU/g, 50 IU/g, and 100 IU/g of enzyme, respectively, and Table 3 summarizes total COS yield. Total COS yield, as presented in Table 3, was calculated by summing the C2 and >C3 values from Table 2, in order to facilitate statistical comparisons. .

Glucose was produced in low amounts, likely due to the limited β-glucosidase activity in the Celluclast enzyme cocktail. For untreated cotton, glucose yields were 1.44%, 0%, and 0.61% using 20, 50 and 100 IU/g of enzyme, respectively. For alkali and biological pretreatments using 20 IU/g of enzyme charge, the glucose yield did not differ significantly from untreated, except for the 10% NaOH (w/w) at 60 ºC condition, which yielded 3.48%. With 50 and 100 IU/g, most pretreatments resulted in glucose yields that were statistically different from the untreated control. The only exception was the 50% NaOH (w/w) at 60 °C condition, which yielded 1.46% and 4.62% at 50 and 100 IU/g, respectively.Glucose production increased with enzyme load for all pretreatments, reaching a maximum of 19.41% in the ball-milled sample after 6 hours of milling and hydrolysis at 100 IU/g.

The C2 yield from untreated cotton was negligible (0.01% at 20 IU/g), and no C2 was detected at 50 or 100 IU/g. In contrast, ball milling pretreatment led to the highest C2 yield, reaching 18.33% after 6 hours of milling and hydrolysis at 100 IU/g. All ball-milled samples produced C2 yields significantly higher than the untreated control.

Alkali pretreatment using 10% NaOH (w/w) at 60 ºC and 20 IU/g yielded 0.65% of C2, increasing to 2.57% at 100 IU/g under the same chemical condition. At 20 IU/g, this 0.65% yield was the only one not significantly different from the untreated control [statistically similar to the untreated cotton], while the value of 1.07% for 25% NaOH (w/w) at 60 ºC was the only C2 yield statistically similar to untreated at 50 IU/g. The highest C2 yield with alkali pretreatment was 6.89%, using 50% NaOH (w/w) at 100 ºC. Biological pretreatment followed a different trend, with the highest C2 yield at 20 IU/g (2.55%) and slightly lower yield at 100 IU/g (2.26%), suggesting a nonlinear response to enzyme load. For >C3 yields, untreated cotton reached 5.98%, 6.58%, and 8.68% at 20, 50, and 100 IU/g, respectively.. These values were statistically higher than those observed for alkali and biological pretreatments at all enzyme loads. In alkali pretreatment, >C3 yields remained relatively constant, regardless of NaOH

concentration and temperature, indicating minimal influence of these variables. Notably, in most cases, no statistical differences were observed between 60 °C and 100 °C, suggesting that temperature had little effect on COS yield under the tested conditions. Maximum values observed were: 1.64% (25% NaOH at 60 °C, 20 IU/g), 2.73% (10% NaOH at 60 °C, 50 IU/g), and 5.47% (10% NaOH at 60 °C, 100 IU/g).

In biological pretreatment, >C3 yields were statistically different from untreated at all enzyme loads, with a maximum of 6.86% at 100 IU/g. Ball milling pretreatment consistently produced higher >C3 yields, except for the 1 h sample at 100 IU/g. The best >C3 yield was 11.78% after 6 h of milling at 100 IU/g.Considering total COS yield (Table 3), untreated cotton showed 5.99%, 6.56%, and 8.68% at 20, 50, and 100 IU/g. The increase between 20 and 50 IU/g was not statistically significant, but yield at 100 IU/g was significantly higher. A similar pattern was observed in the biological pretreatment, where 100 IU/g produced a significantly higher COS yield (9.13%) compared to the lower loads.The samples that were ball milled showed the highest values for COS yield of all pretreatments, reaching 30.11% after 6 h of milling and using 100 IU/g of enzyme charge. For the ball milling method, the values of COS using an enzyme charge of 50 IU/g was statistically higher than the 20 IU/g ones, except for the 1 h milling, and the enzyme charge of 100 IU/g was statistically similar to the 50 IU/g, except for the 3 h milling. The alkali pretreated samples showed statistical differences in COS yield comparing the enzyme charge of 50 IU/g and 20 IU/g, except for the 25% (w/w) of NaOH at 60 ºC, and also showed statistical differences between 50 IU/g and 100 IU/g. However, the COS yield values for alkali pretreated cotton were lower or equal to untreated cotton, except for the 50% NaOH (w/w) at 100 ºC, reaching 11.61% of COS yield. In the literature were found examples of alkali pretreatment where the enzymatic hydrolysis was performed with positive results (MOU et al., 2024; YANG et al, 2021). However, the solid fractions in these studies were directly enzymatic hydrolyzed or passed through a steam-explosion method, while in the present study, the solid fraction was dried before the hydrolysis. A consequence of drying a high cellulose quantity biomass after alkali pretreatment is the cellulose reorganization, recovering the crystallinity and recalcitrance. Literature reports better enzymatic hydrolysis results for alkali pretreatments, especially when the biomass is processed without drying or combined with steam explosion (MOU et al., 2024; YANG et al., 2021). In this study, drying the solid fraction prior to hydrolysis may have induced cellulose reorganization and recrystallization, increasing recalcitrance.

Overall, although COS yields were modest, even for ball milling and biological pretreatments (AUSTAD, 2018), it is important to consider the influence of biomass origin, composition, and fungal strain. While 100 IU/g enzyme load led to significantly higher COS production, the fivefold increase in enzyme amount did not result in a proportional yield gain. This raises important questions about the cost-effectiveness of high enzyme dosages in industrial applications.

Table 3.2 - Glucose, C2 and >C3 yield after enzymatic hydrolysis using cellulase

Sample			Enzymatic charge of 20 IU/g						Enzymatic charge of 50 IU/g						Enzymatic charge of 100 IU/g					
			Glucose (%)	Glucose (g/L)	C2 (%)	C2 (g/L)	>C3 (%)	>C3 (g/L)	Glucose (%)	Glucose (g/L)	C2 (%)	C2 (g/L)	>C3 (%)	>C3 (g/L)	Glucose (%)	Glucose (g/L)	C2 (%)	C2 (g/L)	>C3 (%)	>C3 (g/L)
Untreated			1.44 ± 1.27^{a}	0.15 ± 0.13	0.01 ± 0.03^{a}	0	5.98 ± 0.83^{a}	0.63 ± 0.02	0^{a}	0	0^{a}	0	6.58 ±0.44^{a}	0.75 ± 0.05	0.61 ± 0.74^{a}	0.06 ± 0.08	0^{a}	0	8.68 ± 1.48^{a,b}	0.91 ± 0.08
BM (1 h)			2.95 ± 0.19^{b,c}	0.31 ± 0.02	2.77 ± 0.54^{b}	0.29 ± 0.06	7.88 ± 1.39^{b}	0.82 ± 0.03	6.15 ± 0.93^{b}	0.64 ± 0.10	3.72 ± 0.97^{b}	0.39 ± 0.10	9.03 ± 0.21^{b}	0.94 ± 0.01	11.96 ± 0.21^{b}	1.24 ± 0.02	6.21 ± 0.88^{b,c}	0.65 ± 0.09	10.03 ± 3.12^{a,c}	1.04 ± 0.08
BM (3 h)			4.22 ± 0.70^{c}	0.44 ± 0.07	4.13 ± 0.33^{c}	0.43 ± 0.03	9.26 ± 0.39^{b}	0.96 ± 0.01	8.72 ± 0.14^{c}	0.90 ± 0.01	6.81 ± 0.66^{d}	0.70 ± 0.07	10.78 ± 0.66^{c}	1.12 ± 0.01	12.68 ± 0.43^{b}	1.31 ± 0.05	8.32 ± 1.65^{d}	0.86 ± 0.17	11.68 ± 0.34^{c}	1.21 ± 0.04
BM (6 h)			6.55 ± 1.10^{d}	0.68 ± 0.12	8.90 ± 0.73^{g}	0.93 ± 0.08	8.30 ± 2.59^{b}	0.87 ± 0.03	9.18 ± 0.08^{c}	0.95 ± 0.01	14.43 ± 0.33^{e}	1.49 ± 0.03	12.44 ± 0.84^{d}	1.29 ± 0.06	19.41 ± 1.91^{g}	2.01 ± 0.21	18.33 ± 2.48^{h}	1.90 ± 0.26	11.78 ± 2.23^{c}	1.22 ± 0.08
AP (NaOH)	60 ºC	10% (w/w)	3.48 ± 1.02^{b,c}	0.41 ± 0.12	0.65 ± 0.16^{a,d}	0.08 ± 0.02	1.22 ± 0.05^{c,d}	0.15 ± 0	3.49 ± 1.75^{d}	0.41 ± 0.21	2.22 ± 0.26^{c}	0.26 ± 0.03	2.73 ± 0.10^{e}	0.33 ± 0.01	8.62 ± 1.25^{b,c}	1.03 ± 0.15	2.57 ± 0.16^{e,f,g}	0.31 ± 0.02	5.47 ± 0.24^{d,e}	0.66 ± 0.01
		25% (w/w)	2.52 ± 1.0^{a,b}	0.33 ± 0.14	1.30 ± 1.08^{d,e}	0.17 ± 0.14	1.64 ± 0.82^{c,d}	0.22 ± 0.05	3.69 ± 2.22^{d,e}	0.49 ± 0.29	1.07 ± 0.91^{a}	0.14 ± 0.12	2.40 ± 0.10^{e}	0.32 ± 0.06	9.58 ± 0.82^{b,d}	1.27 ± 0.11	2.28 ± 0.73^{e}	0.30 ± 0.10	4.70 ± 0.28^{d,e}	0.63 ± 0.02
		50% (w/w)	1.15 ± 0.45^{a}	0.15 ± 0.06	1.89 ± 0.24^{b,e,f}	0.24 ± 0.03	1.15 ± 0.05^{c,d}	0.15 ± 0	1.46 ± 0.59^{a}	0.19 ± 0.08	3.74 ± 0.26^{b}	0.49 ± 0.03	2.58 ± 0.33^{e}	0.34 ± 0.06	4.62 ± 1.55^{a,c}	0.60 ± 0.20	4.47 ± 0.83^{b,f}	0.58 ± 0.11	4.51 ± 0.79^{d}	0.59 ± 0.13
	100 ºC	10% (w/w)	1.91 ± 0.47^{a,b}	0.24 ± 0.06	2.39 ± 0.10^{b,f}	0.30 ± 0.01	1.32 ± 0.13^{c,d}	0.17 ± 0.03	4.72 ± 1.43^{b,d}	0.60 ± 0.18	3.65 ± 0.97^{b}	0.46 ± 0.12	2.55 ± 0.20^{e}	0.33 ± 0.01	8.70 ± 3.44^{b,e}	1.10 ± 0.43	3.63 ± 0.82^{e,f,g}	0.46 ± 0.10	5.01 ± 0.14^{d,e}	0.64 ± 0.01
		25% (w/w)	1.08 ± 0.25^{a}	0.15 ± 0.04	2.08 ± 0.69^{b,e}	0.30 ± 0.10	1.02 ± 0.17^{c}	0.15 ± 0.03	5.42 ± 0.21^{b,e}	0.77 ± 0.03	4.93 ± 0.29^{f}	0.70 ± 0.04	2.12 ± 0.12^{e}	0.31 ± 0.01	10.09 ± 3.12^{b,f}	1.43 ± 0.44	4.88 ± 1.64^{b,c,g}	0.69 ± 0.23	4.62 ± 0.13^{d}	0.66 ± 0.01
		50% (w/w)	2.45 ± 0.94^{a,b}	0.33 ± 0.13	4.21 ± 0.96^{c}	0.57 ± 0.13	0.88 ± 0.55^{c}	0.12 ± 0.03	4.55 ± 0.36^{b,d}	0.61 ± 0.05	5.31 ± 0.68^{g}	0.71 ± 0.09	2.22 ± 0.05^{e}	0.31 ± 0	11.47 ± 4.21^{b}	1.55 ± 0.57	6.89 ± 1.29^{c,d}	0.93 ± 0.17	4.72 ± 0.10^{d,e}	0.64 ± 0.01
BP – *G. trabeum*			2.44 ± 1.31^{a,b}	0.21 ± 0.11	2.55 ± 0.78^{b}	0.22 ± 0.07	2.80 ± 0.11^{d}	0.25 ± 0.01	4.65 ± 1.62^{b,d}	0.41 ± 0.14	2.44 ± 0.99^{c}	0.21 ± 0.09	4.24 ± 0.43^{f}	0.38 ± 0.07	7.25 ± 3.88^{c,d,e,f}	0.63 ± 0.34	2.26 ± 0.98^{e}	0.20 ± 0.09	6.86 ± 0.33^{b,e}	0.61 ± 0.01

Source: author. Significant statistical differences (p-value less than 0.05) were symbolized by different letters in the same column for each pretreatment. BM: ball-milling; AP: alkaline pretreatment; BP: biological pretreatment.

Table 3.3 - Total COS yield after enzymatic hydrolysis using cellulase at 20, 50 and 100 IU/g

Sample			Enzyme charge					
			20 IU/g		50 IU/g		100 IU/g	
			Total COS (%)	Total COS (g/L)	Total COS (%)	Total COS (g/L)	Total COS (%)	Total COS (g/L)
Untreated			5.99 ± 0.82^{aA}	0.63 ± 0.08	6.56 ± 0.44^{aA}	0.76 ± 0.11	8.68 ± 1.46^{aB}	0.91 ± 0.15
BM (1 h)			10.65 ± 1.84^{bA}	1.11 ± 0.19	12.74 ± 1.02^{bA,B}	1.32 ± 0.11	16.24 ± 2.44^{bB}	1.69 ± 0.25
BM (3 h)			13.39 ± 0.71^{cA}	1.38 ± 0.07	17.59 ± 0.81^{cB}	1.82 ± 0.08	20.00 ± 1.39^{cC}	2.08 ± 0.15
BM (6 h)			17.20 ± 2.40^{dA}	1.79 ± 0.25	26.87 ± 1.17^{dB}	2.78 ± 0.12	30.11 ± 3.28^{dB}	3.12 ± 0.35
AP (NaOH)	60 ºC	10% (w/w)	1.87 ± 0.12^{eA}	0.23 ± 0.02	4.96 ± 0.25^{eB}	0.60 ± 0.03	8.03 ± 0.25^{aC}	0.96 ± 0.03
		25% (w/w)	2.95 ± 0.56^{eA}	0.40 ± 0.08	3.47 ± 1.00^{fA}	0.46 ± 0.13	6.98 ± 0.96^{aB}	0.93 ± 0.13
		50% (w/w)	3.04 ± 0.22^{eA}	0.40 ± 0.03	6.33 ± 0.58^{a,eB}	0.82 ± 0.07	8.98 ± 0.82^{a,eC}	1.17 ± 0.11
	100 ºC	10% (w/w)	3.71 ± 0.23^{e,fA}	0.47 ± 0.03	6.20 ± 1.05^{a,eB}	0.79 ± 0.13	8.63 ± 0.70^{aC}	1.10 ± 0.09
		25% (w/w)	3.10 ± 0.53^{eA}	0.44 ± 0.08	7.05 ± 0.37^{aB}	1.01 ± 0.05	9.50 ± 1.77^{a,eC}	1.35 ± 0.26
		50% (w/w)	5.09 ± 1.50^{a,fA}	0.69 ± 0.20	7.53 ± 0.69^{aB}	1.02 ± 0.09	11.61 ± 1.20^{eC}	1.57 ± 0.16
BP – *G. trabeum*			5.35 ± 0.73^{a,fA}	0.47 ± 0.07	6.68 ± 1.34^{aA}	0.59 ± 0.12	9.13 ± 1.19^{a,eB}	0.81 ± 0.10

Source: author. Significant statistical differences (*p*-value less than 0.05) were symbolized by different letters in the same column for each pretreatment. BM: ball-milling; AP: alkaline pretreatment; BP: biological pretreatment.

3.3.3 Crystallinity index (X-ray analysis)

X-ray diffraction was applied to compare the crystallinity of the lignocellulosic structure in untreated and pretreated cotton residues (Figure 1). A notable observation (the most perceptible detail) was that the peak of all samples, including untreated, was shifted from the typical 2θ = 22º. It could be a microcrystalline cellulose Iβ, which typically exhibits a peak is around 2θ = 22.8º. The microcrystalline cellulose Iβ reacts differently with water in comparison to the usual cellulose (MATTHEWS *et al*., 2006). This shift may imply the reduction of structural distortion of cellulose, probably due to the relocation or removal of matrix components from cellulose crystallites (KAFLE *et al*., 2015).

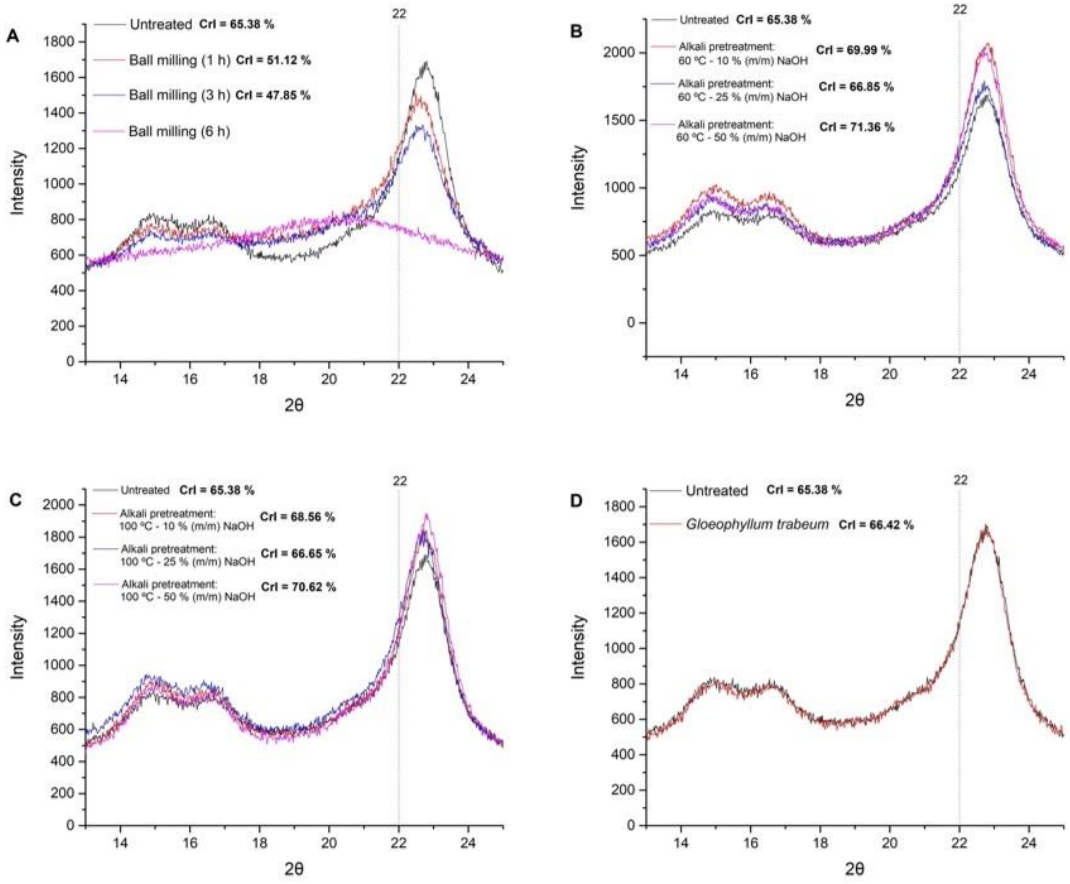
The untreated cotton had a crystallinity index of 65.38%, while the cotton that passed through the ball milling presented a crystallinity index of 51.12% for the 1 h, and 47.85% for the 3 h. The 6 h ball milled cotton completely lost its crystallinity, showing no peak in the analysis. The decrease in the crystallinity index for ball-milled cotton was expected, once previous works with other biomasses showed the same

possible effect (FELIPUCI *et al*., 2024). Also, the reduction in crystallinity was closely related to the cotton recalcitrance, the best results of COS yield were for ball-milled samples.

All cottons that were alkali pretreated showed an increment in the crystallinity index, reaching 71.36% of crystallinity for the 50% (w/w) of NaOH at 60 ºC. This effect of NaOH in the biomass partially solubilizes the lignin and hemicellulose (FELIPUCI *et al*., 2020; FELIPUCI *et al*., 2021; RASTOGI, SHRIVASTAVA, 2017), which may be the reason for the crystallinity increase. Despite the differences in the cellulose, hemicellulose and lignin removal after the alkali pretreatment, the values of crystallinity index remained relatively similar when compared only to the concentration of NaOH. Previous works showed an alkali pretreatment focusing on glucose yield from the enzymatic hydrolysis of cotton. In this case, the study separated the alkali pretreatment in two steps: first, it was pretreated with 10% NaOH at 60 ºC for 8 h in a water bath, then it was pretreated with 10% NaOH at 121 ºC for 45 min in a rotary shaker. The results showed that the pretreatment reached more than 74% of glucose yield and also increased the crystallinity (MOU *et al*., 2024).

The cotton that was biologically pretreated with *G. trabeum* showed 66.42% of crystallinity index, compared to the 65.38% of untreated cotton, which means that the biological pretreatment modestly increased the crystallinity index of cotton. This data was coherent when analyzed with COS yield results (Table 3), which did not significantly change the COS yield. Ramamoorthy *et al* (2019) reported an increase in crystallinity index in cotton after biological pretreatment with *Paecilomyces inflatus*, reaching 47.05%, while the untreated cotton crystallinity index was 36.16%. In contrast, Ranjithkumar *et al* (2022) reported a reduction of ~80-85% in cotton crystallinity after pretreatment with *Pleurotus florida*. Undoubtedly, the methods of measuring the crystallinity index and the fungal cultivation conditions may lead to different results.

Figure 3.1 - X-ray diffraction from cotton residues comparing the untreated and pretreated matrial.



Source: author; Software: OrignPro 2016; A: X-ray diffraction from ball milled cotton residues; B: X-ray diffraction from cotton residues after alkali pretreatment at 60 ºC; C: X-ray diffraction from cotton residues after alkali pretreatment at 100 ºC; D: X-ray diffraction from cotton residues after biological pretreatment

3.3.4 FTIR-ATR analysis

FTIR-ATR was applied to untreated and pretreated samples to evaluate the modifications in the lignocellulosic structure. Table 4 shows wavenamber to the functional groups in biomass residues. The wavenumbers corresponding to 1100 cm^{-1} and 1155 cm^{-1} were evident in all samples, except for the cotton that was ball milled, mainly for the 6 h. These wavenumbers correspond to the cellulose and hemicellulose presence, more specifically C-O-C vibration pyranose rings (1161 cm^{-1}) and C-O-C vibration in the anomeric region of the xylans (1050 cm^{-1}) (JU, *et al*, 2011). The difference between the ball-milled samples to the other pretreatments and untreated indicates that the ball-milling pretreatment changes the lignocellulose structure, possibly reducing its recalcitrance. This finding was supported by the enzymatic hydrolysis and x-ray diffraction results, in which the ball milling method resulted in the

highest COS production and the crystallinity decreased in comparison to the untreated cotton, respectively. Besides that, the ball milling method also modified the lignin structure, observable in the wavenambers of 1320 cm^{-1} and 1425 cm^{-1}, which the band were not evident as in the other pretreatments. Those bands corresponded to the regions of syringyl (1330 cm^{-1}) and aromatic skeleton vibrations (1600 - 1425 cm-1) (GARCIA *et al*., 2012).

Similar to the ball milling pretreatment, the alkali pretreatment also modified the lignin structure of the cotton but in the region of 1615 cm^{-1}, both in the 60 ºC and 100 ºC pretreatments. This region corresponds to the aromatic skeleton vibrations (GARCIA *et al*., 2012). It is known from literature that alkali pretreatment removes lignin and increases the surface area and porosity (KIM, LEE, KIM, 2016; FELIPUCI *et al*., 2020; RASTOGI, SHRIVASTAVA, 2017). Despite the reduced band intensityin the FTIR-ATR analysis, these conditions of alkali pretreatment were not enough to statistically increase the COS production after enzymatic hydrolysis, except for the most aggressive NaOH conditions using the higher quantity of enzyme, as discussed before.

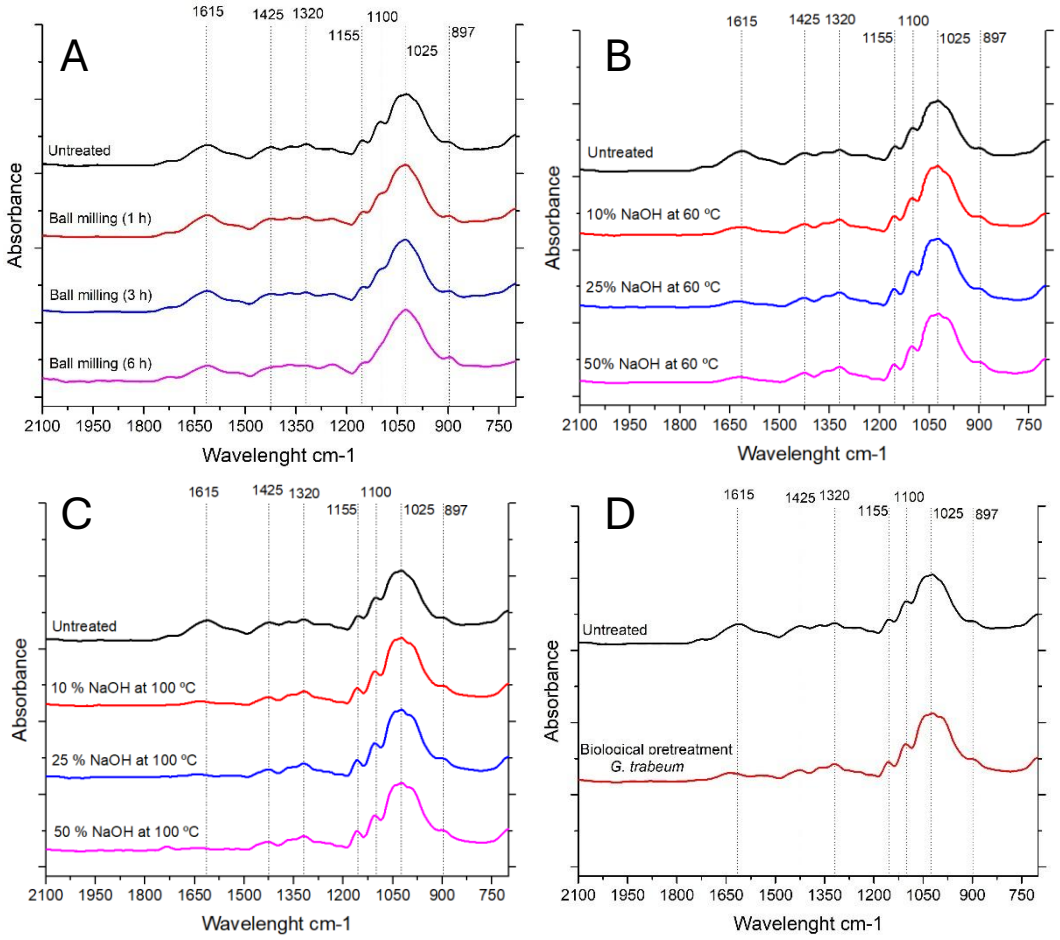
The region concerned to 1025 cm^{-1}, related to polysaccharides (around 1058 cm^{-1}), was evident in all pretreated and untreated, showing no change in the C-O stretching vibrations of cellulose and hemicellulose (HARIPRIYA *et al*., 2014). Despite that, the biological pretreatment was similar to the untreated in all wavenumbers, suggesting minimal structural modification.

Table 3.4 – FTIR-ATR absorbance wavenumbers for lignocellulosic biomass

Wavenumber	Functional group	Polymer	Reference
1600	Aromatic ring vibration	Lignin	GARCIA *et al*, 2012
1425	aromatic skeleton vibrations	lignin	GARCIA *et al*, 2012
1330	regions of syringyl	lignin	GARCIA *et al*, 2012
1161	C–O–C vibration pyranose ring	Cellulose, hemicellulose	OUYANG *et al*, 2015; NAKASONE *et al*, 2016
1058	C–O stretching vibrations cellulose and hemicellulose	Cellulose, hemicellulose	HARIPRIYA *et al*, 2014
1050	C–O–C vibrations in the anomeric region of the xylans	Hemicellulose	JU, *et al*, 2011; HARIPRIYA *et al*, 2014

Source: author

Figure 3.2 - FTIR-ATR spectra of untreated and pretreated samples



Source: author; Software: OriginPro 2016; A: ball milled cotton residues; B: alkali pretreatment at 60 ºC; C: alkali pretreatment at 100 ºC; D: biological pretreatment

3.3.5 Scanning electron microscope (SEM) images

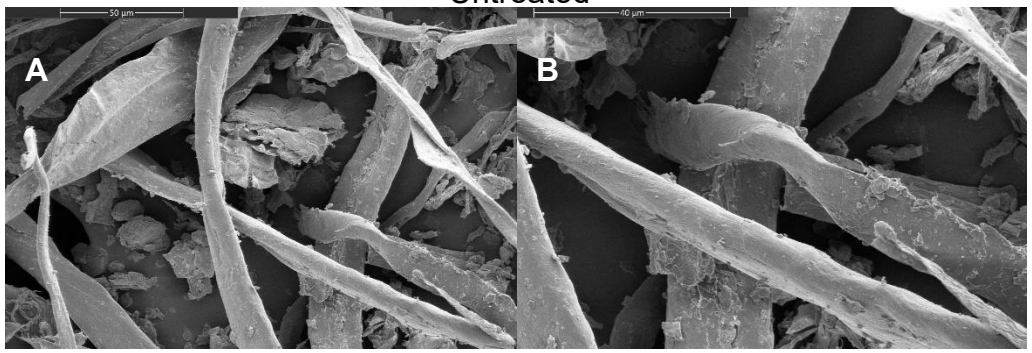
Scanning electron microscopy was applied to untreated and pretreated cotton to compare the structural modifications (Figure 3). The pretreated cotton images analyzed were from ball milling after 1, 3 and 6 h; alkali pretreatment using 10, 25, and 50% (w/w) of NaOH at 60 and 100 ºC; and biological pretreatment using *G. trabeum* with 15 days of growth in a liquid medium.

The untreated cotton appeared morphologically similar to to the alkali and biologically pretreated cotton, particularly when observed in the 500x and 1000x magnification. These pretreatments modified chemically the cotton, changing the polysaccharides and lignin content/percentage. For alkali pretreated cotton it was observed an increment of crystallinity when measured by x-ray analysis. However, this

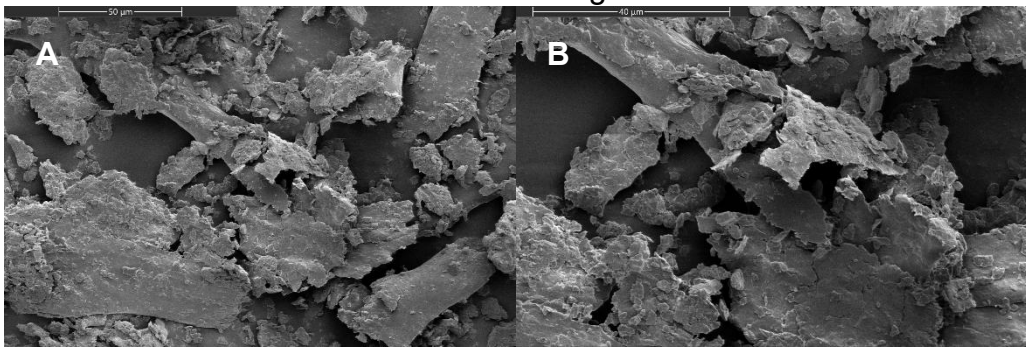
difference was not evident when the samples were observed by the SEM images. The most evident change in lignocellulosic structure by SEM images was the ball-milled cotton, which presented modifications right in the 3 h milling, increasing to a complete powder aspect after 6 h of milling. This image corroborates with x-ray analysis, where the 6 h ball-milled cotton presented no crystallinity at all. This result coincides with other studies reported elsewhere (HERNÁNDEZ-VARELA *et al*., 2020).

Figure 3.3 - SEM images from unetreated and pretreated cotton

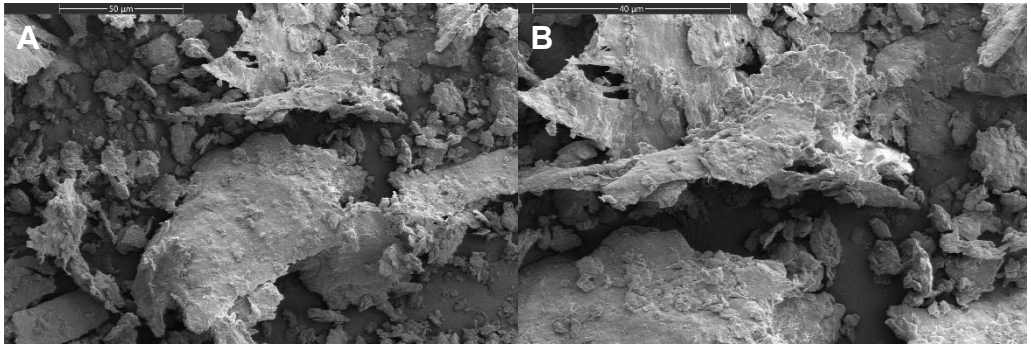
Untreated



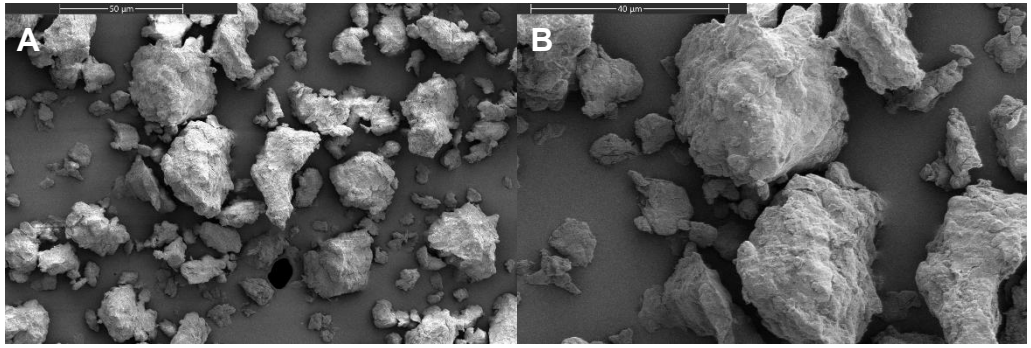
Ball milling 1 h



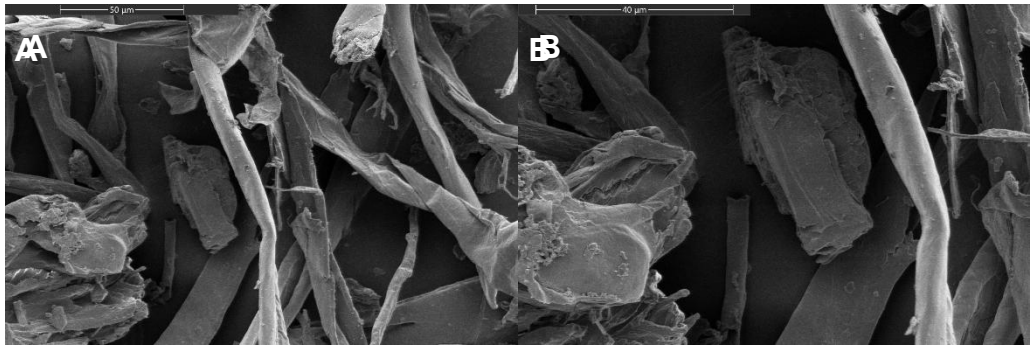
Ball milling 3 h



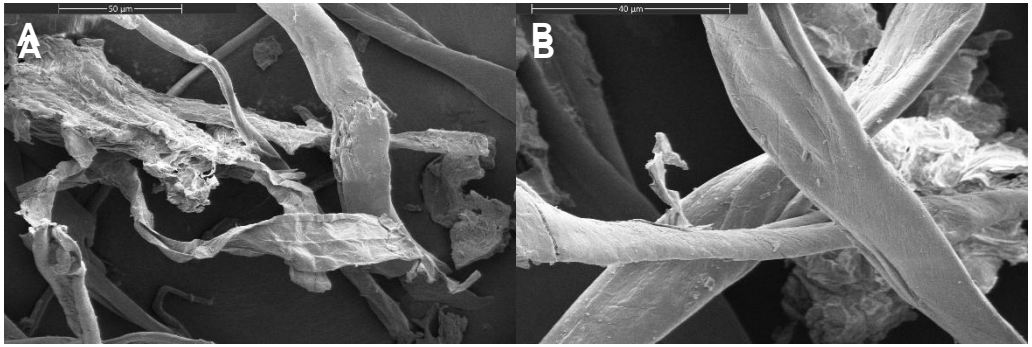
Ball milling 6 h



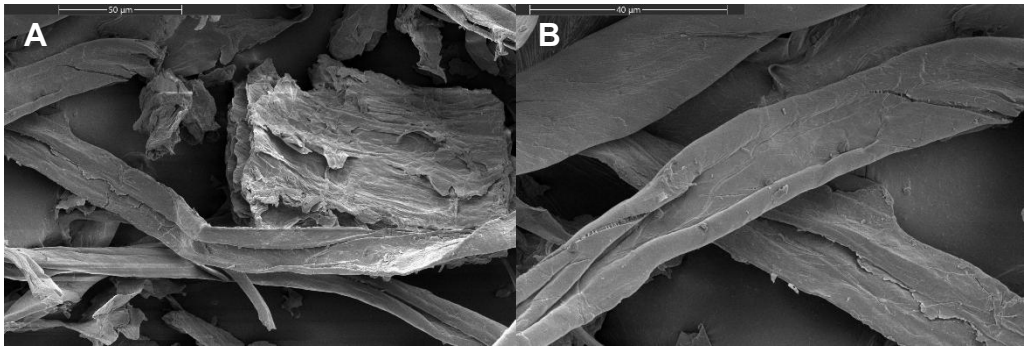
Alkali pretreatment 10 % (m/m) of NaOH at 60 ºC



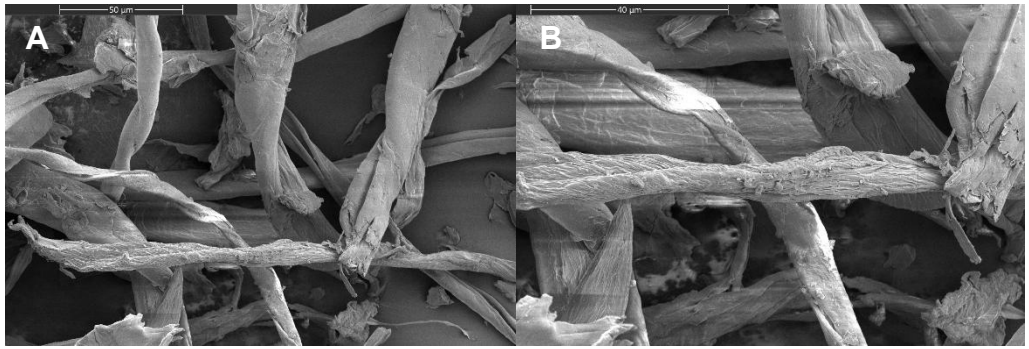
Alkali pretreatment 25 % (m/m) of NaOH at 60 ºC



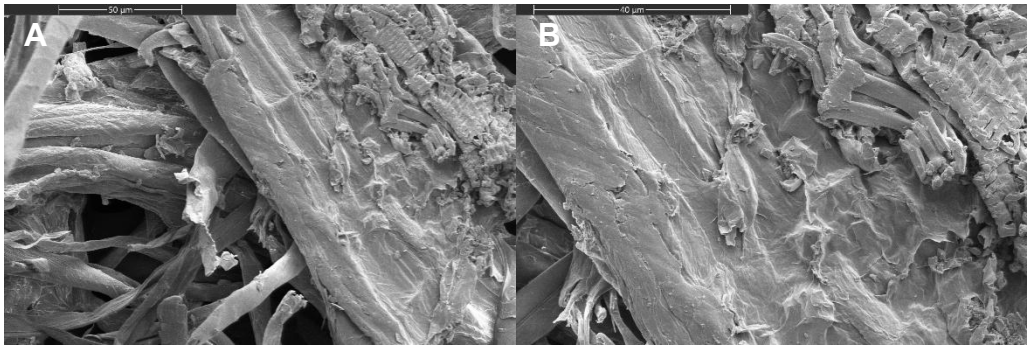
Alkali pretreatment 50 % (m/m) of NaOH at 60 ºC



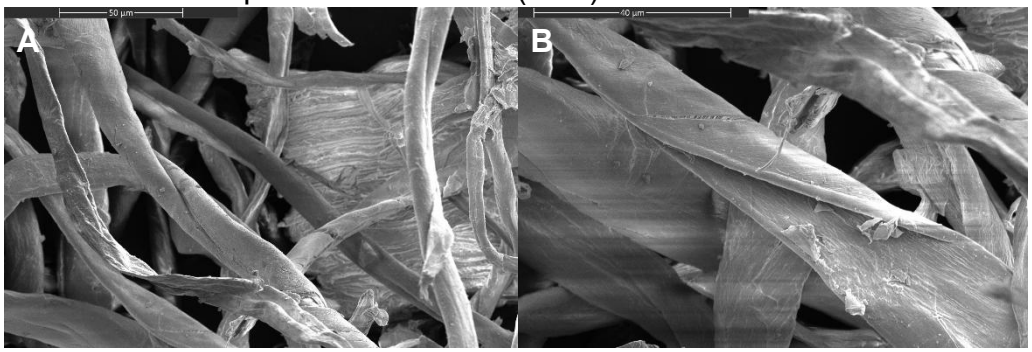
Alkali pretreatment 10 % (m/m) of NaOH at 100 ºC



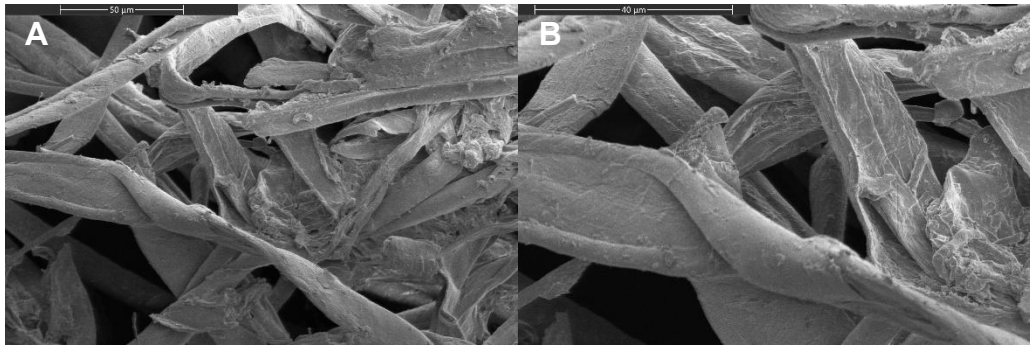
Alkali pretreatment 25 % (m/m) of NaOH at 100 ºC



Alkali pretreatment 50 % (m/m) of NaOH at 100 ºC



Biological pretreatment - *Gloeophyllum trabeum*



Source: Author. A: 1000x magnification, scale bar: 50 µm; B: 2000x magnification, scale bar: 40 µm

3.4 CONCLUSIONS

This study hypothesis was about the modifications in the cotton residue pretreatments aiming for a reduction of recalcitrance with consequent improvement of the COS yield after enzymatic hydrolysis. The modifications in the lignocellulosic structure by SEM are only perceptible in the ball-milled samples. These modifications in the ball-milled cotton are also clear in the x-ray analysis, where the crystallinity index was reduced, also, the results from COS yield confirm a reduction in the cotton recalcitrance after ball milling. However, the data from x-ray analysis confirm an increase in the biomass crystallinity index after alkali pretreatment. On the other side, the biological pretreatment stayed similar to untreated cotton for the COS yield, and for x-ray analysis and FTIR-ATR. This study indicates a great capacity for COS production from cotton residues using ball milling, and this pretreatment has the potential to be used together with alkali or biological pretreatment, for example. Future experiments could work based on the combination of the pretreatments, aiming for a better COS yield, a reduction in the total energy of the process, or a reduction in the chemicals involved.

Author contribution

Conceptualization: Michel Brienzo, Jefferson Felipuci; methodology: Jefferson Felipuci, Luiza Pinto, Danieli Marin; formal analysis and investigation: Jefferson Felipuci, Luiza Pinto; validation: Derlene de Angelis, João Sarava; writing—original draft preparation: Jefferson Felipuci; writing— review and editing: Michel Brienzo, Jefferson Felipuci, Luiza Pinto, Danieli Marin, Derlene de Angelis, João Sarava; supervision: Michel Brienzo and Derlene de Angelis.

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Competing Interest Statement:

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data Availability Statement:

The data supporting this study are available upon reasonable request to the corresponding author.

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THESIS GENERAL CONCLUSION AND FUTURE PERSPECTIVE

The hypothesis behind the thesis was the biomass recalcitrance reduction using different types of pretreatment such as chemical, physical and biological, and a combination of them to maximize the COS and XOS yield by enzymatic hydrolysis. Ball milling methods showed the best influence in the COS yield for both sugarcane bagasse and cotton residue. This pretreatment also decreased the crystallinity index for both biomasses, possibly reducing the recalcitrance. The combination of ball milling and biological pretreatment for COS production from sugarcane bagasse resulted in a yield improvement when compared to the knife mill and biological pretreated combination. Besides that, the XOS production was also possible combining biological pretreatment when milled with knife mill and xylanase applied. On the other side, the biological pretreatment in cotton residue maintained the same COS yield and crystallinity index when compared to the untreated sample. The chemical pretreatment of cotton residue showed a decrease in the crystallinity index, and an equal or less COS yield.

For future studies, could be interesting to optimize the chemical pretreatment such as a combination of alkali and ball milling pretreatment, for example, or use different combinations of concentrations and temperatures of reaction. The biological pretreatment has a huge potential to degrade lignocellulosic biomass, so the study of other microorganisms would be fundamental to the biotechnology and biomass conversion area.

ATTTACHMENTS

Figure 4 - Book published in 2020

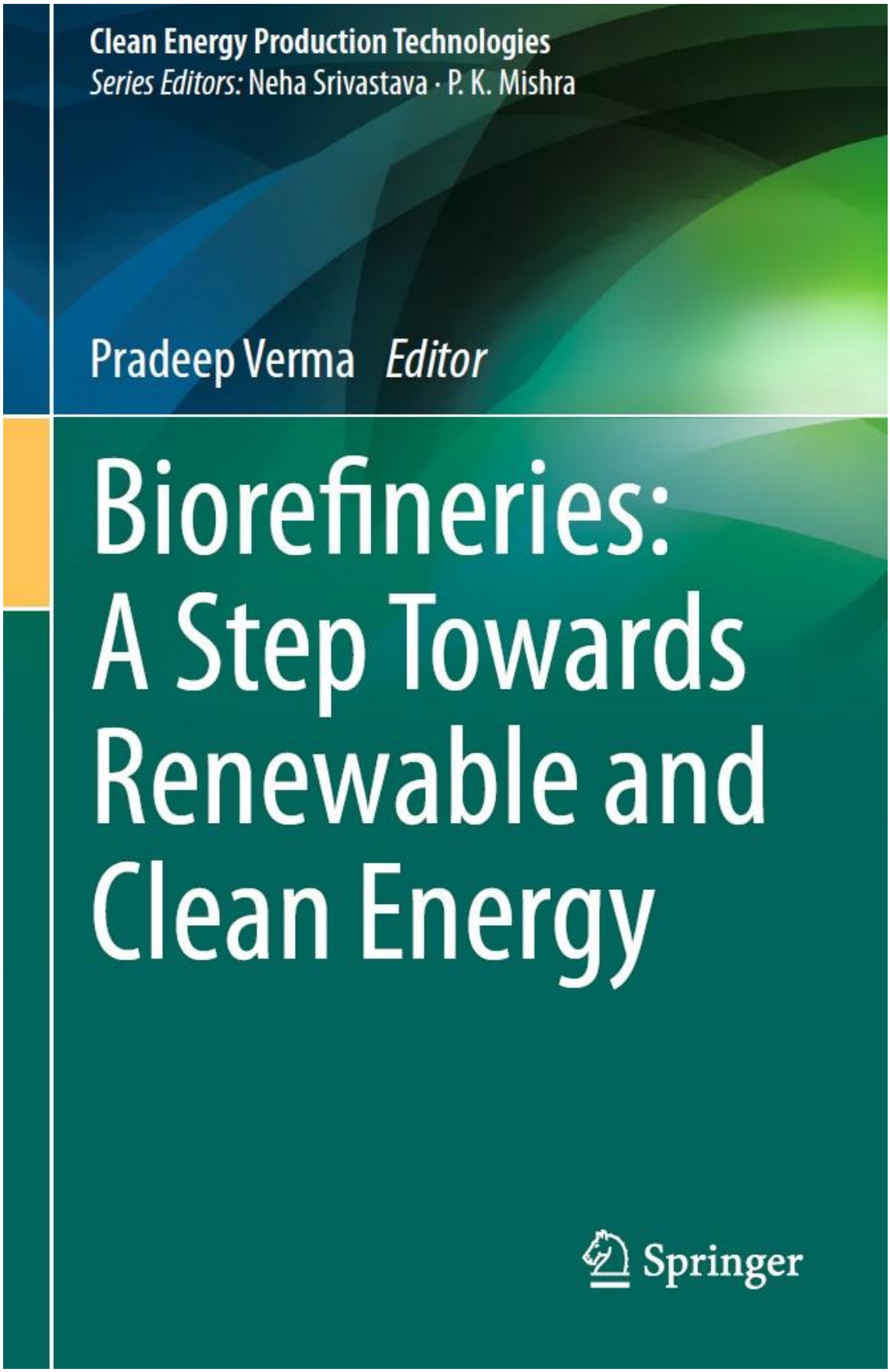

Figure 5 - Book chapter published in 2020

Chapter 6
Biotechnological Aspects of Microbial Pretreatment of Lignocellulosic Biomass

Jefferson Poles Felipuci, Caroline de Freitas, Hernan Dario Zamora Zamora, Derlene Attili Angelis, and Michel Brienzo

Abstract In several areas, products are obtained from lignocellulosic biomass, such as bioethanol and personal items. Notwithstanding, it features high recalcitrance, hence its use often demands pretreatment and hydrolysis stages to reach bio-based final products. Industrially, the most common method is the chemical pretreatment which, as the name implies, involves chemical components with potential environmental risks. This procedure is responsible to increase biomass accessibility and to enhance polysaccharides achieving in subsequent stages. Biological pretreatment presents a new perspective to replace or cooperate with its chemical counterpart, once microorganisms can modify the lignocellulosic structure and facilitate accessibility to macromolecules of interest. According to the above, this chapter covers the potential of biological pretreatment as well as the mechanisms of microbial degradation, their enzymes, and the impacts on the economy worldwide.

Keywords Microbial enzymes · Wood decay · Rot microorganisms · Recalcitrance · Biological delignification

Abbreviations

GHs Glycosyl hydrolases
LiP Lignin peroxidases

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Source: Author

Figure 6 - Paper published in 2024

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ORIGINAL ARTICLE

Efficient production of cellooligosaccharides and xylooligosaccharides by combined biological pretreatment and enzymatic hydrolysis process

Jefferson Poles Felipuci[1] · Caroline de Freitas[1] · Alex Otávio Sanches[2] · Renato Grillo[2] · Derlene Attili-Angelis[3,4] · Michel Brienzo[1]

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Abstract
Xylooligosaccharide and cellooligosaccharide production has been improved over the years using different kinds of methods. Chemical and physical methods are the most used due to their facility in reducing the biomass recalcitrance; however, such methods require a great deal of energy and generate undesirable compounds. On the other hand, biological pretreatment is an option to overcome the high energy demand and pollution issues and optimize production. In this work, sugarcane bagasse was biologically pretreated with *Coniophora puteana* (CBMAI 0870), *Gloeophyllum trabeum* (CBMAI 0872), and *Pleurotus ostreatus* (CCIBt 2338) for 5 months. After the biological pretreatment, part of the material was milled in a knife mill (20-mesh) and another part was milled in a ball mill (powder aspect). The material was enzymatic hydrolyzed with cellulase (20, 50, and 100 IU/g) and xylanase (50 IU/g) combined with the milling techniques to produce cellooligosaccharides and xylooligosaccharides. Xylooligosaccharides and cellooligosaccharides were characterized by ATR-FTIR analysis, scanning electron microscopy images and X-ray to evaluate the modifications in the lignocellulosic structure. The enzymatic hydrolysis using cellulase (50 IU/g) combined with knife milling resulted in 26.23% of cellooligosaccharide conversion after 5 months of cultivation with *C. puteana*, while cellulase with the same enzymatic charge combined with ball milling resulted in 36.65% of cellooligosaccharide conversion using the same fungus and the same time of cultivation. Xylooligosaccharide conversion also reached better results when compared to untreated material: the best result was 78.12% of xylooligosaccharide conversion after the biological pretreatment with *G. trabeum*. Finally, scanning electron microscope images made it possible to observe gaps formed by the fungi growth in the biomass in comparison to untreated material; and x-ray analysis showed evidence of the effect of ball milling pretreatment in the biomass, reducing its crystallinity.

Keywords Ball mill · Biomass recalcitrance · Non-digestible oligomers · Prebiotic compounds · Soluble fiber · White-rot fungi

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1 Introduction

In the literature, the process of converting lignocellulosic biomass into value-added products is commonly referred to as lignocellulosic biorefinery. This approach expands potential applications of lignocellulosic materials beyond generating heat energy and ethanol to include the production of by-products and value-added molecules [1–5]. Two value-added molecules are xylooligosaccharides (XOS) and cellooligosaccharides (COS). These molecules comprise xylose and glucose, respectively, and are known to be interesting to human health. XOS is considered healthy due to its capacity to prevent disorders in human nutrition, such as gastrointestinal disorders and infection, obesity control, and

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Source: Author

Attachment Table 2.1 - ANOVA test for chemical characterization comparison between samples. *G. trabeum* (Gt); *C. puteana* (Cp); *P. ostreatus* (Po).

CELLULOSE	untreated	*Gt m1*	*Gt m2*	*Gt m3*	*Gt m4*	*Gt m5*
untreated						
Gt m1	a					
Gt m2	a	b				
Gt m3	a	a	b			
Gt m4	a	a	a	a		
Gt m5	a	a	a	a	a	

CELLULOSE	untreated	*Cp m1*	*Cp m2*	*Cp m3*	*Cp m4*	*Cp m5*
untreated						
Cp m1	a					
Cp m2	a	a				
Cp m3	a	a	a			
Cp m4	b	b	b	b		
Cp m5	b	b	b	b	a	

CELLULOSE	untreated	*Po m1*	*Po m2*	*Po m3*	*Po m4*	*Po m5*
untreated						
Po m1	a					
Po m2	a	a				
Po m3	a	a	a			
Po m4	a	a	a	a		
Po m5	a	a	a	a	a	

HEMICELLULOSE	untreated	*Gt m1*	*Gt m2*	*Gt m3*	*Gt m4*	*Gt m5*
untreated						
Gt m1	b					
Gt m2	b	a				
Gt m3	b	a	a			
Gt m4	b	a	a	a		
Gt m5	b	a	a	a	a	

HEMICELLULOSE	untreated	*Cp m1*	*Cp m2*	*Cp m3*	*Cp m4*	*Cp m5*
untreated						
Cp m1	a					
Cp m2	b	a				
Cp m3	b	a	a			
Cp m4	b	b	a	a		
Cp m5	b	b	a	a	a	

HEMICELLULOSE	untreated	*Po m1*	*Po m2*	*Po m3*	*Po m4*	*Po m5*
untreated						
Po m1	a					
Po m2	b	a				
Po m3	b	a	a			
Po m4	b	a	a	a		
Po m5	b	b	a	a	a	

LIGNIN	untreated	*Gt m1*	*Gt m2*	*Gt m3*	*Gt m4*	*Gt m5*
untreated						
Gt m1	b					
Gt m2	b	a				
Gt m3	a	b	b			
Gt m4	b	a	a	b		
Gt m5	b	b	b	a	b	

LIGNIN	untreated	*Cp m1*	*Cp m2*	*Cp m3*	*Cp m4*	*Cp m5*
untreated						
Cp m1	a					
Cp m2	b	b				
Cp m3	b	a	a			
Cp m4	b	b	a	a		
Cp m5	b	a	a	a	a	

LIGNIN	untreated	*Po m1*	*Po m2*	*Po m3*	*Po m4*	*Po m5*
untreated						
Po m1	a					
Po m2	a	a				
Po m3	a	a	a			
Po m4	b	a	a	a		
Po m5	a	a	a	a	a	

Source: author. Statistical analysis: ANOVA

Attachment Table 2.2 - Yield of glucose and COS (%) after enzymatic hydrolysis of biologically pretreated sugarcane bagasse using three different enzymatic charges of cellulase.

Samples (20-mesh)	Cultivation time	Enzymatic charge																			
		20 IU/g							50 IU/g							100 IU/g						
Fungi	Month	Glucose	C2	C3	C4	C5	C6	>C6	Glucose	C2	C3	C4	C5	C6	>C6	Glucose	C2	C3	C4	C5	C6	>C6
Untreated		45,12	0	0	1,04	0	0,28	1,12	48,11	0	0	3,02	0,61	0,83	1,21	0	0,00	0,001	5,67	0	2,62	1,12
G. trabeum	1	23,17	0	0	0,30	0	4,33	0	13,52	0,92	0	0,10	0	11,36	0	13,13	1,50	1,11	0,10	8,31	5,88	0,02
	2	33,74	0,02	0	0,13	0	3,95	0	20,20	0,16	0	0,09	0	6,81	0	8,03	0,63	0	0,09	4,99	4,42	0
	3	35,01	0,04	0	0,11	0	6,40	0	48,00	0,16	0	0,11	0	12,51	0	6,66	0,005	0,64	0,11	0	14,24	0
	4	45,45	0,02	0	0,09	0	5,86	0	40,58	0,50	0	0,09	0	7,50	0	2,81	0,50	0	0,09	0	8,59	0
	5	71,69	2,17	0,06	0,09	0	12,17	0	41,63	1,51	0	0,09	0	5,09	4,69	2,99	1,00	0	0,09	6,39	2,42	1,63
C. puteana	1	49,86	0	0	0,09	0	2,23	0	29,09	0,19	0	0,09	0	4,60	0	9,08	0,38	0	0,09	3,84	2,26	0,004
	2	62,55	0,46	0	0,09	0	4,16	0	48,55	0,97	0	0,14	0	8,68	0	6,93	0,62	0	0,09	6,06	4,11	0,00
	3	31,86	0,17	0	0,09	0	5,54	0	45,15	0,70	0	0,09	0	8,23	0	8,06	0,70	0	0,10	12,44	0	0,0002
	4	83,13	0,18	0	0,12	0	9,80	0	34,89	0,89	0	0,12	0	11,47	0	3,75	0,99	0	0,12	0	10,69	0
	5	85,27	1,10	0	0,19	0	20,51	0	15,44	2,32	0	0,13	0	23,96	0	20,18	2,52	0	0,13	9,12	13,48	0
P. ostreatus	1	67,33	0	0	0,11	0	5,13	0,007	47,15	0	0	0,11	0	2,56	3,27	0	0,06	0	0,11	0	6,05	0
	2	92,37	0	0	0,10	0	5,53	0	34,76	0,04	0	0,10	0	2,38	1,02	5,79	0,05	0	0,10	0	4,75	0,71
	3	96,68	0	0	0,10	0	7,72	0	41,02	0,05	0	0,15	0	2,19	2,31	0	0,04	0	0,10	0	2,86	1,78
	4	41,51	0	0,12	0,09	0	2,97	0,92	24,20	0,29	0	0,09	0	2,79	3,23	9,53	0,20	0	0,09	0	4,80	1,23
	5	74,34	0	0	0,10	0	3,54	1,62	40,64	0,03	0	0,10	0	2,28	2,28	5,14	0,13	0	0,10	0	3,74	0,58

Source: author. Statistical analysis: ANOVA. *C1* glucose; *C2* celobiose; *C3* celotriose; *C4* celotetraose; *C5* celopentaose; *C6* celohexaose; > C6 chains biggers than celohexaose. *COS* gluco-oligosaccharides = C2 + C3 + C4 + C5 + C6 + > C6

Attachment Table 2.3 - ANOVA test for enzymatic hydrolysis (cellulase) comparison between samples for each enzymatic charge (20, 50 and 100 IU/g). *G. trabeum* (Gt); *C. puteana* (Cp); *P. ostreatus* (Po).

20 IU/g	**untreated**	***Gt m1***	***Gt m2***	***Gt m3***	***Gt m4***	***Gt m5***
untreated						
Gt m1	a					
Gt m2	a	a				
Gt m3	a	a	a			
Gt m4	a	a	a	a		
Gt m5	b	b	b	b	b	

20 **IU/g**	**untreated**	***Cp m1***	***Cp m2***	***Cp m3***	***Cp m4***	***Cp m5***
untreated						
Cp m1	a					
Cp m2	a	a				
Cp m3	a	a	a			
Cp m4	a	a	a	a		
Cp m5	b	b	b	b	b	

20 **IU/g**	**untreated**	***Po m1***	***Po m2***	***Po m3***	***Po m4***	***Po m5***
untreated						
Po m1	a					
Po m2	a	a				
Po m3	b	a	a			
Po m4	a	a	a	b		
Po m5	a	a	a	a	a	

50 IU/g	**untreated**	***Gt m1***	***Gt m2***	***Gt m3***	***Gt m4***	***Gt m5***
untreated						
Gt m1	b					
Gt m2	a	b				
Gt m3	b	a	b			
Gt m4	a	b	a	b		
Gt m5	b	a	b	a	a	

50 **IU/g**	**untreated**	***Cp m1***	***Cp m2***	***Cp m3***	***Cp m4***	***Cp m5***
untreated						
Cp m1	a					
Cp m2	a	a				
Cp m3	a	a	a			
Cp m4	b	b	a	a		
Cp m5	b	b	b	b	b	

50 **IU/g**	**untreated**	***Po m1***	***Po m2***	***Po m3***	***Po m4***	***Po m5***
untreated						
Po m1	a					
Po m2	a	a				
Po m3	a	a	a			
Po m4	a	a	a	a		
Po m5	a	a	a	a	a	

100 IU/g	untreated	*Gt m1*	*Gt m2*	*Gt m3*	*Gt m4*	*Gt m5*
untreated						
Gt m1	b					
Gt m2	a	a				
Gt m3	a	a	a			
Gt m4	a	b	a	a		
Gt m5	a	a	a	a	a	

100 **IU/g**	untreated	*Cp m1*	*Cp m2*	*Cp m3*	*Cp m4*	*Cp m5*
untreated						
Cp m1	a					
Cp m2	a	a				
Cp m3	a	a	a			
Cp m4	a	a	a	a		
Cp m5	b	b	b	b	b	

100 **IU/g**	untreated	*Po m1*	*Po m2*	*Po m3*	*Po m4*	*Po m5*
untreated						
Po m1	a					
Po m2	a	a				
Po m3	b	a	a			
Po m4	a	a	a	a		
Po m5	b	a	a	a	a	

	UI/g		
	20x50	20x100	50x100
untreated	A	A	A
Gt m1	A	B	A
Gt m2	A	A	A
Gt m3	B	B	A
Gt m4	A	A	A
Gt m5	A	A	A
Cp m1	A	A	A
Cp m2	A	A	A
Cp m3	A	B	A
Cp m4	A	A	A
Cp m5	A	A	A
Po m1	A	A	A
Po m2	A	A	A
Po m3	B	A	A
Po m4	A	A	A
Po m5	A	A	A

Source: author. Statistical analysis: ANOVA

Attachment Table 2.4 - ANOVA test for enzymatic hydrolysis (cellulase) comparison between samples for 50 IU/g of enzymatic charge for ball milling method. *G. trabeum* (Gt); *C. puteana* (Cp); *P. ostreatus* (Po).

50 IU/g	**untreated**	***Gt m1***	***Gt m5***
untreated			
Gt m1	b		
Gt m5	a	b	

50 **IU/g**	**untreated**	***Cp m2***	***Cp m5***
untreated			
Cp m2	b		
Cp m5	b	b	

50 IU/g	**untreated**	***Po m1***	***Po m4***
untreated			
Po m1	a		
Po m4	b	b	

Source: author. Statistical analysis: ANOVA

Attachment Table 2.5 - ANOVA test for enzymatic hydrolysis (xylanase) comparison between samples for 50 IU/g of enzymatic charge. *G. trabeum* (Gt); *C. puteana* (Cp); *P. ostreatus* (Po).

50 IU/g	**untreated**	***Gt m1***	***Gt m2***	***Gt m3***	***Gt m4***	***Gt m5***
untreated						
Gt m1	b					
Gt m2	b	b				
Gt m3	b	b	b			
Gt m4	b	b	b	a		
Gt m5	b	b	a	a	a	

50 **IU/g**	**untreated**	***Cp m1***	***Cp m2***	***Cp m3***	***Cp m4***	***Cp m5***
untreated						
Cp m1	b					
Cp m2	b	b				
Cp m3	b	b	b			
Cp m4	b	b	b	a		
Cp m5	b	b	a	b	b	

50 **IU/g**	**untreated**	***Po m1***	***Po m2***	***Po m3***	***Po m4***	***Po m5***
untreated						
Po m1	b					
Po m2	b	b				
Po m3	b	a	b			
Po m4	b	a	b	a		
Po m5	b	b	a	b	b	

Source: author. Statistical analysis: ANOVA

Attachment Table 3.1 - ANOVA test for chemical composition comparison between samples.

CELLULOSE	Untreated	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	Biological pretreatment - Gloeophyllum trabeum
Untreated								
AP 60 ºC - 10 % (m/m) NaOH	B							
AP 60 ºC - 25 % (m/m) NaOH	B	B						
AP 60 ºC - 50 % (m/m) NaOH	B	A	A					
AP 100 ºC - 10 % (m/m) NaOH	B	A	A	A				
AP 100 ºC - 25 % (m/m) NaOH	B	B	A	B	B			
AP 100 ºC - 50 % (m/m) NaOH	B	B	A	A	A	A		
Biological pretreatment - *Gloeophyllum trabeum*	B	B	B	B	B	B	B	

HEMICELLULOSE	Untreated	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	Biological pretreatment - Gloeophyllum trabeum
Untreated								
AP 60 ºC - 10 % (m/m) NaOH	B							
AP 60 ºC - 25 % (m/m) NaOH	B	A						
AP 60 ºC - 50 % (m/m) NaOH	B	A	A					
AP 100 ºC - 10 % (m/m) NaOH	B	A	A	A				
AP 100 ºC - 25 % (m/m) NaOH	B	A	A	A	A			
AP 100 ºC - 50 % (m/m) NaOH	B	A	A	A	A	A		
Biological pretreatment - *Gloeophyllum trabeum*	B	A	A	A	A	A	A	

LIGNIN	Untreated	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	Biological pretreatment - Gloeophyllum trabeum
Untreated								
AP 60 ºC - 10 % (m/m) NaOH	B							
AP 60 ºC - 25 % (m/m) NaOH	B	A						
AP 60 ºC - 50 % (m/m) NaOH	B	A	A					
AP 100 ºC - 10 % (m/m) NaOH	B	B	A	A				
AP 100 ºC - 25 % (m/m) NaOH	B	B	B	B	A			
AP 100 ºC - 50 % (m/m) NaOH	B	A	A	A	B	B		
Biological pretreatment - *Gloeophyllum trabeum*	B	B	B	B	B	B	B	

Source: author. Statistical analysis: ANOVA

Attachment Table 3.2 - ANOVA test for glucose using 20, 50 and 100 IU/g of enzyme charge

Glucose 20 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	A									
Ball-milling (6 h)	B	B	B								
AP 60 ºC - 10 % (m/m) NaOH	B	A	A	B							
AP 60 ºC - 25 % (m/m) NaOH	A	A	B	B	A						
AP 60 ºC - 50 % (m/m) NaOH	A	B	B	B	B	A					
AP 100 ºC - 10 % (m/m) NaOH	A	A	B	B	A	A	A				
AP 100 ºC - 25 % (m/m) NaOH	A	B	B	B	B	A	A	A			
AP 100 ºC - 50 % (m/m) NaOH	A	A	B	B	A	A	A	A	A		
BP - Gloeophyllum trabeum	A	A	B	B	A	A	A	A	A	A	

Glucose 50 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	B									
Ball-milling (6 h)	B	B	A								
AP 60 ºC - 10 % (m/m) NaOH	B	B	B	B							
AP 60 ºC - 25 % (m/m) NaOH	B	B	B	B	A						
AP 60 ºC - 50 % (m/m) NaOH	A	B	B	B	B	B					
AP 100 ºC - 10 % (m/m) NaOH	B	A	B	B	A	A	B				
AP 100 ºC - 25 % (m/m) NaOH	B	A	B	B	B	A	B	A			
AP 100 ºC - 50 % (m/m) NaOH	B	A	B	B	A	A	B	A	A		
BP - Gloeophyllum trabeum	B	A	B	B	A	A	B	A	A	A	

Glucose 100 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	A									
Ball-milling (6 h)	B	B	B								
AP 60 ºC - 10 % (m/m) NaOH	B	A	A	B							
AP 60 ºC - 25 % (m/m) NaOH	B	A	A	B	A						
AP 60 ºC - 50 % (m/m) NaOH	A	B	B	B	A	B					
AP 100 ºC - 10 % (m/m) NaOH	B	A	A	B	A	A	B				
AP 100 ºC - 25 % (m/m) NaOH	B	A	A	B	A	A	B	A			
AP 100 ºC - 50 % (m/m) NaOH	B	A	A	B	A	A	B	A	A		
BP - Gloeophyllum trabeum	B	B	B	B	A	A	A	A	A	B	

Source: author. Statistical analysis: ANOVA

Attachment Table 3.3 - ANOVA test for C2 using 20, 50 and 100 IU/g of enzyme charge

C2 20 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	B									
Ball-milling (6 h)	B	B	B								
AP 60 ºC - 10 % (m/m) NaOH	A	B	B	B							
AP 60 ºC - 25 % (m/m) NaOH	B	B	B	B	A						
AP 60 ºC - 50 % (m/m) NaOH	B	A	B	B	B	A					
AP 100 ºC - 10 % (m/m) NaOH	B	A	B	B	B	B	A				
AP 100 ºC - 25 % (m/m) NaOH	B	A	B	B	B	A	A	A			
AP 100 ºC - 50 % (m/m) NaOH	B	B	A	B	B	B	B	B	B		
BP - Gloeophyllum trabeum	B	A	B	B	B	B	A	A	A	B	

C2 50 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	B									
Ball-milling (6 h)	B	B	B								
AP 60 ºC - 10 % (m/m) NaOH	B	B	B	B							
AP 60 ºC - 25 % (m/m) NaOH	A	B	B	B	B						
AP 60 ºC - 50 % (m/m) NaOH	B	A	B	B	B	B					
AP 100 ºC - 10 % (m/m) NaOH	B	A	B	B	B	B	A				
AP 100 ºC - 25 % (m/m) NaOH	B	B	B	B	B	B	B	B			
AP 100 ºC - 50 % (m/m) NaOH	B	B	B	B	B	B	B	B	B		
BP - Gloeophyllum trabeum	B	B	B	B	A	B	B	B	B	B	

C2 100 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	B									
Ball-milling (6 h)	B	B	B								
AP 60 ºC - 10 % (m/m) NaOH	B	B	B	B							
AP 60 ºC - 25 % (m/m) NaOH	B	B	B	B	A						
AP 60 ºC - 50 % (m/m) NaOH	B	A	B	B	A	B					
AP 100 ºC - 10 % (m/m) NaOH	B	B	B	B	A	A	A				
AP 100 ºC - 25 % (m/m) NaOH	B	A	B	B	A	B	A	A			
AP 100 ºC - 50 % (m/m) NaOH	B	A	A	B	B	B	B	B	A		
BP - Gloeophyllum trabeum	B	B	B	B	A	A	B	A	B	B	

Source: author. Statistical analysis: ANOVA

Attachment Table 3.4 - ANOVA test for >C3 using 20, 50 and 100 IU/g of enzyme charge

>C3 20 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	A									
Ball-milling (6 h)	B	A	A								
AP 60 ºC - 10 % (m/m) NaOH	B	B	B	B							
AP 60 ºC - 25 % (m/m) NaOH	B	B	B	B	A						
AP 60 ºC - 50 % (m/m) NaOH	B	B	B	B	A	A					
AP 100 ºC - 10 % (m/m) NaOH	B	B	B	B	A	A	A				
AP 100 ºC - 25 % (m/m) NaOH	B	B	B	B	A	A	A	A			
AP 100 ºC - 50 % (m/m) NaOH	B	B	B	B	A	A	A	A	A		
BP - Gloeophyllum trabeum	B	B	B	B	A	A	A	A	B	B	

>C3 50 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	B									
Ball-milling (6 h)	B	B	B								
AP 60 ºC - 10 % (m/m) NaOH	B	B	B	B							
AP 60 ºC - 25 % (m/m) NaOH	B	B	B	B	A						
AP 60 ºC - 50 % (m/m) NaOH	B	B	B	B	A	A					
AP 100 ºC - 10 % (m/m) NaOH	B	B	B	B	A	A	A				
AP 100 ºC - 25 % (m/m) NaOH	B	B	B	B	A	A	A	A			
AP 100 ºC - 50 % (m/m) NaOH	B	B	B	B	A	A	A	A	A		
BP - Gloeophyllum trabeum	B	B	B	B	B	B	B	B	B	B	

>C3 100 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 ºC - 10 % (m/m) NaOH	AP 60 ºC - 25 % (m/m) NaOH	AP 60 ºC - 50 % (m/m) NaOH	AP 100 ºC - 10 % (m/m) NaOH	AP 100 ºC - 25 % (m/m) NaOH	AP 100 ºC - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	A										
Ball-milling (3 h)	B	A									
Ball-milling (6 h)	B	A	A								
AP 60 ºC - 10 % (m/m) NaOH	B	B	B	B							
AP 60 ºC - 25 % (m/m) NaOH	B	B	B	B	A						
AP 60 ºC - 50 % (m/m) NaOH	B	B	B	B	A	A					
AP 100 ºC - 10 % (m/m) NaOH	B	B	B	B	A	A	A				
AP 100 ºC - 25 % (m/m) NaOH	B	B	B	B	A	A	A	A			
AP 100 ºC - 50 % (m/m) NaOH	B	B	B	B	A	A	A	A	A		
BP - Gloeophyllum trabeum	A	B	B	B	A	A	B	A	B	A	

Source: author. Statistical analysis: ANOVA

Attachment Table 3.5 - ANOVA test for enzyme charge comparison used for COS production

20 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 °C - 10 % (m/m) NaOH	AP 60 °C - 25 % (m/m) NaOH	AP 60 °C - 50 % (m/m) NaOH	AP 100 °C - 10 % (m/m) NaOH	AP 100 °C - 25 % (m/m) NaOH	AP 100 °C - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	B									
Ball-milling (6 h)	B	B	B								
AP 60 °C - 10 % (m/m) NaOH	B	B	B	B							
AP 60 °C - 25 % (m/m) NaOH	B	B	B	B	A						
AP 60 °C - 50 % (m/m) NaOH	B	B	B	B	A	A					
AP 100 °C - 10 % (m/m) NaOH	B	B	B	B	A	A	A				
AP 100 °C - 25 % (m/m) NaOH	B	B	B	B	A	A	A	A			
AP 100 °C - 50 % (m/m) NaOH	A	B	B	B	B	B	B	A	B		
BP - Gloeophyllum trabeum	A	B	B	B	B	B	B	A	B	A	

50 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 °C - 10 % (m/m) NaOH	AP 60 °C - 25 % (m/m) NaOH	AP 60 °C - 50 % (m/m) NaOH	AP 100 °C - 10 % (m/m) NaOH	AP 100 °C - 25 % (m/m) NaOH	AP 100 °C - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	B									
Ball-milling (6 h)	B	B	B								
AP 60 °C - 10 % (m/m) NaOH	B	B	B	B							
AP 60 °C - 25 % (m/m) NaOH	B	B	B	B	B						
AP 60 °C - 50 % (m/m) NaOH	A	B	B	B	A	B					
AP 100 °C - 10 % (m/m) NaOH	A	B	B	B	A	B	A				
AP 100 °C - 25 % (m/m) NaOH	A	B	B	B	B	B	A	A			
AP 100 °C - 50 % (m/m) NaOH	A	B	B	B	B	B	A	A	A		
BP - Gloeophyllum trabeum	A	B	B	B	B	B	A	A	A	A	

100 IU/g	Untreated	Ball-milling (1 h)	Ball-milling (3 h)	Ball-milling (6 h)	AP 60 °C - 10 % (m/m) NaOH	AP 60 °C - 25 % (m/m) NaOH	AP 60 °C - 50 % (m/m) NaOH	AP 100 °C - 10 % (m/m) NaOH	AP 100 °C - 25 % (m/m) NaOH	AP 100 °C - 50 % (m/m) NaOH	BP - Gloeophyllum trabeum
Untreated											
Ball-milling (1 h)	B										
Ball-milling (3 h)	B	B									
Ball-milling (6 h)	B	B	B								
AP 60 °C - 10 % (m/m) NaOH	A	B	B	B							
AP 60 °C - 25 % (m/m) NaOH	A	B	B	B	A						
AP 60 °C - 50 % (m/m) NaOH	A	B	B	B	A	A					
AP 100 °C - 10 % (m/m) NaOH	A	B	B	B	A	A	A				
AP 100 °C - 25 % (m/m) NaOH	A	B	B	B	A	A	A	A			
AP 100 °C - 50 % (m/m) NaOH	B	B	B	B	B	B	A	B	A		
BP - Gloeophyllum trabeum	A	B	B	B	A	A	A	A	A	A	

Source: author. Statistical analysis: ANOVA

Attachment Table 3.6 - ANOVA test for pretreatment comparison used for COS production

Untreated	20	50	100
20			
50	A		
100	B	B	

Ball-milling (1 h)	20	50	100
20			
50	A		
100	B	A	

Ball-milling (3 h)	20	50	100
20			
50	B		
100	B	B	

Ball-milling (6 h)	20	50	100
20			
50	B		
100	B	A	

AP 60 ºC - 10 % (m/m) NaOH	20	50	100
20			
50	B		
100	B	B	

AP 60 ºC - 25 % (m/m) NaOH	20	50	100
20			
50	A		
100	B	B	

AP 60 ºC - 50 % (m/m) NaOH	20	50	100
20			
50	B		
100	B	B	

AP 100 ºC - 10 % (m/m) NaOH	20	50	100
20			
50	B		
100	B	B	

AP 100 ºC - 25 % (m/m) NaOH	20	50	100
20			
50	B		
100	B	B	

AP 100 ºC - 50 % (m/m) NaOH	20	50	100
20			
50	B		
100	B	B	

BP - Gloeophyllum trabeum	20	50	100
20			
50	A		
100	B	B	

Source: author. Statistical analysis: ANOVA