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INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

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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
2020

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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).

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Coorientador: Prof. Dr. Jonas Contiero.

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TÍTULO DA TESE: ANTIOXIDANTS FAVOR LIGNIN REMOVAL FROM SUGARCANE BIOMASS AND INCREASE ENZYME HYDROLYSIS YIELDSAND ETHANOL VIA SIMULATANEUS FERMENTATION AND SACCHARIFICATION

AUTOR: ALISON ANDREI SCHMATZ
ORIENTADOR: MICHEL BRIENZO

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Prof. Dr. MICHEL BRIENZO (Participaçao Virtual)
Laboratório de Caracterização de Biomassa / Instituto de Pesquisa em Bioenergia- IPBEN

Prof. Dr. ADILSON ROBERTO GONÇALVES (Participaçao Virtual)
IPBEN / Instituto de Pesquisa em Bioenergia

Prof. Dr. GEORGE JACKSON DE MORAES ROCHA (Participaçao Virtual)
Laboratório Nacional de Biorrenováveis (LNBR) / Centro Nacional de Pesquisa em Energia de Materias

Prof. Dr. PEDRO DE OLIVA NETO (Participaçao Virtual)
Departamento de Biotecnologia / Faculdade de Ciências e Letras - UNESP - Assis

Prof. Dr. SÍLVIO VAZ JR. (Participaçao Virtual)
Embrapa Agroenergia / Embrapa

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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.

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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