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"JÚLIO DE MESQUITA FILHO"
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SYNERGY BETWEEN CHEMICAL ADDITIVES AND PRETREATMENTS OF SUGARCANE BIOMASS FAVORING ENZYMATIC HYDROLYSIS AND PRODUCTION OF FUEL ETHANOL

ALISON ANDREI SCHMATZ

Rio Claro – SP
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Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Microbiologia Aplicada).

Orientador: Prof. Dr. Michel Brienzo.
Coorientador: Prof. Dr. Jonas Contiero.

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Você não sabe o quanto eu caminhei
Pra chegar até aqui
Percorri milhas e milhas antes de dormir
Eu nem cochilei
Os mais belos montes escalei
Nas noites escuras de frio chorei, ei, ei, ei
Ei, ei, ei, ei, ei, ei, ei

A vida ensina e o tempo traz o tom
Pra nascer uma canção
Com a fé do dia a dia encontro a solução
Encontro a solução

Quando bate a saudade eu vou pro mar
Fecho os meus olhos e sinto você chegar
Você chegar
Psicon! Psicon! Psicon! Psicon!

Quero acordar de manhã do teu lado
E aturar qualquer babado
Vou ficar apaixonado
No teu seio aconchegado
Ver você dormindo e sorrindo
É tudo que eu quero pra mim
Tudo que eu quero pra mim
Quero!

Você não sabe o quanto eu caminhei
Pra chegar até aqui
Percorri milhas e milhas antes de dormir
Eu nem cochilei
Os mais belos montes escalei
Nas noites escuras de frio chorei, ei, ei, ei
Ei, ei, ei, ei, ei, ei, ei

Together, together

Meu caminho só meu pai pode mudar
Meu caminho só meu pai
Meu caminho só meu pai

Together, together

A estrada – Cidade Negra

ABSTRACT

Sugarcane bagasse is a feedstock material in the production of second-generation ethanol (2G) and value-added products with biotechnological potential. However, biomass needs pretreatments to break down the cellulose-hemicellulose-lignin complex. During acid and hydrothermic pretreatments, can occur formation of sugar degradation products (toxic to fermenting microorganisms), which can condense with lignin fragments forming the pseudo-lignin, harmful to enzymatic hydrolysis. Therefore, this study aimed evaluate the hemicellulose and lignin removal from sugarcane biomass during diluted acid and organosolv pretreatment with addition of low-cost antioxidants, and their influences on the suppression the inhibitors formation and fermentable sugars production in simultaneous saccharification and fermentation (SSF). Different sugarcane fractions showed different recalcitrance. External fraction showed lower yields in the enzymatic hydrolysis, and 5 g L^{-1} of acetic acid in the hydrolysate from acid pretreatment. The content of extractives and lignin were identified as key factor contributing to the formation of pseudo-lignin. Partial delignified and acid pretreated biomass showed yields of approximately 90% of cellulose conversion by enzymatic hydrolysis (up to 12 g L^{-1}). Butylated hydroxytoluene (BHT) demonstrated a potential effect on the removal of lignin from biomass in acid pretreatment (121 °C). With 50% (v/v) ethanol, BHT induced 10 g 100 g^{-1} more lignin removal. Addition of BHT in acid pretreatment resulted in lower formation of inhibitors in liquid fraction (0.01 g L^{-1} of furfural, 0.01 g L^{-1} of HMF and 0.68 g L^{-1} of acetic acid) and low residual hemicellulose and lignin content in the pretreated biomass. All the 6 chemical compounds (tert-butylhydroquinone; 3-tert-butyl-4-hydroxyanisole; methyl 3,4,5-trihydroxybenzoate; Tween 20; Tween 80 and dimethyl sulfoxide) individually improved lignin removal and enzyme hydrolysis yield. The 3-tert-butyl-4-hydroxyanisole showed 71% lignin removal, while Tween 80 used in the pretreatment resulted in a material with high digestibility, 98.9% glucose yield by enzymatic hydrolysis. After 48 h of SSF, 17.06 g L^{-1} of glucose and 15.17 g L^{-1} of ethanol were observed in delignified-acid pretreated biomass (DL-PT). Removal of hemicellulose and lignin resulted in higher yields conversion of sugars. Antioxidants and surfactants were identified as potential lignin removal effect, indicating that they can be applied in biotechnological process.

Keywords: Saccharification. Fermentation. Bioethanol. Pseudo-lignin. Biorefinery. Lignocellulosic biomass.

RESUMO

O bagaço de cana-de-açúcar é matéria-prima na produção de etanol de segunda geração (2G) e produtos de valor agregado com potencial biotecnológico. No entanto, a biomassa precisa de pré-tratamentos para quebrar o complexo celulose-hemicelulose-lignina. Durante os pré-tratamentos ácido e hidrotérmico, pode ocorrer a formação de produtos de degradação de açúcares (tóxico para microrganismos fermentadores), que podem se condensar com fragmentos de lignina formando a pseudo-lignina, prejudicial à hidrólise enzimática. Portanto, este estudo teve como objetivo avaliar a remoção de hemicelulose e lignina da biomassa da cana-de-açúcar durante o pré-tratamento ácido diluído e organosolv com adição de antioxidantes de baixo custo, e suas influências na supressão da formação de inibidores e produção de açúcares fermentáveis na fermentação e sacarificação simultânea (SSF). Diferentes frações da cana-de-açúcar apresentaram diferentes recalcitrâncias. A fração externa apresentou menores rendimentos na hidrólise enzimática e 5 g L^{-1} de ácido acético no hidrolisado do pré-tratamento ácido. O conteúdo de extrativos e lignina foram identificados como fatores chave contribuindo para a formação de pseudo-lignina. A biomassa parcialmente deslignificada e pré-tratada com ácido apresentou rendimentos de aproximadamente 90% de conversão de celulose na hidrólise enzimática (até 12 g L^{-1}). O hidroxibutil tolueno (BHT) demonstrou potencial efeito na remoção de lignina da biomassa no pré-tratamento ácido (121 ° C). Com 50% etanol (v/v), o BHT induziu 10 g 100 g^{-1} a mais de remoção de lignina. A adição de BHT no pré-tratamento ácido resultou em menor formação de inibidores na fração líquida (0,01 g L^{-1} de furfural, 0,01 g L^{-1} de HMF e 0,68 g L^{-1} de ácido acético) e baixo teor residual de hemicelulose e lignina na biomassa pré-tratada. Todos os 6 compostos químicos (terc-butil hidroquinona; 3-terc-butil-4-hidroxianisol; 3,4,5-trihidroxi benzoato de metila; Tween 20; Tween 80 e dimetilsulfóxido) melhoraram individualmente a remoção de lignina e o rendimento da hidrólise enzimática. O 3-terc-butil-4-hidroxianisol apresentou 71% de remoção de lignina, enquanto o Tween 80 utilizado no pré-tratamento resultou em um material com alta digestibilidade, 98,9% de rendimento de glicose na hidrólise enzimática. Após 48 h de SSF, 17,06 g L^{-1} de glicose e 15,17 g L^{-1} de etanol foram observados na biomassa deslignificada e pré-tratada com ácido (DL-PT). A remoção de hemicelulose e lignina resultou em maiores rendimentos de conversão de açúcares. Antioxidantes e surfactantes foram identificados como potenciais removedores de lignina, indicando que podem ser aplicados em processos biotecnológicos.

Palavras-Chave: Sacarificação. Fermentação. Bioetanol. Pseudo-lignina. Biorrefinaria. Biomassa lignocelulósica.

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ABBREVIATIONS

2G ……..…..Second generation

ATR……...……..Attenuated total reflectance

BHT ……..Butylated hydroxytoluene

BHT-PT……...…….Butylated hydroxytoluene pretreated

CCoAOMT……..CaffeoylCoA *O*-methyltransferase

COMT……..……..Caffeic acid *O*-methyltransferase

DL…….……..Partial delignified

DL-PT……..……..Partial delignified acid pretreated

DMSO / DS……..Dimethyl sulfoxide

EF……..……..Extractive-free

EF-PT……...……..Extractive-free acid pretreated

F5H……...……..Ferulate 5-hydroxylase

FPU……..Filter paper units

FTIR……..……..Fourier Transform Infrared

HPLC ……..……..High Performance Liquid Chromatography

HMF ……..5- hydroxymethylfurfural

IN……..*In natura*

IN-PT ……..*In natura* acid pretreated

IU……..……International Units

MR…….……..Mass recovery

MT……..Methyl 3,4,5-trihydroxybenzoate

PT…….……..Diluted acid pretreatment

SEM……..……..Scanning electron microscopy

SSF..……..Simultaneous Saccharification and Fermentation

T2……..……..Tween 20

T8……..……..Tween 80

TB……...……..Tert-butylhydroquinone

TH……..……..3-tert-butyl-4-hydroxyanisole

WT……..Wild-type

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CHAPTER 1 INTRODUCTION AND MOTIVATION

The burning of fossil fuels (oil, natural gas and coal) increases the atmospheric concentrations of gases that cause global warming (SO_2, CO_2 and NO_x). Oil, gas and coal may continue to exist for decades to come, but the shift in energy matrices to low-carbon fuels is critical to slow climate change. Renewable energy sources are fundamental alternatives in the energy transition, migrating from fossil fuels to sustainable alternatives. Biotechnological processes have made it possible to convert biomass (plant and animal waste) into biofuels, which have become popular around the world (ABAS; KALAIR; KHAN, 2015).

Lignocellulosic biomass is abundant in the world and consists of carbohydrates (cellulose and hemicellulose) and lignin that can be converted into second generation-ethanol and value-added products (2G ethanol) (VIEIRA et al., 2020). Brazil is the largest producer and consumer of sugarcane in the world. In 2018, the country was responsible for the production of 746 million tons, almost double the second place (India, with 376 million tons) (FAOSTAT, 2018). For each ton of cane processed, 140 kg of dry bagasse and 140 kg of dry straw are generated (CARVALHO et al., 2014). In the state of São Paulo, the largest national producer, it was stipulated the gradual elimination of sugarcane straw burning during the harvest period, until 2021 in mechanizable areas and until 2031 in non-mechanizable areas (State Decree n ° 47.700 /2003; State Law n° 11241/2002), reducing the suspension of soot in the air and reducing the emission of carbon dioxide into the atmosphere (GONÇALVES et al., 2017). For this reason, Brazil is faced with a new abundant lignocellulosic material, the straw, which has potential in the production of 2G ethanol.

Due complex and recalcitrant structure of lignocellulosic biomass, pretreatments are necessary to fractionate the cellulose-hemicellulose-lignin complex and solubilize the sugar fraction of the biomass, converting it into fermentable sugars (glucose), an energy currency for various metabolic routes of microorganisms with biotechnological application (VIEIRA et al., 2020). However, in acidic and hydrothermal pretreatments, particularly in conditions of high severity (temperature, reaction time and acid loading), the formation of sugar degradation products can occur that have a negative impact on hydrolysis and fermentation yields. The degradation of glucose can generate 5-hydroxymethylfurfural (HMF), which can undergo reactions resulting in formic and levulinic acid, while the degradation of xylose can generate furfural. Aldehydes, aliphatic

and aromatic acids can also form and these compounds have an inhibitory impact on enzymes and yeasts (RASMUSSEN; SØRENSEN; MEYER, 2014).

Repolymerization of polysaccharide degradation products (furfural and HMF) and/or polymerization with lignin monomers can form pseudo-lignin. However, the formation of pseudo-lignin can be generated from carbohydrates without the contribution of lignin in diluted acid pretreatments. This molecule formed of carbonyl, aromatics, methoxy and aliphatic structures is harmful in the conversion of sugars. Pseudo-lignin tends to irreversibly bind to enzymes through hydrophobic interactions, resulting in loss in their activities. Therefore, to avoid pseudo-lignin in reaction medium could contribute to the process success (HU; JUNG; RAGAUSKAS, 2012).

Hemicellulose and lignin also have a direct influence on yields of enzymatic hydrolysis, acting as a barrier protecting cellulose. The diluted acid pretreatment partially removes hemicelluloses and modifying the structure of lignin (SHIMIZU et al., 2020). Lignin is responsible for the plant's structural integrity. Polysaccharides are naturally "protected" in the plant cell walls by lignin, which acts as a chemical barrier against external threats. Organosolv pretreatments can remove lignin, and this complete removal from biomass results in extremely digestible material. Delignification yields can be improved with addition of chemicals in pretreatment. The removal or modification of hemicellulose and lignin is essential to increase cellulose accessibility of enzymes (SHIMIZU et al., 2020; ZENG et al., 2014).

The removal of lignin from biomass is a fundamental step for the development of biofuels. Separating the components of sugarcane bagasse under mild conditions in addition to increasing the accessibility of cellulose, also enables the recovery and extraction of lignin, which can be used in the synthesis of new resins to replace those based on chemical compounds derived from the petrochemical industry (lignin valorization) (VALDIVIA et al., 2016).

Therefore, this study aimed to evaluate the contribution of biomass heterogeneity (fractions of internode, node, external fraction and leaf) in pseudo-lignin formation and sugar degradation products. Furthermore, the effect of low-cost antioxidants and surfactants (butylated hydroxytoluene; tert-butylhydroquinone; 3-tert-butyl-4-hydroxyanisole; methyl 3,4,5-trihydroxybenzoate; Tween 20; Tween 80 and dimethyl sulfoxide) were evaluated in lignin removal during organosolv and diluted acid pretreatment and subsequent conversion of sugars by enzymatic hydrolysis and SSF.

Removal of hemicellulose and lignin can generate value-added products, improving pretreatments efficiency and collaborating with the biorefinery use of biomass.

CHAPTER 2 OBJECTIVES

2.1 General objective

- This study aimed to evaluate the effect of antioxidant compounds added to the pretreatment in the removal of hemicellulose and lignin from biomass, and consequences on enzymatic hydrolysis and ethanol yield in the Simultaneous Saccharification and Fermentation (SSF).

2.2 Specific objectives

- Evaluate the lignin and hemicellulose removal effect on the cellulose accessibility and enzymatic hydrolysis yield;
- Evaluate the effect of butylated hydroxytoluene (BHT), a low-cost antioxidant, in the organosolv e diluted acid pretreatment for the removal of lignin;
- Evaluate the effect of Tert-butylhydroquinone, 3-tert-butyl-4-hydroxyanisole, methyl 3,4,5-trihydroxybenzoate, Tween 20, Tween 80 and dimethyl sulfoxide (antioxidants and surfactants) on lignin and hemicellulose removal from biomass under different pretreatment conditions and subsequent enzymatic hydrolysis;
- Evaluate the SSF of pretreated biomass in the presence of BHT (sugarcane bagasse *in natura*, extractive-free and partial delignified).

CHAPTER 3 SUGARCANE BIOMASS CONVERSION INFLUENCED BY LIGNIN

Abstract

Plant biomass residues are renewable sources for the production of biofuels and high-value macromolecules. Sugarcane bagasse is one such plant biomass residue that is available from the sugar processing industry. It is used as a raw material for biobased ethanol production. However, some of its properties and its behavior during processing have a major inhibitory effect on its successful conversion. Among these inhibitory properties are the lignin content, its distribution in plant tissues, and its chemical properties. These make the materials naturally resistant to bioconversion processes. Furthermore, lignin and carbohydrate degradation products can be formed during acid pretreatment, which is one of the major steps during biomass conversion to bioethanol. These products negatively affect the liberation of fermentable sugars and the yield of ethanol during the fermentation stage of the conversion process. Other factors that also have an influence on the production of fermentable sugar are related to the different structural arrangement of plant tissues (sugarcane fractions of the node, internode, and external fraction), as well as biomass variety. Biomass varieties with low lignin content result in an improved yield of fermentable sugars, which in turn contributes to improved viability of the second-generation bioethanol production processes. By selecting sugarcane varieties with the best properties, ethanol production can be increased without increasing the total area under cultivation. Efforts have been dedicated to reducing biomass recalcitrance by classical and precision breeding. Genetic modification of sugarcane alters the genes responsible for the encoding enzymes for lignin biosynthesis, generating sugarcane with low recalcitrance.

Keywords: pseudo-lignin; recalcitrance; pretreatment; second-generation ethanol; precision breeding; classical breeding.

3.1 Pretreatments effects on biomass components

Biomass pretreatment is a major step employed during the conversion of biomass to ethanol. It has the sole aim of reducing its recalcitrance. During this step, the glycosidic bonds linking the individual sugar molecular chains are broken down, resulting in changes in the physical and chemical structure of the cell walls of the lignocellulosic material (BRIENZO et al., 2015; HIMMEL et al., 2007; SANTOS et al., 2012). Furthermore, during this step the pretreatment chemicals penetrate the biomass and solubilize lignin from the secondary walls, making it possible for the enzymes to access and hydrolyze the polysaccharides. Different strategies may be required to pretreat different biomass raw materials (ZENG et al., 2014). Physical methods, such as ball milling or steam explosion, mechanically fractionate the lignocellulosic material,

reducing its particle size, while acidic or alkaline (chemical) methods promote structural breakdown and improvement in the recovery of glucose from cellulose by removing hemicellulose or lignin (MOSIER et al., 2005).

However, such strategies as the commonly used dilute acid pretreatment do not only cause the breakdown of the biomass but also contribute to lignin reorganization. This may vary according to the severity of the pretreatment and the nature of the lignocellulosic material (BRIENZO et al., 2017; PU et al., 2013). The breakdown of the cellulose-hemicellulose-lignin complex during acid pretreatment increases the surface area of the fiber and decreases the degree of polymerization and crystallinity of the cellulose (SANTOS et al., 2012). This type of pretreatment can break cellulose chains but hemicellulose is more susceptible due to its amorphous character, its high polydispersity, and its lower degree of polymerization (BRIENZO et al., 2017; MOSIER et al., 2005).
Other pretreatment strategies, including organosolv pretreatment, which involves the use of organic solvents and acids as catalysts, have shown good selectivity for sugarcane bagasse, resulting in high physicochemical modification, with improved accessibility to cellulose by cellulase enzymes (SUN et al., 2016).

During organosolv pretreatment a reaction occurs between an alcohol (methanol, isobutanol, cyclohexanol, or benzyl alcohol) and a mineral acid, thereby forming the soluble alcoholic lignin with 30% yield (SALIBA et al., 2001). This process has been observed to generate relatively low amounts of fermentation inhibitor compounds (SUN et al., 2016).

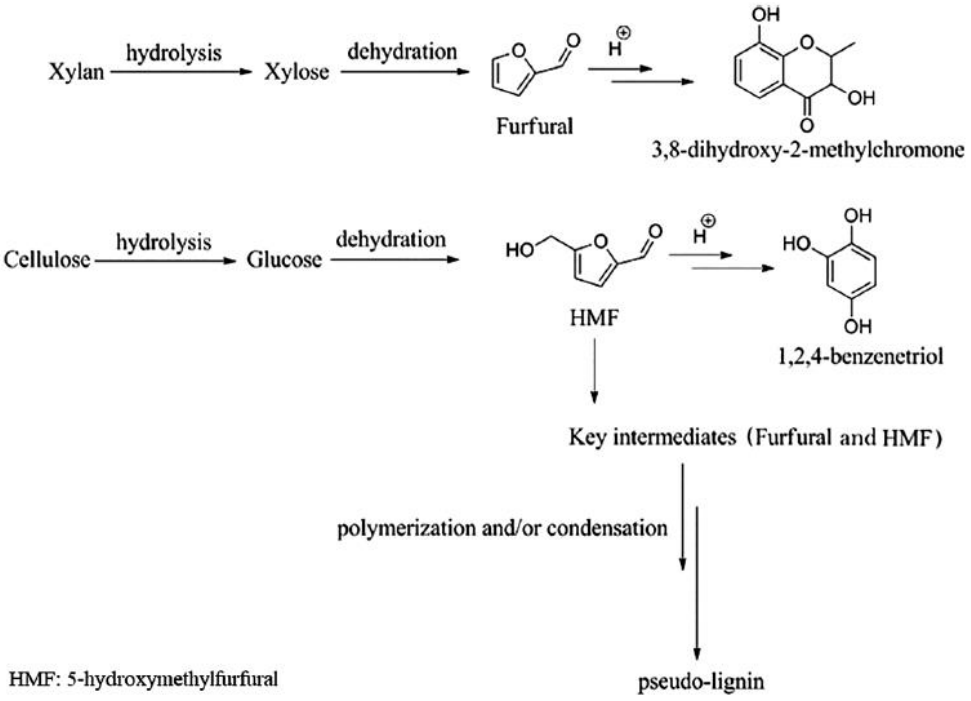
The presence of lignin in the biomass restricts enzymatic hydrolysis of sugars by inhibiting the physical access of cellulase enzymes to cellulose. This causes an irreversible and unproductive binding. Some studies indicate that the lignin content observed in the biomass after pretreatment is equal to or greater than the starting material for some pretreatment technologies. These values can be attributed to loss of polysaccharides and formation of pseudo-lignin (Klason lignin) (PU et al., 2013).

Six genetically distinct varieties of sugarcane were evaluated for glucose yield obtained after alkaline-sulfite (Na_2SO_3 10% plus NaOH 5%) pretreatment and enzymatic hydrolysis. The variety with lower lignin content in its chemical composition showed lower solids yield after pretreatment, suggesting that the low original lignin content facilitated the degradation of polysaccharides most exposed during alkaline sulfite reactions. The hybrid with lower original lignin content also presented a higher initial rate of cellulose hydrolysis, achieving 75% conversion in the first 8 h of reaction. In contrast,

hybrids with high lignin content require 64–72 h of reaction to achieve 70% conversion. Comparison of the hydrolysis efficiency of these hybrids after 24 h of reaction indicated that varieties with lower lignin content require less chemical loading during the pretreatment stage to achieve high levels of cellulose conversion (LAURITO-FRIEND et al., 2015).

During dilute acid pretreatments (DAP), pseudo-lignin can be formed from the degradation of carbohydrates without the contribution of lignin, especially in conditions of high severity. During pretreatment, acetic acid is generated from the hydrolysis of the acetyl groups of hemicellulose. This contributes to the hydrolysis of polysaccharides, leading to the formation of monosaccharides, which, after dehydration reactions, form furfural and hydroxymethylfurfural (HMF), respectively, from pentoses and hexoses (Fig. 1) (LORENCINI et al., 2016; SANNIGRAHI et al., 2011). Furfural and 5-hydroxymethylfurfural formed by acid pretreatments and steam explosion have little effect on enzyme inhibition. However, they make a major contribution to the inhibition of the fermentation process (SANT'ANNA; SOUZA; BRIENZO, 2014).

Figure 1 - Briefly explanation of the reaction pathways of pseudo-lignin formation under dilute acid pretreatment conditions.



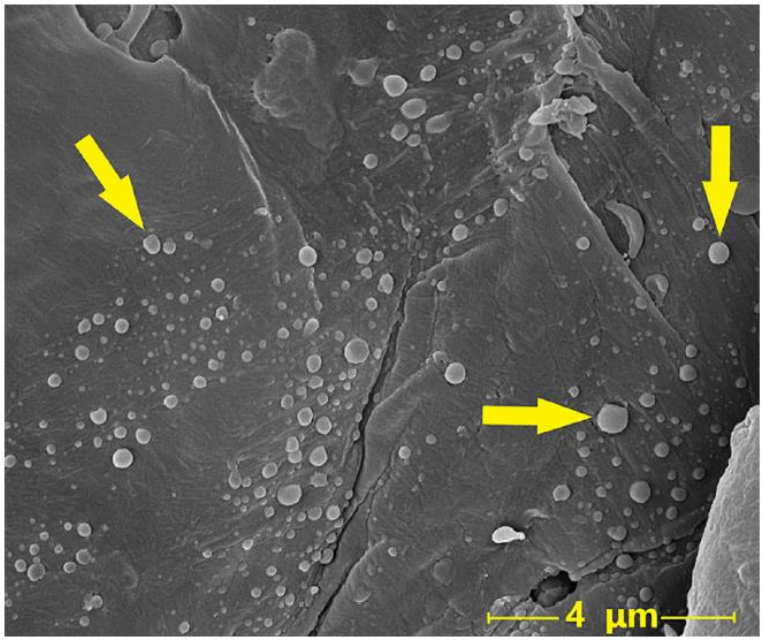
Source: Prepared by the author

However, during DAP in temperatures between 120 °C and 200 °C, the lignin reaches its glass transition, becoming fluid, and condenses in the form of drops / beads in the cell wall. This procedure does not preferentially remove lignin but contributes to its modification and reallocation / redistribution (FENGEL; WEGNER, 1984; PINGALI et al., 2010; SANNIGRAHI; RAGAUSKAS; MILLER, 2008).

Therefore, an understanding of the effects of the behavior of lignin and sugars degradation products is important for the development of an economical feasibility process (JÖNSSON; MARTÍN, 2016). Further, the development of an optimized pretreatment process can reduce the formation of pseudo-lignin, resulting in lower enzymatic requirements and lower losses of fermentable sugars in the enzymatic hydrolysis process, thus improving the economic aspect of the process (SANNIGRAHI et al., 2011).

Pseudo-lignin is aromatic material resembling lignin. This compound is a macromolecule form and contains carbonyl, carboxylic, aromatic, and aliphatic structures, which are formed from the degradation of cellulose and hemicellulose during DAP (Fig. 2). These compounds are considered even more harmful to the enzymatic deconstruction of the cellulose than lignin (HU; JUNG; RAGAUSKAS, 2012).

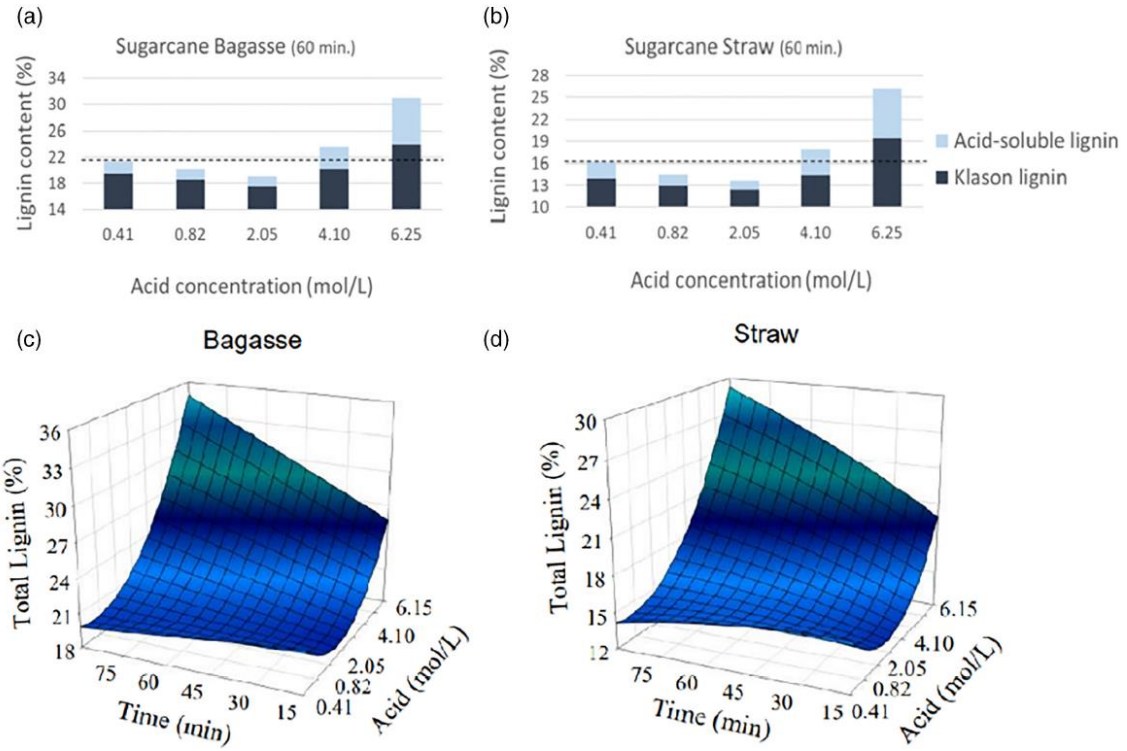
Figure 2 - Scanning electron microscopy images of the delignified sugarcane epidermis (with sodium chlorite) after diluted acid pretreatment (121 °C for 30 min) highlighting the pseudo-lignin droplets formed on the surface of the material.



Source: Prepared by the author

The measurable amounts of lignin (Klason and soluble lignin) are more susceptible to variations in acid load (mol L^{-1}) than the reaction time variation during the acid hydrolysis of sugarcane bagasse and straw. At acid concentrations greater than or equal to 4.10 mol L^{-1} H_2SO_4, the hydrolysis of lignin occurs in smaller proportions than the formation and deposition of lignin and pseudo-lignin. Consequently, the measurable amount of lignin increases (Fig. 3) (CARVALHO; COLODETTE, 2017).

Figure 3 - The effect of acid concentration H_2SO_4 on sugarcane bagasse (a) and straw (b) after two acid hydrolysis steps: the first at 12 mol L^{-1} 30 °C for 30 min and the second at 60 min and temperature of 121 °C under different acid conditions. The dotted line indicates that the rate of hydrolysis of lignin occurred to a great degree than the formation of pseudo-lignin. Total lignin content in sugarcane bagasse (c) and straw (b) after acid hydrolysis at different time and acid concentration.



Source: CARVALHO; COLODETTE, (2017).

Due to the low severity of the pretreatment process (170 °C 1 h^{-1}), the carbohydrates, furfural, and HMF degradation products accounted for only 10.62% of the pseudo-lignin (SANTOS et al., 2012). The application of pretreatments on xylan and avicel substrates resulted in a higher formation of pseudo-lignin when compared with

avicel-only pretreatments, which results in a decrease in the enzymatic hydrolysis yield of more than 25% (KUMAR et al., 2012).

Although the formation of pseudo-lignin during pretreatment with dilute acid has been observed, it is also likely to occur in all pretreatment processes performed at low pH and high temperature, such as liquid hot water and steam explosion (SHINDE et al., 2018).Understanding the effects of pretreatments applied on biomasses and implementing optimized process conditions can therefore help prevent the formation of pseudo-lignin. Understanding the mechanisms leading pseudo-lignin formation can provide insights into how to suppress these molecules, thereby improving the efficiency of enzymatic hydrolysis of cellulose for future bioethanol production (MENG; RAGAUSKAS, 2017). However, literature detailing the formation of pseudo-lignin is still scarce, especially on sugarcane bagasse and its negative effects on enzymatic hydrolysis.

3.2 Lignin effects on enzymatic hydrolysis

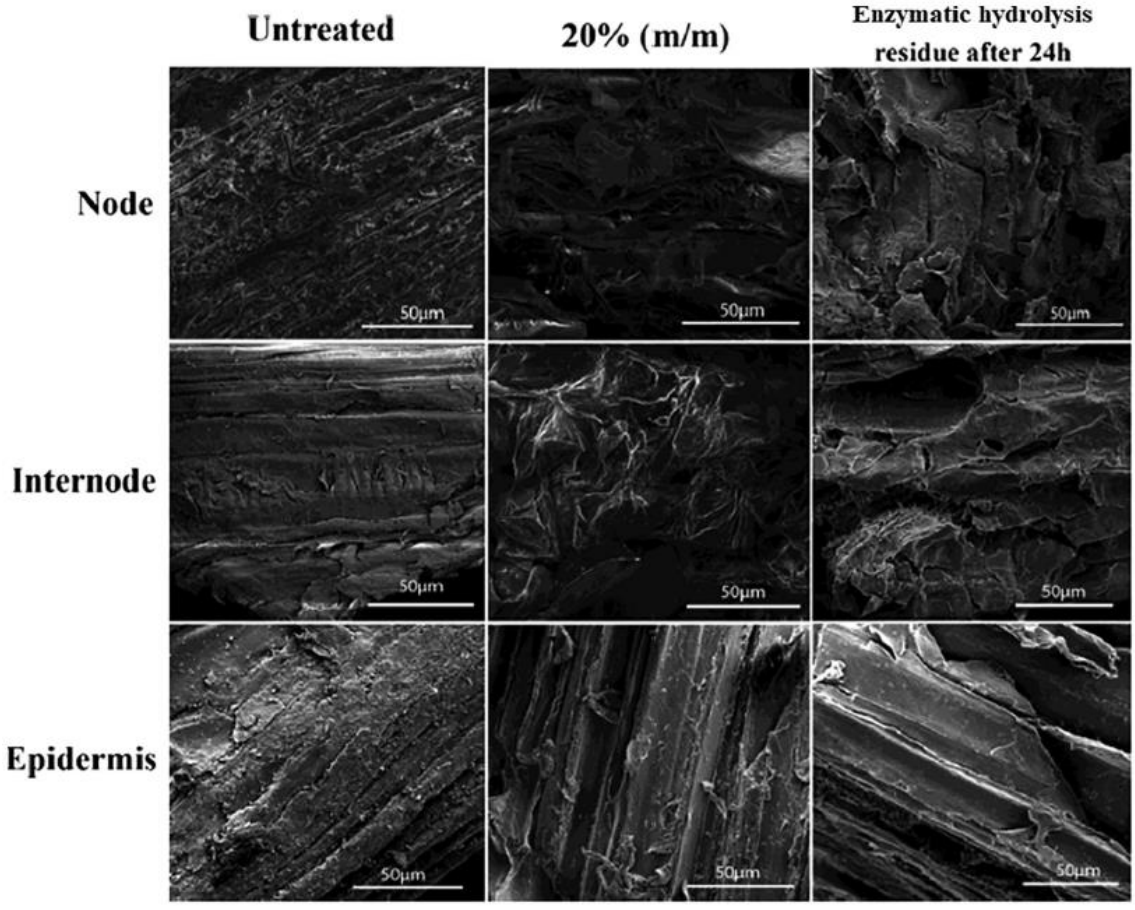
Biomass polysaccharides are naturally ‘protected’ by the lignin in the cell walls, making it difficult for microorganisms or their enzymes to reach them (ZENG et al., 2014). The variability in lignin content in various parts of plant biomass leads to variability with regard to the degree of cell-wall recalcitrance. In wood, the variability in lignin content varies from pith to bark, base to the top of the stem roots, branches, and includes reaction wood as well as juvenile wood and mature wood (BRIENZO et al., 2016).

Lignin content varies in amount in different fractions of sugarcane tissues. For example, node and internode, fragmented into various fractions (pith, interface, rind and outermost fraction), were observed different contents of the total amount of lignin. Lignin represented between 18 and 25% in fractions of hybrid H140 and approximately 13 and 20% between fractions hybrids H58 and H89. The evaluation of the four fractions (pith, interface, rind and outermost fraction) of each hybrid revealed variations in tissue chemical composition, where the maximum and minimum lignin contents were 12.6% (pith of hybrid H58) and 25.2% (outermost fraction of hybrid H140). In general, lignin and hemicellulose content increases from the pith towards the rind region (COSTA et al., 2013).

To compare recalcitrance of the sugarcane culm, isolated fractions (epidermis, node and internode) were evaluated individually. After acid pretreatment, epidermis showed less damage in its structure, while the node and internode presented higher

solubilization of xylose, demonstrating that epidermis was more resistant to pretreatment than node and internode. The enzymatic hydrolysis of these materials proved that this culm fraction was also less susceptible when compared to internode and node (18, 56 and 75%, respectively). Scanning electron microscopy (SEM) images revealed that the epidermis had less structural damage after enzymatic hydrolysis (Fig. 4). The chemical composition of these fractions revealed similarities between the lignin content of the node and internode (22%), while the epidermis presented 29% of lignin in its composition. The lower lignin content of the internode was probably responsible for the higher glucose yield from hydrolysis (BRIENZO et al., 2014). In fact, there is a difference in vascular bundles between node and internode, which in turn has an impact on more lignified structures. The heterogeneity of lignin content can therefore influence the bagasse quality generated in the sugar/ethanol industry.

Figure 4 - Scanning electron microscope images of untreated (extractive-free) and acid-pretreated sugarcane (20% m/m acid at 121 °C 30 min^{-1}) node, internode, and epidermis and the solid residue from enzymatic hydrolysis.



Source: BRIENZO et al., (2014).

To determine the effects of sugarcane culm heterogeneity, the internode and node fractions were pretreated with sulfuric acid (2.9% m/v at 130 °C 30 min^{-1}) after which approximately 1 g of untreated and pretreated sugarcane fractions were submitted to enzymatic hydrolysis. The enzymatic digestibility of cellulose was then determined at different reaction times (2, 4, 6, 8, 24, 48, and 72 h). The chemical characterization of these fractions revealed that the internode was composed of 39.5% cellulose, 27.8% hemicelluloses, and 19.8% lignin, and the node contained 41.1% cellulose, 25.5% hemicelluloses, and 21.3% lignin (m/m). After 72 h of enzymatic hydrolysis, internode and node yielded 26.8 and 20.4% of glucose respectively, while the glucose yields for the fractions that were submitted to acid pretreatment were 73% (internode) and 57% (node).

The internode was more susceptible to the acid pretreatment than the node due to its lower lignin content and better digestibility. However, some pretreatments may overcome recalcitrance and improve biomass digestibility. These results suggest that the internode and node isolated from the sugarcane stem responded differently to pretreatment technologies due to differences in their chemical composition, and lignin distribution along cell wall and vascular bundles (morphological and physicochemical properties) (BRIENZO et al., 2016).

Milled sugarcane bagasse of 11 varieties (from classical and precision breeding) with different lignin contents in its chemical composition (between 13.3% and 20.9%) were submitted to diluted acid pretreatment. After enzymatic hydrolysis with low cellulase loading (1.5 FPU/g supplemented with 1.5 U/g β-glycosidase) it was possible to observe that the varieties with low lignin content in their chemical composition (approximately 15%) presented higher glucose yields after 24, 48, and 72 h of reaction when compared with varieties with high lignin content (approximately 20%). The same variation in glucose yield was also observed among the varieties when a high cellulase enzyme load was applied in the enzymatic hydrolysis (15 FPU/g cellulase enzyme supplemented with 15 U/g β- glycosidase). After 24, 48, and 72 h of reaction, the varieties that presented lower content of lignin also presented higher yields of glucose (BRIENZO et al., 2015).

Different sugarcane cell types were evaluated for their lignin content and their influence on enzymatic hydrolysis. The plant internodes were separated and cut into the longitudinal axis in circular pieces, and the central fraction (pith) and the peripheral fraction (rind) were delignified in aqueous sodium chlorite/acetic acid reagent. After chemical characterization, rind presented 19% m/m of lignin content, whereas the pith

presented only 12% of lignin content. Cellular ultraviolet (UV) microspectrophotometry (UMSP) revealed that parenchymal cells predominate in the pith region (lower lignin content), which is consistent with the results. The enzymatic hydrolysis (10 FPU of cellulase and 20 IU of β -glucosidase per gram of substrate) of the untreated fractions revealed that the pith was readily hydrolyzed to glucose after 72 h of reaction (63% conversion) while the rind samples showed only 20% conversion, indicating that the less lignified (pith) cells were significantly less recalcitrant than the predominant fibers in the rind. Chlorite treatment did not result in an increase in cellulose conversion in pith cells, but delignification markedly increased glucose conversion in the rind cells, indicating an inverse correlation between the cellulose conversion levels and the lignin content of the cell types present in the rind (SIQUEIRA et al., 2011).

In fact, the lignin content has been identified as a parameter for feedstock selection to produce second-generation (2G) ethanol (BRIENZO et al., 2015). Lignin content has been reported in various studies to be negatively correlated with biomass digestibility and conversion. An increase in the ethanol yield with a decrease in the lignin content was reported in studies of hydrolysis followed by fermentation of 15 different wood species (VINZANT et al., 1997). The heterogeneity and different types of organization of sugarcane tissues and lignin contents may influence the viability of bioethanol produced from sugarcane bagasse, because the intrinsic recalcitrance of biomass impairs its conversion into bioethanol (BRIENZO et al., 2016).

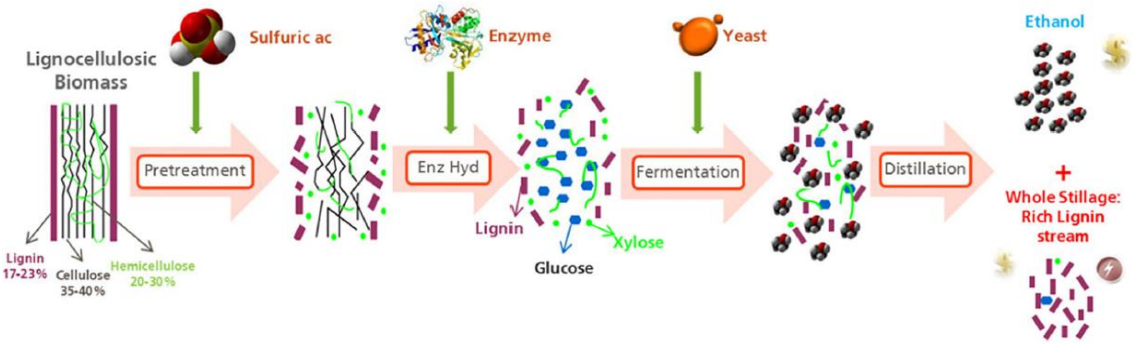
Only a few microorganisms, including bacteria such as *Streptomyces* sp., *Nocardia* sp., and basidiomycetes, can degrade lignin, depending on the oxidative action of enzymes such as lignin peroxidase, manganese peroxidase, and laccase. However, biorefineries must process biomass efficiently on a large scale, and the deconstruction of biomass by microorganisms is a slow process. The pretreatment step is thus critical to remove or displace the lignin prior to the enzymatic hydrolysis of the biomass. The overall efficiency of the biomass conversion therefore depends on the improvement of pretreatment technologies (ZENG et al., 2014).

The techniques for processing lignocellulosic biomass are not yet clearly defined. Although many advances have been made, 2G ethanol biorefineries have many challenges to overcome to become stable and competitive. The process of conversion of biomass into bioethanol can be divided into four stages (Fig. 5): (i) pretreatment, which makes polysaccharides accessible; (ii) enzymatic hydrolysis, where enzymes break polysaccharides into smaller molecules; (iii) fermentation, where bioethanol is produced

from monomers of five and six carbons (pentoses and hexoses, respectively); and (iv) distillation, where the fuel is obtained in pure form. Throughout the procedure there is still a need for improvement of the production technology (VALDIVIA et al., 2016).

The purpose of pretreatment is to make the polysaccharides susceptible to enzymatic hydrolysis and to release fermentable sugars (ZENG et al., 2014). Each pretreatment process has a different effect on the various wood fractions, mainly cellulose, hemicellulose, and lignin. Different methods and pretreatment conditions must therefore be chosen and applied according to the composition of the selected material. The correct choice of pretreatment procedure may be critical in subsequent conversion steps in terms of cellulose digestibility, generation of inhibitory toxic compounds for yeasts, energy demand, and treatment of by-products generated in the process (ALVIRA et al., 2010).

Figure 5 - Conversion process of lignocellulosic biomass into 2G ethanol.



Source: VALDIVIA et al., (2016).

The degree of polymerization and the crystallinity of the cellulose are important parameters in the determination of hydrolysis rates. Any decrease in crystallinity may be caused by the alteration of other characteristics of the substrate, such as particle size reduction and consequently increase in the surface area of the material. The accessibility of the substrate to cellulolytic enzymes is one of the main factors that influences the hydrolysis process. The pore size of the substrate relative to the size of the enzymes is a limiting factor in the hydrolysis of the biomass (ALVIRA et al., 2010).

However, the irreversible bonds between lignin and cellulolytic enzymes are a main cause of the inefficiency of pretreatment processes. Some strategies have been evaluated to overcome the unproductive binding between lignin and cellulases, such as extraction with alkaline solutions and the addition of proteins or other additives. These can lead to an additional cost in the bioethanol production process (ALVIRA et al., 2010).

The content, distribution, and composition of lignins are responsible for the recalcitrance of biomass where the S:G ratio of biomass is a significant parameter in the delignification process. Recent studies have shown that this relationship is also important for the determination of how much ethanol can be obtained from fermenting the hydrolyzed biomass (GOVENDER et al., 2009). In sugarcane bagasse, proportions of 2:38:60 were found for *p*-hydroxyphenyl:guaiacyl:syringyl (H:G:S), while in straw the respective molar composition was 4:68:28. In this context, the lignin structures present in these materials suggest that bagasse is more susceptible to pretreatment than straw, due to its higher syringyl content and lower degree of condensation. Efficiency in the pretreatment processes is highly dependent on the content and structure of the lignins. Knowledge of the constituent of the lignins in the straw and bagasse therefore becomes necessary for the development of appropriate pretreatment and delignification methodologies (DEL RÍO et al., 2015).

The costs of the enzymatic pretreatment of lignocellulosic biomass (about 25% of the total cost), the conversion of biomass and microbial tanks limit the viability of biofuels in relation to fossil fuels. This underscores the importance of genetic improvement of biomass composition to reduce processing costs (HOANG et al., 2015).

One of the main focuses in biofuels research is the study of lignin biosynthesis and deposition in plant cell walls, as this molecule is the main factor that causes recalcitrance in plant cell walls. However, results from studies targeting sugarcane are still scarce (BOTTCHER et al., 2013). In the production of 2G ethanol, the availability and viability of the biomass should be economical enough for the industry and for the final consumer (VALDIVIA et al., 2016). The optimization of the processing of all available biomass resources could improve the efficiency of biorefineries, which could lead to the sustainable production of lower carbon emission biofuel compared with fossil fuels (DEL RÍO et al., 2015).

Sugarcane is the main biomass cultivated to produce sugar and ethanol in Brazil. Brazil is the largest producer of sugarcane in the world and, as such, this is an important crop for bioenergy purposes. It is also important for other tropical and subtropical countries due to its high biomass yield and high sucrose content. After juice extraction, the remaining bagasse along with leaves has the potential to be converted into 2G ethanol. However, the chemical composition of the bagasse can vary according to the genotype, harvest location, planting year, crop age, and environmental parameters. The available information on the variability of agronomic properties combined with the chemical

composition of bagasse versus the combined ethanol yield of sugarcane varieties produced in different crops is limited. This available information implies that the selection of sugarcane cultivars to produce ethanol and 2G ethanol can be determined by genetic differences between the varieties, thus improving the energy efficiency of the crop (BENJAMIN; GARCÍA-APARICIO; GÖRGENS, 2014).

3.3 Classical and precision breeding effect on sugarcane bagasse conversion

Worldwide, sugarcane is the most commonly used biomass in the production of bioethanol, due to the high content of fermentable sugars and high yields of biomass. The integration of first-generation (1G) and 2G technology can improve the sustainability and economy of the process (BENJAMIN; GÖRGENS; JOSHI, 2014). To improve the production of biofuels, sugarcane breeding programs need to concentrate their efforts, not only on important characteristics such as biomass yield, adaptability, resistance to drought and diseases, and high biomass yields per hectare (classical breeding), but also on characteristics such as improved cell-wall chemical composition. This relates to improved sucrose content, reduced lignin concentrations, or both, through genetic engineering (precision breeding) (BENJAMIN; GÖRGENS, 2015; HOANG et al., 2015). Studies on different sugarcane varieties have shown that bagasse with lower lignin content resulted in higher yields of fermentable sugars and consequently high ethanol yields (combined ethanol yield, from sucrose and glucose/xylose obtained after pretreatment hydrolysis of bagasse, in liters per hectare). However, this is also dependent on the use of optimized dilute-acid pretreatment processes, use of lower enzyme dosages, which in turn could reduce the operational cost without harming the production of ethanol from sucrose. The different varieties of sugarcane also demonstrated variable results in cane yields (wet ton/ha), sucrose content (kg ton^{-1} wet cane), sugar juice content (kg ton^{-1} wet cane) (BENJAMIN; GÖRGENS; JOSHI, 2014).

Varieties of sugarcane clones (transgenic and wild type) showed differences between agronomic properties and conversion of sugars. Transgenic clones produced higher stature (144 cm), higher stalk population (129 603), and larger quantities of soluble sugars in the juice (147 kg ton^{-1} of cane) compared to the wild type (123 cm in stature, population of 104 039 stalks, and 140 kg of soluble sugars in juice per ton of sugarcane) (BENJAMIN; GÖRGENS, 2015).

The transgenic (classical breeding) sugarcane also generated bagasse with higher enzymatic digestibility, higher sugar yield after pretreatment, and better enzymatic hydrolysis than the wild genotype. As for ethanol yield, the transgenic variety yielded 29.6 g L^{-1}, corresponding to 75.8% of potential glucose in pretreated material. The wild variety yielded 26.8 g L^{-1}, corresponding to 69.2% of potential glucose. These variations can be attributed to the different lignin content found in the transgenic genotype (16.4% dry weight) and wild type (21.3%). The bagasse generated from the transgenic sugarcane also showed higher digestibility, sugar yield after pretreatment, and better enzymatic hydrolysis than the wild genotype (BENJAMIN; GÖRGENS, 2015).

In studies carried out with 115 varieties of sugarcane (100 from classical breeding and 15 from precision breeding), the potential of the genotypes was evaluated for the capacity to produce fermentable sugar after dilute acid pretreatment and enzymatic hydrolysis. Significant differences were observed in the chemical compositions of the various bagasse samples, which can be attributed to the variety type and breeding technology. Varieties produced by precision breeding showed a higher content of arabinoxylan and a lower lignin and ash content. Those produced by classical breeding and showing low lignin content did not exhibit increased amounts of structural carbohydrates. During processing – i.e. pretreatment and enzymatic hydrolysis – the samples with a lower lignin content showed a higher glucose yield. Most varieties produced through precision breeding techniques exhibited higher digestibility than many classical breeding varieties. However, the optimization of pretreatment processes for the selected varieties is necessary to maximize the yield of combined sugars produced through hydrolysis and subsequently bioethanol after fermentation (BENJAMIN; CHENG; GÖRGENS, 2013).

Modification was performed in the genome of sugarcane, reducing the lignin content without altering its agronomic performance. By intragenic RNAi suppression of the gene *Sh4CL1* (responsible for lignin biosynthesis) a reduction of up to 16.5% of lignin (m/m) was obtained along with altered proportions of monolignol without reduction in biomass production. Intragenic sugarcane exhibited significantly improved saccharification efficiency by 52% and 76% (after dilute acid pretreatment and enzymatic hydrolysis), compared to wild-type (WT) controls. After 7 months of growth in the field, none of the intragenic and transgenic lines differed significantly in stem diameter compared to WT, but all lines were significantly shorter than WT. Six of the eight evaluated intragenic and transgenic lines did not differ significantly in dry biomass yield

from WT, but two lines displayed significantly reduced dry biomass compared to WT. Juice volume per g of fresh stem samples in intragenic and transgenic lines presented a volume of 2–18% higher than WT, although this was not statistically significant (JUNG et al., 2016).

The efficiency and maximization of the conversion of sugarcane bagasse to bioethanol requires improvements in cultivar production, not only in biomass yield per planted area and fiber content but also in better degradability of biomass during pretreatment and enzymatic digestion. The conversion can be facilitated if the feedstock genotype and composition are optimized for this purpose (HOANG et al., 2015). Different biomass characteristics require specific pretreatment conditions to overcome the intrinsic recalcitrance (ALVIRA et al., 2010) In fact, low lignin contents in the biomass represent decreased recalcitrance and physicochemical properties for the conversion process (BRIENZO et al., 2015, 2017)

Biotechnology can assist in producing favorable genetically modified sugarcane plants with high cellulose content, resistance to diseases and pests, better agronomic performance, or even with enzymes (*in planta*) capable of degrading lignin before its conversion into ethanol. Genetic engineering allows the selective sequencing of genomic loci of interest, defining goals for potential manipulation of the DNA molecule, and better exploitation of sugarcane biomass for biofuels, which may lead to a reduction in bioethanol production costs (HOANG et al., 2015).

3.4 Sugarcane genetic engineering and genes role

There is a relatively high genetic variability within hybrid sugarcane cultivars due to their heterozygosity and high polyploidy. The size of the sugarcane genome is about 10 Gb, and the complexity of its genome is due to the mixture of euploid and aneuploid chromosome sets with homologous genes present from 8 to 12 copies. Recently, the transcription factors that govern the monolignol biosynthesis in the lignin pathway have received attention. This allows for the reduction and modification of the lignin content and composition, thus reducing the recalcitrance of the biomass (HOANG et al., 2015).
The composition of the sugarcane cell wall varies according to the plant genotype and external environmental factors such as the period of harvest, location, solar and rain intensity, and availability of soil nutrients, among others (BENJAMIN; GÖRGENS; JOSHI, 2014). In addition, lignin biosynthesis may also be induced by several external

factors such as water stress, temperature, ultraviolet-B radiation, mineral deficiency, and pathogen infection. Some studies report that genes expressing enzymes related to lignin deposition in the cell wall can be regulated differentially during water stress. Two sugarcane genotypes (IACSP94-2094 and IACSP95-5000) were evaluated for lignin deposition in the stem under conditions of water stress. In response to the stress there were significant changes in the levels of gene expression of enzymes responsible for lignin biosynthesis. As a result, there was an increase of up to 60% in lignin content in the bark of new internodes in both genotypes. Greater accumulation of lignin in the stem implies a lower quality biomass raw material for the production of biofuels on the one hand (SANTOS et al., 2015). On the other hand, a low concentration of lignin in the sugarcane and any modifications in its recalcitrance, as well as low ash content are fundamental parameters for the efficiency of the conversion of lignocellulosic material into ethanol (BRIENZO et al., 2017).

The composition of the bagasse from different varieties differs, suggesting that the modification and the reallocation of lignin is a decisive factor in improving the accessibility of acid-pretreated sugarcane bagasse (BRIENZO et al., 2017). A common approach to genetic engineering is to modify the expression of enzymes responsible for lignin biosynthesis. However, drastic reductions in lignin levels may have negative effects on plant growth and development. The incorporation of atypical phenolic monomers into the lignin by genetic engineering can produce a lignin that is easily degradable in the pretreatment process (CESARINO et al., 2012)

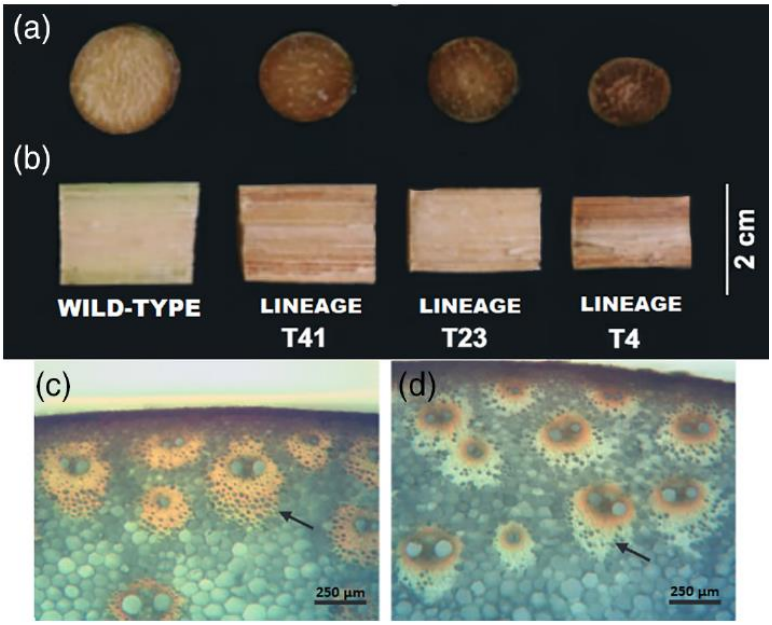
The lignin content in cultivated plants has already been reduced through conventional breeding, natural and induced mutations, and insertion of transgenes. In general, a significant reduction in lignin content represents low yields, lodgings, and long-term survival reduction in some perennial species (PEDERSEN; VOGEL; FUNNELL, 2005). On the other hand, the reduction of lignin biosynthesis, besides exerting negative effects on the growth, development, and lodging of the plant, can also reduce biotic and abiotic resistance and stresses, which could result in a serious threat in agricultural production (LIU; LUO; ZHENG, 2018). However, it is possible to reduce lignin in some populations without causing damage to their physical fitness (PEDERSEN; VOGEL; FUNNELL, 2005). Genetic modifications can improve the varieties of sugarcane to obtain cultivars with high cellulose contents and less recalcitrance (low lignin content) to bioconversion, without affecting the development of the plant under controlled conditions (BENJAMIN; GÖRGENS; JOSHI, 2014).

Many genes are involved in the production of lignin monomers. These include phenylalanine, with CaffeoylCoA *O*-methyltransferase (CCoAOMT) being one of the main enzymes in the synthesis of G and S monomers of lignin. The down-regulation of CCoAOMT results in a decrease in the G monomers in some plant species. The ferulate 5-hydroxylase (F5H) gene codes for an enzyme that has the necessary function in the production of the lignin S monomer. A decrease in the expression of this gene results in the reduction of S monomer in some vegetables. A third key gene in lignin biosynthesis is caffeic acid *O*-methyltransferase (COMT). Using interfering RNA (RNAi), down-regulation of COMT expression results in the decrease in S monomers and total lignin in alfalfa, switchgrass, and sugarcane. By applying RNAi (three events for each analysis) to specifically reduce CCoAOMT, F5H, and COMT gene expression, with the ultimate objective of altering the composition and deposition of lignin in sugarcane bagasse and leaves, it was observed that, for the CCoAOMT lines, only one line presented improved glucose release. No line had a reduction in lignin content for the F5H gene. However, one line resulted in a significant increase in glucose release. For the COMT gene, one line presented lower lignin content and, along with another line, elevated levels of glucose were observed during enzymatic hydrolysis. Two of the improved glucose release lines (F5H-2 and COMT-2) also showed reduced S : G ratios (BEWG et al., 2016).

Research has shown that it is possible to reduce sugarcane lignification successfully through RNA interference, despite its highly complex and polyploid genome. Suppression of COMT expression by 67% to 97% reduced the lignin content by 3.9% to 13.7%, respectively. The S/G ratio in the lignin was reduced from 1.47% in the wild type to values ranging from 1.27% to 0.79%. Without pretreatment, the fermentable sugar yield of the biomass increased by up to 29%. When dilute acid pretreatment was applied, the glucose yield increased up to 34%. Microscopic and histochemical analyses (Fig. 6) demonstrated a slight delignification of biomass in transgenic lineages (3.9, 8.4, and 13.7% in varieties T41, T23, and T4 respectively) when compared with the WT sugarcane. This could result in the reduction of recalcitrance without compromising plant performance under controlled conditions. This, in turn, indicates that genetically engineered transgenics are more likely to perform better during the conversion of biomass to bioethanol (JUNG et al., 2012).

Figure 6 - Phenotypic differences in sugarcane stalks through suppression of the COMT gene and the reduction in lignin content. (a) Transversal cuts; (b) longitudinal cuts. (c and

d) wild-type and T4 lineage, respectively, stained with Mäule reagent, indicating a reduction in lignin S units in sclerenchyma fiber cells.



Source: JUNG et al., (2012).

The agronomic performance of transgenic sugarcane (with modified lignin), as well as cell-wall characteristics and enzymatic saccharification efficiency were evaluated (6 months after initiation of the field trial) by suppression of the COMT gene by RNAi. Stably suppressed gene resulted in a reduction in gene transcription (80%, 89%, 92% and 91% in transgenic lines T41, T23, T31 and T4, respectively), when compared with WT, resulting in a reduction between 6% and 12% in total lignin content in different transgenic lines. Consequently, there was also a reduction in the incorporation of *S*- and *p*-coumarate units into the lignin molecules. This resulted in significantly lower S/G molar ratios of 1.17 (T41) and 0.72 (T4), compared with WT (1.48). A 6% reduction in the total lignin in sugarcane transgenic (line T41) resulted in improved effectiveness of enzymatic saccharification, resulting in bioethanol yields ranging between 19% and 23%. No significant differences were observed in biomass yield, plant height and diameter, tiller number, and brix value when compared with control plants. Strains with 6% to 12% less lignin required one-third less hydrolysis time and 3–4 times fewer enzymes to release equal or higher amounts of fermentable sugars when compared with non-transgenic plants (JUNG et al., 2013).

Reductions in lignin content of 8%, 11%, and 12% (lines T23, T31, and T4) gave 21%, 64%, and 65% reductions in biomass, respectively, compared with the control

transgenic varieties. However, the number of internodes per stalk was not statistically different from WT, indicating that the development of the genetically altered plants was similar until the time of harvest. Only the T31 line presented a lower number of stems per plant and, together with the T4 varieties, presented significantly reduced concentrations of soluble solids in their stalks when compared with the control plants. Diseases, pests, and lodging were monitored monthly in the crop, where a small occurrence of orange rust (*Puccinia kuehnii*) was observed in all varieties during the growth period, with no significant differences between varieties. Another pink sugarcane infestation (*Saccharicoccus sacchari*) was observed and eliminated by the application of agrochemicals after scoring and without significant differences between varieties. It was only observed the presence of lodgings in the tillage until the moment a storm hit the field with winds of 68 km h^{-1}. The most severe damage was observed on the face of the variety facing the prevailing wind direction, however, without statistical differences between the varieties (JUNG et al., 2013)

There is still a lot of work that needs to be done to describe fully the genes that encode enzymes, which are involved in the biosynthesis of cell-wall structural molecules, and to develop new methods for manipulating their physicochemical properties. These advances will enable the realization of new approaches in the engineering of cell-wall components aimed at improving the biomass use efficiency in biofuel production and perhaps other applications (BURTON; FINCHER, 2014). The manipulation of the composition of lignin in sugarcane through genetic engineering should improve economic competitiveness in the production of bioethanol (JUNG et al., 2013). Most of the knowledge about lignin metabolism comes from herbaceous and dicotyledonous plants; however, mechanisms underlying biosynthesis (polymerization and regulation) are conserved among all vascular plants. Despite recent advances, there is still much to be explored in terms of gene expression and regulation of the lignin biosynthesis pathway in sugarcane (CESARINO et al., 2012).

The reduction of costs in 2G ethanol production could be achieved by combining the selection of desirable cultivars and the optimization of the pretreatment processes. These combined factors can increase efficiency in sugar conversion and increase ethanol yields per ton of sugarcane produced (BENJAMIN; GÖRGENS; JOSHI, 2014). The yield of sugarcane and the sugar content (structural and non-structural) are the main parameters in the application of this plant in the production of bioethanol. The selection of varieties should therefore consider the production per hectare and the combination of sugars

present in the plant (BENJAMIN; CHENG; GÖRGENS, 2013). Reducing the lignin content is considered the best way to reduce the natural recalcitrance of biomass to biological conversions (CHANG; HOLTZAPPLE, 2000).

3.5 Concluding remarks

Among the various lignocellulosic residues that can be used in the production of 2G ethanol and commercial value-added compounds, sugarcane bagasse can be viewed as a hugely viable option. Its integral / total use can increase the production of 2G ethanol without the necessity to increase the cultivated area, and with no need to compete with food crops. Due to genotypic and environmental factors, the chemical composition of the plant cell wall can vary within the same species, where the proportions of cellulose, hemicelluloses, and lignin are found in different amounts in different plant tissues. The chemical composition of bagasse, mainly lignin, is an influential factor in the conversion process. Several pretreatments that alter the physicochemical properties in order to increase the enzymatic digestibility, like steam explosion, acidic or alkaline routes, or the use of organic solvents can be applied to biomass to remove / displace lignin. Through biotechnology, the modification of gene coding for enzymes responsible for the biosynthesis of the lignin can result in sugarcane varieties with different proportions of G/S lignin, which in turn would generate hybrids that are more favorable to pretreatment. Genetically modified varieties with low lignin content can also contribute to the success of the sugar hydrolysis process and subsequent fermentation in the production of 2G ethanol.

CHAPTER 4 PSEUDO-LIGNIN CONTENT DECREASED WITH HEMICELLULOSE AND LIGNIN REMOVAL, IMPROVING CELLULOSE ACCESSIBILITY, AND ENZYMATIC DIGESTIBILITY

Abstract

The sugarcane bagasse is a heterogeneous material and needs a pretreatment to breakdown its complex structure to make cellulose accessible to enzyme action. This study aimed to evaluate pseudo-lignin formation, enzymatic hydrolysis of sugarcane fractions (leaf, external fraction, internode, and node), and bagasse after partial delignification and acid pretreatment. The leaf and external fraction presented the highest content of lignin, and external fraction was the most recalcitrant material resulting in lower glucose release. Pretreatment with diluted sulfuric acid (20% m/m or 2% m/v) generated 5 g L^{-1} of acetic acid and 2.07 g L^{-1} of 5-hydroxymethylfurfural (external fraction *in natura* and leaf extractive-free, respectively). Furfural ranged between 0.11 g L^{-1} (node delignified) and 0.06 g L^{-1} (leaf, external fraction, and node *in natura*). A decrease was observed in pseudo-lignin formed with extractive-free and delignified biomasses, with different structure compared with non-delignified samples. The biomass partial delignification and subsequent pretreatment with dilute acid generate a material with fragmented anatomical structure, with improved cellulose accessibility, favoring enzymatic hydrolysis achieving more than 90% of glucose yield (up to 12 g L^{-1}). This study has shown strategies to decrease material heterogeneity and avoid pseudo-lignin formation as it results in lower recalcitrance and better efficiency of the enzymatic hydrolysis.

Keywords: Extractives; Diluted acid pretreatment; Cellulose; Glucose; Sugarcane fraction.

4.1 Introduction

The high demand for energy has led to the consumption of large quantities of fossil fuels, which have raised environmental concerns along with energy security issues. Global climate change caused by greenhouse gas emissions stimulated researchers to develop alternative fuels based on sustainable resources. Agro-energy crops and plant residues are the most promising, sustainable, low-cost feedstock for biofuel production and energy co-generation. Given the concern for sustainability and the need to maximize the use of natural resources, the use of sugarcane bagasse is receiving significant attention in biorefining applications as it is a promising resource for conversion into biofuels such as second-generation ethanol and co-generation (BEZERRA; RAGAUSKAS, 2016). However, the sugarcane biomass is heterogeneous with different tissue organization (anatomic fractions) and recalcitrance (BRIENZO et al., 2014, 2016).

Brazil is the main producer and consumer of sugarcane in the world. In 2018, Brazilian production was 746 million tons, while India, the world's second largest producer, cultivated 376 million tons (FAOSTAT, 2018). Large amounts of bagasse are generated in ethanol and sugar industries. One of the main challenges in the application of lignocellulosic biomass is the conversion of the complex polysaccharides into fermentable sugars, which can serve as a source of carbon for microbial fermentation process. Two steps are required to produce fermentable sugars: pretreatment and enzymatic hydrolysis. The purpose of some types of lignocellulosic material pretreatment is to remove hemicellulose and lignin, reducing recalcitrance (biomass resistance to fragmentation) and increasing the accessibility of cellulose for enzymatic hydrolysis, which is the most critical step in the biomass conversion process (SHIMIZU et al., 2018).

Lignin-like compounds (pseudo-lignin) can be formed under conditions of high severity in hydrothermal/acid pretreatments. This occurs due to the formation of carbohydrate degradation products that can condense with lignin fragments generating pseudo-lignin (MA et al., 2015; ZHUANG et al., 2017). These unwanted products such as sugars and lignin degradation compounds are inhibitors of the fermentation process (RASMUSSEN; SØRENSEN; MEYER, 2014). High concentrations of inhibitors, like acetic acid, furfural, and 5-hydroxymethylfurfural (HMF), negatively influence ethanol production. Acetic acid is ubiquitous in the hydrolysate, so it is desirable for the fermenting microorganism to be tolerant to this compound. Even at low concentrations (e.g., 5 g L^{-1}), these acid products affect the growth and fermentable productivity of both gram-negative and gram-positive bacteria. Acetic acid may inhibit biotechnological processes such as vinification or fermentation of biomass hydrolysate in which *Saccharomyces cerevisiae* plays a central role. This yeast can use acetate as a source of carbon and energy; however, in a concentrated glucose medium, the enzymes required for acetate catabolism are strongly suppressed and acetic acid becomes a stressor. In general, the toxic effects are like those in bacteria, which enters the cell cytosol inhibiting the activity of metabolic enzymes, inducing oxidative stress, and depleting ATP cells (TRČEK; MIRA; JARBOE, 2015).

In the dilute acid pretreatment, condensation between HMF and furfural occurs when the monomer products of lignin degradation condense and precipitate on the fiber surface. HMF and furfural are results of pentose and hexose dehydration, respectively (SCHMATZ; TYHODA; BRIENZO, 2020; ZHUANG et al., 2017), while the degradation of lignin forms monomers *p*-coumarilic alcohol, *p*-hydroxyphenyl

predecessor (H), *trans*-coniferyl alcohol, guaiacyl predecessor (G), and *trans*-synaptic alcohol, predecessor of syringyl (S) (GOVENDER et al., 2009). HMF and furfural are known as key intermediates to form pseudo-lignin, which hinder enzymatic hydrolysis of the pretreated material (HU; JUNG; RAGAUSKAS, 2012). High temperatures, low pH value, and oxygen presence were found to be crucial conditions for pseudo-lignin formation (ZHUANG et al., 2017). Pseudo-lignin can be broadly defined as an aromatic material that is not derived from native lignin, consisting of carbonyl, carboxylic, aromatic, and aliphatic structures that, in an acidic medium, condense and precipitate on the biomass fibers. Its presence produces a positive value of Klason lignin and also inhibits significantly the enzymatic hydrolysis of cellulose (HU; JUNG; RAGAUSKAS, 2012).

Lignin acts as a natural glue for cellulosic fibers and is produced by enzyme-mediated radical coupling from three monolignols. The syringyl unit (S) has two methoxy groups; the guaiacyl unit (G) has a methoxy group, while the third monolignol contains a *p*-hydroxyphenylpropane unit (H). The syringyl to guaiacyl ratio (S/G) is a significant parameter in delignification processes and is also important in determining the amount of ethanol that can be obtained from the fermentation of hydrolyzed biomass. Studies have shown a significant decrease in lignin content with an increase in S/G ratio. The xylose yield was also high with an increase in S/G ratio in some types of biomass. Therefore, an increase in ethanol production would be expected with an increase in the S/G ratio (GOVENDER et al., 2009).

Lignin modification or removal increases the digestibility of the plant cell wall. In fact, selection of sugarcane varieties with low lignin content is a strategy to generate a low recalcitrance biomass (BRIENZO et al., 2017). Recalcitrance in grasses varies according to cell type and maturation. It was observed that enzymatic digestibility can occur unevenly in different regions of the same internode. The outermost fraction and the shell are more recalcitrant, while the pith-rind interface and the pith are more digestible, with an inversely proportional correlation between the area occupied by vascular bundles and the efficiency of cellulose hydrolysis (COSTA et al., 2013). Studies conducted with 11 sugarcane hybrids revealed that the conversion of glucan to glucose by commercial cellulase was increased in samples with low lignin content, and chemical delignification increased the conversion to values above 80%. In general, experimental hybrids with low lignin content showed the highest digestibility (MASARIN et al., 2011, 2013).

Understanding the lignin contribution on the recalcitrance of sugarcane biomasses/tissue is essential in the biomass conversion into biofuels and chemicals. Lignin and pseudo-lignin limits enzymatic hydrolysis by inhibiting cellulase enzymes (LI; PU; RAGAUSKAS, 2016; SCHMATZ; TYHODA; BRIENZO, 2020). Due to differences in chemical composition, inhibition may have a different impact between sugarcane fractions (node, internode, leaf, external fraction, and bagasse) (BRIENZO et al., 2014). Therefore, this study evaluated the behavior of sugarcane fractions exposed to diluted acid pretreatment with prior partial delignification, presence of inhibitors, and pseudo-lignin formation in enzymatic hydrolysis glucose yield.

4.2 Materials and Methods

4.2.1 Sugarcane Sample Preparation

Sugarcane biomasses (culm, leaf, and bagasse) harvested manually without burning leaves were kindly donated by Sugarcane Technology Center (CTC-Piracicaba, SP, Brazil). Sugarcane bagasse was received after juice extraction at the refinery and was successively submerged in distilled water, with constant water renewal for 3 days, in order to remove sucrose.

The biomasses (external fraction, node, and internode) were separated from the culm of the plant, as described elsewhere (BRIENZO et al., 2014; COSTA et al., 2013). The external fraction (containing epidermis) was separated by hand cutting using a stainless-steel knife to obtain a 2-mm-thick strips. Epidermis-free culms were subsequently manually cut with a stainless-steel knife into transverse sections by visual identification to obtain the node fractions (connection point between internode) and internode (epidermis-free culm region). Node and internode were mill mechanically pressed for juice extraction and submerged in distilled water, with constant water renewal for 3 days, in order to remove remaining sucrose. These obtained biomasses/fractions, together with the sugarcane leaves, were oven dried at 55 °C for 48 h and then ground in a 20-mesh knife mill (825 μm), obtaining the untreated *in natura* biomass (IN) and stocked to assay (BRIENZO et al., 2014).

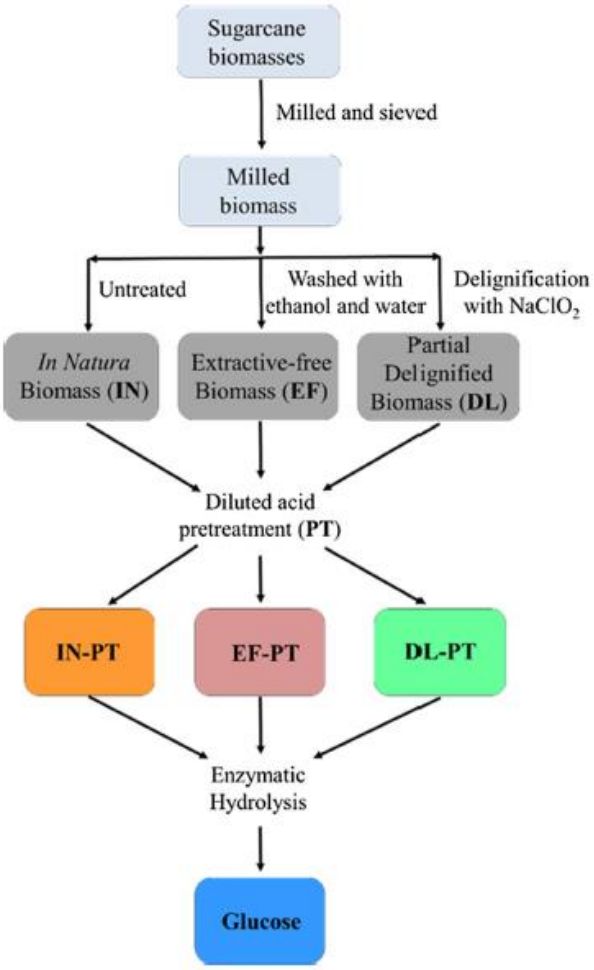
4.2.2 Pretreatments

Extractive-Free Biomass: Approximately 3 g of IN biomass was subjected to constant washing in Soxhlet apparatus with ethanol 92.8% (v/v) for 8 h and subsequent water-washing for the same period. After drying (55 °C for 24 h) and weighing, 300 mg of each fraction of the biomass was used individually in the chemical characterization process, as described later, and the remaining of EF biomasses was stocked for essays (ABNT, 2018).

4.2.3 Partial Delignified Biomass

The delignification process was reproduced following α-cellulose extraction (BRIENZO et al., 2015). Approximately 5 g of IN biomass was placed in a Schott flask containing 200 mL deionized water, 1.88 g sodium chlorite, and 0.63mL glacial acetic acid. Three additional doses, equivalent to the first dosage of sodium chlorite and glacial acetic acid, were added hourly after 2, 3, and 4 h, totaling 7.52 g of sodium chlorite, 2.52 mL of acetic acid after 5 h. The reaction was conducted in a water bath at 70 °C. The biomass was filtered, the liquid fraction was discarded, and the mass recovery (MR) was washed with deionized water (800 mL heated water at 70 °C and 2000 mL room temperature water) until the pH value of the filtrate was neutral (BRIENZO et al., 2015). After drying the biomass, 300 mg of each DL fraction was used for chemical characterization and remaining was stored for future analysis. Each sugarcane fraction (bagasse, leaf, external fraction, node, and internode) in its three forms (IN, EF, and DL) was pretreated (PT) with diluted sulfuric acid (20% mass acid/mass of material or 2% m/v) for 30 min at 121 °C in autoclave (1:10 solid-liquid ratio) (Fig. 7). After the reaction, the pretreated biomass sample was vacuum-filtered and the liquid fraction was collected for quantification of released sugars and inhibitors in acid medium. MR was washed with distilled water until the pH value of the filtrate reached neutrality. After the MR was dried at 55 °C for 24 h, the material was stocked in plastic flask for future analyses.

Figure 7 - Flowchart of biomass pretreatment. IN: intreated *in natura* biomass (original); EF: extractive-free biomass; DL: partial delignified biomass; PT: pretreatment; IN-PT: *in natura* (original) pretreated; EF-PT: extractive-free pretreated; DL-PT: partial delignified pretreated.



Source: Prepared by the author

4.2.4 Chemical Characterization

Untreated biomasses (EF and DL) and acid pretreated of each sugarcane fraction (IN-PT, EF-PT, and DL-PT) were chemically characterized to determine lignin content, cellulose, and hemicellulose. Approximately 300 mg of dry material was hydrolyzed with 3 mL of 72% sulfuric acid (m/m) at 30 °C for 1 h, with periodic homogenization with glass stick. The reaction was stopped with 84 mL of distilled water and autoclaved at 121 °C for 1 h. The reaction mixture (solids in the solution) was filtered with a porous plate crucible. The liquid fraction was collected to determine soluble lignin by spectrophotometer UV-Vis at 215 and 280 nm and sugar quantification by HPLC

(hexoses and pentoses). Solid residue was oven-conditioned at 105 °C to determine insoluble lignin values (ABNT, 2018). All treatments and analyses were performed in triplicate. Values obtained in HPLC were used to calculate the anhydrous sugars, for example, glucose released converted in glucan/cellulose present in the biomass, with hydration factor of 0.9. For xylose and arabinose, the factor was 0.88 and acetic acid of 0.72.

4.2.5 Pseudo-Lignin Content

One gram of MR, from the acid pretreatment, was inserted into filter paper bags that were extracted in Soxhlet with 1,4-*p*-dioxane/water solution (9:1) for 8 h. After solvent recovery and evaporation, the residual mass of pseudo-lignin was weighed (HU; JUNG; RAGAUSKAS, 2013). The pseudo-lignin percentage was determined by the relation between mass solubilized per the amount of material used. Infrared (FTIR) attenuated total reflectance (ATR) of the pseudo-lignin extracted samples was examined between 4000 and 400 cm^{-1} at 25 °C with 2-cm^{-1} resolution, 32 scans per spectrum. The ATR method used in a FTIR-VERTEX 70/BRUKER spectrophotometer with a source: HeNe laser (emits radiation in the mid-infrared region); Detector: DLaTGS.

4.2.6 Cellulose Accessibility

Sugarcane fractions (bagasse, leaf, external fraction, node, and internode) acid-untreated (EF and DL) and diluted acid pretreated (IN-PT, EF-PT and DL-PT) were evaluated for the exposed cellulose area, accessible to enzymatic action. The quantification occurred by the adsorption of the dye of Direct Blue (internal specific surface) and Direct Orange (external specific surface). For this, 0.5 mL of phosphate buffer (pH 6) was added together with 50 mg of dry biomass in six centrifuge tubes. To each tube, both blue and orange dyes were added, in six different concentrations (0.06, 0.25, 0.375, 0.5, 0.75, and 1 mL each), and the volume was completed up to 5 mL with distilled water. After constant agitation at 120 rpm at 70 °C for 6 h, the samples were centrifuged at 5600 rpm for 5 min and the supernatant was evaluated in a spectrophotometer at 624 and 244 nm. The amount of dye was assessed by the difference between the final and initial concentration (SHIMIZU et al., 2020). Direct Orange and Direct Blue concentrations were obtained through the following equations:

$$(1)\quad A_{455nm} = \mathcal{E}_{O/455}\,LCO + \mathcal{E}_{B/455}\,LCB$$
$$A_{624nm} = \mathcal{E}_{O/624}\,LCO + \mathcal{E}_{B/624}\,LCB$$

A is the solution adsorption at 450 or 624 nm, $\mathcal{E}$ is the extinction coefficient of each component in its respective wavelength, *L* is the cuvette's length (1 cm). CO is direct orange concentration and CB is direct blue concentration. The extinction coefficients were determined through a standard curve of the dyes and the angular coefficient of their absorptions at 455 and 624 nm. Extinction coefficients used in this study were: $E_{O/455}$ = 25.61; $E_{B/455}$ = 0.86; $E_{O/624}$ = 3.1; and $E_{B/624}$ = 16.45 $Lg^{-1}\ cm^{-1}$ (SHIMIZU et al., 2020).

4.2.7 Enzymatic Hydrolysis

Sugarcane biomass untreated (EF and DL) and acid pretreated (IN-PT, EF-PT, and DL-PT) were subjected to enzymatic hydrolysis in triplicate using 15 FPU g^{-1} of material (Cellic® Cetec–Novozymes, 60 FPU mL^{-1}). The reaction was performed with 0.1 g of material in 5 mL (2% solid loading) of 0.05 mol L^{-1} sodium citrate buffer, pH 4.8, 50 °C for 24 h at 120 rpm (BRIENZO et al., 2017). After this reaction period, the hydrolysate was water boiled for 5 min and centrifuged (2500 rpm for 15 min at 4 °C), and the liquid fraction was evaluated by high performance liquid chromatography (HPLC). The values obtained were used to calculate the enzymatic digestibility of the material (anhydroglucose released in relation to the glucan/cellulose content) from the cellulose content present in the biomass. The equation used to obtain the glucose yields follows:

(2) Cellulose conversion (%) = 100 x glucose concentration / (1.11 x *f* x mass biomass)

where glucose is the concentration of glucose released during enzymatic hydrolysis (g L^{-1}); biomass is the dry biomass concentration at the beginning of the enzymatic hydrolysis (g L^{-1}); *f* is the cellulose fraction in dry biomass (g^{-1}); 1.11 is the conversion factor of cellulose to glucose equivalents.

4.2.8 High-Performance Liquid Chromatography

Sugar monomers and acid acetic were quantified using a HPLC with an Aminex® column (Bio-Rad) HPX-87 H 300 × 7.8 mm, mobile phase 0.050 mol L^{-1} H_2SO_4, flow of 0.4 mL min^{-1}, oven at 65 °C and RID detector, isocratic method, and dilute acid treatment. Sugar degradation products (furfural and hydroxymethylfurfural) were evaluated in a C18 column (NST) 150 mm × 4.6 mm × 0.5 μm, mobile phase water/acetonitrile (8:1) with 1% acetic acid, 0.8 mL min^{-1} flow, 35 °C and 20 mL injection volume, detector UV-Vis 274 nm—isocratic method. All treatments and analyses were performed in triplicate.

4.2.9 Scanning Electron Microscopy

For scanning electron microscopy (SEM) analysis, untreated and pretreated sugarcane bagasse samples were washed with deionized water and dried at 45 °C for 24 h. Samples were mounted onto stubs using carbon double-sided tape, coated with 5 nm platinum, and examined in a FEI Quanta FEG 450 scanning electron microscope, operating at an accelerating voltage of 1 kV (SHIMIZU et al., 2018).

4.2.10 Statistical Analysis

Results were reported as average of at least three replicates and standard deviation shown. Tukey test was applied to the main results (pseudo-lignin content and enzymatic hydrolysis) to identify similar statistical values (identified with the same letter in figures/tables). DX6Trial-expert software was used (ANOVA), with 95% of significance level ($p < 0.05$).

4.3 Results and Discussion

4.3.1 Chemical Characterization of Untreated and Pretreated Material

The composition of the biomass (m/m) revealed cellulose contents of 37.4% for bagasse, 37.7% for leaf, and 37.5% for external fraction, while node and internode presented 36.4% and 42.6%, respectively. The content of hemicellulose in bagasse was 26.1% for bagasse, 27.4% for leaf, and 27.8 % for external fraction, while node presented 31.5% and internode 29.1%. The lignin contents for bagasse were 19.4%, leaf 33.7%, external fraction 31.0%, node 23.4%, and internode 25.3% (Table 1).

The data showed that the highest content of cellulose was in the internode, while in the other fractions, its level was equivalent. On the other hand, the node presented the highest values of hemicellulose, with similar level compared with other fractions. The highest content of lignin was found in leaf and the smallest was in bagasse. The fractions showed different values of extractives in its composition. Bagasse showed 6.6%, leaf 11.4%, external fraction 12.2%, node 7.2%, and internode 10.6% of extractives (Table 1). Pretreated biomasses were chemically characterized to determine changes in their components. Partial delignified and diluted acid pretreated (DL-PT) fractions presented higher cellulose content compared with the other treatments (highest content was observed with internode, with 61.7% and the lowest with leaf, with 53.6%). Sodium chlorite removed the lignin in the biomass while sulfuric acid mainly removed the hemicellulosic compound. The delignification process applied preserved hemicellulosic fraction (Table 1). Delignification with sodium chlorite partially removed lignin from biomass, in the condition applied. However, this procedure probably removed other compounds present in biomass such as extractives (pigments, waxes, alkaloids, terpenes, flavonoids, ashes, silica, and sugars) that may contribute to the formation of pseudo-lignin (CARVALHO et al., 2015).

The highest lignin content was observed with bagasse (35.4%) and leaf (51.9%) EF-PT and with the external fraction (53.7%), node (45.7%), and internode (50.4%) IN-PT. However, the lowest content was observed with bagasse (5.4%) and node (15.2%) partially delignified (DL), leaf (16.8%), external fraction (11.2%), and internode (15.2%) DL-PT (Table 1).

Heterogeneity of sugarcane fractions can influence its conversion into value-added products. Studies reported that epidermis-free internode was composed of 39.58% cellulose, 27.87% hemicellulose, and 19.88% lignin, while node contained 41.17% cellulose, 25.58% total hemicellulose, and 21.32% lignin (similar lignin content compared with internode and node) (BRIENZO et al., 2016). Different regions of the same internode of sugarcane have differences in their chemical compositions. After dividing the internode into four parts, from the periphery to the center, it was observed that the outermost fraction showed higher levels of lignin (20–25%) while the innermost fraction (pith) showed higher levels of glucan (41–54%), compared with the other sections (COSTA et al., 2013). In the present study, similar results were found as those

Table 1 - Chemical composition of pretreated sugarcane biomass fractions after partial delignification and dilute acid pretreatment.

Fraction	Treatment	Chemical composition (%, m/m dry basis)				MR	Component Removal (g 100 g^{-1})		
		Cellulose	Hemicellulose	Total Lignin	Extractives		Cellulose	Hemicellulose	Lignin
Bagasse	EF	37.4 ± 1.3	26.1 ± 1.6	19.4 ± 1.0	6.63 ± 0.6	100	-	-	-
	IN-PT	53.6 ± 0.5	11.3 ± 1.9	33.7 ± 1.2	-	63.0 ± 2.4	9.6	72.8	0
	EF-PT	46.3 ± 1.4	8.2 ± 0.6	35.4 ± 1.4	-	58.7 ± 0.2	27.2	81.6	0
	DL	49.1 ± 0.4	31.2 ± 0.8	5.4 ± 1.1	-	69.5 ± 1.3	8.6	16.8	80.8
	DL-PT	56.7 ± 1.2	8.35 ± 0.5	13.5 ± 0.8	-	62.2 ± 1.0	5.5	80.1	56.8
Leaf	EF	37.7 ± 0.8	27.4 ± 1.6	33.7 ± 1.7	11.38 ± 0.4	100	-	-	-
	IN-PT	42.3 ± 1.5	6.5 ± 1.5	49.4 ± 1.9	-	62.9 ± 0.1	29.4	85.0	8.0
	EF-PT	40.5 ± 1.0	8.2 ± 1.0	51.9 ± 0.6	-	63.5 ± 0.8	31.8	80.9	2.3
	DL	39.4 ± 0.1	25.6 ± 1.9	23.7 ± 0.9	-	64.0 ± 1.8	33.1	40.1	55.1
	DL-PT	53.6 ± 1.0	7.8 ± 0.3	16.8 ± 1.8	-	60.4 ± 0.8	14.1	82.7	70.0
External fraction	EF	37.5 ± 1.7	27.8 ± 1.5	31.0 ± 0.6	12.19 ± 0.2	100	-	-	-
	IN-PT	41.2 ± 0.7	7.6 ± 0.9	53.7 ± 0.7	-	36.6 ± 0.9	40.1	90.0	9.8
	EF-PT	41.4 ± 0.1	7.0 ± 0.7	47.7 ± 1.3	-	68.6 ± 0.4	24.2	82.8	0
	DL	38.0 ± 1.9	26.6 ± 0.9	17.2 ± 0.2	-	73.8 ± 1.4	25.3	29.5	59.0
	DL-PT	55.4 ± 1.0	8.2 ± 1.0	11.2 ± 0.5	-	60.9 ± 0.1	10.1	82.0	78.1
Node	EF	36.4 ± 1.7	31.5 ± 1.0	23.4 ± 1.3	7.18 ± 0.7	100	-	-	-
	IN-PT	45.5 ± 0.4	8.2 ± 0.7	45.7 ± 0.6	-	59.5 ± 0.2	25.7	84.6	0
	EF-PT	41.1 ± 0.5	10.6 ± 1.0	41.3 ± 1.0	-	59.5 ± 0.1	32.9	80.0	0
	DL	43.4 ± 1.2	32.0 ± 1.0	15.2 ± 0.9	-	69.7 ± 0.6	17.0	29.3	54.7
	DL-PT	56.0 ± 1.9	7.9 ± 0.4	15.4 ± 1.6	-	53.5 ± 0.5	17.7	86.7	64.7
Internode	EF	42.6 ± 1.6	29.1 ± 1.3	25.3 ± 1.1	10.58 ± 0.4	100	-	-	-
	IN-PT	45.9 ± 1.0	7.3 ± 0.9	50.4 ± 0.2	-	51.2 ± 0.1	44.9	87.1	0
	EF-PT	44.4 ± 1.2	8.3 ± 1.0	50.4 ± 1.7	-	59.8 ± 0.1	37.7	83.0	0
	DL	47.3 ± 1.4	30.5 ± 1.5	19.4 ± 0.7	-	57.9 ± 0.3	35.8	39.4	55.6
	DL-PT	61.8 ± 1.4	6.9 ± 0.7	15.2 ± 1.1	-	56.0 ± 1.2	18.8	86.8	66.3

EF: Extractive-free; IN-PT: *In natura* (original) acid pretreated; EF-PT: Extractive-free acid pretreated; DL: Partial delignified biomass; DL-PT: Partial delignified acid pretreated; MR: Mass Recovery.

Source: Prepared by the author

reported in the literature, indicating that fractions share similarities in their chemical compositions.

Seven sugarcane varieties were extractives determined and showed a range from 1.9 to 7.5% (MASARIN et al., 2011). Higher values of extractives were reported, 15% and 12% for sugarcane bagasse and straw, respectively (CARVALHO et al., 2015). The extractives can be influenced by the presence of soluble sugars or environmental contamination. The content of extractives, as well as the chemical composition of structural sugars and lignin present in sugarcane, may vary according to their genotype, tissue, and environmental conditions during planting (location, rainfall, available nutrients, temperatures, etc.); this reflects on their development and provokes diversity in its constitution. Sugarcane bagasse presents great heterogeneity and extractive represents on average 6.61% of its composition (MELATI et al., 2017). Studies with sugarcane bagasse diluted acid pretreated (175 °C, 40 min reaction time, and acid load 1.25% m/m H_2SO_4) revealed 48% cellulose, 19% xylan, and 26% lignin (m/m) (MESA et al., 2014). Cellulose content was similar to that found in the present study, but differences in hemicellulose removal may have been caused by differences in pretreatment severity (acid content, temperature, and time reaction).

Sugarcane bagasse and straw pretreated with diluted acid (H_2SO_4 4.5% m/m for 15 min at 175 °C) showed an increase in the lignin content to 25.70% and 27.78%, respectively (CARVALHO et al., 2015). Probably, the lignin increase was due to the formation of pseudo-lignin. Moreover, the hemicellulose removal increases the cellulose and lignin percentage. In fact, studies with node and internode extractive-free acid pretreated (2.9% sulfuric acid m/v at 130 °C for 30 min) showed that most of the hemicellulose was removed and enriched the lignin content. The residual lignin content was enriched 6.64% for the internode (19.88% initial) and 8.33% for the node (21.32% initial) (BRIENZO et al., 2016). In the present study, the biomasses that were subjected to acid treatment had higher lignin values when compared with untreated biomasses (EF), while biomasses that were subjected to partial delignification and pretreated with acid (DL-PT) had higher cellulose values (Table 1).

This study demonstrated that after acid PT, bagasse showed a higher recovery yield (MR) among *in natura* (original) (IN-PT) with 62.94%, while external fraction presented greater recovery yield among the extractive-free and dilute acid pretreated biomasses (EF-PT) with 68.61%. Effective acid pretreatment aimed to solubilize most of the hemicellulosic fraction of the biomass, which resulted in high concentration of xylose

in the liquid fraction. On the other hand, the effect of delignification had the principle of extracting large amounts of lignin present in the biomass.

4.3.2 Sugars and Inhibitors in the Diluted Acid Hydrolysate

Hydrolysate (liquid fraction) obtained after the PT of the biomasses was used to quantify released hexoses, pentoses, and degradation products (inhibitors). Untreated and diluted acid pretreated fractions (IN-PT) presented higher soluble glucose contents compared with other biomasses. Extractive-free and diluted acid pretreated (EF-PT) presented the highest glucose release with bagasse (2.57 g L^{-1}) and the lowest with the external fraction (0.63 g L^{-1}). DL-PT had the highest release of glucose with the internode and the lowest with the external fraction (2.09 and 0.50 g L^{-1}, respectively) (Table 2).
The highest concentration of xylose was observed with the external fraction (24.5 g L^{-1}) and the lowest with the leaf (18.37 g L^{-1}) (IN-PT). The pretreatment of extractive-free biomass (EF-PT) resulted in higher concentration of xylose in the node and the lower in the leaf (24.14 g L^{-1} and 17.61 g L^{-1}, respectively). Among the delignified biomasses, the largest removal of xylose occurred in the node (23.69 g L^{-1}) and the lowest occurred in the external fraction (18.27 g L^{-1}) (Table 2).

Acetic acid (6.0 g L^{-1}) was observed in the node hydrolysate (EF-PT) in highest concentration, while the lowest concentration was observed in the leaf (DL-PT) with 1.67 g L^{-1}. In the external fraction hydrolysate (IN-PT), the highest HMF concentration (2.07 g L^{-1}) was observed and the lowest HMF concentration occurred in the node (0.05 g L^{-1}). Among the EF-PT treatments, HMF concentrations ranged from 0.04 g L^{-1} (bagasse and internode) to 0.01 g L^{-1} (leaf, external fraction, and node), while DL-PT biomass hydrolysate showed 0.01 g L^{-1} (bagasse, leaf, external fraction, and node) and 0.02 g L^{-1} (internode) of HMF. Concentrations of furfural observed between treatments and fractions varied between 0.11 g L^{-1} (node DL-PT) and 0.06 g L^{-1} (leaf, external fraction, and node, INPT) (Table 2).

Table 2 - Monosaccharides and degradation products released in the hydrolysate from the diluted acid pretreatment (20 % m/m H_2SO_4, 121 °C/30 min, solid-to-liquid ratio 1:10).

Sample	Component (g L^{-1})	Bagasse	Leaf	External fraction	Node	Internode
IN-PT	Glucose	3.35 ± 0.15	1.02 ± 0.03	10.30 ± 0.23	2.39 ± 0.07	5.32 ± 2.33
	Xylose	23.25 ± 1.59	18.37 ± 0.57	24.5 ± 0.33	19.64 ± 0.56	23.2 ± 2.81
	HMF	1.35 ± 0.53	0.19 ± 0.04	2.07 ± 0.38	0.05 ± 0.05	1.41 ± 0.15
	Furfural	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.10 ± 0.01
	Acetic acid	4.09 ± 0.44	3.61 ± 1.29	4.15 ± 1.94	3.57 ± 0.31	3.88 ± 1.21
EF-PT	Glucose	2.57 ± 0.02	0.65 ± 0.27	0.63 ± 0.10	1.85 ± 0.35	2.31 ± 0.01
	Xylose	22.02 ± 0.24	17.61 ± 1.68	18.22 ± 2.92	24.14 ± 1.11	21.21 ± 0.05
	HMF	0.04 ± 0.01	0.01 ± 0.02	0.01 ± 0.05	0.01 ± 0.01	0.04 ± 0.01
	Furfural	0.08 ± 0.01	0.06 ± 0.04	0.06 ± 0.03	0.10 ± 0.02	0.08 ± 0.03
	Acetic acid	4.07 ± 0.56	5.35 ± 1.84	4.01 ± 1.17	6.00 ± 0.99	3.80 ± 0.05
DL-PT	Glucose	0.74 ± 0.07	0.95 ± 0.03	0.50 ± 0.07	1.99 ± 0.26	2.09 ± 0.06
	Xylose	21.18 ± 2.08	22.63 ± 0.47	18.27 ± 2.61	23.69 ± 2.97	23.21 ± 0.64
	HMF	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
	Furfural	0.10 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.08 ± 0.02
	Acetic acid	3.95 ± 0.40	1.67 ± 0.04	3.37 ± 0.48	4.31 ± 0.52	4.42 ± 0.13

IN-PT: *In natura* (original) acid pretreated; EF-PT: Extractive-free acid pretreated; DL-PT: Partial delignified acid pretreated; HMF: 5-hydroxymethylfurfural.

Source: Prepared by the author

Studies with diluted acid pretreatment of sugarcane bagasse (0.5–4% H_2SO_4 v/v 121 °C for 1 h, solid-to-liquid ratio 1:15) revealed glucose concentrations ranging from 1.7 to 3.41 g L^{-1} (for 1.0 and 2.0 H_2SO_4 v/v, respectively) (RAI et al., 2014). Sugarcane bagasse pretreated using 1% sulfuric and acetic acid (m/v) at 190 °C 10 min^{-1} min showed concentrations of 3.67 g L^{-1} and 3.09 g L^{-1} glucose in the hydrolysate for solid-to-liquid ratios of 1.5:10 and 1:10, respectively (ROCHA et al., 2011). In the present study, the glucose values obtained for the fractions were similar to those reported in the literature (except for the external fraction). The low concentrations of glucose in the hydrolysate (DL-PT) correspond to the amorphous cellulose that was hydrolyzed, which is in accordance with acid pretreatment to remove the hemicellulosic fraction, retaining in MR a high cellulose content.

Xylose concentration of 41.54 g L^{-1} was determined in the hydrolysate of sugarcane bagasse dilute sulfuric acid pretreated (1% w/w, at 121 °C for 150 min, 20% solids) (MARTINS; RABELO; DA COSTA, 2015). However, sugarcane bagasse pretreated with 1% sulfuric acid and 1% acetic acid (m/v) (at 190 °C/10 min, 1.5:10 and 1:10 solid-to-liquid ratios) showed 9.04 (1.5:10) and 9.33 (1:10) g L^{-1} xylose (ROCHA et

al., 2011). Xylose rates observed in the present study are among the values reported in the literature. The concentration of xylose released in the hydrolysate undergoes direct action of the acid concentration applied to biomass. However, severe pretreatment conditions (high acid loads, temperature, and reaction time, etc.) may favor the formation of sugar degradation products that are precursors to the formation of pseudo-lignin (SCHMATZ; TYHODA; BRIENZO, 2020) and other undesirable toxic compounds for fermentation process (CANDIDO et al., 2020).

In the hydrolysate of sugarcane bagasse pretreated with sulfuric acid (1% H_2SO_4, at 121 °C/150 min, 20% solids load), 2.95 g L^{-1} of acetic acid was detected (MARTINS; RABELO; DA COSTA, 2015). However, pretreatment conducted with 1% sulfuric acid and 1% acetic acid (m/v) at 190 °C 10^{-1} min 1:10 solid-to-liquid ratios showed 2.89 g L^{-1} of acetic acid (ROCHA et al., 2011). As the pretreatment severity increased (time and temperature), a gradual increase in the concentration of acetic acid was observed in the hydrolysate (HONGDAN; SHAOHUA; SHUBIN, 2013). Studies conducted with 64 different microorganisms revealed that only 5 were able to grow in the presence of 5 g L^{-1} acetic acid (SOARES et al., 2016). The acetic acid contents observed in the present study were slightly above the average reported in the literature. Thus, some acetic acid contents (leaf and node EF-PT) could be potential inhibitors of microbial growth, while other contents might be close to the considered inhibitory. The detoxification of the lignocellulosic biomass hydrolysate is an important step that can improve the efficiency of the industrial process, mitigating the effects caused by toxic compounds on the metabolism of microorganisms (CANDIDO et al., 2020).

Temperature is an important factor in degradation products formation. At 180 °C and 200 °C, HMF concentrations increased from 0.01 to 0.11 g L^{-1}, while furfural increased from 0.26 to 2.42 g L^{-1}, respectively (hot water pretreatment/20 min, 1:20 m/v). However, increasing the reaction time to 30 and 40 min increased the furfural to 0.51 and 0.73 g L^{-1}, respectively. The increase of temperature and reaction time provoked a greater degradation of sugars (HONGDAN; SHAOHUA; SHUBIN, 2013). In fact, higher concentrations (9.6%) of furfural were observed with high severity treatments (1.25% H_2SO_4 m/m, 185 °C/40 min) (MESA et al., 2014). In the present study, the concentrations of HMF observed can be explained by the presence of sulfuric acid in the reaction medium, while the furfural concentrations were due to the shorter time and reaction temperature.

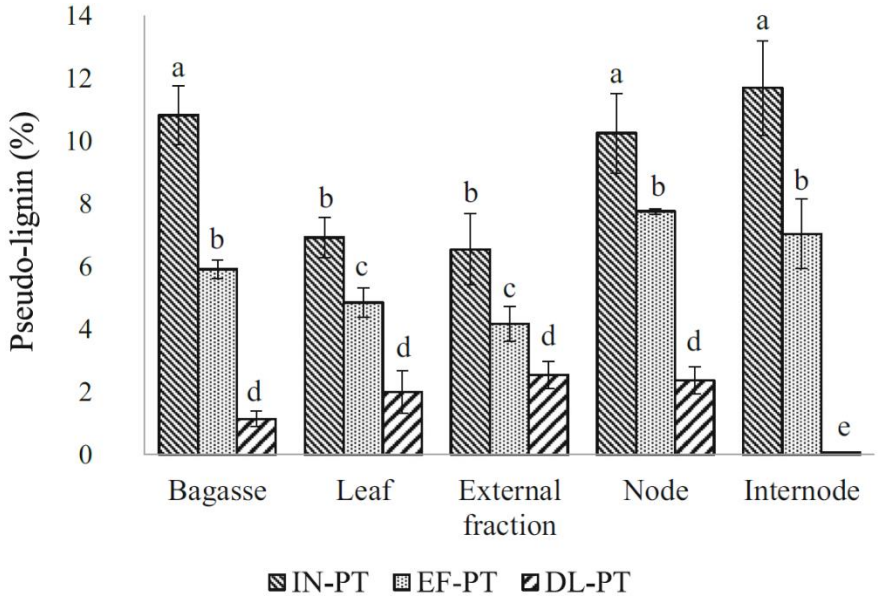
Furfural and HMF were observed in low concentration, independent of the sugarcane biomasses, compared with the literature (Table 2). Pretreatment severity can

interfere in sugar release and its degradation. Higher content of HMF was observed in the external fraction (2.07 g L^{-1}) and internode (1.41 g L^{-1}) IN-PT, if compared with EF-PT and DL-PT. Furfural content was lower than some literature studies for all the pretreatments and biomasses. A large amount of glucose was observed in the hydrolysate of external fraction INPT (10 g L^{-1}). This higher content was probably because of sucrose present in the biomass. The external fraction was hand cut from sugarcane, and probably, it comes with certain sucrose content. Studies with external fraction reported a washing step before processing, removing sucrose content (BRIENZO et al., 2014). Concerned with industrial process of material, in the present study, the external fraction was not washed. These data corroborate to the low MR after dilute acid pretreatment (Table 1) and higher HMF values caused by the degradation of glucose present in sucrose (Table 2). This glucose concentration was not observed in the other external fraction pretreatment because extractive removal and chlorite delignification probably solubilized sucrose before PT.

4.3.3 Pseudo-Lignin Content

The pseudo-lignin formation was observed in the highest content for all fraction IN-PT, followed by the EF-PT and in smaller amount DL-PT (Fig. 8). Pseudo-lignin content (m/m) in IN-PT was 10.82% for bagasse, 6.92% for leaf, 6.55% for external fraction, 10.25% for node, and 11.70% for internode. However, pseudo-lignin content decreased with EF-PT, with 6.91% for bagasse, 4.85% for leaf, 4.17% for external fraction, 7.76% for node, and 7.04% for internode. The DL-PT showed the lower pseudo-lignin contents of 1.14%, 2%, 2.55%, 2.38% and 0.07%, respectively for bagasse, leaf, external fraction, node, and internode (Fig. 8). The results suggested that to remove extractives before the acid pretreatment may reduce pseudo-lignin formation. The previous delignification with sodium chlorite was more effective in suppressing pseudo-lignin formation (Fig. 8).

Figure 8 - Pseudo-lignin from sugarcane biomasses pretreated with dilute acid (20% m/m or 2% m/v, at 121 °C/30 min). IN-PT: in natura (original) acid pretreated; EF-PT: extractive-free acid pretreated; DL-PT: partial delignified acid pretreated. Equal letters indicate statistical similar values.



Source: Prepared by the author

Partial delignification prior to dilute acid pretreatment may reduce the concentration of secondary compounds forming pseudo-lignin by removing an amount of lignin (Table 1). Biomass extractives and silica were suggested to contribute to the formation of pseudo-lignin during pretreatment with sulfuric acid (CARVALHO et al., 2015). According to pretreatment applied to biomass, the increase in severity (temperature, reagent loading, time, etc.) results in an impact on the amount of xylan removed, increasing also the possibility of generating furfural. A study showed that the increase in acid loading in sugarcane bagasse pretreatment (above 2% v/v) decreases the glucose and xylose in the hydrolysate, while furfural and acetic acid increase (RAI et al., 2014). Degradation of xylose may contribute to the formation of pseudo-lignin, which may impair the enzymatic hydrolysis process. In acid medium, the pseudo-lignin formed contributed with a significant increase of the lignin in bagasse and sugarcane straw (CARVALHO et al., 2015). Due to the low lignin content in biomass, which condenses with sugar degradation products (largely responsible for the formation of pseudo-lignin) during acid pretreatment, there was also a smaller amount of pseudo-lignin formation in the biomasses. Possibly, the IN-PT biomass had higher pseudo-lignin content due to the

presence of extractives in the material, which may undergo reactions with furfural and HMF and lignin during dilute acid pretreatment. The contribution of extractives in pseudo-lignin formation can be evidenced by comparing the same biomass between IN-PT and EF-PT.

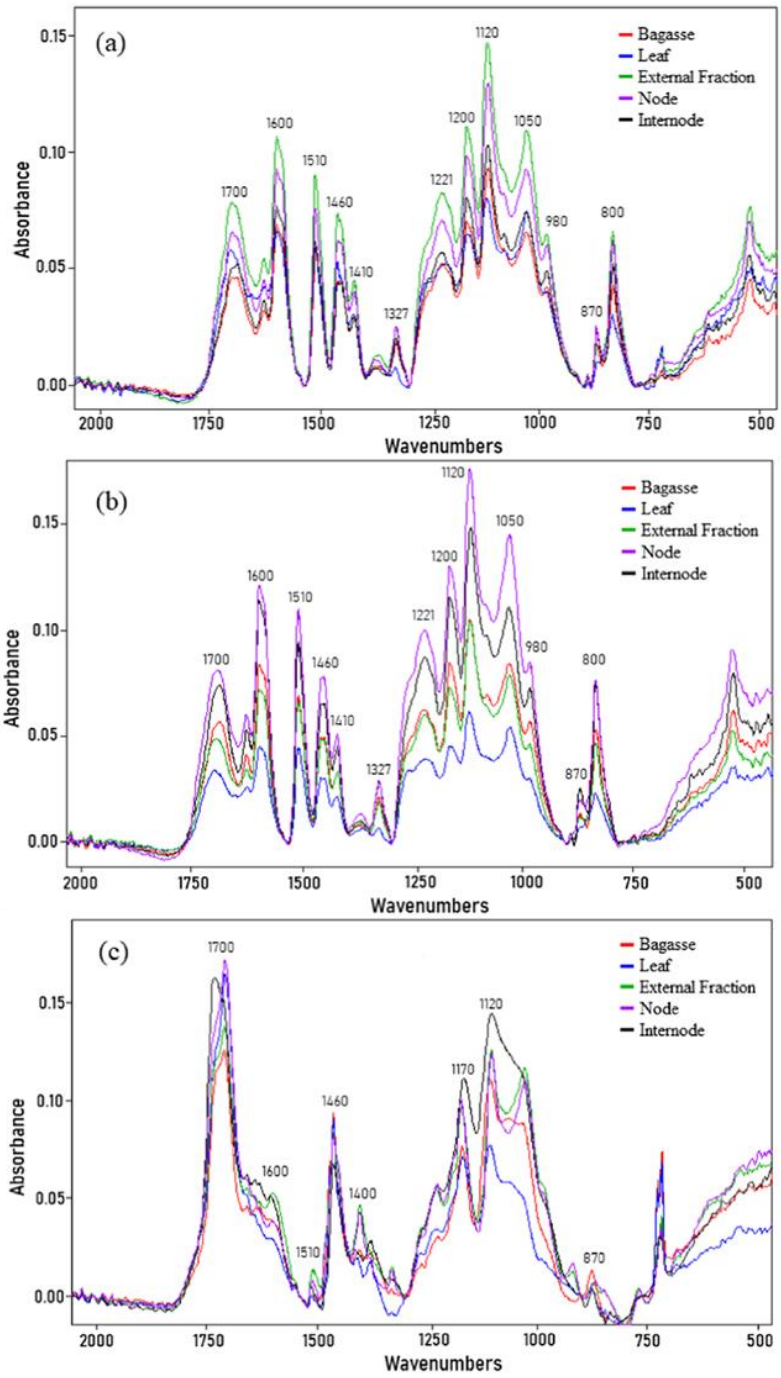
4.3.4 FTIR

The extracted pseudo-lignin was evaluated using FTIR. FTIR analyses of the pseudo-lignin of the fractions evaluated in this study were consistent with those reported in the literature. Bands between 1750 and 1700 cm^{-1} represent the C=O stretching in carboxylic acids, ketones, ester groups of carbohydrates, conjugated aldehydes; region between 1600 and 1510 cm^{-1} represents aromatic ring skeletal vibration (C=O stretching); region between 1460 cm^{-1} represents the C–H deformation, and 1410 cm^{-1} represents aromatic skeletal vibrations combined with deformation C–H in plane. The band at 1327 cm^{-1} corresponds to the syringyl ring condensed with guaiacyl ring (G ring replaced at position 5), C–C, C–O, C=O stretch, secondary OH aromatic, C–H in plane deformation (typical for G units), and primary OH; 1221 cm^{-1} represents G ring (C=O stretch), 1200 cm^{-1} and 1050 cm^{-1} represent C–O stretching in alcohols, ethers, or carboxylic acids. Band at 800 cm^{-1} represents C–H out-of-plane bending at positions 2, 5, and 6 (G units), while 870 cm^{-1} represents C–H out-of-plane at positions 2 and 6 (S units) (Fig. 9) (HU; JUNG; RAGAUSKAS, 2012; SINGH et al., 2005).

Figures 9 (a) and (b) correspond to *in natura* (IN-PT) and extractive-free (EF-PT) pseudo-lignin bands, respectively. Fractions represented in these spectra showed similar band patterns, assuming similarity between the structures. However, pseudo-lignin extracted from partially delignified with sodium chlorite (Fig. 9c) showed changes in the bands between 1750 and 1700 cm^{-1} indicating an increase in the number of C=O in conjugated and unconjugated, respectively. Strong bands in 1700 cm^{-1} region represent carbonyl and carboxylic groups present in the pseudo-lignin (stretching in ketones). Band at 1600 cm^{-1} is attributed to the stretching of the aromatic ring; however, this band was not clearly identified in pseudo-lignin, probably due to the delignifying effect (Fig. 9c). The reduction in the band intensity corresponding to 1600 cm^{-1} and 1510 cm^{-1} indicates a reduction in the stretching of the C=O bonds (aromatic ring vibrations, the latter mainly in G units), probably due to the effect of the delignification. The absence of the band at 1270–1260 cm^{-1} indicates a lower proportion of unit G in the delignified fractions (Fig.

9c) compared with the pseudo-lignin from IN-PT and EF-PT (Fig. 9 a and b). The band in the region of 1120 cm^{-1} represents higher levels of S units in the delignified material (Fig. 9c).

Figure 9 – FTIR-ATR spectra of pseudo-lignin extracted from sugarcane biomass partial delignified and acid pretreated. (a) *In natura* (original) acid pretreated (IN-PT). (b) Extractive-free acid pretreated (EF-PT). (c) Partial delignified acid pretreated (DL-PT).



Source: Prepared by the author

4.3.5 Cellulose Accessibility

The exposed cellulose surface on sugarcane biomasses was evaluated by adsorption of Direct Orange and Direct Blue dyes (Table 3). Untreated fractions showed low dye adsorption. External fraction showed total adsorption of 11 mg g^{-1}, node of 24 mg g^{-1}, while internode and leaf showed 106 and 145 mg g^{-1}, respectively. After the acid pretreatment (IN-PT), all the materials increased the dye adsorption from 1.5 to 22 times. Pretreatment carried out with 20% m/m of sulfuric acid reported total adsorption of dye of 86 mg g^{-1} by the external fraction. Node showed 69 mg g^{-1} of adsorption, while internode and leaf presented total adsorption of dye of 628 mg g^{-1} and 666 mg g^{-1}, respectively (SHIMIZU et al., 2020).

Fractions that had a greater adsorption of dyes suggest greater accessibility to cellulose, probably due to the structure modification (BRIENZO et al., 2015). The removal of hemicellulose and lignin generates pores and allows the adsorption of Direct Orange dye on the exposed cellulose. The partial delignification process provoked an increase in the cellulose accessibility by removal of lignin. The DL provoked an increase in the dye adsorption on to bagasse sample of 27 times compared with untreated (EF). The acid pretreatment of the de DL bagasse resulted in a decrease of the dye adsorption, however adsorbed 13 times more dye than untreated bagasse. The decrease of dye adsorption after the pretreatment of partial delignified bagasse could be related to a reorganization of cellulose component and effect of dry process (JUNIOR et al., 2013). Direct Blue dye has less affinity for cellulose than Direct Orange dye, penetrating areas where cellulases cannot reach. Therefore, the accessibility to cellulose can be measured not only by the total amount of adsorbed dye, but also by the greater amount of Direct Orange adsorbed in relation to Direct Blue (higher concentration of adsorbed Direct Orange means greater cellulose surface exposed to cellulases) (SHIMIZU et al., 2020).

Studies carried out with the sugarcane fractions (external fraction, node, internode, and leaves) submitted to oxidative pretreatment (1.5 g of NaClO2 and 0.5 mL of anhydrous acetic acid added in 50 mL of distilled water with 5 g of biomass, reaction temperature 70 °C and reaction time 30 min, 1, 2, and 3 h, with new loads/doses of sodium chlorite and acetic acid added at 1, 2, and 3 h) revealed that the highest rates of total dye adsorption were observed in more severe treatments. After 3 h of reaction, the external fraction showed total dye adsorption of 2076 mg g^{-1}, while the node showed 609 mg g^{-1}.

Internode and leaf showed total adsorption of 1327 mg g^{-1} and 567 mg g^{-1}, respectively (SHIMIZU et al., 2020).

Table 3 - Maximum adsorption of Direct Orange and Direct Blue dyes at each sugarcane biomass.

Sample	Biomass/fraction	Max adsorption DO (mg g^{-1})	Max adsorption DB (mg g^{-1})	Ratio (DB/DO)	Total (mg g^{-1})
EF	Bagasse	14.8	49.2	3.3	64.0
	Leaf	85.6	60.0	0.7	145.6
	External Fraction	5.1	6.2	1.2	11.3
	Node	5.4	19.5	3.6	24.9
	Internode	89.3	17.5	0.2	106.8
IN-PT	Bagasse	162.7	60.5	0.4	223.2
	Leaf	133.2	94.0	0.7	227.2
	External Fraction	96.5	74.2	0.8	170.7
	Node	270.8	275.0	1.0	545.8
	Internode	846.3	511.4	0.6	1357.8
DL	Bagasse	1133.3	621.8	0.5	1755.1
	Leaf	757.3	578.6	0.8	1335.9
	External Fraction	60.7	58.0	1.0	118.7
	Node	405.0	229.5	0.6	634.5
	Internode	87.3	67.1	0.8	154.5
DL-PT	Bagasse	483.8	372.9	0.8	856.7
	Leaf	235.7	178.0	0.8	413.6
	External Fraction	525.7	446.4	0.8	972.0
	Node	208.2	149.1	0.7	357.3
	Internode	845.4	323.2	0.4	1168.6

DO Direct Orange, DB Direct Blue, EF extractive-free biomass, DL partial delignified biomass, IN-PT in natura (original) acid pretreated, EF-PT extractive-free acid pretreated, DL-PT partial delignified acid pretreated.

Source: Prepared by the author

The porosity/accessibility of lignocellulosic biomass is a fundamental characteristic for enzymatic hydrolysis of polysaccharides, directly affected by the lignin content (BRIENZO et al., 2017). Higher levels of lignin in the biomass represent low porosity and worse enzymatic conversion of the polysaccharides. Consequently, the removal of lignin improves enzymatic saccharification (ROCHA et al., 2015). A more severe pretreatment removing lignin could increase the cellulose accessibility. In fact, a

chlorite pretreatment with harsh condition (two more loads of chemicals) increased the total dye adsorption to 2076 mg g^{-1} (LI; PU; RAGAUSKAS, 2016).

4.3.6 Enzymatic Hydrolysis

The untreated sugarcane biomasses (EF) showed lower glucose yields (Fig. 10). The pretreatment is necessary to breakdown the biomass structure and increase the cellulose accessibility, improving enzymatic action (BRIENZO et al., 2015, 2017). The lowest conversion of the cellulose into glucose occurred in the external fraction (5%) and the highest in the internode (12%). The partial delignified samples (DL) improved the cellulose conversion, resulting in an increase of the yield of 4.4 and 3.3 times, respectively, to external fraction and internode. A partial lignin removal process increased glucose conversion yield, indicating that there was an increase in the cellulose surface area to which the cellulase enzymes had access (Fig. 10). DL-PT of biomasses had glucose yields in the range of 90% and showed similar statistical value (Fig. 10). Partial delignification followed by diluted acid pretreatment was fundamental in the biomass conversion process. The combination of these procedures resulted in the high surface area (due to the removal of hemicellulose and lignin) to the cellulase enzyme action.

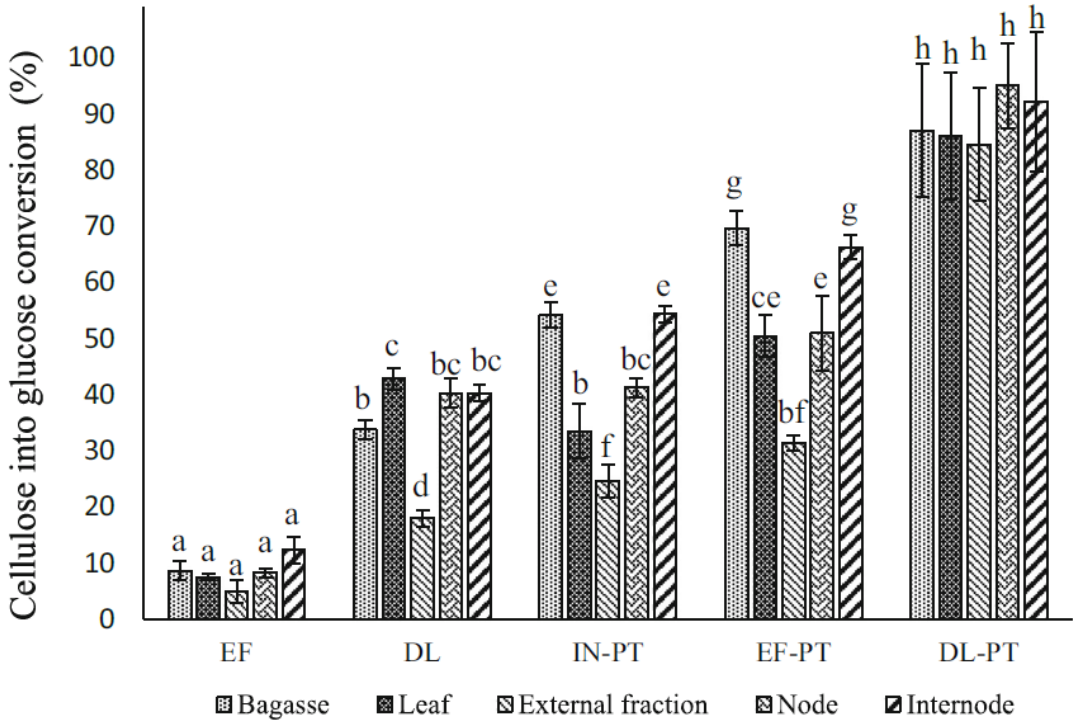
The DL-PT biomass reached the highest glucose yields, suggesting that the removal of the lignin (DL) and the hemicellulose (PT) together is the best strategy to improve cellulose conversation. Bagasse and internode IN-PT had similar statistical value, with 54% glucose yield after enzymatic hydrolysis, and the highest conversion applying IN-PT.

The EF-PT treatment of bagasse and internode showed similar statistical value, reaching the highest levels of glucose conversion (69% to 66%, respectively). Leaf (50%) and node (51%) were also similar statistical value, and the lowest glucose conversion rate was observed again in the external fraction (31%) (Fig. 10). The difference in the increase in cellulose conversion between IN-PT and EF-PT biomasses can be explained by the absence of extractives in the biomass, which contributes to the formation of pseudo-lignin which is detrimental in enzymatic hydrolysis.

Sugarcane bagasse of 11 different varieties that were submitted to dilute acid pretreatment (0.5% m/m H_2SO_4 at 180 °C/15 min) and subsequent enzymatic hydrolysis showed glucose yield between 39 and 78% after 24 h of hydrolysis. After 72 h of reaction, the glucose yield reached between 61 and 94% with low recalcitrance varieties identified

(BRIENZO et al., 2015). The present study reached similar conversion yield for bagasse with pretreatments of DL, IN-PT, EF-PT, and DL-PT (Fig. 10).

Figure 10 - Glucose yield from enzymatic hydrolysis of untreated and acid pretreated materials. EF: extractive-free biomass; DL: partial delignified biomass; IN-PT: *in natura* (original) acid pretreated; EF-PT: extractive-free acid pretreated; DL-PT: partial delignified acid pretreated. Equal letters indicate statistical similar values.



Source: Prepared by the author

A different approach could be an acid pretreatment followed by a partial delignification. Sugarcane bagasse pretreated with dilute acid (1% m/v H_2SO_4 at 121 °C/10min) and further delignification with NaOH (1–1.5 % m/v at 100 °C/1 h) revealed glucose yield 56% for diluted acid pretreated material and 79% after delignified step (ROCHA et al., 2015).

The cellulose conversion into glucose results obtained in the present study is close or higher than that reported in the literature. However, the severity of the pretreatment influences the final hydrolysis process by the amount of hemicellulose removed and the exposed surface area of the cellulose. Factors such as the presence of extractives, lignin content (Table 2), and pseudo-lignin formation (Fig. 8) interfered with the results of glucose enzymatic hydrolysis yield (Fig. 10). The biomasses that underwent the

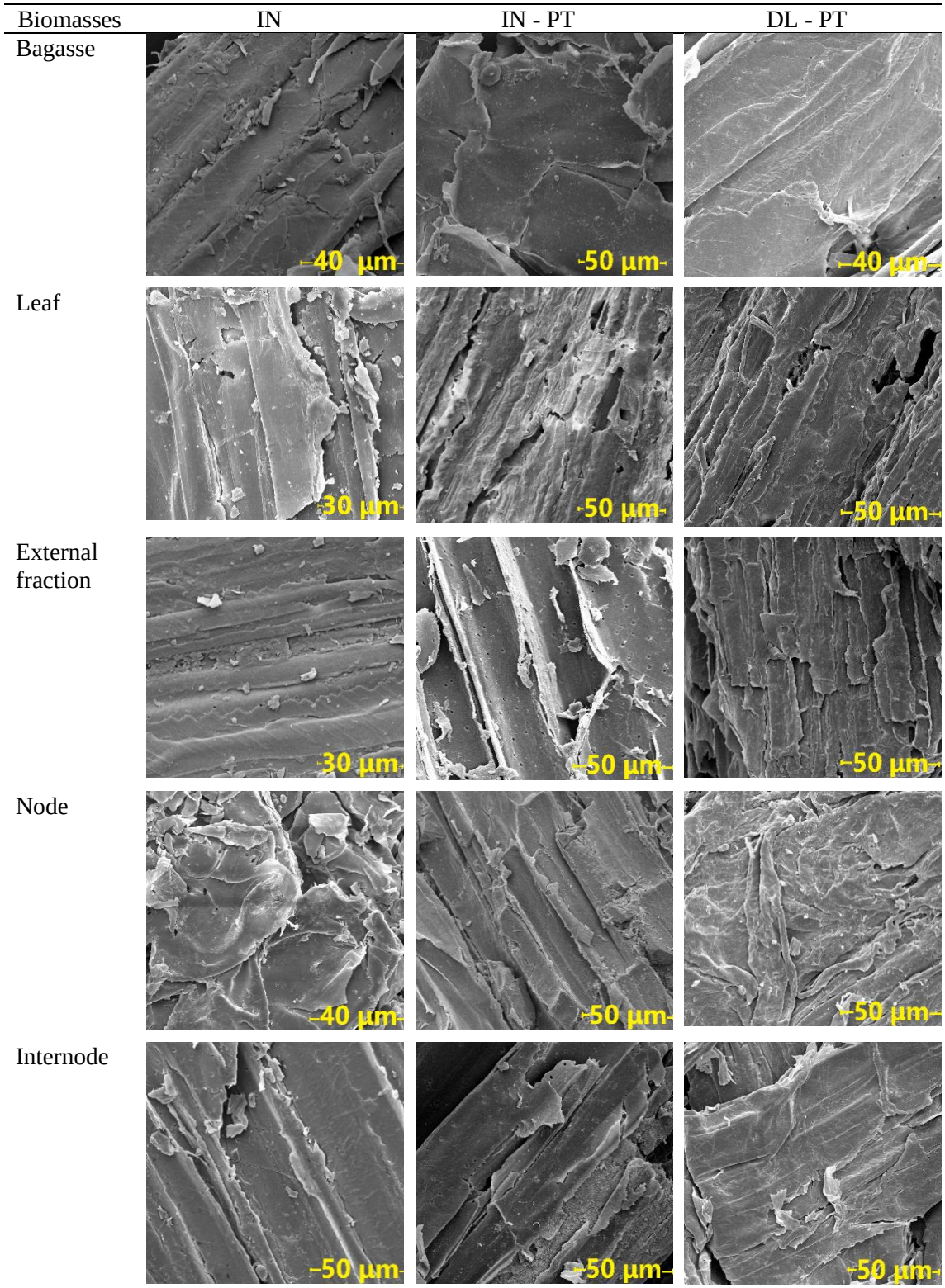
extractive removal/pretreated (EF-PT) and delignification/pretreated (DL-PT) showed higher values of glucose yield compared with IN-PT biomass (Fig. 10). The partial delignification alone was enough to modify the biomass structure, improving the glucose yield slightly close to the IN-PT for some biomasses, such as leaf and node. Therefore, the removal of extractives and partial delignification of biomass may be a key factor in the conversion of biomass to fermentable sugars via acid pretreatment. The results suggest that the presence of these compounds during dilute acid pretreatment could generate higher levels of pseudo-lignin.

4.3.7 Scanning Electron Microscopy

Sugarcane biomasses were investigated for morphological changes after acid pretreatment and delignification. SEM images of untreated biomasses (IN) showed an integral rigid structure pattern among the fractions. The surfaces were characterized by the absence of degradation or fragmentation. Damage on the structure of the untreated material could be result of the milling process but revealing a tissue integrity (Fig. 11).

Sulfuric acid acts on plant cell walls, causing the fibrous mesh to loosen. Acid pretreatment removed on average 45% m/m of biomass components (IN-PT), while delignification removed on average 33% m/m (DL) and 41% m/m more followed by acid pretreatment (DL-PT). The materials pretreated with sulfuric acid presented deformed and fractured structure after pretreatment application (IN-PT) (Fig. 11). The damage of the cell wall structure of biomass by removing hemicellulose (IN-PT) is not as aggressive as pretreatment of partial delignification followed by acid pretreatment (DL-PT). For some biomasses, it was possible to observe a progressive damage between the IN-PT and DL-PT biomasses (leaf, external fraction, and node). The biomasses suffered major changes in their structure due to the removal of hemicellulose and lignin by the action of chemical agents, resulting in loss of initial mass (Table 1) and making the cellulose fibers exposed to enzymatic action (Table 3). In fact, delignified and acid pretreated biomass (DL-PT) presented more open and fragmented fibers compared with *in natura* acid pretreated (IN-PT) and untreated (IN) biomasses. Removal of xylan caused structural changes in the biomass surface. However, with the additional removal of lignin, the structural damage was more evident (Fig. 11).

Figure 11 - Scanning electron microscope images of sugarcane biomasses untreated and dilute acid pretreated. IN: untreated in natura; IN-PT: in natura (original) acid pretreated; DL-PT: partial delignified acid pretreated.

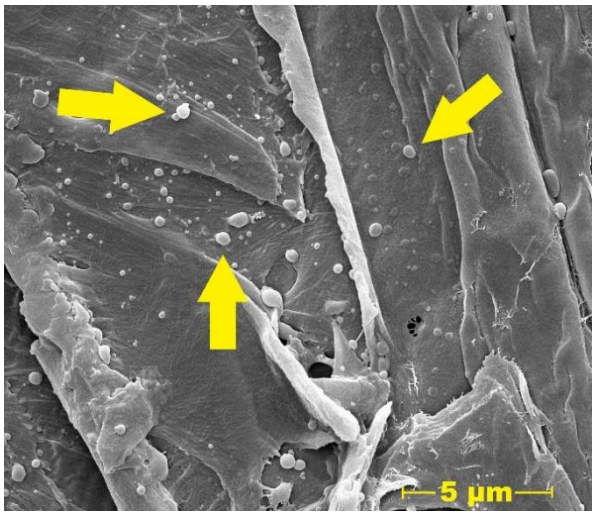


IN: Untreated *in natura*; IN-PT: *In natura* (original) acid pretreated; DL-PT: Partial delignified acid pretreated.

Source: Prepared by the author

Higher magnification of SEM images was possible to visualize pseudo-lignin droplets that were formed during dilute acid pretreatment and condensed onto the material fibers (Fig. 12, arrows). Pseudo-lignin was observed even on the DL-PT material, which was previously partially delignified (Fig. 12, arrows). These droplets are formed from the condensation of sugar degradation products and lignin fragments (CARVALHO et al., 2015), with interference of extractives that can increase the content. Pseudo-lignin is detrimental to enzymatic hydrolysis because it covalently and irreversibly binds to cellulase enzymes during enzymatic hydrolysis, reducing its sugar conversion capacity. Therefore, inhibition of its formation is desired in the lignocellulosic ethanol production process.

Figure 12 - Pseudo-lignin droplets on the external fraction of sugarcane bagasse partial delignified and acid pretreated (DL-PT).



Source: Prepared by the author

4.4 Conclusions

This study evaluated different biomasses/fractions of sugarcane response to delignification and acid pretreatment, in regard to pseudo-lignin formation, cellulose accessibility, and enzymatic hydrolysis glucose yield. The heterogeneity of the biomasses was evidenced by the difference in the chemical composition and response to the pretreatment. Hemicellulose was removed in higher amount from all the biomasses by acid pretreatment. The partial delignification distinguished biomass response to lignin removal. Both the hemicellulose and lignin removal provoked modification in the

material structure increasing the cellulose accessibility revealed by dye adsorption. The pseudo-lignin showed to be influenced by extractives, increasing its amount. Pseudo-lignin formed by the acid pretreatment showed structure similarity, except for those from delignified material that showed modification in the aromatic ring spectra profile. As a result, removing part of the hemicellulose and lignin decreased the pseudo-lignin formation, improving the cellulose accessibility improving enzymatic hydrolysis glucose yield.

CHAPTER 5 BUTYLATED HYDROXYTOLUENE IMPROVES LIGNIN REMOVAL BY ORGANOSOLV PRETREATMENT OF SUGARCANE BAGASSE

Abstract

The diluted acid pretreatment preferably removes hemicellulose, while the organosolv pretreatment also removes lignin. However, in an acidic medium, sugars and lignin degradation products can occur. These compounds can condense forming the pseudo-lignin, which is harmful in the enzymatic hydrolysis stage. Advantages in the pretreatment could be reached by improving lignin removal. In this study was evaluated the effect of the antioxidant butylated hydroxytoluene (BHT) in lignin removal from biomass. BHT had a positive effect on the removal of lignin from biomass in pretreatments performed at 121 °C. With 50% (v/v) ethanol as solvent, the presence of BHT resulted in 45.27 % of lignin removal and 72.53 % of hemicellulose, against its absence that resulted in 35.11 % and 55.73 % of lignin and hemicellulose removal, respectively. FTIR revealed that BHT promotes a reduction in HO-C=O in carboxylic acids; C=O in ketones and C=O bonds in aromatic ring vibrations, while the pseudo-lignin formed only in the presence of ethanol (without BHT) showed a reduction in Syringyl and Guaiacyl units. Tests performed at 160 ° C removed approximately 98% of the hemicellulosic fraction of the biomass. The enzymatic hydrolysis of the biomass showed varied glucose conversions, which may have suffered the influence hemicellulose and lignin removal. Pretreated material at 160 ºC with the addition of 0.3% BHT, resulted in 61.63% glucose yield after enzymatic hydrolysis. Removal of hemicellulose and lignin favored enzymatic hydrolysis and can improve the recovery of hemicellulose and lignin.

Keywords: Cellulose; enzymatic hydrolysis, pseudo-lignin

5.1 Introduction

The cellulosic ethanol production process comprises pretreatment steps, enzymatic hydrolysis, fermentation and distillation. However, there is still a need to improve production technologies mainly in the pretreatment stage. The purpose of pretreatment is breakdown of the lignocellulose structure, reducing the crystallinity of the cellulose and increasing the accessible surface area for action of enzymes. Several pretreatment techniques can be applied to biomass, individually or in combination, increasing the yield and productivity of the desired products. The appropriate choice of the type of pretreatment and applied conditions can be critical in terms of conversion, generation of inhibitory toxic compounds, energy demand and treatment of by-products generated in the process (SCHMATZ; TYHODA; BRIENZO, 2020; SOLARTE-TORO et al., 2019).

Physical methods, such as ball milling or steam blast, mechanically fractionate the biomass, reducing its particle size. Chemical methods result in structural breakdown of the cell wall, removing hemicellulose or lignin and promoting improvement in cellulose recovery (SCHMATZ; TYHODA; BRIENZO, 2020). Among the pretreatment technologies organosolv use of organic solvents and partially removes the lignin from the material. (ESPIRITO SANTO et al., 2018). The application of ethanol in the organosolv pretreatment is considered economically attractive, due to the recovery and recycling of ethanol, generating substrates rich in cellulose, with good enzymatic digestibility and producing high purity lignin that can be used as feedstock material (ZHANG; WU, 2015). On the other hand, pretreatment with diluted acid acts on the disruption of the lignocellulosic matrix, cleaving glycosidic bonds, converting polysaccharides into oligomers and monomeric sugars. Hemicellulose is the fraction most affected in this pretreatment (depolymerization into xylose and inhibitory compounds), however, a fraction of lignin and cellulose can be affected (SOLARTE-TORO et al., 2019).

Under conditions of high severity in the pretreatment (high temperatures, pressure and reaction time), sugar-degrading compounds such as furfural, 5-hydroxymethylfurfural (HMF), levulinic acid, formic acid, etc., are usually produced. These compounds are described as potential inhibitors of the fermenting microorganisms (SOLARTE-TORO et al., 2019). Degradation products can undergo additional polymerization and/or polycondensation reactions, forming pseudo-lignin. This molecule is undesirable in the pretreatments, since its presence is more harmful to enzymatic hydrolysis of cellulose than soluble acid lignin (HU; JUNG; RAGAUSKAS, 2013). Droplets of pseudo-lignin tend to bind irreversibly to enzymes through hydrophobic interactions that cause loss in their activities. This unproductive bond may result in the requirement for high enzyme charges (HU; JUNG; RAGAUSKAS, 2012).

Usually, lignin is a by-product in the pretreatment for cellulose conversion into glucose by saccharification, as well as a co-product in the cellulose industry. Specific uses and processes of biomass biorefineries make lignin a potential source of raw material for value-added products in the fields of chemical, food, textile, pharmaceutical and cosmetic research. Only 5% of the world's production of lignin is used in the formulation of adhesives, dispersants, surfactants, antioxidants and rubbers. The other 95% is used mainly to produce energy in cogeneration systems (CARVAJAL; GÓMEZ; CARDONA, 2016). Lignin isolated from sugarcane bagasse and chemically modified lignins (hydroxymethylation reaction) can have antibacterial and antioxidant properties. In

addition, modified lignins can be used to synthesize resins that are used in adhesives and composites and coatings (KAUR; UPPAL; SHARMA, 2017). Therefore, techniques to increase the extraction of lignin from biomass and its industrial application can optimize the use of this high-value molecule.

Therefore, this study aimed to evaluate a low-cost antioxidant butylated hydroxytoluene (BHT) in diluted acid pretreatment and organosolv, in order to improve lignin removal from sugarcane bagasse. BHT is a phenolic antioxidant widely used in foods to slow the auto-oxidation of polyunsaturated fatty acids. The effects of BHT were investigated because radical reactions have been reported for acidolysis conducted in dioxane-water. Electrophilic free-radicals would probably increase aromatic nuclei at the C-6 ring position, where BHT action can eliminate these free-radicals (GOVENDER et al., 2009).

5.2 Materials and methods

Sugarcane bagasse was kindly donated by São João plant (Araras - SP). The biomass was successively submerged in distilled water, with constant water renewal for 3 days, in order to remove reserve sugars (sucrose). Sugarcane bagasse was oven dried at 55 °C for 48 hours and then ground in a 20-mesh knife mill (0.825 mm).

5.2.1 Dilute acid and Organosolv pretreatment

Approximately 5 grams of bagasse (solid-to-liquid ratio 1:10) was added in Schott bottles with 0.1%, 0.25%, 0.5%, 1% and 2% of BHT m/v (mass/volume) containing 100 mL of dilute sulfuric acid solution 20% m/m (mass acid/material mass, equivalent to approximately 2% mass/volume). The solution was pretreated in autoclave for 30 minutes at 121 °C.

Pretreatment were also performed in stainless steel reactors at 160 °C for 30 minutes with concentrations of 2%, 0.3% and 0.1% of BHT (m/v), 20% sulfuric acid (m/m), and 5 grams biomass (10% m/v). After cooling to room temperature, the hydrolysate from both pretreatments, was filtered using filter paper. The liquid fraction followed for sugar analysis by High Performance Liquid Chromatography (HPLC), while the solid fraction was washed excessively with distilled water until the pH value of the filtrate reached neutrality, and then oven dried at 55 °C for 24h.

Organosolv pretreatments were performed using ethanol as organic solvent, with solutions of 25%, 50% and 75% of ethanol (v/v), with 1% BHT (m/v) and without BHT, 20% sulfuric acid (m/m) and 30 min reaction time at 121 °C, with 5 grams biomass (10% m/v), performed in stainless steel reactors. Resulting hydrolysate was filtered, the liquid and solid fraction was prepared as described previously.

5.2.2 Pseudo-lignin extraction and FTIR analysis

One gram of pretreated sugarcane bagasse was inserted into filter paper bags that were extracted in Soxhlet with 1,4-*p*-dioxane/water solution (9:1) for 8 h. After solvent recovery and evaporation, the residual mass of pseudo-lignin was weighed (HU; JUNG; RAGAUSKAS, 2013). The pseudo-lignin percentage was determined by the relation between mass solubilized per the amount of material used.

Infrared (FTIR)-attenuated total reflectance (ATR) of the pseudo-lignin extracted samples was examined between 4000 and 400 cm^{-1} at 25 °C with 2-cm^{-1} resolution, 32 scans per spectrum. The ATR method used in a FTIR-VERTEX 70/BRUKER spectrophotometer with a source: HeNe laser (emits radiation in the mid-infrared region); Detector: DLaTGS

5.2.3 Chemical characterization

Untreated and pretreated sugarcane bagasse were chemically characterized to determine lignin content, cellulose, and hemicellulose. Approximately 300 mg of dry material was hydrolyzed with 3 mL of 72% (m/m) sulfuric acid at 30 °C for 1 h, with periodic homogenization with glass stick. The reaction was stopped with 84 mL of distilled water and autoclaved at 121 °C for 1 h. The reaction mixture (solids in the solution) was filtered with a porous plate crucible. The liquid fraction was collected to determine soluble lignin by spectrophotometer UV-Vis at 215 and 280 nm and sugar quantification by HPLC (hexoses and pentoses). Solid residue was oven-conditioned at 105 °C to determine insoluble lignin values (ABNT, 2018). All treatments and analyses were performed in triplicate. Values obtained in HPLC were used to calculate the anhydrous sugars, for example, glucose released converted in glucan/cellulose present in the biomass, with hydration factor of 0.9. For xylose and arabinose, the factor was 0.88 and acetic acid of 0.72.

5.2.4 Enzymatic hydrolysis

Sugarcane biomass untreated *in natura* and pretreated sugarcane bagasse were subjected to enzymatic hydrolysis in triplicate using 15 FPU/g of material (Cellic® Cetec–Novozymes, 60 FPU/mL). The reaction was performed with 0.1 g of material in 5 mL (2% solid loading) of 0.05 mol L^{-1} sodium citrate buffer, pH 4.8, 50 °C for 24 h at 120 rpm (BRIENZO et al., 2017). After this reaction period, the hydrolysate was water boiled for 5 min and centrifuged (2500 rpm for 15 min at 4 °C), and the liquid fraction was evaluated by high performance liquid chromatography (HPLC). The values obtained were used to calculate the enzymatic digestibility of the material (anhydroglucose released in relation to the glucan/cellulose content) from the cellulose content present in the biomass. The equation used to obtain the glucose yields follows:

(3) Cellulose conversion (%) = 100 x glucose concentration / (1.11 x *f* x mass biomass)

where glucose is the concentration of glucose released during enzymatic hydrolysis (g L^{-1}); biomass is the dry biomass concentration at the beginning of the enzymatic hydrolysis (g L^{-1}); *f* is the cellulose fraction in dry biomass (g g^{-1}); 1.11 is the conversion factor of cellulose to glucose equivalents.

5.2.5 HPLC analyzes

Sugar monomers and acid acetic were quantified using a HPLC with an Aminex® column (Bio-Rad) HPX-87 H 300 × 7.8 mm, mobile phase 0.050 mol L^{-1} H_2SO_4, flow of 0.4 mL min^{-1}, oven at 65 °C and RID detector, isocratic method, and dilute acid treatment. All treatments and analyses were performed in triplicate.

5.2.6 Statistical analyzes

Results were reported as average of at least three replicates and standard deviation shown. Tukey test was applied to the main results (pseudo-lignin content and enzymatic hydrolysis) to identify similar statistical values (identified with the same letter in figures/tables). DX6Trial-expert software was used (ANOVA), with 95% of significance level ($p < 0.05$).

5.3 Results and discussion

5.3.1 Chemical characterization and solubilized sugars

Untreated *in natura* sugarcane bagasse (raw material) had a chemical composition of 37.13% cellulose, 25.91% hemicellulose and 19.45% lignin. The bagasse pretreated at 121 ºC presented in its chemical composition 53.60% cellulose, 11.30% hemicellulose and 33.70% lignin. Biomass pretreated at 121 °C with the addition of 2% BHT (m/v) showed a chemical composition of 51.9% cellulose, 11.23% hemicellulose and 18.42% lignin. In pretreatments performed with the addition of 25% ethanol (v/v), 50.53% cellulose, 16.84% cellulose and 21.67% lignin were observed in assays without BHT, while in pretreatment with the addition of 1% BHT 59.09% of cellulose, 13.54% of hemicellulose and 21.2% of lignin were observed. In tests performed with 50% ethanol (v/v) the pretreated biomass without BHT presented 56.41% cellulose, 17.90% hemicellulose and 19.70% lignin, while tests conducted with the action of BHT resulted in biomass with 45.83% cellulose, 11.36% hemicellulose and 16.99% lignin, indicating that under these conditions BHT showed an effect in to remove structural components. However, in alcoholic concentrations of 75% (v/v), a slight difference in the chemical composition of the biomass was observed by the addition of BHT in the reaction medium, with 50.99% cellulose, 14.73% hemicellulose and 15.94% in the assays with 1% BHT, and 54.35% cellulose, 15.28% hemicellulose and 15.18% lignin in the absence of BHT (Table 4).

The control pretreatment carried out at 160 ° C, without adding BHT, resulted in biomass with 55.51% cellulose, 0.71% hemicellulose and 40.58% lignin. Addition of 0.1% BHT in pretreatment resulted in a chemical composition of 60.04% cellulose, 1.22% hemicellulose and 34.83% lignin. In pretreatment containing 0.3% BHT, 52.67% cellulose, 0.72% hemicellulose and 37.47% lignin were observed in the pretreated biomass, while in pretreatments with the addition of 2% BHT, the chemical composition of the bagasse was 53.41% cellulose, 0.79% hemicellulose and 33.45% lignin. Pretreatment performed at 160 °C removed large amounts of hemicellulose from biomass compared to pretreatments performed at 121 °C, however, assays performed in alcoholic concentrations (121 °C) showed greater removal of lignin (Table 4).

Table 4 - Chemical characterization of sugarcane bagasse in diluted acid-organosolv pretreatment (20% H_2SO_4 m/m, for 30 min).

Ethanol (% v/v)	BHT (% m/v)	Reaction temperature	Cellulose	Hemicellulose	Total lignin	Solid recovery (%)	Component Removal (g 100 g^{-1})		
							Cellulose	Hemicellulose	Lignin
0	0	Untreated	37.13 ± 1.3	25.91 ± 1.60	19.45 ± 1.00	100	-	-	-
0	0	121 °C	53.60 ± 0.5	11.30 ± 1.9	33.70 ± 1.2	63.00 ± 2.4	9.61	72.82	0
0	2	121 °C	51.9 ± 1.96	11.23 ± 0.31	18.42 ± 0.72	68.11 ± 3.53	5.38	70.48	35.49
25	0	121 °C	50.53 ± 2.36	16.84 ± 0.85	21.67 ± 0.8	70.93 ± 2.51	4.06	53.89	20.97
25	1	121 °C	59.09 ±1.36	13.54 ± 1.91	21.26 ± 0.52	66.06 ± 4.66	0	65.47	23.35
50	0	121 °C	56.41 ± 2.32	17.90 ± 0.6	19.70 ± 0.43	64.07 ± 3.01	3.26	55.73	35.11
50	1	121 °C	45.83 ± 0.28	11.36 ± 0.15	16.99 ± 0.78	62.65 ± 1.77	23.14	72.53	45.27
75	0	121 °C	50.99 ± 2.82	14.73 ± 0.94	15.94 ± 0.66	56.02 ± 2.25	23.54	68.15	54.09
75	1	121 °C	54.35 ± 1.05	15.28 ± 0.5	15.18 ± 0.56	56.38 ± 3.45	17.98	66.75	56.00
0	0	160 °C	55.51 ± 1.56	0.71 ± 0.39	40.58 ± 0.48	45.95 ± 1.99	31.72	98.74	4.13
0	0.1	160 °C	60.04 ± 2.32	1.22 ± 0.21	34.83 ± 0.94	53.23 ± 2.03	14.45	97.49	4.68
0	0.3	160 °C	52.67 ±2.67	0.72 ± 0.08	37.47 ± 1.48	48.80 ± 2.24	31.20	98.64	5.99
0	2	160 °C	53.41 ± 2.64	0.79 ± 0.04	33.45 ± 1.67	56.54 ± 1.98	19.17	98.28	2.76

Source: Prepared by the author

Sugarcane bagasse organosolv pretreated with 50% ethanol (v/v) (190 °C for 50 minutes) revealed a chemical composition of 55% cellulose, 14% hemicellulose and 21% lignin. However, biomass previously pretreated in a hydrothermal medium (30 minutes at 160 °C) and combined with the organosolv pretreatment, resulted in a chemical composition of 64% cellulose, 9.7% hemicellulose and 21% lignin (m/m), indicating greater removal of hemicellulose in the combined treatments (ESPIRITO SANTO et al., 2018). In the present study, the removal of the hemicellulosic fraction was superior, which may be the result of the individual properties of the biomass, such as recalcitrance (resistance to pretreatment), which is influenced by the chemical composition and amount of initial biomass lignin.

Pretreated sugarcane bagasse in ethanol solution (60% ethanol, 5% acetic acid at 160 ° C for 1 hour) showed 43% of glucose, xylose 24%, and 19% lignin (m/m) (ZHANG; WU, 2015). In this study, the presence of diluted sulfuric acid in the reaction medium favors the removal of hemicellulose from the biomass, resulting in greater structural fracturing after pretreatment, and better xylose removal even in shorter reaction times.

Studies carried out with ethanol-organosolv pretreated sugarcane bagasse (157 °C for 90 minutes, with 50% v/v ethanol and formic acid 0.05 mol L^{-1}, liquor-biomass ratio 20:1 v/m) revealed a chemical composition of 41% cellulose, 21% xylan and 24% lignin (m/m). After 120 minutes of reaction, cellulose concentrations of 39%, 19% xylan and 25.7% lignin were observed (m/m). (AGNIHOTRI et al., 2015). In the present study, the removal of pentoses (hemicellulose) was greater than that observed in the literature, even using higher reaction time. This can be left over from the action of sulfuric acid compared to other weak acids, and also due to individual biomass characteristics, such as chemical composition and heterogeneity.

In fact, pretreatments using diluted acids mainly remove the hemicellulosic fraction of the biomass, however, under conditions of high severity, the formation of sugar degradation products that contribute to the formation of pseudo-lignin may occur.

5.3.2 Component removal

Pretreatment of sugarcane bagasse at 121 °C revealed removal of 9.61 g 100 g^{-1} of cellulose, 72.82 g 100 g^{-1} of hemicellulose and without removing the lignin from the biomass. This is due to sulfuric acid reacting with the biomass hemicellulose. However, the addition of 2% (m/v) of BHT to the medium resulted in the removal of 5.38 g 100 g^{-}

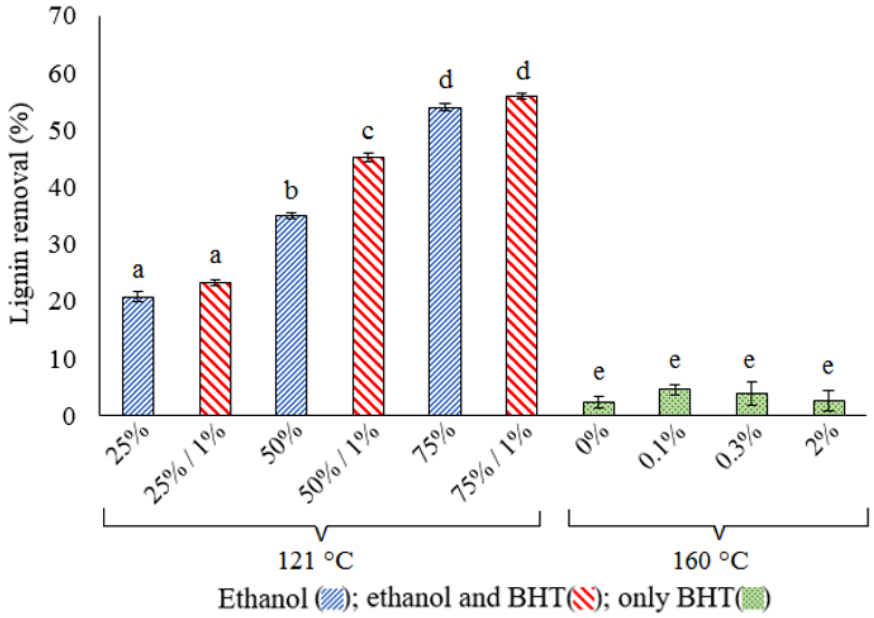
^{1} of cellulose, 70.48 g 100 g $^{-1}$ of hemicellulose and 35.49 g 100 g^{-1} of lignin, indicating that BHT has an effect on the removal of lignin. Greater cellulose removal among the tests performed was observed in pretreatment at 160 º C, without ethanol and BHT (1.72 g 100 g^{-1}). This result may be due to the high temperature applied to the pretreatment (Table 4).

In pretreatments carried out at 121 ºC the removal of hemicellulose varied between 53.89 g 100 g^{-1} and 72.53 g 100 g^{-1}, for the tests carried out with 25% ethanol without BHT and 50% ethanol 1% BHT, respectively. In tests carried out at 160 ° C, the removal of hemicellulose reached yields close to 100% (between 97.49 g 100 g^{-1} and 98.74 g 100 g^{-1}), indicating that temperature has a fundamental influence on the removal of hemicellulose from biomass, with little effect BHT's action (Table 4).

Pretreatment at 121 ºC with 2% BHT removed 35.49 g 100 g^{-1} of lignin, indicating that BHT may have an effect on lignin removal when compared to the control assay. Organosolv pretreatment (121 °C) revealed that with 25% (v/v) ethanol lignin removal was 20.89 g 100 g^{-1} without BHT and 23.35 g 100 g^{-1} with 1% BHT (m/v). With 50% (v/v) ethanol there was 35.11 g 100 g^{-1} removal of lignin without BHT, while the addition of 1% (m/v) BHT resulted in a removal of 45.27 g 100 g^{-1}, an increase of more than 10% due to BHT action. Slight increase in the lignin removal can be observed in alcoholic medium containing 75% ethanol (54.09 g 100 g^{-1} without BHT and 56.0 g 100 g^{-1} with BHT) (Figure 13).

Pretreatment of sugarcane bagasse in organosolv medium (50% v/v ethanol with 0.05 mol L^{-1} formic acid for 90 minutes and solid-to-liquid ratio 1:20) at 160 ° C resulted in biomass delignification of approximately 30%. However, under reaction at 175 ºC, a delignification of 75% was observed, with the highest delignification rate (approximately 80%) occurring at 210 ºC (AGNIHOTRI et al., 2015). According to the literature, temperature is a limiting factor in the delignification of biomass, however it occurs only in the presence of organic solvent. High reaction temperatures can favor the formation of inhibitors generated from the degradation of sugars. Therefore, small concentrations of BHT added to the reaction medium (organosolv) can optimize lignin removal rates, even in milder temperatures.

Figure 13 - Effects of lignin removal with ethanol (25, 50 and 75% v/v) and BHT (1%) in diluted acid and organosolv pretreatment (20% H_2SO_4 m/m, for 30 min). Equal letters indicate statistical similar values.



Source: Prepared by the author

Lignins isolated from biomass can act as a natural and safe source of antioxidants compared to the cost and efficiency of synthetic antioxidants. Natural antioxidants can be used to preserve foods from lipid peroxidation and oxidative damage that occurs in living systems, as well as inhibiting or delaying oil rancification in industrial systems. Natural antioxidants also act to stabilize molecules involved in the loss of color, taste and content of active vitamins in food (FAUSTINO et al., 2010).

5.3.3 Pseudo-lignin extraction

The control pretreatment showed formation of 6.64% of pseudo-lignin in the biomass. The increasing addition of BHT in the pretreatment (0.1, 0.25, 0.50, 1 and 2% m/v) resulted in pseudo-lignin concentrations of 7.96, 7.33, 8.45, 7.06, 8.59, respectively. However, in organosolv pretreatments (25% ethanol), were observed 7.68% pseudo-lignin (without BHT) and 8.82% pseudo-lignin (with 1% BHT). At alcoholic concentrations of 50% (v/v), 7.57% and 12.59% pseudo-lignin were observed in assay without and with BHT, respectively. In the pretreatment with 75% ethanol (1% BHT),

5.37% pseudo-lignin was observed. Organosolv pretreatment with 75% ethanol and BHT was extremely aggressive to the physical aspect of the biomass. Visually, the bagasse became a creamy-looking paste that hindered filtration due to the clogging of the pores of the filter paper.

Due to the different conditions of the pretreatments (acid and organosolv) and the presence or absence of BHT in the reaction medium, the amount of pseudo-lignin formed varied, the control test had statistical similar values to the tests containing 0.1, 0.25 and 1% BHT, and in alcoholic concentrations of 25, 50 (without BHT) and 75% (with BHT) of ethanol (Table 5)

Table 5 - Pseudo-lignin formation by diluted acid and organosolv pretreatment of sugarcane bagasse (20% H_2SO_4 m/m, at 121 °C for 30 min).

Ethanol (%)	BHT (% m/v)	Pseudo-lignin (%)
0	0	6.64 ± 0.68 [a]
0	0.10	7.96 ± 0.84 [a]
0	0.25	7.33 ± 0.55 [a]
0	0.50	8.45 ± 1.20 [b]
0	1	7.06 ± 1.06 [a]
0	2	8.59 ± 0.98 [b]
25	0	7.68 ± 0.14 [a]
25	1	8.82 ± 0.71[b]
50	0	7.57 ± 1.02 [a]
50	1	12.59 ± 0.77 [c]
75	1	5.37 ± 0.98 [a]

Equal letters indicate statistical similar values.

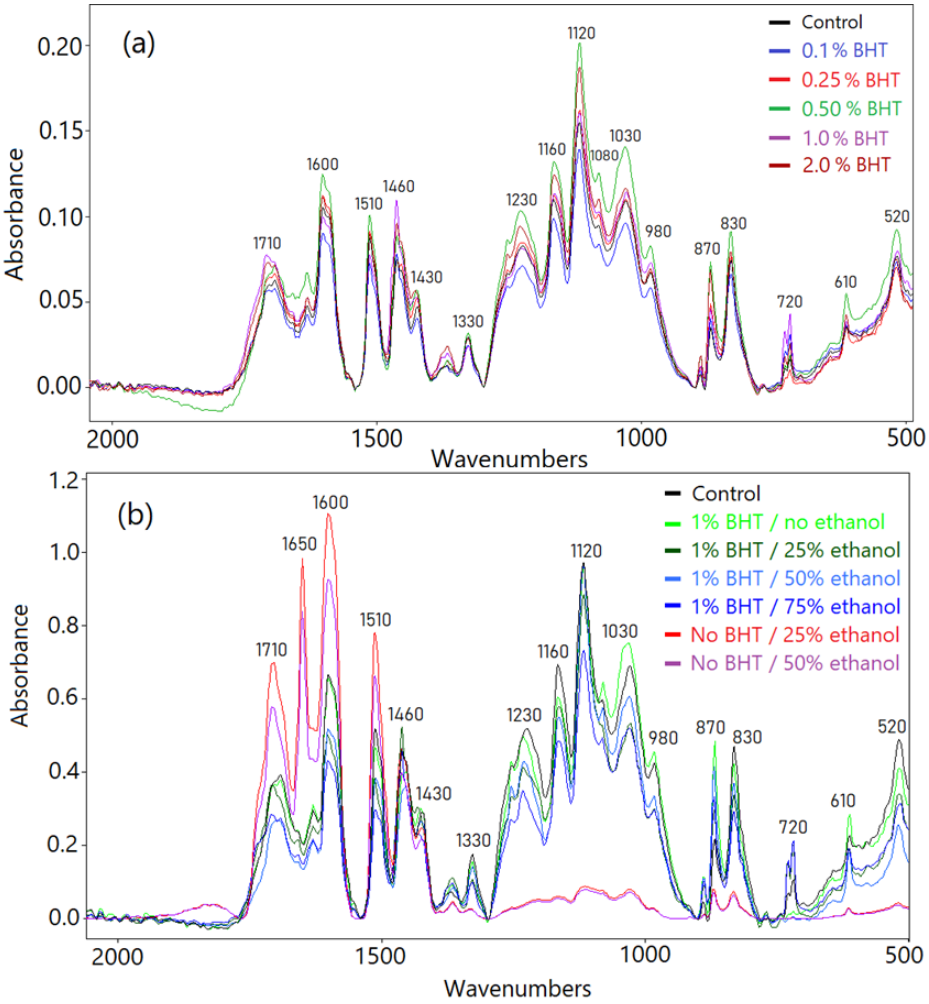
Source: Prepared by the author

Recent study indicate that the content of pseudo-lignin is influenced by extractives and lignin present in sugarcane bagasse. In acid pretreatments (20% H_2SO_4, at 121 °C for 30 min), 10.8% of pseudo-lignin was observed in the pretreated *in natura* biomass, and 6.91% was formed in the extractive-free bagasse, and 1.14% in the material previously delignified with sodium chlorite before the acid pretreatment (SCHMATZ et al., 2020). The results obtained in this study are in accordance with those observed in the literature for untreated biomass.

BHT was previously employed to eliminate electrophilic free radicals, which would likely increase aromatic nuclei at the C-6 ring position in *E. grandis* biomass hydrolyzed (GOVENDER et al., 2009) For this, 0.1g BHT in 100 mL of solution (dioxane:water) was showed to decrease the S:G ratio from 1.59 to 1.44. The degradation/modification/condensation of diketone G is assumed to occur at higher rates than diketone S, and the addition of BHT probably decreased the rate of such reactions (GOVENDER et al., 2009).

The extracted pseudo-lignin evaluated using FTIR analyses agreed with the reported in the literature. Bands at 1710 cm^{-1} represent the C=O stretching in carboxylic acids, ketones, ester groups of carbohydrates, conjugated aldehydes; band in the region between 1600 and 1510 cm^{-1} represents aromatic ring skeletal vibration (C=O stretching); band in region between 1460 cm^{-1} represents the C–H deformation, and 1430 cm^{-1} represents aromatic skeletal vibrations combined with deformation C–H in plane. The band at 1330 cm^{-1} corresponds to the syringyl ring condensed with guaiacyl ring (G ring replaced at position 5), C–C, C–O, C=O stretch, secondary OH aromatic, C–H in plane deformation (typical for G units), and primary OH; 1230 cm^{-1} represents G ring (C=O stretch). Bands between 1200 cm^{-1} and 1050 cm^{-1} represent C–O stretching in alcohols, ethers, or carboxylic acids. Band at 800 cm^{-1} represents C–H out-of-plane bending at positions 2, 5, and 6 (G units), while 870 cm^{-1} represents C–H out-of-plane at positions 2 and 6 (S units) (Fig. 14) (GUILHERME et al., 2015; HU; JUNG; RAGAUSKAS, 2012; SCHMATZ et al., 2020; SINGH et al., 2005).

Figure 14 – FTIR-ATR spectra of pseudo-lignin extracted from sugarcane bagasse pretreated with 20% H_2SO_4 m/m at 121 °C for 30 min. (a) Different BHT concentrations (m/v); and (b) different BHT (m/v) and ethanol (v/v) concentrations.



Source: Prepared by the author

Figure 14 (a) represents tests performed with different loads of BHT in an acid medium, while figure 14 (b) represents tests performed with the addition of ethanol (organosolv) and BHT. Infrared bands of the pseudo-lignin formed in the pretreatments with BHT did not show differences to the control test. This result suggests that the different loads of BHT do not interfere in the composition of the pseudo-lignin (Fig. 14 a). Pseudo-lignin generated form organosolv pretreatment without BHT addition (Figure 14 b) showed strong bands between 1710 cm^{-1}. This band indicated an increase in the HO-C=O in carboxylic acids (conjugated and unconjugated, representing carbonyl and carboxylic groups present in the pseudo-lignin, stretching in ketones); 1650 cm^{-1} C=O in ketones; 1600 and 1500 cm^{-1} C-H vibration in aromatic ring (C=O bonds in aromatic ring

vibrations, the latter mainly in G units). Absence of bands was observed in the region between 1400 and 900 cm^{-1} indicated that units S and G.

5.3.4 Enzymatic hydrolysis

Untreated sugarcane bagasse showed a yield of 8.46% glucose in enzymatic hydrolysis. Low sugar conversion corresponds to the hydrolysis of the non-crystalline region of the cellulose. Pretreatment is necessary to disrupt the biomass and increase the cellulose surface area (SHIMIZU et al., 2020), increasing the yields of enzymatic hydrolysis. Pretreatments carried out at 121 ° C without the addition of BHT showed 36.4% of glucose in enzymatic hydrolysis, while with the addition of 2% of BHT (m/v) it presented 37.55%, statistical similar values (Table 6).

With ethanol concentrations of 25% (v/v) and 121°C pretreatment, 51.52% and 36.82% glucose were observed, respectively for tests without and with the addition of BHT (1% m/v). With 50% ethanol (v/v) 39.10% and 51.72% conversion were observed, respectively in the tests without and with BHT. At 121 °C the highest conversion in the enzymatic hydrolysis was observed 75% ethanol and without the addition of BHT (58.8%), while the addition of BHT in the reaction medium (75% ethanol) resulted in 41.26% glucose (Table 6).

Pretreatments performed at 160 °C, control assay, showed 45,98% glucose in the enzymatic hydrolysis (control). The addition of 0.1, 0.3 3 2% BHT resulted in yields of 48.29%, 61.63% and 46.98%, respectively (Table 6). Low yields in the conversion of sugars may be the result of the removal of the non-crystalline region of the cellulose (component removal) during pretreatments (Table 4).

Table 6 - Enzymatic hydrolysis (cellulose into glucose conversion) of sugarcane bagasse organosolv pretreated with BHT (20% H_2SO_4 m/m, for 30 min).

Ethanol (%)	BHT (% m/v)	Reaction temperature	Glucose conversion (%)
-	-	Untreated	8.46 ± 1.79 [a]
0	0	121 °C	36.40 ± 2.49 [b]
0	2	121 °C	37.55 ± 0.38 [b]
25	0	121 °C	51.62 ± 2.18 [c]
25	1	121 °C	36.82 ± 2.69 [b]
50	0	121 °C	39.10 ± 1.16 [d]
50	1	121 °C	51.72 ± 2.47 [e]
75	0	121 °C	58.80 ± 0.04 [f]
75	1	121 °C	41.26 ± 0.07 [g]
0	0	160 °C	54.98 ± 1.80 [h]
0	0.1	160 °C	48.29 ± 1.32 [i]
0	0.3	160 °C	61.63 ± 0.03 [j]
0	2	160 °C	46.98 ± 0.77 [i]

Equal letters indicate statistical similar values.

Prepared by the author

Enzymatic hydrolysis (72 h, substrate at 4% m/v, in 50 mmol L^{-1} sodium citrate buffer, pH 5.0, at 150 rpm, 50 °C) of untreated and organosolv pretreated sugarcane bagasse (50% ethanol at 190 °C for 50 minutes) with 10 FPU of Accellerase 1500 and Beta-Glucanase15 U/g of biomass resulted in 22% (untreated) and 51% cellulose conversion (ESPIRITO SANTO et al., 2018). In the present study, similar levels of cellulose conversion were reached, even under milder pretreatment conditions and shorter enzymatic hydrolysis time. The heterogeneity of biomass is a limiting factor in the conversion of sugars, and for conversion of sugars, biomasses with less recalcitrance are desirable.

Sugar conversion of sugarcane bagasse after organosolv pretreatment (60% ethanol, 5% acetic acid at 160 ° C for 1 hour) and enzymatic hydrolysis (20 FPU/g at 50 °C for 24 hours, in sodium acetate buffer pH 4.8) revelated that there was a conversion of cellulose of approximately 15%. As the hydrolysis time increased to 72 hours the conversion reached 35% (ZHANG; WU, 2015). The present study showed better

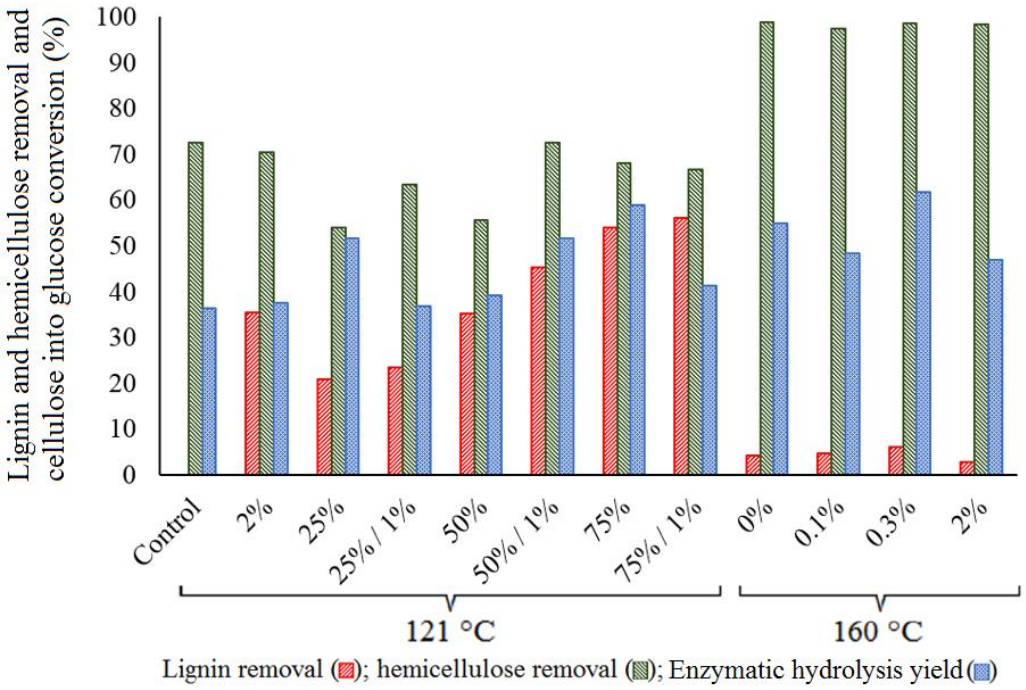
conversion results. the removal of lignin and hemicellulose from biomass favors enzymatic hydrolysis. The sulfuric acid in the medium also results in greater removals of hemicellulose from the biomass, exposing the cellulosic fibers. However, under conditions of high severity they may favor the formation of inhibitors.

Sugarcane bagasse pretreated with diluted sulfuric acid (20% H_2SO_4 m/m at 121 °C for 30 min) and enzymatic hydrolysis (15 FPU/g at 50 °C in sodium acetate buffer pH 4.8 for 24 h) showed a conversion of cellulose into glucose of approximately 54% (SCHMATZ et al., 2020), values close to those obtained in this study.

Success in enzymatic hydrolysis (high conversion yield) can be achieved through a combination of desirable factors for the process, such as removing large amounts of hemicellulose and lignin from biomass (Figure 15). Pretreatments applied sequentially to remove hemicellulose and lignin generate cellulose-rich biomass. However, the variation in the severity of the pretreatments applied to the sugarcane bagasse removed part of the cellulose from the biomass, possibly corresponding to the non-crystalline fraction that is more susceptible to the activity of the enzymes, reducing yields of enzymatic hydrolysis. Formed inhibitors may also have contributed to reduced efficiency in enzymatic hydrolysis.

Increase in the accessibility of the cellulosic fraction to cellulose enzymes and minimization in the formation of inhibitors generated under conditions of high severity conditions (load of acids, temperature, reaction time, etc.) can result in the integral conversion of biomass.

Figure 15 - Combined effect of hemicellulose and lignin removal in the enzymatic hydrolysis of diluted acid pretreated materials whit BHT (0.1, 0.3, 1 and 2%) and organosolv with ethanol (25, 50 and 75%) (20% H_2SO_4 m/m, for 30 min).



Source: Prepared by the author

5.4 Conclusion

This study evaluated the effect of the BHT action on organosolv pretreatment of sugarcane bagasse. Pretreatment conducted at 121 ºC with BHT showed potential removal of lignin from biomass. Acid pretreatment, with at 160 ° C mainly removed hemicellulose from the biomass resulting in a material rich in cellulose and lignin. The addition of BHT in the organosolv process improved the removal of lignin for different solvent concentration. Pretreatment with 50% ethanol and with BHT, resulted in higher lignin removal than without BHT. These results indicate that BHT may have industrial applications in lignin removal in biotechnological processes. Among several applied pretreatments, the conversion of cellulose into glucose by enzymatic hydrolysis varied between 36.4% and 61.6%, and may have influenced by the hemicellulose and lignin removal.

CHAPTER 6 ANTIOXIDANT ADDITIVES TO ORGANOSOLV PRETREATMENT IMPROVED LIGNIN REMOVAL

Abstract

Annually, tons of sugarcane bagasse are generated in the sugar and alcohol industries. This biomass has great potential in the use of converting cellulose into glucose, an energy currency for various biotechnological processes. However, lignin is considered a limiting factor in the accessibility to cellulose, due to the recalcitrance of biomass pretreatments are necessary. Acid and hydrothermal pretreatments provoke degradation of sugars, which condense with lignin fragments and precipitate on the fiber, forming pseudo-lignin. This study aimed to improve the lignin removal, evaluating antioxidant effect on biomass pretreatment. Among the additives tested, tert-butylhydroquinone, 3-tert-butyl-4-hydroxyanisole, methyl 3,4,5-trihydroxybenzoate, Tween 20, Tween 80 and dimethyl sulfoxide (DMSO), all of them showed improvement in the lignin removal. The 3-tert-butyl-4-hydroxyanisole reached 71% lignin removal. On the other hand, tween 80 and tert-butylhydroquinone showed a higher extraction yield of sugars from pretreated biomass. Use of antioxidants/surfactants during organosolv pretreatment improved enzymatic hydrolysis with all the antioxidants applied, reaching almost complete conversion of the cellulose into glucose (tween 80). The use of chemical compounds during pretreatment beneficed the removal of lignin from biomass, and consequently the cellulose hydrolysis.

Key words: Surfactants; antioxidants; pseudo-lignin; cellulose; Tween80.

6.1 Introduction

Lignin is considered a limitation in the accessibility of cellulose, as it acts as a physical barrier in the protection of cellulose and can also adsorb proteins in a non-productive way, decreasing the availability of cellulases (SCHMATZ et al., 2020). Hemicellulose hydrolysis and delignification contribute to increasing cellulose accessibility (SHIMIZU et al., 2020), however, delignification was suggested as more important for exposing the surface of carbohydrates, especially in moderate pretreatment conditions (ZHAO et al., 2018).

Lignocellulosic fraction of sugarcane bagasse can be used as raw material in the production of several biotechnological products, such as xylitol, industrial enzymes, organic acids, high value-added products (antibiotics, single cell protein, bio-hydrogen, aroma, pigments, etc.) and second generation ethanol production (ethanol 2G) by converting cellulose to glucose and subsequent fermentation (CHANDEL et al., 2012).

Major challenge in converting sugars is to overcome the natural recalcitrance of biomass (MELATI et al., 2019). Pretreatments are necessary to reduce crystallinity, fiber

size, increase porosity and surface area and reduce hemicellulose and lignin contents. Organosolv and diluted acid pretreatments solubilize hemicellulose and altering or degrading lignin, making cellulose more accessible (PHITSUWAN; SAKKA; RATANAKHANOKCHAI, 2013). Organosolv process applied with ethanol improves cellulose digestibility, promotes efficient extraction of hemicellulosic sugars and produces hydrolysate with a low concentration of compounds derived from the degradation of sugars and lignin. In addition, the use of ethanol promotes greater solids recovery, protection of the cellulosic fraction, increase porosity of the pretreated biomass and allow to recover the lignin fraction with superior quality and with potential use in several applications (MESA et al., 2016). Diluted acid treatment is widely used because it is cheap, convenient and effective in a wide variety of lignocellulosic biomass. The diluted sulfuric acid weakens the hydrolyzed glycosidic bonds in hemicellulose, lignin-hemicellulose bond and lignin bond that leads to the dissolution of sugar from hemicellulose, generating high hydrolysis yields and increasing the porosity of the plant cell wall (BRIENZO et al., 2017; SRITRAKUL; NITISINPRASERT; KEAWSOMPONG, 2017). However, during acid and hydrothermal pretreatments, can occurs generation of inhibitors and/or deactivators of enzymes and toxic substances with affect the quality of enzymatic hydrolysis (CANDIDO et al., 2020; PHITSUWAN; SAKKA; RATANAKHANOKCHAI, 2013).

Degradation products of pentoses (furfural) and hexoses (5-hydroxymethylfurfural) have little effect on the inhibition of cellulase enzymes, but are toxic in the fermentation process (SCHMATZ; TYHODA; BRIENZO, 2020). However, furfural and 5-hydroxymethyl furfural are key compounds in the formation of pseudo-lignin. This molecule is not derived from native lignin, but consists of carbonyl, carboxylic and aliphatic structures, which condense on the cellulosic fibers of plant biomass in an acidic medium. Presence of pseudo-lignin in the reaction medium should be avoided as it significantly inhibits cellulose hydrolysis, due to the irreversible and unproductive binding with cellulase enzymes (HU; JUNG; RAGAUSKAS, 2012).

Dimethyl sulfoxide (called DMSO) was reported as pseudo-lignin suppressor (HU; RAGAUSKAS, 2014), an amphipathic molecule, with a highly polar domain and two apolar methyl groups, soluble in both aqueous and organic media. DMSO is a hydrogen-bound disrupter, a cell-differentiating agent, which eliminates hydroxyl radicals, solubilizing agent used in the preparation of samples for electron microscopy, exercising many applications in the pharmaceutical industry in the *in vivo* administration

of various water-insoluble substances (SANTOS et al., 2003). Based on the DMSO action, the hypothesis of this study was that cheaper and non-toxic antioxidant could collaborate to pseudo-lignin formation or improve the lignin solubilization. Tert-butylhydroquinone (TBHQ) is reported stabilize of biofuels (JAIN; SHARMA, 2010). 3-tert-butyl-4-hydroxyanisole (BHA) has great potential as an antioxidant compound, used as a parameter in tests of natural antioxidants (KAUR; UPPAL, 2015). Methyl-3,4,5-trihydroxybenzoate (Methyl gallate) is a bioactive phenolic compound widely distributed in herbs and plant foods. It is a gallotanine that can be found in medicinal and food plants, such as Galla rhois *(Rhus chinensis*), Chinese toon (*Toona sinensis*), Shantung maple (*Acer truncatum*), mushrooms (*Pholiota adiposa*) and mangoes (*Mangifera indica*) (JIAMBOONSRI et al., 2015). The non-ionic surfactant Tween 20 is used as a catalyst, emulsifier and complexing agent in aqueous and non-aqueous media, in addition as the most widely used detergents in the industry as a solubilizer with a wide range of applications in biological systems (PRASAD et al., 2015), while Tween 80 has specific cellobiohydrolase activating potential. The suppression of cellobiohydrolase inhibitors (lignin, hemicellulose and its derivatives that compete for the active site) keeps the enzyme in high activity during the enzymatic hydrolysis process (XIN et al., 2017).

Improvement of organosolv pretreatment of biomass conversion can collaborate to the process feasibility. This study aimed to evaluate and the effectiveness of chemical compounds in organosolv pretreatment, which may have antioxidant effects that act in the removal of lignin from biomass. The effect of the antioxidant auxiliary the lignin removal was evaluated for enzymatic hydrolysis improvement.

6.2 Material and methods

Sugarcane bagasse was donated by the CTC (Centro de Tecnologia Canavieira – Piracicaba - SP, Brazil), received after juice extraction at the refinery and then was successively washed with distilled water. Sugarcane bagasse was oven dried at 55 °C for 48 hours and then ground in 20-mesh a knife mill (825 μm). The biomass was conditioned in plastic bags and stocked to assay.

6.2.1 Organosolv pretreatment

Pretreatments were carried out in stainless steel reactors with 5 grams of sugarcane bagasse (solid-to-liquid ratio 1:10 mass/volume) in 50% ethanol solution and 20% of sulfuric acid (mass acid/mass of material, or 2% m/v).

Pretreatments were conducted without (control sample) and with addition of chemicals purchased by the Sigma-Aldrich® company. Six antioxidants were evaluated, called TB (tert-butylhydroquinone); TH (3-tert-butyl-4-hydroxyanisole); MT (methyl 3,4,5-trihydroxybenzoate); T2 (Tween 20); T8 (Tween 80); and DS (dimethyl sulfoxide – DMSO) (Attachment A). TB, TH and MT were used 1% mass/mass in reaction medium, while T2, T8, and DS were used 2% (mass/volume). The reactor was inserted in an oil bath previously heated to 160 °C. After 30 min of pretreatment the reactor was cooled to room temperature in ice bath. Pretreated sugarcane bagasse was vacuum-filtered, and the liquid fraction was used for sugars and degradation products determination. The solid fraction resulting from the pretreatment was washed with distilled water until the neutral pH. The resulting biomass was oven dried at 55 °C for 48 hours and stored in plastic flasks for future analysis. All tests were performed in triplicate.

6.2.2 Chemical characterization

Untreated and pretreated sugarcane bagasse were chemically characterized to determine lignin, cellulose and hemicellulose content. Approximately 300 mg (dry basis) were hydrolyzed with 3 mL of 72% sulfuric acid at 30 °C for 1 h, with periodic homogenization with glass stick. The reaction was stopped with 84 mL of distilled water and autoclaved at 121°C for 1 h. The reaction mixture (solids in the solution) was filtered with a porous plate crucible. The liquid fraction was collected to determine soluble lignin by spectrophotometer UV-VIS (215 and 280 nm) and sugar quantification by HPLC (hexoses and pentoses). Solid residue was oven-conditioned at 105 ºC to determine insoluble lignin values (ABNT, 2018). All treatments and analyzes were performed in triplicate. Values obtained in HPLC were used to calculate the anhydrous sugars, for example, glucose released converted in glucan/cellulose present in the biomass, with hydration factor of 0.9. For xylose and arabinose, the factor was 0.88 and acetic acid of 0.72.

6.2.3 Pseudo-lignin determination

Approximately 1 g of the pretreated biomass was inserted into filter paper bags,

which were extracted in soxhlet with 1,4-dioxane:water solution (9:1) for 8 h. After solvent recovery and evaporation, the residual mass of pseudo-lignin was weighed (HU; JUNG; RAGAUSKAS, 2013). The pseudo-lignin percentage was determined by the relation between mass solubilized per the amount of material used. Pseudo-lignin was evaluated in Infrared (FTIR).

6.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

Infrared (FTIR) attenuated total reflectance (ATR) of the pseudo-lignin extracted samples were examined between 4000 and 400 cm^{-1} at 25 °C with 2 cm^{-1} resolution, 32 scans per spectrum. The ATR method used in a FTIR-VERTEX 70/BRUKER spectrophotometer with a source: HeNe laser (emits radiation in the mid-infrared region); Detector: DLaTGS.

6.2.5 Enzymatic hydrolysis

Untreated and pretreated biomasses were subjected to enzymatic hydrolysis in triplicate using 15 FPU/g of material (Cellic® Cetec – Novozymes, 60 FPU/mL). The reaction was performed with 0.1 g of material in 5 mL of 0.005 mol L^{-1} sodium citrate buffer (2 % solid loading), pH 4.8, 50 °C for 24 hours at 140 rpm (BRIENZO et al., 2017). After this reaction period, the hydrolysate was water boiled for 5 min, centrifuged (2500 rpm for 15 min at 4 ºC), and filtered on cellulose acetate membrane 0.22 µm. Liquid fraction was evaluated by High Performance Liquid Chromatography (HPLC). The values obtained were used to calculate the enzymatic digestibility of the material (anhydroglucose released in relation to the glucan/cellulose content) from the cellulose content present in the biomass as follows:

(4) Cellulose conversion (%) = 100 x glucose concentration / (1.11 x *f* x mass biomass)

where glucose is the concentration of glucose released during enzymatic hydrolysis (g L^{-1}); biomass is the dry biomass concentration at the beginning of the enzymatic hydrolysis (g L^{-1}); *f* is the cellulose fraction in dry biomass (g g^{-1}); 1.11 is the conversion factor of cellulose to glucose equivalents.

6.2.6 High Performance Liquid Chromatography (HPLC)

Pentoses, hexoses and acid acetic resulting from the liquid fraction of the organosolv and chemical characterization treatments were quantified by HPLC, with Aminex® column (Bio-Rad) HPX-87 H 300 x 7.8 mm, mobile phase 0.05 mol L^{-1} H_2SO_4, flow of 0.4 ml min^{-1}, oven 65ºC and RID detector, isocratic method. All treatments and analyzes were performed in triplicate.

6.2.7 Statistical analyzes

Results were reported as average of at least three replicates and standard deviation showed. Tukey test was applied to the main results (pseudo-lignin content and enzymatic hydrolysis) to identify similar statistical values (identified with the same letter in figures). DX6Trial-expert software was used (ANOVA), with 95 % of significance level (p<0.05).

6.3 Results

6.3.1 Chemical characterization

Untreated sugarcane bagasse presented in its chemical composition 37.36% cellulose, 26.1% hemicellulose, 19.43% lignin and 6.63% extractives (washed in soxhlet with ethanol 92.8% for 8 h, and subsequent water washed for the same period). After organosolv pretreatment, the control sample showed 72.98% cellulose, 7.61% hemicellulose and 22.62% lignin in its chemical composition. Among the tests carried out with the addition of antioxidants and surfactant, cellulose content between 71.27% and 77.89% was observed for the experiments TB, TH, MT, T2 and DS. However, experiment performed with Tween 80 (T8) showed 57.97% of cellulose in its chemical composition. Regarding the hemicellulose present in the biomass after organosolv pretreatment, it was observed variation between 5.59% (T8) and 8.83% (MT). Lower percentage of lignin was observed in the pretreatments with TH (16.12%), MT (16.81%) and DS (17.09%), while the highest percentage with TB (23.79%), T2 (22.36%) and T8 (20.28%) (Table 7).

Table 7 - Chemical composition of pretreated sugarcane bagasse after organosolv pretreatment at 160 °C for 30 min with 20% (m/m) of acid.

	Chemical composition (%)				Component Removal (g 100 g^{-1})		
	Cellulose	Hemicellulose	Total Lignin	MR (%)	Cellulose	Hemicellulose	Lignin
Untreated	37.36 ± 1.30	26.10 ± 1.60	19.43 ± 1.03	100	-	-	-
Control	72.98 ± 2.47	7.61 ± 0.59	22.62 ± 1.21	44.2 ± 2.12	13.64	87.11	48.53
TB	73.58 ± 3.05	7.09 ± 0.42	23.79 ± 0.43	38.2 ± 1.37	23.53	89.54	58.86
TH	77.89 ± 0.86	8.41 ± 1.32	16.12 ± 1.13	40.26 ± 0.25	15.96	87.03	71.21
MT	75.84 ± 2.15	8.83 ± 1.12	16.81 ± 0.92	43.98 ± 0.61	10.69	85.12	66.49
T2	71.27 ± 1.34	6.52 ± 0.81	22.36 ± 1.45	47.16 ± 0.62	10.02	88.21	48.96
T8	57.97 ± 3.32	5.59 ± 0.45	20.28 ± 0.60	49.53 ± 1.56	23.14	89.39	51.56
DS	74.23 ± 2.23	6.46 ± 0.9	17.09 ± 0.88	44.63 ± 1.64	11.32	88.94	64.45

MR: Mass recovery; TB: Tert-butylhydroquinone; TH: 3-tert-butyl-4-hydroxyanisole; MT: Methyl 3,4,5-trihydroxybenzoate; T2: Tween 20; T8: Tween 80; and DS: Dimethyl sulfoxide – DMSO.

Source: Prepared by the author

Lower percentage of xylan was reported in chemical composition of organosolv pretreated sugarcane bagasse (60% v/v ethanol, solid-to-liquid ratio 1:10, at 160 ºC for 30 minutes), with 70% cellulose, 3% xylan and 19% lignin. However, the reaction was $FeCl_3$-catalyzed (0.05 mol L^{-1}, 60% v/v ethanol, at 160 ºC for 10 minutes), resulting in all the xylan and 59% of the lignin removal, causing increase in cellulose content to 75%. Delignification and the removal of xylan increased gradually as the $FeCl_3$ concentration increased (ZHANG et al., 2018).

In the present study, the control assay showed 87.11% removal of the hemicellulosic fraction. The tests performed with the addition of chemical compounds showed little or no difference in relation to the control assay, with averages between 85.12% (MT) and 89.54% (TB), indicating that only the organosolv pretreatment under the applied conditions in this study are effective in removing most of the hemicellulose present in the biomass. The large amount of glucose observed in residual pretreated biomass (mass recovery) is due to the partial removal and/or degradation of the hemicellulosic fraction.

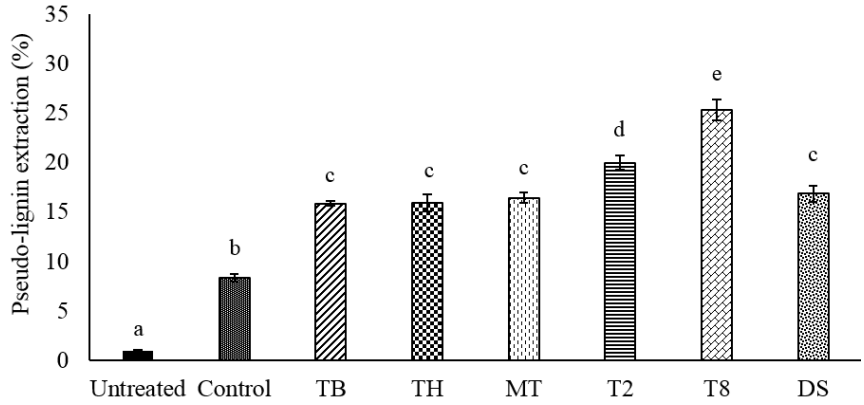
Higher lignin content observed in the pretreated biomass compared to untreated biomass can be attributed mainly to the loss of polysaccharides and the formation of pseudo-lignin, which occurs in acid and hydrothermal treatments (PU et al., 2013). However, organosolv process causes the removal of lignin from biomass and the tested

antioxidants/surfactants potentiated this effect. As shown in table 7, the control test showed lignin removal of 48.53 g 100 g^{-1}. The lowest lignin removal were observed with the addition of T2, T8 and TB (48.96, 51.56 and 58.86 g 100 g^{-1}, respectively), while the highest lignin removal were observed with the addition of TH, MT and DS (71.21, 66.49 and 64.45 g 100 g^{-1}, respectively). These results indicate that chemical compounds applied during the organosolv pretreatment can increase lignin removal yields from sugarcane biomass. Among the antioxidants tested, TH showed a removal of approximately 23 g 100 g^{-1} higher than that observed in the control test.

6.3.2 Pseudo-lignin extraction

Pretreated samples (adding antioxidants) showed pseudo-lignin extraction of 15.82% (TB); 15.89% (TH); 16.41% (MT); 19.95% (T2); 25.28% (T8); and 16.8% (DS) (Figure 16). Untreated and extractive-free sugarcane bagasse resulted in 0.9% of extraction (not identified material), while the control test (no antioxidant added) showed 8.32% pseudo-lignin, indicating that the extracted content was formed during organosolv pretreatment. Compounds TB, TH, MT and DS produced approximately twice pseudo-lignin compared to the control test and showed similar statistical value. Pretreatment T2 presented approximately two and a half times more pseudo-lignin than control assay, while pretreatment T8 presented a three times pseudo-lignin more than the control assay (Figure 16).

Figure 16 - Pseudo-lignin extraction from sugarcane bagasse after organosolv pretreatment at 160 °C for 30 min.



TB: Tert-butylhydroquinone; TH: 3-tert-butyl-4-hydroxyanisole; MT: Methyl 3,4,5-trihydroxybenzoate; T2: Tween 20; T8: Tween 80; and DS: Dimethyl sulfoxide – DMSO. Equal letters indicate statistical similar values.

Source: Prepared by the author

Studies conducted with untreated sugarcane bagasse submitted to diluted acid pretreatment (20% H_2SO_4, 30 min at 121 °C, solid-to-liquid ratio 1:10) revealed a formation of 10.82% pseudo-lignin. However, applied the process of extractives removal with ethanol and water prior the diluted acid pretreatment (extractive-free bagasse), pseudo-lignin content observed after diluted acid pretreatment was 6.91% (SCHMATZ et al., 2020). Some antioxidants/surfactants may have facilitated the breakdown of sugars in furfural and HMF.

DMSO has a solvation effect on HMF protection, on rehydration and humine formation reactions (except dehydration reaction on HMF, key intermediate in pseudo-lignin formation), that result in acyclic dehydration and fragmentation products, insoluble humines and reversion products. The addition of DMSO to the reaction medium and the use of an organic phase can extract HMF from the aqueous medium. It is suggested that the presence of DMSO in the reaction medium play a role in protecting HMF from additional rehydration to levulinic acid and formic acid. The addition of non-aqueous solvents such as DMSO affects the selective dehydration of carbohydrates in furan compounds (MUSHRIF; CARATZOULAS; VLACHOS, 2012).

Higher levels of pseudo-lignin observed in the tests performed with Tween 80 (T8) compared to the other pretreatments, can be explained by greater degradation of sugars during the pretreatment. Higher yields of sugars degradation may result in higher pseudo-lignin formation (tween 80). As observed in the chemical characterization, assay T8 presented a lower cellulose and hemicellulose content in the pretreated biomass and greater removal of sugars (component removal).

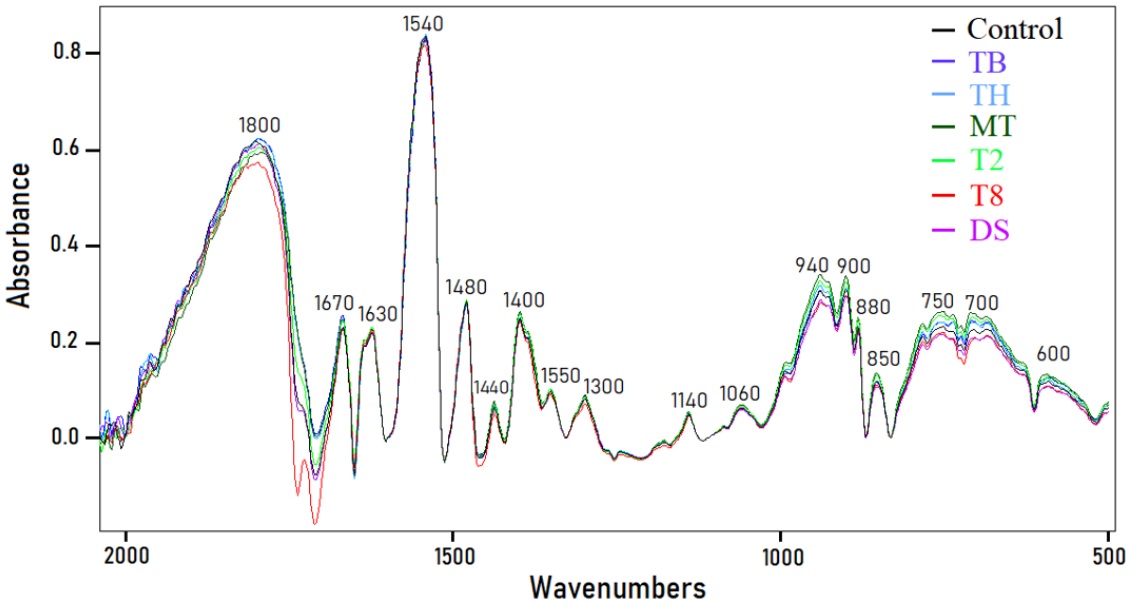
6.3.3 Fourier Transform Infrared Spectroscopy (FTIR)

Pseudo-lignin extracted from pretreated sugarcane bagasse (with or without antioxidants) showed similarity among the bands observed in the spectra (Figure 17). Specific bands for each region indicate similarity in the composition of the pseudo-lignin, with no distinct differences among samples.

A wide band was observed in the region between 1900 cm^{-1} and 1600 cm^{-1}, which corresponds to the carbonyl groups bond C=O (stretching in carboxylic acids, ketones, ester groups of carbohydrates, conjugated aldehydes); region between 1600 and 1510 cm^{-1} represents aromatic ring skeletal vibration (C=O stretching) corresponding to

aromatic ring of lignin; band at 1300 cm^{-1} represents S ring and G ring condensed (G ring substituted at position 5) C–C, C–O, C=O stretch, G condensed > G etherified, secondary OH aromatic C–H in plane deformation, typical for G units and primary OH (HU; JUNG; RAGAUSKAS, 2012; SINGH et al., 2005; YANG et al., 2016) (Figure 17).

Figure 17 – FTIR-ATR spectra of pseudo-lignin extracted form sugarcane bagasse organosolv pretreated at 160 °C for 30 min.



TB: Tert-butylhydroquinone; TH: 3-tert-butyl-4-hydroxyanisole; MT: Methyl 3,4,5-trihydroxybenzoate; T2: Tween 20; T8: Tween 80; and DS: Dimethyl sulfoxide – DMSO.

Source: Prepared by the author

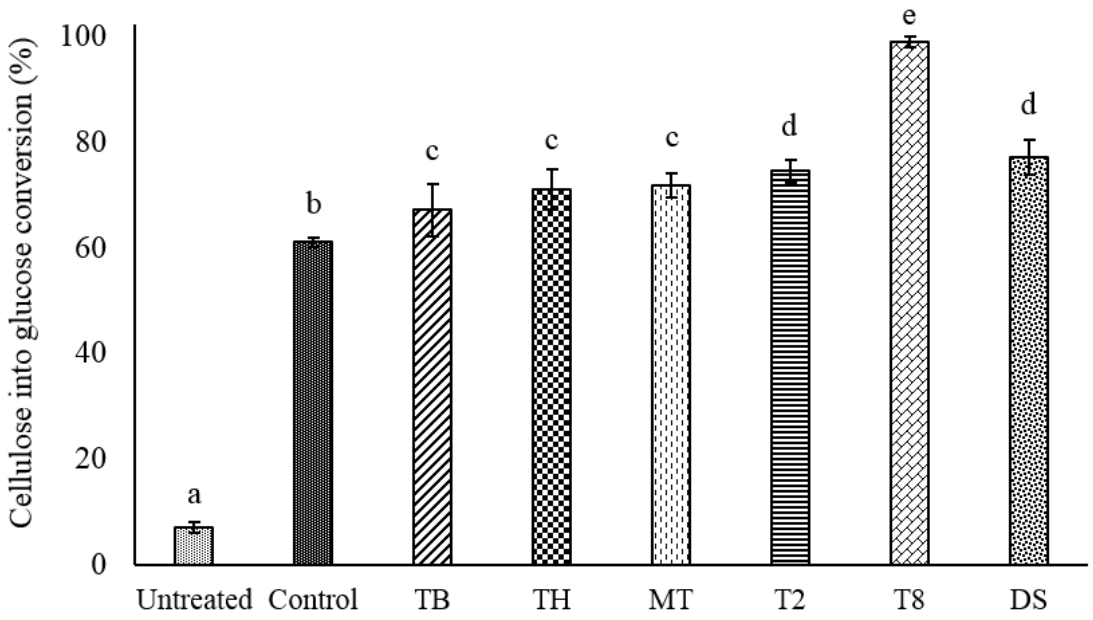
Similarity between the spectra can be attributed to the combination of temperature (160 °C), acid (20% m/m), time (30 min) and the concentration of ethanol in the reaction medium (50% v/v), indicating that the composition of the pseudo-lignin depends of process conditions applied to the pretreatment, and it was not influenced by the chemical compounds applied. Pseudo-lignin spectrum can be attributed to the removal and degradation of compounds from lignin and hemicellulose during pretreatment, resulting in the fragmentation of the structure composed of cellulose, hemicellulose and lignin.

6.3.4 Enzymatic hydrolysis

Untreated sugarcane bagasse resulted in lower glucose yields (7.08%) after enzymatic hydrolysis, showing that the pretreatment is necessary to breakdown the

biomass structure and increase the cellulose accessibility, improving enzymatic action (BRIENZO et al., 2015; SHIMIZU et al., 2020). The control pretreatment (no antioxidant) showed 61% of glucose after enzymatic hydrolysis, resulting from the exposure of cellulosic fibers to cellulase enzymes, due to the fractionation of biomass by the pretreatment. Addition of chemical compounds in the pretreatment caused an increase in the conversion of cellulose into glucose. Pretreatment with TB showed a conversion of 67.15%, while pretreatment with TH showed a conversion of 71.12% and the addition of MT resulted in a conversion of 71.85%. The biomasses pretreated with T2, T8 and DS showed conversion of 74.45%, 98.9% and 77.16%, respectively (Figure 18). Improvement in the enzymatic hydrolysis yield may be the result of the lignin removal by the addition of antioxidants/surfactants in the organosolv pretreatment. However, other mechanisms must be related to action of surfactants in saccharification process (T2 and T8), by reducing the non-specific enzyme-lignin binding.

Figure 18 - Glucose yield from enzymatic hydrolysis of untreated and organosolv pretreated sugarcane bagasse.



TB: Tert-butylhydroquinone; TH: 3-tert-butyl-4-hydroxyanisole; MT: Methyl 3,4,5-trihydroxybenzoate; T2: Tween 20; T8: Tween 80; and DS: Dimethyl sulfoxide – DMSO. Equal letters indicate statistical similar values.

Source: Prepared by the author

Sugarcane bagasse pretreated at 160 °C for 60 minutes (60% ethanol v/v and 5% mass acetic acid, solid-to-liquid ratio 1:8) and subsequent hydrolysis enzymatic activity

for 72 hours (2 % m/m loading, at 50 °C, with 20 FPU/g of Cellulase enzyme) revealed glucose yields of approximately 35% (15 g based on 100 g raw material), while untreated bagasse showed less than 10% (4 g based on 100 g raw material) conversion in the same hydrolysis time (ZHANG; WU, 2015). The difference in glucose conversion obtained in the present study can be attributed to the presence of sulfuric acid in the reaction medium, which extracts part of the hemicellulosic fraction of the biomass exposing the cellulose fibers, as well as by the chemical action caused by the addition of antioxidants/surfactants, due lignin removal in organosolv pretreatment.

Tween is commonly added to the enzymatic hydrolysis study. Organosolv pretreated sugarcane bagasse was subsequent enzymatic hydrolyzed (20 FPU/g, 2% solids m/m at 50 °C/24 h) resulting in glucose yield of 80%. Supplementation with 150 mg g^{-1} of Tween 80 (30 minutes before the addition of cellulose enzymes – 20 FPU/g) in $FeCl_3$-catalyzed organosolv pretreatment, resulted in 43.9 g of glucose after 24 hours os hydrolysis. In this case, the total glucose yield can reach up to 97.9%, which was ascribed to the $FeCl_3$-catalyzed ethanol pretreatment and the addition of Tween 80 (ZHANG et al., 2018). On the other hand, in the present study the Twen 80 was applied during organosolv treatment, showing effect on the pretreated material, improving cellulose conversion in the enzymatic hydrolysis.

Enzymatic hydrolysis of organosolv pretreated sugarcane bagasse (50% v/v ethanol with 1.5% NaOH m/m at 175 °C for 90 minutes, solid-to-liquid ratio 1:5) with the addition of 2.5% Tween 20 (m/m) resulted in a significant influence on glucose yield. Surfactant can change the structure of the substrate, increasing the stability of the enzyme, reducing denaturation, and affecting the enzyme-substrate interaction (MESA et al., 2010). In the present study, the decrease of lignin content could minimize of the non-specific enzyme bonds to the substrate (such as lignin), favoring the performance of cellulolytic enzymes. Furthermore, the pretreatment resulted in a material that with standard enzymatic hydrolysis condition resulted in high cellulose conversion into glucose.

6.4 Conclusion

Some chemical compounds were tested for their effectiveness in removing the lignin fraction from sugarcane bagasse. In general, all tests conducted showed hemicellulose removal greater than 85%. However, among six chemical compounds

tested, addition of the surfactant Tween 80 in the reaction medium demonstrated better efficiency in the removal/degradation of hemicellulose and cellulose. Addition of chemical compounds in the organosolv pretreatment resulted in similar chemical composition of pseudo-lignin formed. All the antioxidants tested showed improvement in the removal of lignin from the biomass, tert-butylhydroquinone reached 71% of lignin removal. This lignin removal corroborates to the higher glucose yields observed in the enzymatic hydrolysis, due to cellulose accessibility improvement. Pretreatment adding Tween 80 showed the highest glucose yield in the enzymatic hydrolysis, reaching 98%. This surfactant when applied in the pretreatment can generate benefits and increase the yield of the process, as well as assist in the removal of compounds of interest from biomass.

CHAPTER 7 INFLUENCE OF CHEMICAL ADDITIVES IN SUGARCANE BAGASSE ACID PRETREATMENT WITH PRIOR REMOVAL OF EXTRACTIVES AND DELIGNIFICATION IN ENZYMATIC HYDROLYSIS AND ETHANOL FUEL PRODUCTION

Abstract

Second generation ethanol is turning into an important alternative to supply bioenergy. This technology is associated with lignocellulosic biomass, agro-industrial residues as resource of fermentable sugars. However, it is necessary to overcome the biomass recalcitrance by pretreatments. Simultaneous Saccharification and Fermentation (SSF) was evaluated as effect of the acid pretreatment of the bagasse adding 3,5-Di-tert-4-butylhydroxytoluene (BHT). This pretreatment, named BHT-PT, showed lower yields of inhibitors in liquid fraction of the acid pretreatment (0.01 g L^{-1} of furfural, 0.01 g L^{-1} of HMF and 0.68 g L^{-1} of acetic acid). The previously delignified bagasse (DL-PT) and the bagasse pretreated with BHT (BHT-PT) resulted in a biomass with low hemicellulose and lignin contents, indicating that BHT influences the lignin removal. The formation of pseudo-lignin decreased with removal of extractives (EF-PT) and lignin (DL-PT). Previous removal of extractives from biomass resulted in practical and global ethanol yields of 84.22% and 90.03% in SSF (EF-PT). DL-PT resulted in the highest glucose and ethanol yield in the SSF. Enzymatic hydrolysis of DL-PT (24 h with 15 FPU/g) showed 87% conversion. After 48 h of SSF (75 FPU/g), 17.06 g L^{-1} of glucose and 15.17 g L^{-1} of ethanol were observed in this biomass. The low content of extractives, lignin, pseudo-lignin and hemicellulose, resulted in better cellulose accessibility and consequently ethanol yield.

Keywords: Extractives, antioxidants, pseudo-lignin, hydrolysis.

7.1 Introduction

Excessive consumption of fossil fuels has led to an increase in the use of oil reserves, increasing emissions of greenhouse gases and deepening environmental concerns. Changes in the environment motivated researchers to explore alternative fuels based on sustainable biological resources (BEZERRA; RAGAUSKAS, 2016). Lignocellulosic materials are among the most abundant biomasses generated in the world. They contain carbohydrates that can be converted into second-generation ethanol (2G ethanol) (VIEIRA et al., 2020). Among the various lignocellulosic residues employed in the production of 2G ethanol and commercial added-value compounds, sugarcane bagasse is a viable option. The use of co-products generated in the sugar and alcohol industry can increase the production of 2G ethanol without the need to increase the

cultivated area and without the need to compete for physical space (arable land) with food crops (SCHMATZ; TYHODA; BRIENZO, 2020).

Brazil is the largest producer of sugarcane in the world. In 2018, the country was responsible for the production of 746 million tons, almost double the second place (India, with 376 million tons) (FAOSTAT, 2018). For each ton of sugarcane processed, 140 kg of dry bagasse and 140 kg of dry straw are generated. Therefore, for every 8.4 million hectares of sugarcane cultivated, 80 million tons of bagasse and 80 million tons of straw are produced. A large amount of residue of sugar and ethanol industries was incinerated inside the plants to produce water steam that feeds an electric power generation turbine. About 10% of the remainder of the bagasse is available for the production of second generation bioproducts and biofuels, such as cellulosic or 2G ethanol, especially when integrated with biorefinery processes (CARVALHO et al., 2014). It is possible to produce between 149 and 192 liters of ethanol per ton of bagasse, varying according to differences in pretreatments, enzymatic hydrolysis and fermentation (BEZERRA; RAGAUSKAS, 2016).

In the production of 2G ethanol, due to the highly recalcitrant nature of the biomass, pretreatments are required to remove/modify the hemicellulose/lignin. The pretreatment exposes cellulose to enzymatic hydrolysis (SCHMATZ et al., 2020; SHIMIZU et al., 2020), solubilizing fermentable sugars (MELATI et al., 2019) to the fermentation step (VIEIRA et al., 2020). In acid pretreatments associated with high temperatures and pressures, sugars degradation products are formed, such as furfural, 5-hydroxymethylfurfural (HMF) and acetic acid, resulting from the degradation of pentoses and hexoses (VIEIRA et al., 2020). These degradation products are inhibitors of the fermentation process, interfering with microorganism growth and ethanol production (CANDIDO et al., 2020).

Sugars degradation products react with lignin fragments solubilized in acid pretreatment by polycondensation and/or polymerization, producing pseudo-lignin. This molecule consists of carbonyl, carboxylic, aromatic and aliphatic structures that significantly inhibit enzymatic hydrolysis of cellulose. Due to hydrophobic interactions, cellulases are irreversibly bound to droplets of pseudo-lignin, resulting in loss of enzyme activity and increasing enzyme charges (HU; JUNG; RAGAUSKAS, 2012). Thereby, pseudo-lignin can influence the enzymatic hydrolysis or the Simultaneous Saccharification and Fermentation (SSF).

Enzymatic hydrolysis can be performed separated from the fermentation stage or simultaneously. The execution of two steps in ideal conditions generates benefits, such as recycling cells after fermentation, but the capital cost is higher because two-unit steps are necessary, and the efficiency of the process can be limited by inhibiting enzymatic activity in the final product, especially when there is a high content of total solids. On the other hand, SSF process enzymes and fermenting microorganisms are working together, and glucose is fermented as it is produced. This procedure is carried out in a single reactor, but one of the steps does not occur in its optimum condition (NEVES; PITARELO; RAMOS, 2016).

Therefore, this study aimed to evaluate the ethanol production by SSF effected by the pseudo-lignin content in the pretreated material, lignin content and pretreated material digestibility. The biomass was diluted acid pretreated (20% H_2SO_4 m/m at 121 °C for 30 min) in the presence of 3,5-Di-tert-4-butylhydroxytoluene (BHT) as antioxidant compound, and partial delignified with sodium chlorite and acid acetic (70 °C for 5 h). SSF was evaluated with a pre-hydrolysis using enzyme charge of 75 FPU/g for 48 h.

7.2 Materials and methods

7.2.1 Raw material

Sugarcane bagasse was kindly donated by São João Plant (Araras-SP). The material was dried at 55 °C for 24 h, and ground in a 20-mesh (screen/mesh opening: 0.85 mm) sieve knife mill, obtaining the milled-*In natura* (IN) biomass.

7.2.2 Biomass preparation

Approximately 10 grams of the IN biomass was subjected to removal of extractives in soxhlet apparatus. The biomass was packed in filter paper envelopes and the extraction took place for 8 h, consecutively using ethanol and water, obtaining extractive-free biomass (EF).

IN biomass was subjected to the partial delignification process, using 10 grams of bagasse in Schott flasks in 400 mL of distilled water, together with 3.76 grams of sodium chlorite and 1.26 mL of glacial acetic acid. The glass flasks were sealed with Teflon cap and heated in a water bath at 70 °C for 5 h. Three additional doses of sodium chlorite and acetic acid were added after 2, 3 and 4 h of reaction. After 5 h of reaction, biomass was

filtered on filter paper and the liquid fraction discarded, while the solid fraction was washed with 1600 mL of water heated at 70 °C and 4000 mL of water at room temperature. Partially delignified biomass (DL) was oven dried at 55 °C for 24 h. Biomasses (IN, EF and DL) were stored in plastic bottles for future analysis (BRIENZO et al., 2015).

7.2.3 Diluted acid pretreatment

The three biomass samples produced (IN, EF and DL) were subjected to sulfuric diluted acid pretreatment. First, 5 grams of samples were soaked in 20% H_2SO_4 m/m (acid mass/bagasse mass equivalent to 2% acid mass/reactional volume), solid-to-liquid ratio 1:10. Reaction time was 30 min in an autoclave at 121 °C/1 atm. A fourth test was carried out with IN biomass, under the same conditions, adding of Butylated hydroxytoluene (BHT), 20% m/m (2% m/v) in the reaction medium.

After natural cooling, the reaction medium was filtered using filter paper. The liquid fraction was stored for sugar and degradation products quantification by High Performance Liquid Chromatography (HPLC), while the solid fraction was washed with distilled water until the pH of the filtrate reached neutrality. The pretreated biomass (IN-PT, EF-PT, DL-PT and BHT-PT) was then oven dried at 55 °C for 24 h and stored for future procedures.

7.2.4 Chemical characterization

The untreated and pretreated biomasses were subjected to chemical composition to determine the cellulose, hemicellulose and lignin contents. To achieve it, 300 mg of the biomass was conditioned in glass flasks coupled with 3 mL of 72% sulfuric acid and heated at 30 °C for 1 h, with constant maceration using a glass stick. The reaction was stopped with 84 mL of distilled water and autoclaved at 121 °C for 1 h. The biomass was filtered using porous plate crucibles previously dried at 105 °C and tared. The resulting liquid fraction was used to determine the soluble lignin (UV-VIS 215 and 280 nm) and sugars by HPLC. The crucibles containing the remaining biomass residue were dried in an oven at 105 °C for 24 to determine the insoluble lignin (ABNT, 2018).

7.2.5 Pseudo-lignin extraction

Pretreated biomass (1 g) was used to determine pseudo-lignin content. The sample was inserted in filter paper bags and extracted occurred in soxhlet, using a solution of 1,4 *p*-dioxane:water (9:1) for 8 h. After the recovery of the solvent by evaporation, the residual mass of pseudo-lignin was weighed and stored for future analysis (HU; JUNG; RAGAUSKAS, 2013).

Fourier Transform Infrared (FTIR) attenuated total reflectance (ATR) of the pseudo-lignin extracted samples were examined between 4000 and 400 cm−1 at 25 °C with 2 cm−1 resolution, 32 scans per spectrum. The ATR method used in a FTIR-VERTEX 70/BRUKER spectrophotometer with a source: HeNe laser (emits radiation in the mid-infrared region); Detector: DLaTGS.

7.2.6 Microorganism and culture media

Saccharomyces cerevisiae (PE-2) was obtained from the stock culture of São Paulo State University (Unesp), Rio Claro, SP, Brazil. It was maintained in solid medium YEPD with agar: yeast extract (10 g L^{-1}); peptone (20 g L^{-1}); dextrose (20 g L^{-1}); Agar (18 g L^{-1}); chloramphenicol (5 ppm). The YEPD medium with the cell culture was preserved in mineral oil (-4 °C). For cell culture activation, 50 mL of YEPD liquid medium contained in Erlenmeyer flasks (30 ± 2 °C, 130 rpm for 72 h) was used. The culture suspension was used in the fermentation medium.

7.2.7 Enzymatic hydrolysis

Sugarcane acid pretreated bagasse were subjected to enzymatic hydrolysis using 15 FPU/g of material (Cellic® Cetec -Novozymes, 60 FPU/mL). The reaction was performed with 0.1 g of pretreated biomass (IN-PT, EF-PT, BHT-PT and DL-PT) in 5 mL of 0.05 mol L^{-1} sodium citrate buffer, pH 4.8, 50 °C for 24 h at 120 rpm (BRIENZO et al., 2017). Sequentially, the hydrolysate was water boiled for 5 min, and centrifuged (2500 rpm for 15 min at 4 ºC). Liquid fraction was evaluated by HPLC, and values obtained were used to calculate the enzymatic digestibility of sugarcane bagasse (anhydroglucose released in relation to the glucan/cellulose content) from the cellulose content. The equation used to obtain the glucose yields follows:

(5) Cellulose conversion (%) = 100 x glucose concentration / (1.11 x f x mass biomass)

where glucose is the concentration of glucose released during enzymatic hydrolysis (g L^{-1}); biomass is the dry biomass concentration at the beginning of the enzymatic hydrolysis (g L^{-1}); *f* is the cellulose fraction in dry biomass (g 100 g^{-1}); 1.11 is the conversion factor of cellulose to glucose equivalents.

7.2.8 Simultaneous saccharification and fermentation (SSF)

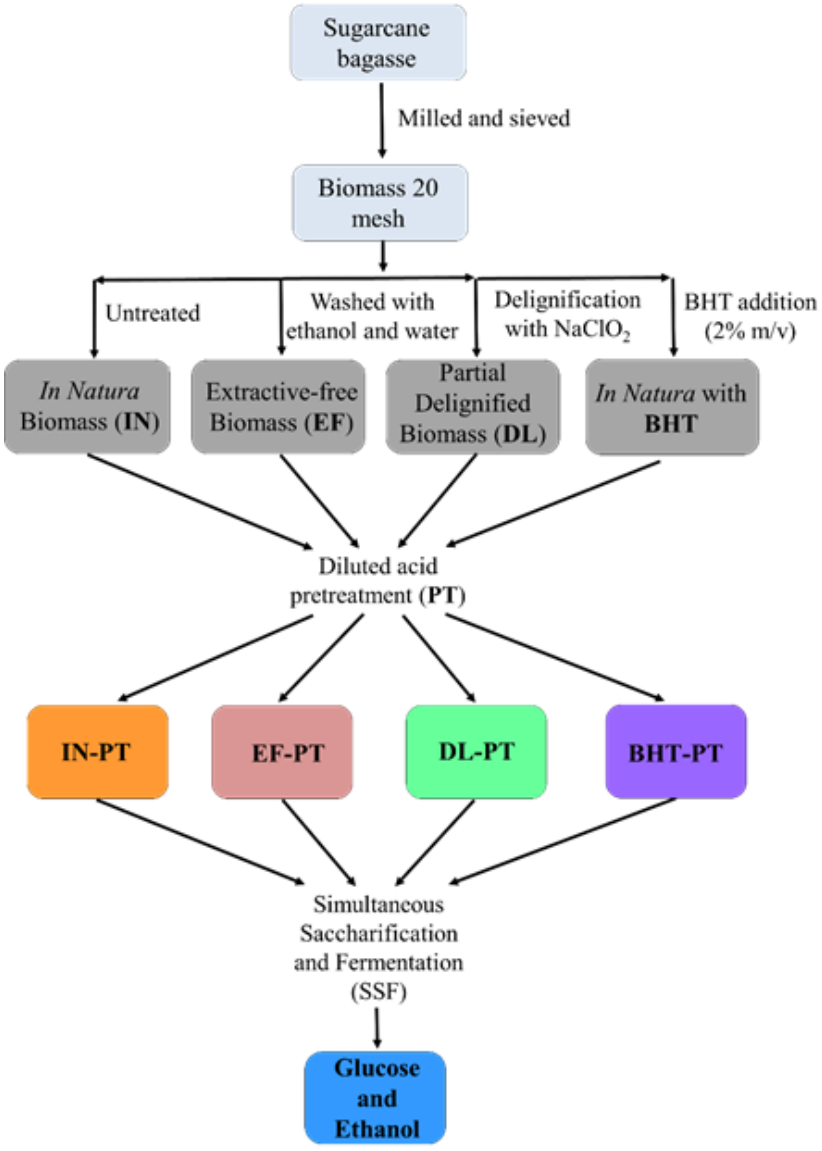
The four pretreated biomasses were subjected to SSF (Fig. 19). YEPD medium was prepared with yeast extract, peptone, dextrose, and chloramphenicol (in the same concentrations mentioned above) in volumetric flasks, and the desired volume was completed with sodium citrate buffer (0.05 mol L^{-1}, pH 4.8), and then autoclaved at 121 ºC for 15 min. Around 3 grams of acid-pretreated sugarcane bagasse (IN-PT, EF-PT, DL-PT and BHT-PT) were placed in fermenters containing 60 mL of YEPD medium (solid-to-liquid ratio 0.5:10). The mixture was incubated at 50 °C with 75 FPU (25 FPU/g substrate) Cellic® Cetec – Novozymes (60 FPU/mL), under constant agitation of 120 rpm for 6 h. After cooling the reactors to room temperature, 10% v/v of fermenting yeasts (*S. cerevisiae*) were inoculated. The equivalent cell suspension was 1.4×10^9 cells mL^{-1} or 35 g L^{-1} in dry weight (105 °C / 24 h / until constant weight). After subtle manual stirring to homogenize the medium, an aliquot of 100 µl was removed for counting viable microorganisms in Neubauer chamber using erythrosine as differential dye. The percentage of cell viability was determined according (CANDIDO et al., 2020). The fermenters were immediately incubated at 30 °C, starting the fermentation time count (time 0).

The fermentation was carried out for 48 h, and every 12 h aliquots were collected to count viable cells (survival percentage > 95%) and quantified sugars and ethanol in the liquid fraction by HPLC. The samples were previously centrifuged, and the supernatant was filtered by polyvinylidene fluoride membrane (PVDF 0.22 µm - Millipore) and stored in a freezer (-5 °C) for subsequent analysis. During fermentation analyzes, the pH of the medium was verified, with a variation of 4.8 (time 0) to 4.5 (12, 24, 36 and 48 h). As a preliminary measure, it was used a Hand-held optical refractometer to determine the sugar concentration in Brix units (soluble solids content determined from the refraction index of the light passed through the sample) (SERPEN, 2012).

7.2.9 High Performance Liquid Chromatography

Sugar monomers were evaluated in HPLC Shimadzu, with Aminex® column (Bio-Rad) HPX-87 H 300 x 7.8 mm, mobile phase 0.050 mol L^{-1} H_2SO_4, flow of 0.4 mL min^{-1}, oven 65ºC and RID detector, isocratic method. HMF and furfural from diluted acid pretreatment were quantified in column C18 (NST) 150 mm x 4.6 mm x 0.5 µm, mobile phase water:acetonitrile (8:1) with 1% acetic acid, flow of 0.8 ml min^{-1}, oven 35 °C, UV-VIS detector, isocratic method.

Figure 19 - Flowchart of Simultaneous Saccharification and Fermentation (SSF). IN: *in natura* (original) bagasse; EF: extractive-free bagasse; DL: partial delignified bagasse; BHT: Butylhydroxytoluene; PT: pretreatment; IN-PT: *in natura* (original) pretreated; EF-PT: Extractive-free pretreated; DL-PT: partial delignified pretreated; BHT-PT: Butylhydroxytoluene pretreated.



Source: Prepared by author

7.3 Results and discussion

7.3.1 Hydrolysate from acid pretreatment (liquid fraction)

Bagasse IN-PT resulted in the highest glucose concentrations in the liquid fraction (3.35 g L^{-1}) and bagasse DL-PT the lowest (0.74 g L^{-1}). Xylose concentrations varied between 23.35 g L^{-1} and 21.18 g L^{-1} in the BHT-PT and DL-PT biomasses, respectively. IN-PT bagasse presented 1.35 g L^{-1} of HMF in the hydrolysate, a concentration higher than that observed in the other pretreatments (0.4 g L^{-1} in EF-PT and 0.01 in BHT-PT and DL-PT). Furfural concentration was between 0.1 g L^{-1} in DL-PT and 0.01 g L^{-1} in BHT-PT, while acetic acid was observed in concentrations between 4.09 g L^{-1} (IN-PT) and 0.68 g L^{-1} (BHT-PT). Bagasse IN-PT showed the highest concentrations of inhibitors (HMF and acetic acid), while the experiments with BHT-PT showed the lowest concentrations of inhibitors evaluated among the treatments (Table 8).

Table 8 - Sugar and inhibitors in liquid fraction after acid pretreatment (g L^{-1})

Treatment	Glucose	Xylose	HMF	Furfural	Acid acetic
IN-PT	3.35 ± 0.15	23.25 ± 1.59	1.35 ± 0.53	0.07 ± 0.01	4.09 ± 0.44
EF-PT	2.57 ± 0.02	22.02 ± 0.24	0.04 ± 0.01	0.08 ± 0.01	4.07 ± 0.56
BHT-PT	1.24 ± 0.28	23.35 ± 0.12	0.01 ± 0.01	0.01 ± 0.01	0.68 ± 0.11
DL-PT	0.74 ± 0.07	21.18 ± 2.08	0.01 ± 0.01	0.10 ± 0.01	3.95 ± 0.40

HMF: 5-hydroxymethylfurfural; IN-PT: *In natura* (original) acid pretreated; EF-PT: Extractive-free acid pretreated; BHT-PT*:* Butylhydroxytoluene pretreated; DL-PT: Partial delignified acid pretreated.

Source: Prepared by the author

Sugarcane bagasse pretreated with diluted acid medium (1% m/v acetic and sulfuric acid at 190 °C for 10 min and solid-to-liquid ratio 1:10) resulted in 3.09 g L^{-1} of glucose; 9.33 g L^{-1} of xylose; 0.02 g L^{-1} of HMF; 0.1 g L^{-1} of furfural; and 2.98 g L^{-1} of acetic acid in hydrolysate (ROCHA et al., 2011). Glucose values observed in this study are close to those cited in the literature, however the xylose values obtained are more than double than presented in literature. This difference may be a result of the acid load and the pretreatment time applied to biomass, which can also interfere with the formation of inhibitors.

Sugarcane bagasse pretreated with 1% sulfuric acid (v/v) for 60 min at 121 °C (solid-to-liquid ratio 1:15 m/v) resulted in hydrolysate containing 1.7 g L^{-1} of glucose;

7.12 g L^{-1} xylose; 0.11 g L^{-1} of furfural and 1.56 g L^{-1} of acetic acid. Using a 2% acid medium (v/v), 3.41 g L^{-1} of glucose was observed; 8.36 g L^{-1} of xylose; 0.19 g L^{-1} of furfural and 1.49 of acetic acid in the pretreatment liquid fraction (RAI et al., 2014). In the present study, glucose concentrations are close to those found in the literature, while high xylose values can be attributed to the difference in biomass heterogeneity and recalcitrance (resistance to pretreatment). The lower amounts of furfural are due to the shorter exposure time of the biomass in the pretreatment.

Sugarcane bagasse pretreated with 1% sulfuric acid at 121 ºC for 150 min (20% solids loading) presented in its hydrolysate 41 g L^{-1} of xylose, 0.12 g L^{-1} of HMF, 0.26 g L^{-1} of furfural and 2.95 g L^{-1} of acetic acid (MARTINS; RABELO; DA COSTA, 2015). Long exposure times at high temperatures during pretreatment, result in higher yields of sugar degradation and the formation of inhibitors. High severity conditions can degrade HMF and furfural into formic and levulinic acid.

7.3.2 Chemical characterization

Pretreatment with diluted acid changed the chemical composition of the biomass (raw material). IN-PT presented 53.64% of cellulose, 11.59% of hemicellulose and 34.41% lignin, EF-PT presented 46.27% of cellulose, 8.18% hemicellulose and 33.13% lignin, while BHT-PT presented 51.35% cellulose, 10.63% hemicellulose and 16.81% total lignin. Delignification with sodium chlorite and acid pretreated bagasse (DL-PT) showed 56.73% glucose, 8.35% hemicellulose and 12.34% total lignin. Component removal indicated that the diluted acid treatment removed large amounts of hemicellulose (among 71% and 81%), while BHT and sodium chlorite removed lignin from biomass (41% and 60%, respectively). However, sodium chlorite removed one third more lignin than BHT (g 100 g^{-1} based on raw material) (table 9). These results show a positive action of BHT in removing lignin compared to IN-PT and EF-PT, indicating that this compound has a potential effect on the lignin fraction of the biomass.

Table 9 - Chemical composition of sugarcane bagasse from different pretreatment.

	Raw material	IN-PT	EF-PT	BHT-PT	DL-PT
Cellulose	37.36 ± 2.30	53.64 ± 2.76	46.27 ± 1.44	51.35 ± 2.72	56.73 ± 1.25
Hemicellulose	25.91 ± 2.58	11.59 ± 1.32	8.18 ± 0.59	10.63 ± 0.10	8.35 ± 0.46
Total lignin	19.45 ± 1.08	34.41 ± 3.16	33.13 ± 1.32	16.81 ± 0.72	12.34 ± 1.59
Extractives	6.63	-	-	-	-
Mass recovery	100	62.94 ± 2.45	58.74 ± 0.20	68.11 ± 3.85	62.20 ± 1.0
Cellulose removal (g 100 g^{-1})	-	5.16	12.60	3.26	3.14
Hemicellulose removal (g 100 g^{-1})	-	8.32	6.66	7.66	6.67
Lignin removal (g 100g-1)	-	0	0	6.91	7.47

IN-PT: *In natura* acid pretreated; EF-PT: Extractive-free acid pretreated; BHT-PT: Butylhydroxytoluene pretreated; DL-PT: Partial delignified acid pretreated; (-) not determined/detected.

Source: Prepared by the author

Sugarcane bagasse (raw material) composed of 39% cellulose, 36% hemicellulose, 23% lignin and 5% extractives, pretreated with diluted sulfuric acid (1% v/v H_2SO_4, at 121 °C for 30 min) show chemical composition of 53% cellulose, 14% hemicellulose and 34% acid-insoluble lignin (SRITRAKUL; NITISINPRASERT; KEAWSOMPONG, 2017). Higher yields of hemicellulose removal can be obtained with higher acid loads in the reaction medium, which is observed in the hemicellulose content of the pretreated bagasse in this study. Cellulose and lignin values are close to those found in the literature.

Chemical composition of sugarcane bagasse *in natura* was 47% cellulose, 25% hemicellulose and 23% lignin. After diluted acid pretreatment (10% H_2SO_4 m/v, at 120 °C for 10 min), 59% cellulose, 15% hemicellulose and 22% lignin were observed. Pretreated biomass subjected to delignification in an alkaline medium (1.5% NaOH m/v, at 100 °C for 1 h, solid-to-liquid ratio 1:20 m/v), and after chemical characterization revealed a composition of 83% cellulose, 5% hemicellulose and 7% lignin (MILÉO et al., 2016). As observed in this study, lower levels of hemicellulose in the pretreated biomass correspond to the longer reaction time that the bagasse was subjected.

Great reduction in hemicellulose content occurred combining acid and alkaline pretreatments. Sugarcane bagasse with chemical composition of 38% cellulose, 27%

hemicellulose and 17% lignin, after pretreatment (2% v/v sulfuric acid at 121 °C for 30 min and 20% m/v of residual biomass immersed in a 4% m/m sodium hydroxide solution at 121 °C for 30 min) resulted , values of 65% glucose, 11% hemicellulose and 8% lignin (GUILHERME et al., 2019). Combination of acid pretreatment with delignification techniques can generate cellulose-rich biomass. However, mild conditions should be used to minimize the formation of inhibitors.

Pretreatment parameters not only cause biomass deconstruction, but also contribute to the reorganization of lignin, which can vary according to the severity of the pretreatment. The breakdown of the cellulose-hemicellulose-lignin complex during the acid pretreatment increases the surface area of the cellulose. The main constituents of biomass agree with data observed in the literature. However, chemical composition of the bagasse can vary according to the cultivated genotype, location of the harvest, year of planting and environmental parameters, such as rainfall index, for example (SCHMATZ; TYHODA; BRIENZO, 2020).

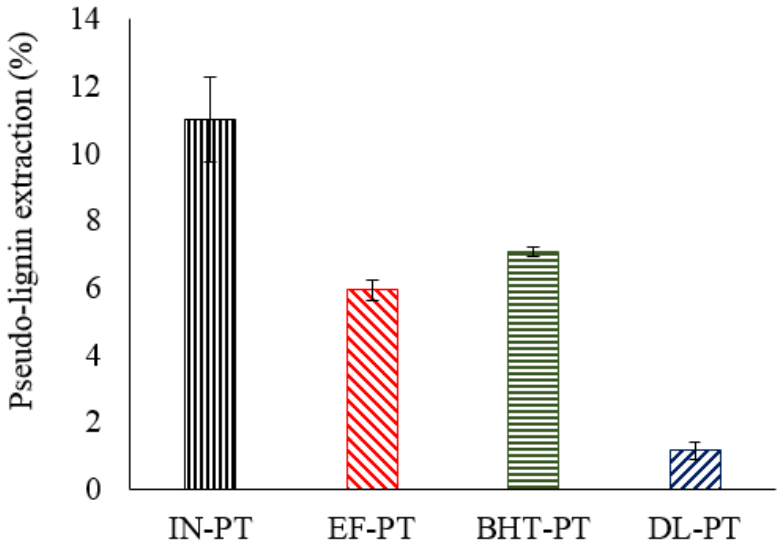
7.3.3 Pseudo-lignin extraction

Highest levels of pseudo-lignin formation were observed in bagasse *in natura* (IN-PT) with 10.99%, followed by BHT-PT with 8.59%, EF-PT with 5.91% and DL-PT with 1.14%, as observed in Fig. 19. Sugarcane bagasse (diluted acid pretreated) revealed the presence of structures containing lignin fragments that have precipitated on the fibers but can be removed by the action of organic solvents. Diluted acid pretreatment (4.5% H_2SO_4 m/m for 15 min at 175 °C, solid-to-liquid ratio 7:1) increase total lignin of the sugarcane bagasse and straw (in the range of 49-54% and 76-128%, respectively, based on the whole biomass), probably due hemicellulose removal (CARVALHO et al., 2015). Under conditions of high severity in pretreatment, occur higher yields of sugar degradation products that can form pseudo-lignin.

Sugarcane straw submitted to hydrothermal pretreatment (high severity conditions) showed loss of hydrolysable sugars, formation of inhibitors and pseudo-lignin. Pretreatment at 220 °C for 15 min increase lignin content in approximately 18% (compared to the initial biomass) (BATISTA et al., 2019). In concentrations above 2% v/v H_2SO_4 in acid hydrolysis was observed decrease in yields of glucose and xylose in biomass and continuous increase in levels of acetic acid and furfural (RAI et al., 2014). Conditions of high severity increases generation of furfural from xylan, contributing to

pseudo-lignin formation (CARVALHO et al., 2015). However, in diluted acid medium, pseudo-lignin can be formed from the degradation of carbohydrates without the contribution of lignin, especially in conditions of high severity (SCHMATZ; TYHODA; BRIENZO, 2020).

Figure 20 - Pseudo-lignin from sugarcane bagasse pretreated with diluted sulfuric acid (20% m/m).



IN-PT: *In natura* acid pretreated; EF-PT: Extractive-free acid pretreated; BHT-PT; Butylhydroxytoluene pretreated; DL-PT: Partial delignified acid pretreated.

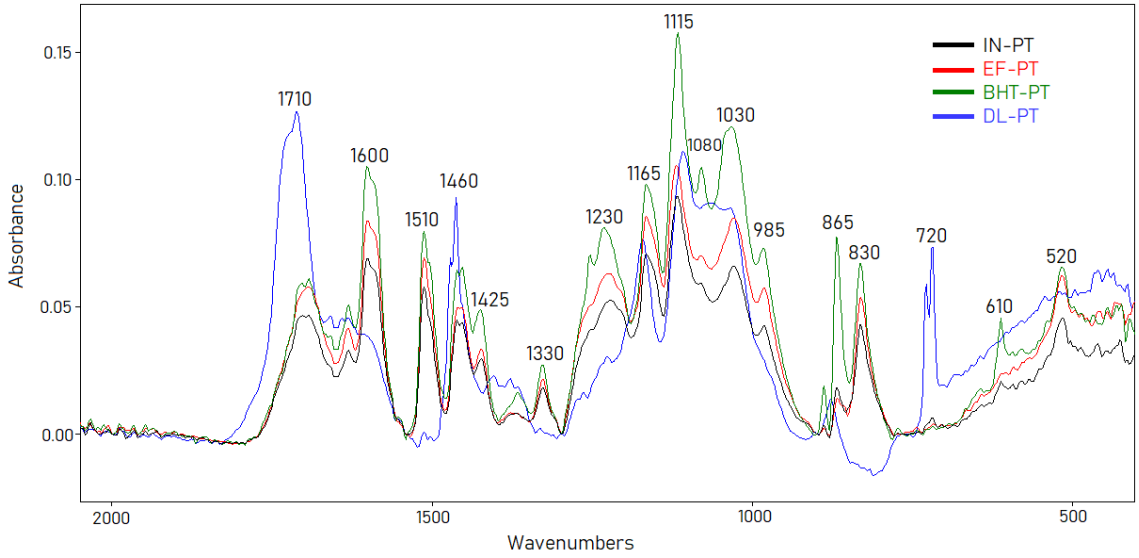
Source: Prepared by the author

The IN-PT material showed higher levels of pseudo-lignin compared to other pretreatments applied. This may be due to the extractives present in the biomass, which can react with HMF, furfural and lignin derivatives under acid condition. This extractives contribution is evident when comparing IN-PT with EF-PT. BHT influences the removal of lignin (table 9), and condensation of monomers with sugar degradation products and subsequent precipitation on the fiber can occur at lower rates (BHT-PT), but the reallocation of lignin in the fiber may have favored the formation of pseudo-lignin. Effects of lignin removal caused by BHT can be seen by comparing IN-PT and BHT-PT. Similar results were observed in DL-PT due to the removal of lignin by sodium chlorite in greater amounts before acid pretreatment, resulting in lower yield of pseudo-lignin formation in the biomass.

7.3.4 FTIR

Pseudo-lignin spectra evaluated in FTIR are consistent with data reported in the literature. Strong band at 1710 cm^{-1} represent the C=O stretching in carboxylic acids, ketones, ester groups of carbohydrates, conjugated aldehydes, while bands at 1600 cm^{-1} and 1510 cm^{-1} represents aromatic rings skeletal vibration (aromatic C=O bonds); Band at 1460 cm^{-1} represents the C-H deformation, while bands at 1410 cm^{-1} represents aromatic skeletal vibrations combined with deformation C-H in plane. Band at 1330 cm^{-1} corresponds to the Syringyl ring condensed with Guaiacyl ring (G ring replaced at position 5), C-C, C-O, C=O stretch, secondary OH aromatic, CH in plane deformation (typical for G units) and primary OH; Band at 1230 cm^{-1} represents G ring (C=O stretch). Bands at 1163 cm^{-1} and 1020 cm^{-1} represent C−O stretching in alcohols, HMF, or carboxylic acids, while 870 cm^{-1} represents C–H out-of-plane at positions 2 and 6 (S units) (Figure 20) (HU; JUNG; RAGAUSKAS, 2012; SINGH et al., 2005; WAN et al., 2019).

Figure 21 – FTIR-ATR spectra of pseudo-lignin extracted form sugarcane bagasse.



IN-PT: *In natura* acid pretreated; EF-PT: Extractive-free acid pretreated; BHT-PT; Butylhydroxytoluene pretreated; DL-PT: Partial delignified acid pretreated.

Source: Prepared by the author

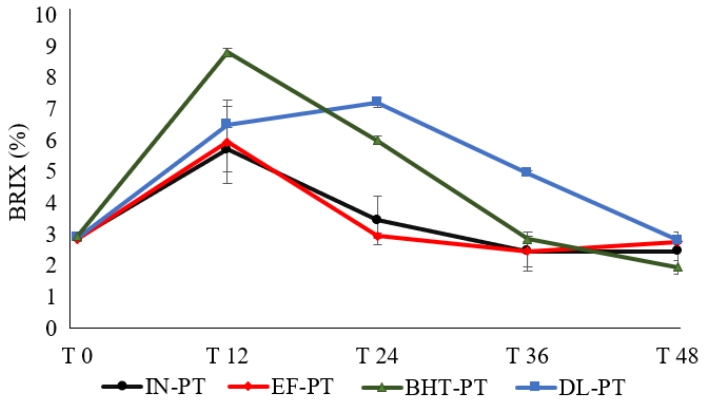
IN-PT and EF-PT biomasses presented similar spectra, indicating similarity between the pseudo-lignin extracted from these pretreated materials. However, the spectrum referring to DL-PT showed a strong band in the region of 1710 cm^{-1}, indicating concentrations of

C=O stretching in carboxylic and/or carboxylic, higher than other biomasses. Also, in DL-PT, there are no band in the region between 1600, 1510, corresponding in part to C=C stretching in aromatic ring; 830 cm^{-1} corresponding to −CH3 hydroxymethyl group (C-H out of plane vibration in lignin). Bands at 1165 cm^{-1} and 1020 cm^{-1} were attributed to C-O stretching (furans, alcohols, ethers or carboxylic acids), observed in higher intensity in BHT-PT material. Based on FTIR characterization, pseudo-lignin consists of hydroxyl, carbonyl and aromatic structures.

7.3.5 Simultaneous Saccharification and Fermentation (SSF)

The starting sugar concentration (T0) was 2.8% for all biomasses. In the first 12 h of fermentation, all treated materials showed an increase in the sugar release (BRIX), resulting from enzymatic hydrolysis, ranged from 5.7% (IN-PT) and 8.8% (BHT-PT). From 12 to 24 h only DL-PT had an increase in the amount of BRIX, indicating that the enzymatic conversion still occurred at higher yield than the consumption of sugars by fermenting microorganisms, reaching 7.2% brix. From 24 to 36h, all treated materials showed a reduction in their BRIX number, until reached lower levels in the sugar BRIX after 48h, between 1.95% and (BHT-PT) and 2.8% (DL-PT) (Fig 21).

Figure 22 - BRIX variation during SSF using sugarcane bagasse from different pretreatment.



IN-PT: *In natura* acid pretreated; EF-PT: Extractive-free acid pretreated; BHT-PT: Butylhydroxytoluene pretreated; DL-PT: Partial delignified acid pretreated.

Source: Prepared by the author

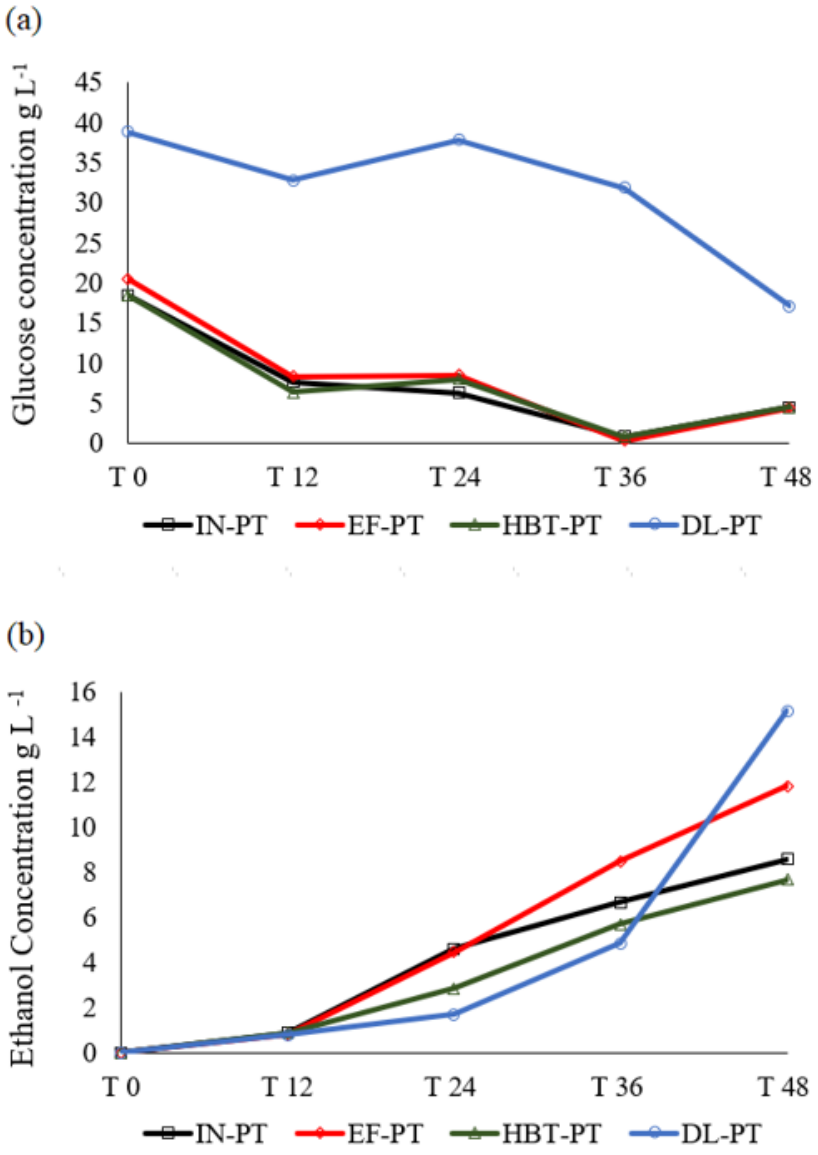
An enzymatic hydrolysis (15 FPU/g) of the materials was performed during 24 for material comparison. The cellulose conversion into glucose was 54.14% was observed

in IN-PT, 69.66% in EF-PT, 37.96% in BHT-PT and 87% in DL-PT. This result suggested that the material DL-PT shows low recalcitrance to enzymatic hydrolysis. The SSF process was performed with a pre-hydrolysis of 6 h, using 75 FPU/g (Cellic® Cetec–Novozymes, 60 FPU/mL).

After 6 h of enzymatic hydrolysis (T0), 18.4 g L^{-1} of glucose was observed in the IN-PT hydrolysate, and 7.65 g L^{-1}, 6.23 g L^{-1}, 0.78 g L^{-1}, and 4.43 g L^{-1}, successively after T12, T24, T36 and T48 h in SSF. Bagasse EF-PT presented glucose at 20.46 g L^{-1} at T0 and concentrations of 8.31 g L^{-1}, 8.43 g L^{-1}, 0.29 g L^{-1}, and 4.35 g L^{-1} of glucose, respectively at T12, T24, T36 and T48. BHT-PT pretreated bagasse showed glucose values of 18.38 g L^{-1} at T0, 6.28 g L^{-1} at T12, 7.93 g L^{-1} at T24, 0.74 g L^{-1} at T36 and 4.41 g L^{-1} at T48. Biomasses EF-PT and BHT-PT had values close to those of IN-PT, observed a stability in glucose content between T12 and T24, indicating a balance in enzymatic hydrolysis and uptake by yeast. However, DL-PT biomass showed 38.77 g L^{-1} of glucose at T0, 32.72 g L^{-1} at T12, 37.74 g L^{-1} at T24, 32.78 g L^{-1} at T36 and 17.06 g L^{-1} at T48 (Figure 22).

After 12 h of SSF, values of 0.8 g L^{-1} and 0.9 g L^{-1} of ethanol were observed for all biomasses, indicating that yeasts converted sugars homogeneously in the first 12 h due to the availability of glucose. IN-PT presented 4.61 g L^{-1} of ethanol in T24, 6.67 g L^{-1} in T36 and 8.60 g L^{-1} in T48. EF-PT after 24 h (T24) of SSF showed 4.48 g L^{-1} of ethanol, 8.49 g L^{-1} in T36 and 11.84 g L^{-1} in T 48. Sugarcane bagasse BHT-PT presented 2.85 g L^{-1} of ethanol in T24, 5.72 g L^{-1} in T36 and 7.68 g L^{-1} in T48. However, DL-PT biomass showed 1.72 g L^{-1} of ethanol in T24, 4.85 g L^{-1} and 15.17 g L^{-1} in T36 and T48, respectively. Pretreatment DL-PT showed approximately twice as much ethanol as BHT-PT after 24 h of SSF (Figure 22). On the other hand, there was an increase in yeast cell mass during the 48 h of fermentation (25% from T0 to T48), which in part can be one of consequences to impair ethanol productivity. At the end of the experiment (T 48h), the ethanol curve was rising, indicating that ethanol productivity in the next few h would be increased.

Figure 23- Glucose (a) and Ethanol (b) concentrations of sugarcane bagasse after 48h of SSF (g L^{-1}).



IN-PT: *In natura* acid pretreated; EF-PT: Extractive-free acid pretreated; BHT-PT: Butylhydroxytoluene pretreated; DL-PT: Partial delignified acid pretreated.

Source: Prepared by the author

Sugarcane bagasse pretreated with 1% (v/v) sulfuric acid (approximately 20% m/m) for 30 min at 121 °C (solid-to-liquid ratio 1:10), and later SSF (solid-to-liquid ratio 0.5:10, with 15 FPU/g of cellulases and 7.5 IU/g of β-glucosidase) at 30 °C and 150 rpm (fermenting microorganism *Pichia stipitis*), was reported as the glucose accumulated only in the first 6 h (approximately 4 g L^{-1}), and after 18 h the glucose concentration was close to zero, indicating that the yeast was metabolically active throughout the fermentation.

The maximum ethanol production occurred at 24 h of fermentation at 3.70 g L^{-1} with an ethanol yield of 0.10 g ethanol/g available fermentable sugars and ethanol productivity of 0.15 g L^{-1}/h, theoretical ethanol yield 20% (SRITRAKUL; NITISINPRASERT; KEAWSOMPONG, 2017). Different yeast species have different metabolic rates for converting glucose to ethanol and can also use pentoses as a carbon source.

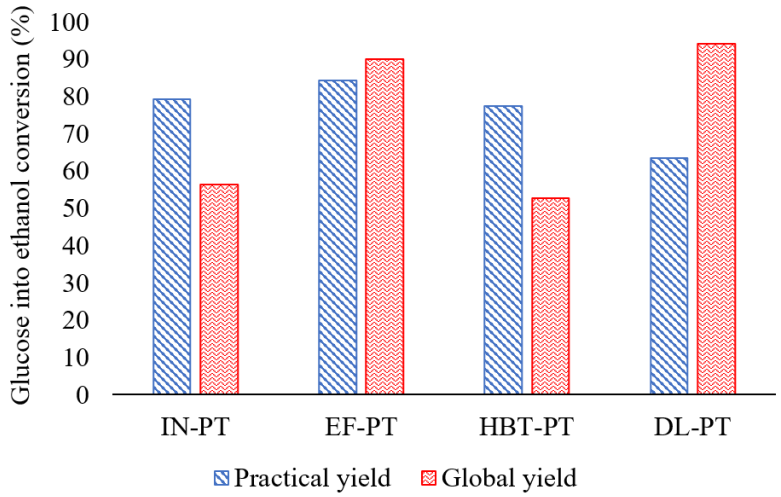
Combined acid and alkaline pretreatment (0.5% m/v H_2SO_4 at 140 °C for 10 min and 1.0% m/v NaOH at 90 °C for 60 min), was applied in the sugarcane bagasse for ethanol production, resulting in biomass with 11% lignin (YU et al., 2018). After 48 h of pre-saccharification SSF of this biomass with *Saccharomyces cerevisiae* resulted in 43 g L^{-1} of ethanol and 81% ethanol yield with an enzymatic loading of 15 FPU/g. In enzymatic loading of 10 FPU/g, the ethanol concentration was 43 g L^{-1} and the ethanol yield was 80%. Similar results in ethanol yield were observed with delignified biomass with Na_2CO_3 (81%) and NaClO (78%), both biomasses with lignin content approximately 11%. However, applying the pre-saccharification of the biomass containing 14% lignin (using a 1.0% NaOH treatment at 80 °C for 60 min), was observed ethanol yield of 64%. Delignification of biomass in addition to reducing the formation of pseudo-lignin also helps in enzymatic hydrolysis, increasing levels of conversion and subsequent fermentation, as lignin is a limiting factor in the saccharification of biomass.

The lignin content was identified as a limiting parameter in the production of second-generation ethanol (2G), negatively correlated with digestibility and conversion of biomass. The increase in yield of 2G ethanol can be obtained by decreasing the lignin content of the biomass (SCHMATZ; TYHODA; BRIENZO, 2020). Some chemical components are applied in enzymatic hydrolysis in order to potentiate enzymatic activity. The BHT action during the pretreatment improved lignin removal, with better enzymatic hydrolysis yield. However, it did not present desired levels in enzymatic hydrolysis and fermentation.

After 48 hours of fermentation, the biomass IN-PT showed 79.14% of practical ethanol yield and 56.38% of global ethanol yield. The EF-PT biomass showed 84.22% and 90.03% of practical and global ethanol yield, respectively. However, the BHT-PT biomass showed 77.32% practical yield and 52.54% global yield, while the DL-PT biomass showed 63.49% theoretical yield and 94.08% global yield in glucose into ethanol conversion (Fig 24).

Fermentation of sugarcane molasses (*S. cerevisiae* 10% v/v, at 30 C for 48 h) showed a practical yield of 50.3% and a real yield of 48.2%. However, detoxification of the substrate improved alcoholic yield (CANDIDO et al., 2020).

Figure 24- Practical and global ethanol yields after 48h SSF (%).



Four different varieties of sugarcane were diluted acid pretreated (H_2SO_4 0.5% m/m, solid-to-liquid ratio 1:10) and then evaluated for SSF ethanol yield (1 h pre-saccharification with 0.15 ml of Cellic Ctec2/g pretreated material and 0.0167 ml of Cellic Htec2/g pretreated material, and then 5 g/l of wet cells *S. cerevisisea* added, solid-to-liquid 10% m/m, at 35 °C for five days). Theoretical maximum ethanol yields of 69.5%, 75.8%, 62.5% and 61.2% were reached, based on glucose content. Increasing the enzyme dosage (0.15 ml Cellic Ctec2/g and 0.213 ml Cellic Htec2/g added at pretreated material) resulted in higher ethanol concentrations. Theoretical maximum ethanol yield was 85.6% and 74.8%. The ethanol concentration showed an inverse correlation to the lignin content (BENJAMIN; GARCÍA-APARICIO; GÖRGENS, 2014).

Practical and global ethanol yields observed in this study are close to those observed in the literature. However, removal of extractives from biomass minimize pseudo-lignin formation, which is harmful to enzymatic activity, benefiting conversion of glucose into ethanol (EF-PT).

7.4 Conclusion

In comparison to the pretreatments IN-PT and EF-PT, pretreated bagasse BHT-PT showed lower glucose yields in the liquid fraction of the hydrolysate, as well as the lower concentrations of inhibitors, indicating that BHT acts by inhibiting the formation of these compounds in solution medium. The chemical characterization of the pretreated bagasse indicated that BHT had a potential effect on the biomass lignin, removing 6.91 g 100 g^{-1}. EF-PT and DL-PT biomasses showed better enzymatic digestion after 24h, indicating that pseudo-lignin may have partially inhibited the process. SSF from bagasse DL-PT showed higher enzymatic digestion reaching 40 g L^{-1} in the T0 of hydrolysis, while the other biomass showed a similar profile. However, the conversion of DL-PT material into ethanol had a slower growth curve compared to the other tests, possibly due to the assimilation of sugars in yeast growth (number of cells), but the highest ethanol yield was observed after 48 h (15.17 g L^{-1}).

8. CONCLUSION

This study evaluated the addition of low-cost antioxidant compounds, applied during the pretreatment, in the removal of hemicellulose and lignin from the sugarcane biomass, pseudo-lignin remotion, enzymatic hydrolysis (glucose yield), and ethanol from SSF. The heterogeneity of sugarcane fractions (bagasse, leaf, external fraction, node and internode) resulted in different responses to the diluted acid pretreatment. The formation of pseudo-lignin was influenced by the presence of extractives and lignin from biomass, and its removal increased glucose yields in the enzymatic hydrolysis. The diversity in glucose yields may reflect the removal of lignin and hemicellulose by effect of BHT. In the organosolv pretreatments (50% ethanol v/v at 121 °C for 30 min) BHT action improved the lignin removal and resulted in lower yields of degradation products/inhibitors. In the pretreatments at 160 °C (50% ethanol), the individual addition of antioxidants/surfactants resulted in a positive effect on the removal of lignin, corroborating with an increase in the enzymatic hydrolysis glucose yield. The addition of Tween 80 in the pretreatment generated benefits in the enzymatic hydrolysis conversion yields, besides help to remove compounds of interest. The partial delignification of biomass, followed by diluted acid pretreatment resulted in high yields in the enzymatic hydrolysis and also in the best ethanol yields in the SSF. Better practical (EF-PT) and global ethanol yields (DL-PT) were observed in the biomass without extractives, indicating that the contribution to the formation of pseudo-lignin can negatively affect alcoholic yield. However, a slower ethanol growth curve may due to the assimilation of sugars during yeast growth (number of cells). In general, the addition of antioxidant/surfactant chemical compounds in the pretreatment resulted in an improvement in the removal of lignin from biomass, reflecting positively on the sugar conversion yields of enzymatic hydrolysis. Its use can bring biotechnological benefits in research and industries.

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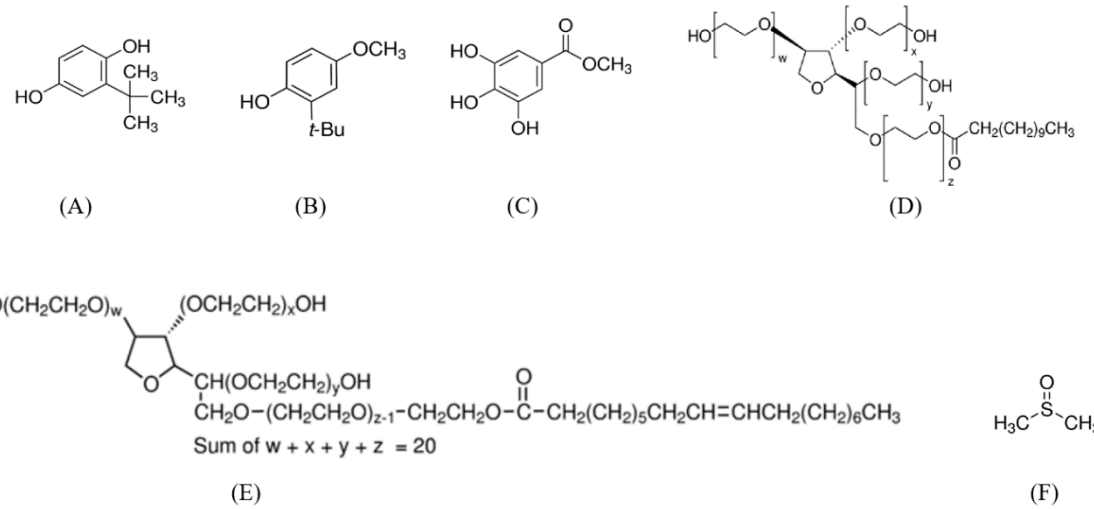
ATTACHMENT A - PRETREATMENT ADDITIVES



Structural formula of antioxidant compounds used in this study. (A) TB: Tert-butylhydroquinone; (B) TH: 3-tert-butyl-4-hydroxyanisole; (C) MT: Methyl 3,4,5-trihydroxybenzoate; (D) T2: Tween 20; (E) T8: Tween 80; and (F) DS: Dimethyl sulfoxide – DMSO.