



UNIVERSIDADE ESTADUAL PAULISTA  
"JÚLIO DE MESQUITA FILHO"

Josiani de Cassia Pereira

Estudo da produção de ligninases e celulases pelo fungo  
*Pycnoporus sanguineus* MCA 16 e uso dos extratos enzimáticos na  
sacarificação do bagaço de cana-de-açúcar para produção de  
bioetanol

São José do Rio Preto

2018

Josiani de Cassia Pereira

Estudo da produção de ligninases e celulases pelo fungo  
*Pycnoporus sanguineus* MCA 16 e uso dos extratos enzimáticos  
na sacarificação do bagaço de cana-de-açúcar para produção de  
bioetanol

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de São José do Rio Preto.

Financiadora: CAPES

Orientador: Prof<sup>a</sup>. Dr<sup>a</sup>. Daniela  
Alonso Bocchini

São José do Rio Preto  
2018

Pereira, Josiani de Cassia.

Estudo da produção de ligninases e celulases pelo fungo *Pycnoporus sanguineus* MCA 16 e uso dos extratos enzimáticos na sacarificação do bagaço de cana-de-açúcar para produção de bioetanol / Josiani de Cassia Pereira . -- São José do Rio Preto, 2018

167 f. : il., tabs.

Orientador: Daniela Alonso Bocchini

Tese (doutorado) – Universidade Estadual Paulista “Júlio de Mesquita Filho”, Instituto de Biociências, Letras e Ciências Exatas

1. Microbiologia. 2. Celulase. 3. Xilanases. 4. Fermentação em estado sólido. 5. Bioetanol. 6. Enzimas microbianas. I. Universidade Estadual Paulista "Júlio de Mesquita Filho". Instituto de Biociências, Letras e Ciências Exatas. II. Título.

CDU – 663.15

Ficha catalográfica elaborada pela Biblioteca do IBILCE  
UNESP - Câmpus de São José do Rio Preto

Josiani de Cassia Pereira

Estudo da produção de ligninases e celulases pelo fungo  
*Pycnoporus sanguineus* MCA 16 e uso dos extratos enzimáticos  
na sacarificação do bagaço de cana-de-açúcar para produção de  
bioetanol

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de São José do Rio Preto.

Financiadora: CAPES

Orientador: Prof<sup>a</sup>. Dr<sup>a</sup>. Daniela Alonso Bocchini

**Comissão Examinadora**

Profa. Dra. Daniela Alonso Bocchini  
UNESP – Câmpus Arararaquara  
Orientadora

Dra. Márcia Maria de Souza Moretti  
UNESP – Câmpus São José do Rio Preto

Profa. Dra. Heloiza Ferreira Alves do Prato  
UNESP – Câmpus Ilha Solteira

Prof. Dr. Luis Henrique de Souza Guimarães  
USP – Câmpus Ribeirão Preto

Profa. Dra. Ariela Veloso de Paula  
UNESP – Câmpus Araraquara

São José do Rio Preto  
27 de julho de 2018

## AGRADECIMENTOS

À Deus por ter me concedido à sorte de ter estudado e passado por todas as adversidades de forma honesta e digna. Aos meus pais José Roberto Pereira e Neide Aparecida Fação Pereira pelo exemplo de vida, de força, perseverança e decência. Ao meu irmão José Roberto Pereira Junior, primeiro amigo, pelo incentivo e ajuda nos momentos mais difíceis. Aos meus avós maternos Nelson Fação (*in memoriam*) e Maria José de Jesus Fação por todo amor sem medida e aos paternos Almerinda Cardoso Pereira e Leonácio Pereira (*in memoriam*) que não pude ter mais tempo com eles.

Ao meu marido Victor Scarpa Neto pelo amor, apoio, companheirismo e compreensão.

A todos os meus animais de estimação (Scooby, Babi, Farofa, Tonico, Tinoco, Titico, Aurora, Porpeta, Porpetinha e Tigrinho) que pelo amor incondicional tornaram os meus dias cada vez melhores.

A CAPES pelo fomento científico durante o doutorado.

À Profa. Dra. Daniela Alonso Bocchini por toda a orientação e disponibilidade em me ajudar e à Profa. Dra. Eleni Gomes pela co-orientação e ajuda.

Aos professores Dr. Maurício Boscolo e Dr. Roberto da Silva pelos auxílios na pesquisa.

Aos amigos do Laboratório de Bioquímica e Microbiologia Aplicada, Laboratório de Sucroquímica e Química Analítica e Laboratório de Enzimas Microbianas: Isabel, Carol Gomes, Gisele, Pedro, Diego, Cissa, Erik, Erick, Janaina, Ana Lúcia, Thiago, Carlos Eduardo, Lorena, Eduardo Simioni, Jaqueline, Roni, Cíntia, Márcio, Raísa, Pâmela, Gabriel Barão, Gabriel, Vanessa, Marília, Lacan, Anelise, Sidnei, Corina, Olavo, Gabriela Salvador, Gabriela Okamura, Josi, Maitê, Yuri, Eduardo Duffeck, Marcos Palka, Marcos Rechi, Julieth, George, Daniela, Naiani, Natália e Luana pelo ambiente agradável.

Aos irmãos sem ser de sangue que conheci nesta caminhada: Natália Paganini Marques, Erick José Galindo Gomes, Isabel Zaparoli Rosa, Ana Carolina dos Santos Gomes, Janaina Pires Borges, Gisele Marta Martins, Pedro Lucas Bueno da Silva e Diego Alves Monteiro.

A todos os funcionários da UNESP.

A todos que de alguma forma colaboraram para o desenvolvimento deste trabalho.

## EPÍGRAFE

*“É muito melhor lançar-se em busca de conquistas grandiosas, mesmo expondo-se ao fracasso, do que alinhar-se com os pobres de espírito, que nem gozam muito menos sofrem muito, porque vivem numa penumbra cinzenta, onde não conhecem nem vitória, nem derrota.”*

Theodore Roosevelt

## Resumo

No presente trabalho, o fungo *Pycnoporus sanguineus* MCA16 foi cultivado em diferentes substratos lignocelulósicos, por fermentação em estado sólido, para obtenção de celulases, xilanases e ligninases. Estas enzimas foram empregadas na sacarificação do bagaço de cana-de-açúcar submetido a pré-tratamento hidrotérmico alcalino realizado na presença ou ausência de agentes oxidantes auxiliares na remoção da lignina, como  $\text{KMnO}_4$ ,  $\text{H}_2\text{O}_2$ ,  $\text{ZnO}$  e  $\text{TiO}_2$  com o intuito de auxiliar na remoção da lignina e desestruturação da lignocelulose. Além disso, as ligninases produzidas pelo bioprocessamento foram estudadas quanto a degradação de compostos fenólicos totais liberados durante a etapa de sacarificação enzimática a fim de que promova um tratamento enzimático. Dentre todos os substratos lignocelulósicos utilizados, a mistura de farelo de trigo e farelo de soja levou à uma maior produção da maioria das enzimas testadas em 96h à 40°C. Após isso as soluções enzimáticas foram aplicadas na sacarificação enzimática do bagaço de cana-de-açúcar pré-tratado, tanto hidrotérmico alcalino quanto empregando agentes oxidantes, utilizando ferramenta de planejamento estatístico para avaliar a influência de diferentes variáveis. Diante de todos os agentes oxidantes avaliados,  $\text{H}_2\text{O}_2$  destacou-se obtendo as maiores liberações de glicose durante a sacarificação enzimática, com 250 unidades de endoglucanase/g de celulose com 11% de bagaço de cana-de-açúcar pré-tratado com 2,5% de  $\text{H}_2\text{O}_2$  durante 106 horas à 50°C. A fermentação alcoólica do hidrolisado nas condições de 288,3 unidades de endoglucanase/g de celulose com 13,5% de bagaço de cana-de-açúcar sob pré-tratamento hidrotérmico alcalino durante 130 horas à 57°C gerou uma conversão de glicose à etanol de 73,5%. Após a sacarificação enzimática, a concentração de compostos fenólicos sofreu uma redução de aproximadamente 90% enquanto que lacase e lignina peroxidase apresentaram uma ativação enzimática de 145% e 226%, respectivamente. Além disso, ácidos orgânicos gerados pela degradação da lignina exerceram influência sobre estas enzimas, ativando-as.

**Palavras-chave:** celulases, xilanases, ligninases, fermentação em estado sólido, sacarificação enzimática, etanol de segunda geração

## **Abstract**

In the present work, the fungus *Pycnoporus sanguineus* MCA16 was grown on different lignocellulosic substrates by solid-state fermentation to obtain cellulases, xylanases and ligninases. These enzymes were used in saccharification of sugarcane bagasse submitted to alkaline hydrothermal pretreatment carried out in the presence or absence of oxidizing agents such as  $\text{KMnO}_4$ ,  $\text{H}_2\text{O}_2$ ,  $\text{ZnO}$  and  $\text{TiO}_2$  in order to assist in the lignin removal and lignocellulose disruption. In addition, the ligninases produced by the bioprocess were studied for the degradation of total phenolic compounds released during the enzymatic saccharification step in order to promote an enzymatic treatment. Among all the lignocellulosic substrates used, the mixture of wheat bran and soybean meal led to a higher production of most of the enzymes tested in 96h at 40 °C. After that, the enzymatic solutions were applied in the enzymatic saccharification of pretreated sugarcane bagasse, both alkaline hydrothermal and using oxidizing agents, using a statistical planning tool to evaluate the influence of different variables. In view of all the oxidizing agents evaluated,  $\text{H}_2\text{O}_2$  was selected by obtaining the highest glucose releases during the enzymatic saccharification with 250 units of endoglucanase/g cellulose with 11% sugarcane bagasse pre-treated with 2.5 %  $\text{H}_2\text{O}_2$  for 106 hours at 50 °C. The alcoholic fermentation of the hydrolyzate under the conditions of 288.3 units of endoglucanase/g cellulose with 13.5% sugarcane bagasse under hydrothermal alkaline pre-treatment for 130 hours at 57 °C generated a conversion of glucose to 73.5% ethanol. After enzymatic saccharification, the concentration of phenolic compounds decreased by approximately 90% while laccase and lignin peroxidase showed an enzymatic activation of 145% and 226%, respectively. In addition, organic acids generated by the degradation of lignin exerted influence on these enzymes, activated them.

**Keywords:** cellulases, xylanases, ligninases, solid state fermentation, enzymatic saccharification, second generation ethanol



## Lista de Figuras

Figura 1. Composição da lignocelulose.....	20
Figura 2. Composição da lignocelulose.....	21
Figura 3. Estrutura química da celulose.....	21
Figura 4. Estrutura da hemicelulose, constituída principalmente por xilana. AGLuc: ácido glucurônico; AF: ácido ferúlico.....	23
Figura 5. Representação de um fragmento da lignina, destacando as ligações entre os monômeros de fenilpropanóides. (A) $\beta$ -O-4, (B) $\beta$ - $\beta$ , (C) 4-O-5, (D) $\beta$ -1, (E) 5-5, (F) $\alpha$ -O-4 e (G) $\beta$ -5. ....	25
Figura 6. Aplicações da lignina como recurso renovável a partir da biomassa.	26
Figura 7. Representação esquemática da ação das celulasas sobre a celulose. ....	28
Figura 8. Representação esquemática da ação das enzimas hemicelulolíticas. ....	29
Figura 9. Representação esquemática do mecanismo catalítico da LiP sobre substrato aromático não-fenólico. ....	32
Figura 10. Álcool veratrílico (VA) como um mediador redox na degradação da lignina.....	32
Figura 11. Mecanismo de ação da lacase em substratos fenólicos. ....	34
Figura 12. Representação esquemática da estrutura do sítio ativo e do ciclo catalítico da lacase. (a) Modelo catalítico de lacase constituído por quatro átomos de cobre. (b) Representação esquemática do ciclo catalítico da lacase produzindo duas moléculas de água a partir da redução de uma molécula de oxigênio molecular e concomitante oxidação (cobre T1) de quatro moléculas de substrato em seus radicais correspondentes. Sub: molécula de substrato; Sub <sup>o</sup> : radical do substrato oxidado.....	35
Figura 13. Mecanismo de ação da lacase em substratos não-fenólicos através de mediador. ....	36
Figura 14. Mediadores redox de lacase. Mediadores redox de lacase. (a) ácido 3-Hidroxi-antranílico (HAA); (b) ácido 2,2'-azino-bis-(3-etilbenzotiazolina-6-sulfônico) (ABTS); (c) N-hidroxi- benzotriazola (HBT); (d) N-hidroxi-ftaimida (HPI); (e) ácido violúrico (VLA); (f) N- hidroxiacetanilida (NHA); (g) metil-éster	

de ácido 4-hidroxi-3,5-dimetoxi- benzóico (ácido siríngico); (h) 2,2,6,6-tetrametilpiperidina-1-iloxi (TEMPO). .....	37
Figura 15. Esquema do mecanismo de ação da lacase na ausência (a) e presença (b, c) de mediadores redox da lacase.....	38
Figura 16. Representação esquemática de alguns processos de micro-escala que ocorrem durante o crescimento fúngico em processos de FES. Gás (g); líquido (l); substrato (s); calor (Q); temperatura (T). .....	40
Figura 17. <i>Pycnoporus sanguineus</i> em meio ágar com extrato de malte.....	45
Figura 18. Pré-tratamento do material lignocelulósico para uso com fins energéticos.....	49
Figura 19. Pré-tratamentos da biomassa lignocelulósica. Tratamentos físicos e químicos.....	50
Figura 20. Fragmentos de lignina originados do pré-tratamento hidrotérmico alcalino. ....	52
Figura 21. Fragmentos de lignina e ácidos da hemicelulose (cumárico e ferúlico) originados do pré-tratamento hidrotérmico alcalino.....	52
Figura 22. Porção da hemicelulose (arabinosilana e ácido ferúlico) que mantém ligação com a lignina.....	53
Figura 23. Porção da hemicelulose (ácido cumárico) que mantém ligação com a lignina.....	53
Figure 1. Time course of cellulases, xylanases (a) and ligninases (b) production by <i>P. sanguineus</i> MCA 16 cultivated by SSF on mixture (1:1 w/w) of wheat bran and soybean meal, at 40 °C.....	86
Figure 2. Pareto chart of effects of the variables solid and enzyme loads.....	89
Figure 3 Response surface using pretreated bagasse and enzyme loads as variables in the enzymatic saccharification of sugarcane bagasse by the extract produced by <i>P. sanguineus fungus</i> MCA 16. ....	90
Figure 4. Pareto chart of effects of the variables time and temperature. ....	92
Figure 5. Fourier Transformed Infra Red (FT-IR) spectroscopies of sugarcane bagasse under different pretreatments. Hyd: hydrothermal; AHyd: alkaline hydrothermal; PP: potassium permanganate; APP: alkaline potassium permanganate; ZO: zinc oxide; AZO: alkaline zinc oxide; TO: titanium dioxide;	

ATO: alkaline titanium dioxide; HP: hydrogen peroxide; AHP: alkaline hydrogen peroxide.....	117
Figure 6. Phenolic compounds present in the liquor obtained after alkaline and non-alkaline hydrothermal pretreatment, in the presence of oxidizing agents. Hyd: hydrothermal; AHyd: alkaline hydrothermal; PP: potassium permanganate; APP: alkaline potassium permanganate; ZO: zinc oxide; AZO: alkaline zinc oxide; TO: titanium dioxide; ATO: alkaline titanium dioxide; HP: hydrogen peroxide; AHP: alkaline hydrogen peroxide. ....	118
Figure 7. Pareto diagram of the estimated effects of the hydrogen peroxide concentration and enzyme loading variables on saccharification of pre-treated sugarcane bagasse. ....	123
Figure 8. Response surfaces generated to the glucose concentration in function of hydrogen peroxide concentration and endoglucanase load (a); hydrogen peroxide concentration and sugarcane bagasse load (b); endoglucanase load and sugarcane bagasse load (c). ....	124
Figure 9. Organic acids present in the liquid fractions obtained from alkaline hydrothermal pretreatments in the presence of different hydrogen peroxide concentrations. (a) Vanilin and ferulic acids concentrations and (b) syringic and p-coumaric acids concentrations. ....	127
Figure 10. Pareto diagram of time and temperature influence on the enzymatic saccharification.....	129
Figure 11. Response surface generated to the glucose concentration obtained after enzymatic saccharification using time and temperature as variable in the statistical planning. ....	130
Figure 12. Scanning electron microscopy of sugarcane bagasse. (a) <i>In natura</i> 500x, (b) <i>in natura</i> 1500x, (c and d) submitted to hydrothermal alkaline pretreatment 500x and 1500x, respectively, (e and f) submitted to hydrothermal alkaline pretreatment with hydrogen peroxide (2.5%) 500x and 1500x, respectively. ....	133
Figure 13. Scanning electron microscopy of sugarcane bagasse submitted to alkaline hydrothermal pretreatment 1500x (a) and alkaline hydrothermal pretreatment with hydrogen peroxide at 2.5% 1500x (b). ....	134
Figure 14. Estimated effects by Pareto Diagram of the variables in the laccase (a), MnP (b) and LiP (c) activities. ....	153

Figure 15. Response surface obtained for pH and temperature effects on ligninases activities.....	154
Figure 16. Effects of pH (a) and temperature (b) on ligninases stability. ....	155
Figure 17. Total phenolic compounds concentration and ligninases activities profiles during the incubation of enzymes solution at 50 °C (a) and 70 °C (b). Enzyme solution was obtained from <i>P. sanguineus</i> MCA 16 cultivation in the mixture of soybean meal and wheat bran (1:1 w/v) for 96 hours at 40 °C. All the enzymatic activities were determined under optimum conditions of pH and temperature.....	157
Figure 18. Profiles of TPC concentration and ligninases activities during enzymatic saccharification of sugarcane bagasse submitted to alkaline hydrothermal pretreatment. Saccharification was conducted at 50 °C and the enzymes activities were determined under optimum conditions.....	159
Figure 19. Scanning electronic micrography (SEM) of pretreated sugarcane bagasse before enzymatic saccharification with 500x (a), after enzymatic saccharification with 500x (b), before enzymatic saccharification with 500x (c), before enzymatic saccharification with 1500x (d), after enzymatic saccharification with 500x (e) and after enzymatic saccharification with 1500x (f).....	162

## Lista de tabelas

Tabela 1. Produção de enzimas celulolíticas e xilanolíticas por fungos utilizando-se resíduos lignocelulósicos. ....	43
Tabela 2. Enzimas de degradação de lignocelulose produzidas por <i>Pycnoporus sanguineus</i> . ....	46
Table 1. Rotational central composite design (RCCD) ( $2^2$ ) used in saccharification of pretreated sugarcane bagasse. ....	82
Table 2. Face centered central composite design (CCD) ( $2^2$ ) used in the saccharification of pretreated sugarcane bagasse. ....	82
Table 3. Cellulases and xylanases activities in the crude enzyme solutions obtained by cultivation of <i>P. sanguineus</i> MCA 16 on different mixtures (1:1 w/w) of lignocellulosic substrates by SSF, for 96 hours, at 40 °C. SB: sugarcane bagasse; WB: wheat bran; RH: rice husk; SM: soybean meal. ....	84
Table 4. Ligninases activities in the crude enzyme solutions obtained by cultivation of <i>P. sanguineus</i> MCA 16 different mixtures (1:1 w/w) of lignocellulosic substrates by SSF, for 96 hours, at 40 °C. SB: sugarcane bagasse; WB: wheat bran; RH: rice husk; SM: soybean meal; MnP: manganese peroxidase; LiP: lignin peroxidase; nd: not detected. ....	85
Table 5. Rotational Central Composite Design (RCCD) model used in the enzymatic saccharification experiments of pre-treated sugarcane bagasse. ...	88
Table 6. Coded and real values of the face-centered design design ( $2^3$ ) used in saccharification of sugarcane bagasse submitted to alkaline hydrothermal pretreatment in the presence of hydrogen peroxide. SB: sugarcane bagasse. ....	112
Table 7. Coded and real values of the rotational central composite design (RCCD) ( $2^2$ ) used in the enzymatic saccharification of the sugarcane bagasse. ....	112
Table 8. Effect of pretreatments on chemical composition of sugarcane bagasse. ....	114

Table 9. Enzymatic saccharification of sugarcane bagasse submitted to different pre-treatments. Assays conducted at 50 °C for 24 hours with bagasse and endoglucanase loads at 3.0% and 100 total units/g cellulose, respectively. ..	120
Table 10. Factorial design of the face-centered type used for the evaluation of the influence, in the saccharification, of the bagasse and hydrogen peroxide loads used in the pretreatment, as well as the endoglucanase loading used in saccharification. An enzymatic extract produced by <i>Pycnoporus sanguineus</i> MCA 16 was used for cultivation on wheat bran and soybean meal (2:3 w/w) at 40 °C for 96 hours. The tests were kept at 50 °C for 24 hours under 300 rpm. ....	122
Table 11. Analysis of the lignocellulosic fractions of sugarcane bagasse subjected to alkaline pre-treatment in the presence of different percentages of hydrogen peroxide.....	126
Table 12. Rotational Central Composite Design (RCCD) used in the enzymatic saccharification experiments of pre-treated sugarcane bagasse.....	129
Table 13. Analyzes of variance (ANOVA) to enzymatic saccharification of sugarcane bagasse. ....	130
Table 14. Coded and real values used in the the 2 <sup>2</sup> type face-centered performed to study the effect of pH and temperature (°C) on enzymes activities. ....	147
Table 15. Coded and real values of the face centered design (2 <sup>2</sup> ) used to evaluate de effect of pH and temperature on laccase and lignin peroxidase activities.....	151
Table 16. Coded and real values of the central composite design (RCCD) used to evaluate the effect of pH and temperature on manganese peroxidase actyity. ....	152
Table 17. Analysis of variance ANOVA of the experimental design for ligninases activities.....	153
Table 18. Influence of metal ions, organic and inorganic compounds on ligninases activities.....	156
Table 19. Effect of organic acids on ligninases activities. All the enzymes activities were determined under optimum conditions of pH and temperature. ....	160

## Sumário

Introdução.....	18
Revisão Bibliográfica .....	20
1.Composição dos resíduos agroindustriais.....	20
1.1.Celulose .....	21
1.2.Hemicelulose.....	23
1.3.Lignina .....	24
2.Degradação enzimática de materiais lignocelulósicos .....	26
2.1.Celulases .....	27
2.2.Hemicelulases .....	29
2.3.Ligninases .....	30
3.Resíduos lignocelulósicos como substratos para produção de enzimas lignocelulolíticas e como fontes de açúcares fermentescíveis.....	39
3.1. Produção de enzimas por fermentação em estado sólido (FES) .....	39
3.2. Bagaço de cana-de-açúcar: substrato para cultivo fúngico e fonte de energia renovável .....	47
4. Pré-tratamentos da biomassa lignocelulósica .....	49
4.1. Pré-tratamento hidrotérmico .....	50
4.1.1. Pré-tratamento hidrotérmico em meio alcalino.....	51
4.1.1.1 Pré-tratamento hidrotérmico na presença de agentes oxidantes .....	53
4.2. Uso de hidrolisados enzimáticos para produção de etanol 2G.....	55
Referências Bibliográficas .....	57
Capítulo 1 .....	76
Ligninases, cellulases and xylanases production by <i>Pycnoporus sanguineus</i> MCA 16 and enzymatic saccharification of sugarcane bagasse for ethanol production .....	76
1.Introduction.....	77
2.Material and methods .....	-
2.1.Microrganism.....	78
2.2.Influence of solid state fermentation (SSF) conditions on the enzymes productions .....	78

2.3. Determination of enzymes activities .....	79
2.4. Sugarcane bagasse hydrothermal alkaline pretreatment .....	80
2.5. Enzymatic saccharification of pretreated sugarcane bagasse.....	80
2.5.1. Influence of enzyme and bagasse loads.....	81
2.5.2. Influence of time and temperature .....	82
2.6. Analytical methods .....	82
2.7. Alcoholic fermentation .....	83
3.Results and discussion .....	84
3.1. Enzymes production by solid-state fermentation.....	84
3.1.1 Influence of lignocellulosic substrates.....	84
3.1.2. Influence of cultivation time on the enzymes production .....	86
3.2.1. Influence of solid and enzyme loads.....	87
3.2.2. Influence of time and temperature .....	91
3.3. Comparative enzymatic saccharifications using filter paper or xylan as substrates .....	94
3.3. Analysis of total phenolic compounds (TPC) .....	94
3.4. Analysis of fermentation inhibitors .....	95
3.5. Alcoholic fermentation .....	95
4.Conclusions .....	96
5.Aknowledgements .....	96
6.References .....	97
Capítulo 2 .....	106
Use of oxidizing agents in the hydrothermal alkaline pretreatment of sugarcane bagasse, enzymatic saccharification and cellulosic ethanol production.....	106
1.Introduction.....	107
2. Material and methods .....	108
2.1. Sugarcane bagasse .....	108
2.2. Hydrothermal Pretreatment .....	109
2.2.1. Use of oxidizing agents .....	109
2.3. Microorganism.....	109



2.4. Enzymes production by solid-state fermentation (SSF) .....	109
2.5. Endoglucanase activity .....	110
2.6. Enzymatic saccharification of pretreated sugarcane bagasse.....	110
2.6.1 Optimization of enzymatic saccharification conditions .....	111
2.7. Analytical methods .....	112
2.8. Scanning electron microscopy .....	113
3. Results and discussion .....	114
3.1. Analysis of sugarcane bagasse lignocellulosic fractions .....	114
3.2. Enzymatic saccharification of pretreated sugarcane bagasse.....	119
3.2.1. Influence of the oxidizing agents used in the hydrothermal pretreatment.....	119
3.2.2. Influence of hydrogen peroxide concentration, sugarcane bagasse and enzyme loading .....	120
3.2.3. Influence of hydrogen peroxide concentration on pretreatment on the composition of sugarcane bagasse .....	125
3.2.4. Influence of time and temperature .....	128
3.3. Alcoholic fermentation inhibitors .....	131
3.2.5. Scanning electron microscopy of sugarcane bagasse .....	132
6. Conclusions .....	134
7. Acknowledgements .....	135
8. References .....	135
Capítulo 3 .....	
Degradation of phenolic compounds by ligninases from <i>Pycnoporus sanguineus</i> MCA 16 during enzymatic saccharification of pretreated sugarcane bagasse.....	142
1. Introduction.....	143
2. Material and methods .....	145
2.1. Lignocellulosic substrates.....	145
2.2. Ligninases activities .....	145
2.3. Ligninases physico-chemical characterization .....	146
2.3.1 Effect of pH and temperature on enzyme activity and stability .....	146
2.3.2. Effect of glucose, ions, organic and inorganic compounds.....	147

2.4. Sugarcane bagasse hydrothermal alkaline pretreatment .....	148
2.5. Enzymes production.....	148
2.6. Degradation of phenolic compounds by ligninases in the enzymatic extract ...	148
2.7. Enzymatic saccharification of pretreated sugarcane bagasse.....	149
2.8. Action of ligninases on phenolic compounds during enzymatic saccharification .....	149
2.9. Analytical Methods .....	149
2.10. Scanning electron microscopy.....	150
3. Results and discussion .....	150
3.1. Ligninases physico-chemical characterization .....	150
3.2. Effect of chemical compounds on ligninases activities.....	156
3.3. Degradation of phenolic compounds by the ligninases in the enzyme solution	156
3.4. Degradation of phenolic compounds by the ligninases during pretreated sugarcane bagasse enzymatic saccharification.....	158
3.5. Morphological analysis of sugarcane bagasse after enzymatic saccharification .....	161
4. Conclusions .....	-
5. Acknowledgements .....	163
6. References .....	163

## Introdução

A produção de celulases, xilanases e ligninases microbianas visando a utilização em processos de obtenção de etanol de segunda geração tem despertado o interesse da comunidade científica nos últimos anos. Tais enzimas podem ser produzidas por microrganismos, especialmente fungos filamentosos, quando cultivados em resíduos lignocelulósicos (agroindustriais, florestais e urbanos) como substratos (MORETTI et al., 2014; PEREIRA et al., 2015; PEREIRA et al., 2016a; PEREIRA et al., 2016b; MARQUES et al., 2018; OLIVEIRA et al. 2018). Entre os diversos resíduos empregados com esta finalidade estão o bagaço de cana-de-açúcar, o farelo de trigo, o sabugo e a palha de milho, a palha e a casca de arroz, o farelo de soja, a cevada e a casca de café e a casca de frutas como maracujá entre outros (FERRAREZI et al., 2014; MORETTI et al., 2012; PEREIRA et al., 2014). Estes resíduos são abundantes no Brasil, especialmente o bagaço de cana-de-açúcar, constantemente gerado em grande volume pela indústria sucroalcooleira.

Os basidiomicetos destacam-se entre os fungos filamentosos produtores de enzimas de degradação da biomassa vegetal, especialmente por serem capazes de produzir, além das celulases e hemicelulases, as ligninases (SIGOILLOT et al., 2012), Tais enzimas são importantes no contexto de obtenção de etanol de segunda geração, uma vez que removem a lignina residual presente no material lignocelulósico pré-tratado, favorecendo o acesso das enzimas sacarificantes aos seus substratos. Além disso, podem remover os compostos fenólicos presentes nos hidrolisados, gerados durante o processo.

Dentre os vários tipos de pré-tratamentos empregados com a finalidade de desorganizar/remover a lignina do material lignocelulósicos citados na literatura está o hidrotérmico alcalino. Neste pré-tratamento, o uso de hidróxido de sódio leva á remoção da lignina, solubilizando-a, além de promover o enturgescimento da celulose nativa resultando em uma celulose menos cristalina, com baixo grau de polimerização e com maior área de superfície, o que facilita a sua exposição e acessibilidade às celulases (HABIBI; LUCIA; ROJAS, 2009; LEE; HAMID; ZAIN, 2014; SANTOS et al., 2012; TALEBNIA; KARAKASHEV; ANGELIDAKI, 2010).

Para facilitar a degradação da lignina, o uso de agentes oxidantes como o permanganato de potássio, óxido de zinco, dióxido de titânio e peróxido de hidrogênio, pode complementar o processo de pré-tratamento hidrotérmico alcalino. Tais agentes têm sido empregados em processos de tratamento de águas residuárias e de detoxicação de ambientes, sendo citados como vantajosos pela eficácia de conversão de compostos orgânicos em inorgânicos (DASH; PATEL; MISHRA, 2009; KADLA; CHANG, 2001; KHAN; NAJEEB; TUIYEBAYEVA, 2014; MA et al., 2015; XU et al., 2005; YU et al., 2015; ZHANG, 2013).

Dentro deste contexto, o presente trabalho objetivou utilizar soluções enzimáticas produzidas pelo fungo *Pycnoporus sanguineus* MCA 16, a partir de cultivos em diferentes substratos lignocelulósicos, na sacarificação enzimática do bagaço de cana pré-tratado. Os pré-tratamentos utilizados foram hidrotérmicos alcalinos com e sem o uso de agentes oxidantes. As sacarificações enzimáticas foram realizadas empregando-se ferramentas de planejamento experimental, tendo como resposta a liberação de glicose nos hidrolisados.

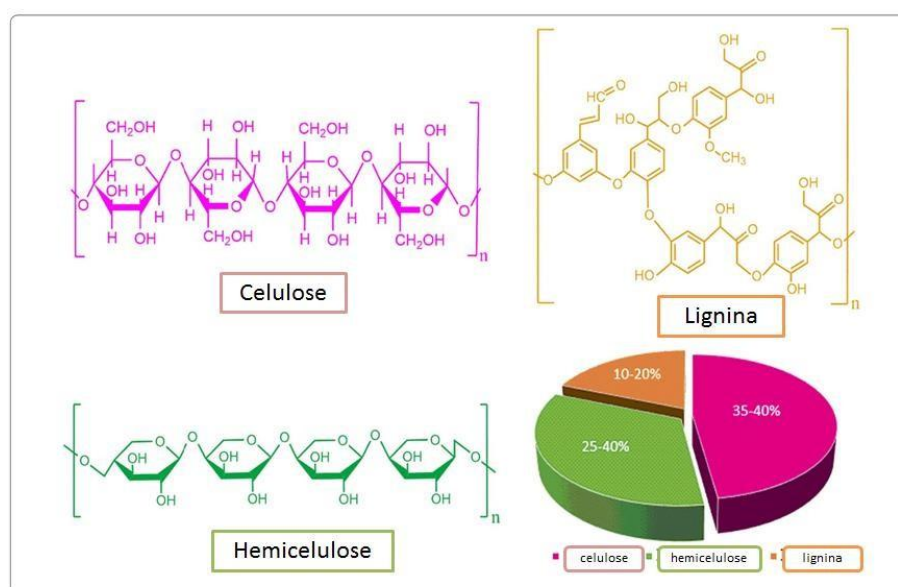
As ligninases produzidas pelo fungo foram avaliadas quanto à eficácia no processo de remoção de compostos fenólicos presentes nos hidrolisados, visando diminuir a influência dos mesmos sobre as celulasas, bem como visando a obtenção de hidrolisados menos tóxicos aos microrganismos fermentadores. Os resultados de todas as análises foram separados em capítulos, sendo o primeiro sobre a produção das enzimas, sacarificação e fermentação alcoólica, o segundo sobre uso de agentes oxidantes sob o pré-tratamento hidrotérmico alcalino e sacarificação e, por fim, o terceiro avaliando a ação das ligninases sobre os compostos fenólicos totais.

## Revisão Bibliográfica

### 1. Composição dos resíduos agroindustriais

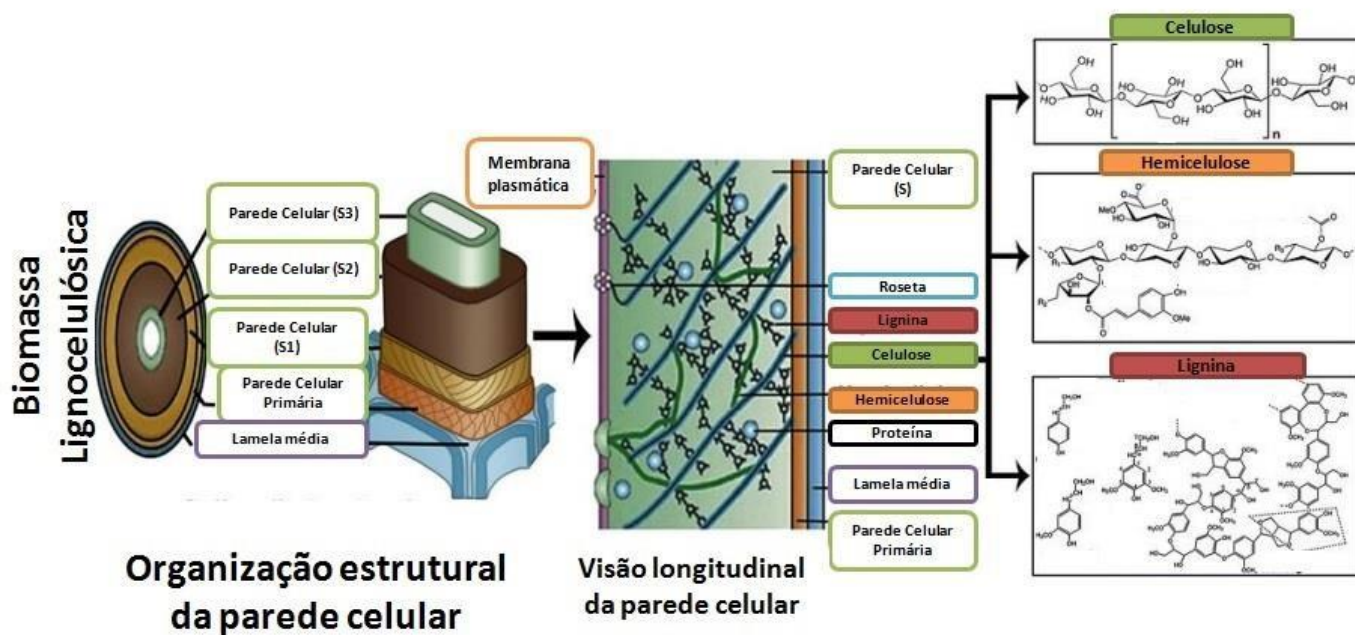
Resíduos agroindustriais são subprodutos como farelos de trigo, soja e algodão, sabugo de milho, palha e bagaço de cana, bagaço de laranja, entre outros, que foram oriundos das atividades agrícolas e compostos principalmente por lignocelulose (LIM, MATU, 2015). Constituída por celulose, hemicelulose e lignina, a lignocelulose é o material estrutural presente na parede celular vegetal e representa a principal fonte de matéria orgânica renovável do planeta (Figuras 1 e 2). A quantidade de cada polímero varia de acordo com a espécie, a época de colheita e, ainda, entre as diferentes partes do vegetal, sendo geralmente 30-50% celulose, 15-35% hemicelulose e 10-20% lignina (GIRIO et al., 2010). Suas propriedades químicas a tornam um material de grande interesse biotecnológico e, há alguns anos, surgiu o conceito de biorefinaria de lignocelulose, que tem recebido atenção crescente devido ao potencial de conversão deste material em diversos produtos de valor agregado, como compostos químicos, substratos para fermentações e, especialmente, biocombustíveis (RAGAUSKAS et al., 2006; ABBASI; ABBASI, 2010).

Figura 1. Composição da lignocelulose.



Fonte: Adaptado e traduzido de Amin et al. (2017).

Figura 2. Composição da lignocelulose.

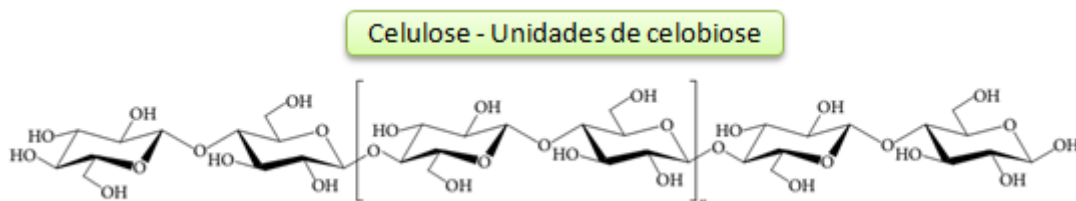


Fonte: Adaptado e traduzido de Manavalan et al. (2015).

### 1.1.Celulose

Descoberta em 1838 pelo químico francês Anselme Payen, o qual também definiu sua estrutura, a celulose é um homopolímero fibroso e insolúvel em água formado por unidades de celobiose (Figura 3) unidas por ligações  $\beta$ -1,4 D-glicosídicas (SAINI et al., 2015).

Figura 3. Estrutura química da celulose.



Fonte: Adaptado e traduzido de Saini et al. (2015).

A grande proporção de celulose na biomassa vegetal seca e sua composição a tornam o maior recurso energético renovável da natureza (CHEN, 2014). Além de plantas, a celulose está presente em alguns animais marinhos, como os tunicatos, em algumas bactérias (como *Acetobacter xylinu*, *Acetobacter xylinum* e *Gluconacetobacter hansenii* e *Gluconacetobacter hansenii*) e alguns fungos (Filos Hyphochytriomycota, Labyrinthulomycota e Oomycota) (DONINI et al., 2010; HABIBI; LUCIA; ROJAS, 2009; JOZALA et al., 2011; KIRK et al., 2008; PÉREZ; SAMAIN, 2010).

Na parede celular vegetal, as moléculas de celulose agrupam-se para formar as microfibrilas. Cada microfibrila, constituída por cerca de 36 moléculas de celulose, apresenta um padrão de torção dependendo da orientação das ligações de hidrogênio estabelecidas entre as unidades de celobiose (HABIBI; LUCIA; ROJAS, 2009; HIMMEL et al., 2007). As microfibrilas agrupam-se dando origem às macrofibrilas e estas, por sua vez, organizam-se dando origem à estrutura fibrosa da celulose (ISIKGOR; BECER, 2015).

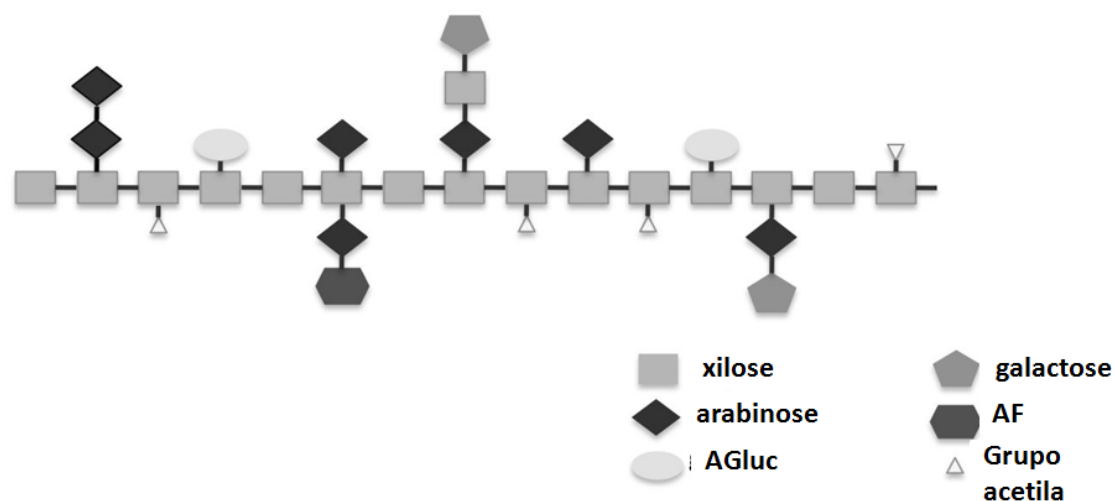
Embora muitos autores reportem a presença de regiões amorfas e cristalinas na celulose (MADADI; TU; ABBAS, 2017; MANAVALAN; MANAVALAN; HEESE, 2015; SUKUMARAN et al., 2010; TAHERZADEH; KARIMI, 2007; TOMÁS-PEJÓ et al., 2011; VLASENKO et al., 2010), Habibi et al. 2009 e Payne et al. 2015 discutem a idéia de que regiões amorfas não existem, sendo um resultado da torção entre as microfibrilas da celulose.

As fibras de celulose exibem extremidades redutoras e não-redutoras, ou seja, extremidades onde apresentam hidroxilas livres. Quanto ao grau de polimerização, ainda não há uma definição estabelecida para a celulose presente em cada vegetal, uma vez que esta determinação requer o isolamento do polímero intacto (o que dificilmente se consegue no processo de extração), além de sua purificação (PÉREZ; SAMAIN, 2010).

## 1.2.Hemicelulose

A hemicelulose é um heteropolissacarídeo composto por xilanas, mananas, xiloglucanas e  $\beta$ -glucanas, compreendendo cerca de 20-27% da biomassa vegetal (CANILHA et al., 2013; EBRINGEROVÁ, 2006) (Figura 4), cujos monômeros incluem hexoses e pentoses (GÍRIO et al., 2010; SCHELLER; ULVSKOV,2010; SOUZA, 2013).

Figura 4. Estrutura da hemicelulose, constituída principalmente por xilana.  
AGluc: ácido glucurônico; AF: ácido ferúlico.



Fonte: Adaptado e traduzido de Souza (2013).

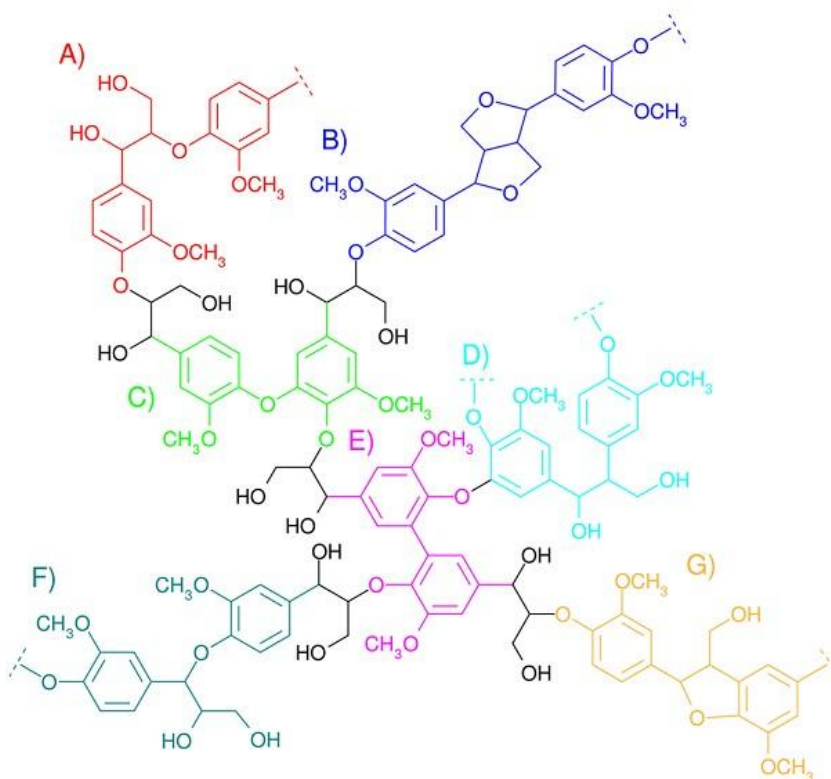
As xilanas constituem o polímero mais abundante da hemicelulose e podem ser agrupadas em subclasses: homoxilanas, glucuronoxilanas, arabinoglucuronoxilanas, arabinoxilanas, glucuronoarabinoxilanas e heteroxilanas (EBRINGEROVÁ, 2006). Suas estruturas baseiam-se em uma cadeia principal constituída por monômeros de xilose unidos por ligações do tipo  $\beta$ -1,4 (SCHELLER; ULVSKOV, 2010). Uma particularidade das xilanas é que estas podem manter ligações com outros polissacarídeos, por meio de ligações laterais. Além disso, xilanas são comumente acetiladas e também fazem ligações ésters com ácido p-cumárico e ferúlico, porção esta ligada à lignina (SCHELLER; ULVSKOV, 2010).



### **1.3.Lignina**

A lignina é um heteropolímero essencial para a estruturação da parede celular de plantas vasculares, sendo o segundo polímero mais abundante da Terra, depois da celulose, representando 20% do carbono total fixado na fotossíntese (HERNÁNDEZ-ORTEGA; FERREIRA; MARTÍNEZ, 2012). É composta por monômeros de fenilpropanóides, sintetizados a partir de monolignóis como os alcoóis *p*-cumarílico, coniferílico e sinapílico que, respectivamente, dão origem às unidades *p*-hidroxifenila, guaiacila e siringila (AZARPIRA; RALPH; LU, 2014). A rede de ligações entre os monômeros incluem os tipos  $\beta$ -O-4-,  $\beta$ -5-,  $\beta$ - $\beta$ -, 5-5- (geralmente como dibenzodioxocinas) e 5-O-4- (Figura 5) (ABDEL- HAMID; SOLBIATI; CANN, 2013a; AZARPIRA; RALPH; LU, 2014; BESTE, 2014). Apesar de a lignina ser constituída por monômeros de compostos fenólicos, o polímero é de natureza não-fenólica, devido à forte predominância de precursores de *p*-hidroxifenilpropanóides (HERNÁNDEZ-ORTEGA; FERREIRA; MARTÍNEZ, 2012).

Figura 5. Representação de um fragmento da lignina, destacando as ligações entre os monômeros de fenilpropanóides. (A)  $\beta$ -O-4, (B)  $\beta$ - $\beta$ , (C) 4-O-5, (D)  $\beta$ -1, E) 5-5, (F)  $\alpha$ -O-4 e (G)  $\beta$ -5.



Fonte: Adaptado e traduzido de Beste (2014).

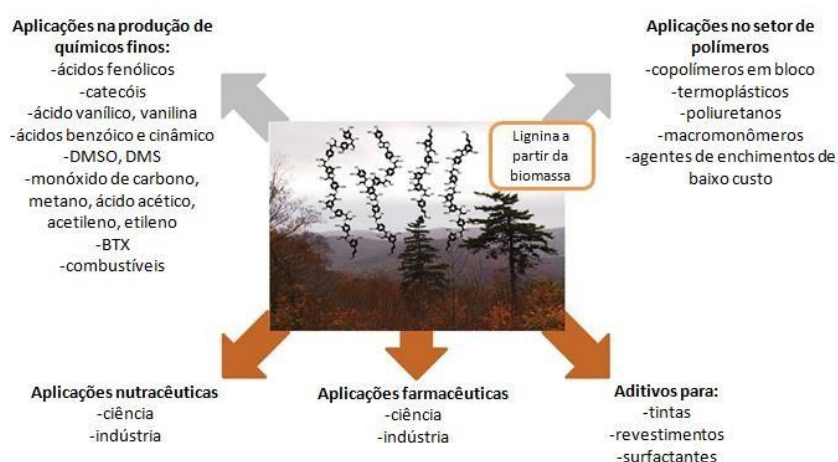
Encontrada nas paredes celulares vegetais primária e secundária, a lignina auxilia na proteção do tecido vegetal como uma barreira física contra patógenos que infectam a planta. A composição média varia entre 20-24% e 28-31% em madeiras macias e duras, respectivamente. Em plantas herbáceas (angiospermas; monocotiledôneas), a lignina representa cerca de 15-19% (SORIEUL et al., 2016).

O tipo de lignina varia entre os grupos de vegetais. Ligninas do tipo guaiacila (álcool coniferílico) são encontradas em gimnospermas. Em dicotiledôneas (angiospermas) tanto o tipo guaiacila (álcool coniferílico) quanto siringila (álcool sinapílico) são encontrados. Em monocotiledôneas, sobretudo as gramíneas, os três tipos de lignina (guaiacila, siringila e *p*-hidroxifenila) estão presentes (BLAND; LOGAN, 1965; ERIKSSON; BERMEK, 2009; LANGE; DECINA; CRESTINI, 2013).

Em alguns processos industriais a lignina deve ser removida, como na

produção de etanol de segunda geração (dificulta o acesso das enzimas sacarificantes aos seus substratos, dá origem a compostos fenólicos que podem inibir o metabolismo da levedura fermentadora, bem como a ação das enzimas sacarificantes), na fabricação de vinhos, cervejas e sucos (altera a coloração, aroma e sabor) e na produção de papel\_(confere cor escura). Entretanto, a partir do processamento da lignina, produtos de valor agregado podem ser obtidos como compostos aromáticos como ácido ferúlico e vanílico, que podem ser aplicados na indústria farmacêutica e de alimentos (ABDELMONSSEF et al., 2016; DWIVEDI et al., 2011; LANGE et al., 2013). A Figura 6 traz uma síntese das diversas aplicações biotecnológicas da lignina.

Figura 6. Aplicações da lignina como recurso renovável a partir da biomassa.



Fonte: Adaptado e traduzido de Lange et al. (2013).

## 2. Degradação enzimática de materiais lignocelulósicos

Enzimas de degradação de materiais lignocelulósicos são produzidas e secretadas por diversos microrganismos, entre os quais destacam-se os fungos filamentosos, cujas enzimas incluem celulasas hidrolíticas (endoglucanases, exoglucanases e  $\beta$ -glicosidases) (YEOMAN et al., 2010), celulasas oxidativas (polissacarídeos mono-oxigenases) (FROMMHAGEN et al., 2017; NAVARRO et al., 2014), hemicelulasas (xilanases,  $\beta$ -xilosidases, mananase, arabinase,

glucuronidases, manosidases e esterases) (SHALLOM; SHOHAM, 2003; SUURNÄKKI et al., 1997; YEOMAN et al., 2010), ligninases (lacases, lignina e manganês peroxidases) (ERIKSSON; BERMEK, 2009; GOMES et al., 2009) e aril-alcool oxidases (aril-alcool desidrogenase, quinona redutase e glioxal oxidases) (ABDEL-HAMID; SOLBIATI; CANN, 2013a; DAOU; FAULDS, 2017; HERNÁNDEZ-ORTEGA; FERREIRA; MARTÍNEZ, 2012; MARTINEZ et al., 2005; SIGOILLOT et al., 2012), estas últimas gerando espécies reativas de oxigênio, cátions metálicos e radicais aromáticos que auxiliam na catálise enzimática das ligninases sobre a lignina.

## **2.1.Celulases**

As enzimas celulolíticas constituem um grupo de glicosil-hidrolases (GH) classificadas em diferentes famílias. Algumas destas enzimas necessitam de um ou mais módulos de ligação ao substrato (ou módulo de ligação ao carboidrato) que correspondem a porções não catalíticas constituídas por aminoácidos e intimamente ligadas à enzima. Celulases hidrolíticas incluem as endoglucanases, exoglucanases e  $\beta$ - glicosidases (Figura 7) que atuam de forma sinérgica sobre a celulose, diminuindo seu grau de polimerização (BORASTON et al., 2004; PEREIRA et al., 2017; SAINI et al., 2015).

Endoglucanases (EG) (E.C.3.2.1.4) catalisam a hidrólise da celulose amorfa e são classificadas em aproximadamente 11 famílias GH (GH5, 6, 7, 8, 9, 12, 44, 45, 48, 51 e 74) (VLASENKO et al., 2010). De forma cooperativa com as outras hidrolases, as EGs agem nas regiões internas da estrutura amorfa da fibra celulósica, liberando celooligossacarídeos de diversos graus de polimerização (GP) e, conseqüentemente, novos terminais redutores e não redutores (CASTRO et al., 2010).

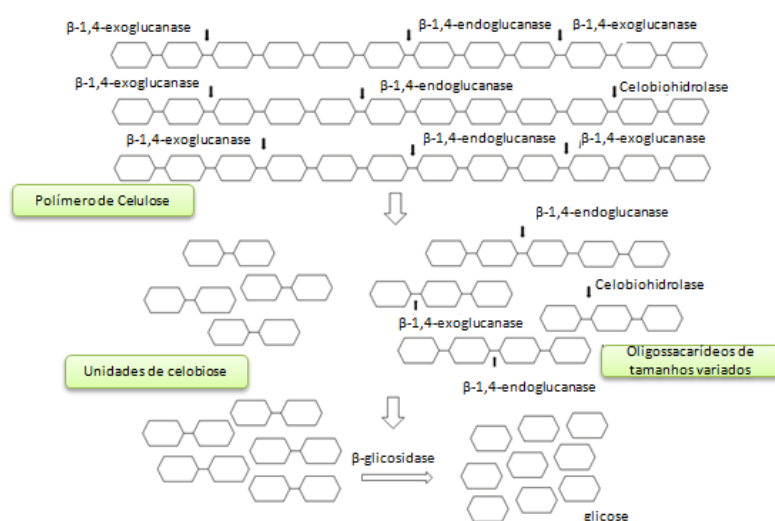
Exoglucanases (ExG) (E.C.3.2.1.74) e celobiohidrolases (CBH) (E.C.3.2.1.91) são enzimas que agem nas extremidades redutoras e não-redutoras da cadeia de celulose, liberando celobiose como produto. Trabalhos mais recentes, como de Hamid et al. (2015); Hsieh et al. (2015) e Payne et al. (2015), consideram tanto exoglucanases quanto celobiohidrolases como sendo a mesma enzima, sendo classificadas como E.C.3.2.1.91, apresentando duas

formas de catálise, uma agindo nas extremidades redutoras, denominadas de celobiohidrolases I (CBHI) e outra com ação nas extremidades não-redutoras, classificadas como celobiohidrolases II (CBHII). As exoglucanases atuam preferencialmente nas regiões cristalinas, mas podem catalisar a hidrólise das ligações glicosídicas das regiões amorfas da celulose (VOUTILAINEN et al., 2007).

As  $\beta$ -1,4-glicosidases ou celobiasas (BG) (E.C.3.2.1.21) hidrolisam celobiose e outras celodextrinas em glicose (LEITE et al., 2008). Agindo em conjunto com EG e ExG (CBHI e CBHII), as BG contribuem para a finalização do processo catalítico da celulose (VOUTILAINEN et al., 2007), sendo responsáveis pela regulação de todo o processo celulolítico (YEOMAN et al., 2010).

A sinergia com que as celulasas atuam depende da natureza do substrato hidrolisado (WOODWARD, 1991). Diferentes graus de sinergismo podem ser encontrados entre celobiohidrolases, entre exo e endoglucanases, entre endoglucanases, entre celobiohidrolases, endoglucanases e  $\beta$ -glicosidases (BOISSET et al., 2000, 2001; LYND et al., 2002; QI; JUN; FORSBERG, 2007; ZHANG; LYND, 2004). O maior grau de sinergismo é geralmente observado na hidrólise da celulose cristalina (ZHANG; LYND, 2004).

Figura 7. Representação esquemática da ação das celulasas sobre a celulose.

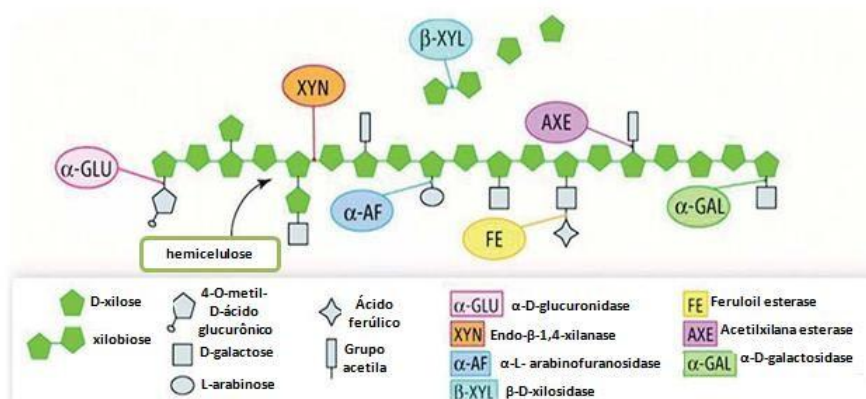


Fonte: Adaptado e traduzido de Saini et al. (2015).

## 2.2.Hemiceluloses

A heterogeneidade da hemicelulose reflete na diversidade das enzimas envolvidas em sua degradação, entre as quais podem ser citadas xilanases,  $\beta$ -xiliosidases, mananases, arabinases, acetilxilana esterases, ácido *p*-cumárico esterases, ácido ferúlico-esterases, galacturonidases (Figura 8) (MADADI; TU; ABBAS, 2017). Assim como as celulases, as hemicelulases podem apresentar módulos de ligação ao substrato, os quais podem ligar-se também à celulose e outros carboidratos (BORASTON et al., 2004; VÁRNAI et al., 2014).

Figura 8. Representação esquemática da ação das enzimas hemicelulolíticas.



Fonte: Adaptado e traduzido de Madadi et al. (2017).

Uma vez que a maior fração da hemicelulose é composta por xilanas, as principais enzimas envolvidas na degradação deste heteropolímero são as xilanases. As  $\beta$ -1,4-xilanase (endoxilanases, 1,4- $\beta$ -D-xylan- xylanohydrolases, endo-1,4- $\beta$ -D-xylanases,  $\beta$ -1,4-xylanases ou  $\beta$ -xylanases) (E.C.3.2.1.8) são glicosidases pertencentes às famílias GH 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 e 62 (COLLINS; GERDAY; FELLER, 2005; LIAO et al., 2015; MOTTA; ANDRADE; SANTANA, 2013). Tais enzimas catalisam a hidrólise de ligações  $\beta$ -1,4-D-xilosídicas de maneira aleatória, liberando xilobiose ou xilooligossacarídeos (SWEENEY; XU, 2012; VAN DYK; PLETSCHE, 2012).

$\beta$ -xiliosidases (1,4- $\beta$ -D-xilana xilohidrolase) (E.C.3.2.1.37) são glicosilhidrolases pertencentes às famílias GH 3, 30, 39, 43, 52 e 54 (BENASSI et al.,

2013) que podem catalisar a hidrólise de xilobiose ou xilooligossacarídeos (BIELY, 1985; SARASWAT; BISARIA, 1997; TERRASAN; GUIBAN; CARMONA, 2016), liberando xilose como produto (KNOB; TERRASAN; CARMONA, 2010; SØRENSEN et al., 2007). Na degradação da xilana, as  $\beta$ -xilosidases são importantes por remover os produtos que poderiam inibir a ação das endoxilanasas (POLIZELI et al., 2005; SUNNA; ANTRANIKIAN, 1997). Na maioria das bactérias e leveduras, as  $\beta$ -xilosidases estão ligadas à célula. Em alguns fungos filamentosos, mantêm-se associadas ao micélio durante os primeiros estágios de crescimento e podem ser liberadas ao meio extracelular ao longo dos processos de lise celular (KNOB; TERRASAN; CARMONA, 2010). Em outros fungos, estas enzimas também podem se manter associadas à célula, não sendo secretadas durante todos os estágios de crescimento (IEMBO et al., 2006; KATAPODIS et al., 2006). Xilanasas são enzimas de grande interesse biotecnológico, podendo ser aplicadas nas indústrias de papel, alimentícia e, mais recentemente, na área de biocombustíveis (BUTT et al., 2008; LIAO et al., 2015).

### **2.3.Ligninases**

Ligninases são produzidas principalmente por fungos basidiomicetos (Abdel-Hamid et al., 2013; Eriksson and Bermek, 2009; Gomes et al., 2009; Gorjup et al., 1999; Górska et al., 2014; Sanghi, 2011, Dashtban et al., 2010). De acordo com Eriksson e Bermek (2009) 40% dos fungos de degradação branca expressam genes que codificam a secreção de ligninases. Dentre estas enzimas estão a fenoloxidase (lacase) e as peroxidases (lignina-peroxidase e manganês-peroxidase).

Lignina-peroxidases (EC 1.11.1.14) são enzimas que atuam sobre compostos fenólicos e não fenólicos na lignina. Essas reações de degradação da lignina são dependentes de peróxido de hidrogênio ( $H_2O_2$ ) resultante de enzimas oxidativas presentes nas membranas biológicas, como aril-alcool-oxidase (AAO), glioxal-oxidase (GLOX), aril-alcool desidrogenase (AAD) e quinona redutase (QR). LiP são hemoproteínas monoméricas, possuindo em sua estrutura molecular um átomo de ferro do tipo III. De acordo com George et

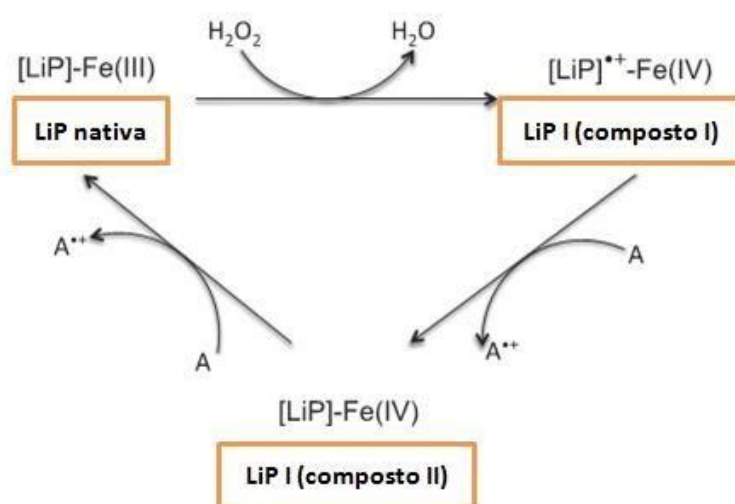
al., (1999), LiPs são secretadas quando o microrganismos encontra-se em estado de privação de alguns nutrientes, como de fontes de nitrogênio, fósforo, enxofre e carbono.

Na degradação de compostos não fenólicos presentes na estrutura da lignina (como ligações do tipo éter  $\beta$ -O-4, do tipo éter aril-glicerol, entre C $\alpha$ -C $\beta$  e compostos aromáticos), o peróxido de hidrogênio advindo de outras vias metabólicas e secretado pelo fungo é oxidado pelo Fe tipo III da LiP, numa reação conhecida como Reação de Fenton, onde o H<sub>2</sub>O<sub>2</sub> é oxidado à água (ABDEL-HAMID; SOLBIATI; CANN, 2013a; BANCI; CIOFI-BAFFONI; TIEN, 1999; BUGG et al., 2011). Sendo assim, o H<sub>2</sub>O<sub>2</sub> é essencial no processo de catálise enzimática pela LiP, pois sua ausência deixa a enzima inativa ou suscetível à inibição por outros compostos presentes no meio, como os compostos fenólicos (HARVEY et al., 1992). O mecanismo de ação da LiP sobre compostos não fenólicos está explicado na Figura 9.

O mecanismo catalítico envolve (1) oxidação de dois elétrons do átomo de ferro contido na enzima nativa (Fe(III)) pelo H<sub>2</sub>O<sub>2</sub>, formando um composto intermediário (composto I) oxo-ferril (na forma de dois elétrons oxidados); (2) o composto I é reduzido por um substrato aromático não-fenólico (A), que é o agente redutor, que recebe um elétron, formando o composto II (um elétron na forma oxidada); (3) composto II recebe o segundo elétron a partir o substrato reduzido, retornando a enzima em sua forma nativa (estado de oxidação do ferro) para completar o ciclo oxidativo (ABDEL-HAMID; SOLBIATI; CANN, 2013; ERIKSSON; BERMEK, 2009). Tanto MnP quanto LiP, são enzimas essenciais para a degradação eficiente da lignina e reduzem o peróxido de hidrogênio à água.



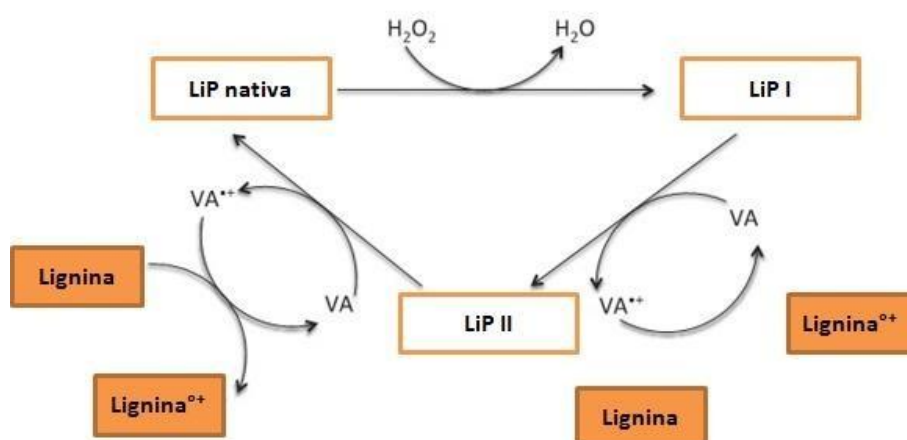
Figura 9. Representação esquemática do mecanismo catalítico da LiP sobre substrato aromático não-fenólico.



Fonte: Adaptado e traduzido de Abdel-Hamid et al. (2013).

Além do H<sub>2</sub>O<sub>2</sub>, à medida que o fungo cresce utilizando lignina como substrato, enzimas como aril-álcool oxidase e glioxal oxidase geram álcool veratrílico (AV), que auxilia na atividade de LiP, aumentando seu poder catalítico. O álcool veratrílico apresenta poder redox muito maior que a LiP (ABDEL-HAMID; SOLBIATI; CANN, 2013(1)) e, dessa maneira, funciona como um mediador redox para a oxidação de substratos da lignina (Figura 10).

Figura 10. Álcool veratrílico (VA) como um mediador redox na degradação da lignina.



Fonte: Adaptado e traduzido de Abdel-Hamid et al. (2013).

A manganês peroxidase (EC 1.11.1.13.; MnP) representa a segunda classe de enzimas oxidativas que despolimerizam a lignina, sobretudo compostos fenólicos, sob concomitante oxidação do peróxido de hidrogênio à água (LANGE; DECINA; CRESTINI, 2013). Assim como as LiP, as MnP apresentam em sua estrutura molecular grupo heme e são moléculas com muitas ligações dissulfeto, as quais dependem da oxidação do manganês para oxidar o substrato (ERTAN et al., 2012).

O ciclo catalítico da MnP não envolve mediadores, como nas LiP e lacases. (1) Ferro da enzima ao reagir com  $H_2O_2$  forma o composto I, que é o complexo radical  $Fe^{+4}$ -oxo-porfirina. (2) o  $Mn^{+2}$  doa seu elétron ao complexo e forma o composto II, e é oxidado à  $Mn^{+3}$ . (3)  $Mn^{+3}$  oxida o substrato e a enzima volta à conformação nativa (com o  $Mn^{+2}$ ).

Lacases (E.C. 1.10.3.2) são fenol-oxidases (oxirredutases; p-difenol: oxirredutases dioxigênicas) capazes de oxidar substratos fenólicos e não-fenólicos incluindo aqueles que compõem a estrutura da lignina. As lacases também são chamadas de proteínas “azuis” por possuírem cobre em sua molécula (ERIKSSON; BERMEK, 2009; GOUVEIA; SANROMÁN; MOLDES, 2013; RIVA; MOLECOLARE; BIANCO, 2006). Dentre todas as enzimas lignolíticas, a lacase destaca-se como uma enzima de alta utilização industrial, por participar de vias de biorremediação, degradando compostos tóxicos que poderiam contaminar mananciais ou até mesmo, os próprios produtos industriais (ERIKSSON; BERMEK, 2009).

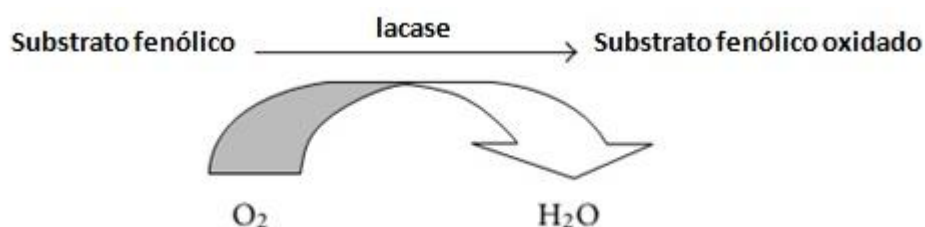
Amplamente distribuídas em plantas superiores, insetos e alguns microrganismos (bactérias e fungos), as lacases são enzimas muito importantes nos processos biológicos. Atuam tanto na polimerização quanto na despolimerização da lignina em plantas, na esclerotização das cutículas em insetos, no mecanismo de defesa à radiação ultravioleta em bactérias por meio da produção de pigmentos como a melanina (SANCHEZ-AMAT; SOLANO; LUCAS-ELÍO, 2010), na pigmentação dos esporos de fungos e na proteção do fungo fitopatogênico contra substâncias nocivas produzidas pela planta hospedeira, como fitoalexinas e taninos (MAYER; STAPLES, 2002).

Juntamente com outras enzimas, como as peroxidases (LiP e MnP), as lacases também atuam na desconstrução da lignina, através da despolimerização em monolignóis (alcoois sinapílico, coniferílico e p-

cumarílico) na presença de  $O_2$  (ABDEL-HAMID; SOLBIATI; CANN, 2013a; LANGE; DECINA; CRESTINI, 2013; MARTINEZ et al., 2005; MAYER; STAPLES, 2002; RIVA; MOLECOLARE; BIANCO, 2006).

Lacases que atuam em substratos fenólicos agem por meio da oxidação dos grupos hidroxila (Figura 11). Enquanto estes grupos são oxidados à radicais fenoxila, oxigênio molecular é reduzido à água. A oxidação do fenol à radical fenoxila é favorecida pela presença de substituintes que liberam elétrons (grupos metoxila) no anel aromático, que facilitam a perda dos prótons ( $Ph-OH \rightleftharpoons Ph-O^- + H^+$ ) (ANDREU; VIDAL, 2013).

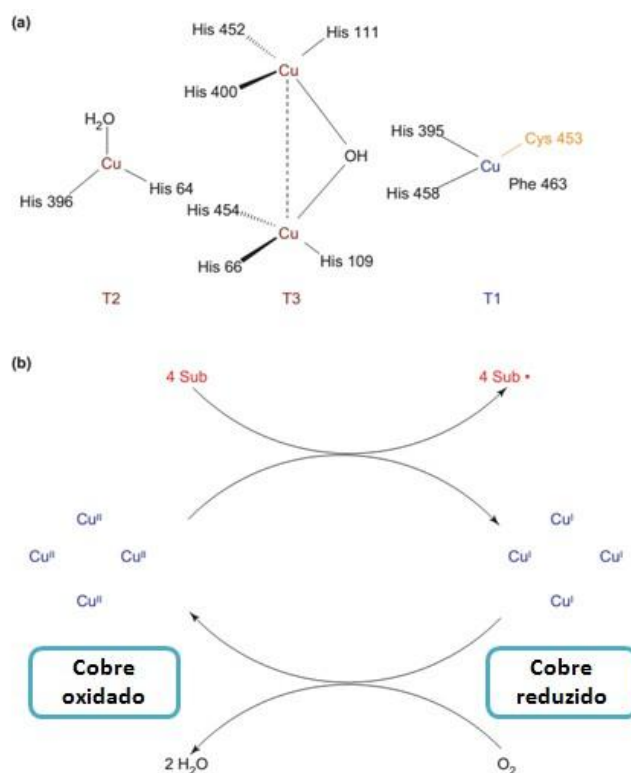
Figura 11. Mecanismo de ação da lacase em substratos fenólicos.



Fonte: Adaptado e traduzido de Brijwani et al. (2010).

A Figura 12 apresenta o modelo catalítico da lacase, formado por quatro átomos de cobre. Neste modelo, cobre do tipo 1 (T1) confere a típica cor azul da proteína e é o sítio onde a oxidação do substrato ocorre. Já cobre do tipo 2 (T2) e do tipo 3 (T3) formam um grupo trinuclear, onde ocorre a redução do oxigênio molecular à água (Figura 12(a)). Dessa forma, os tipos de cobre da molécula de lacase (T1, T2 e T3) ocorrem de forma concomitante, ou seja, à medida que T2 e T3 oxidam o substrato à formação de radical (Sub), há a formação de duas moléculas de água à partir da redução de uma molécula de oxigênio molecular em T1 (Figura 12(b)).

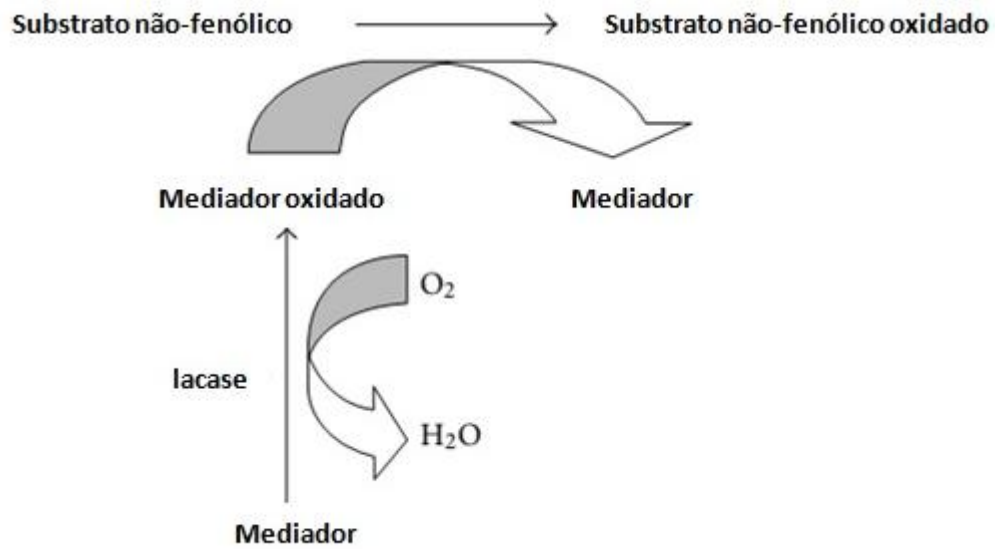
Figura 12. Representação esquemática da estrutura do sítio ativo e do ciclo catalítico da lacase. (a) Modelo catalítico de lacase constituído por quatro átomos de cobre. (b) Representação esquemática do ciclo catalítico da lacase produzindo duas moléculas de água a partir da redução de uma molécula de oxigênio molecular e concomitante oxidação (cobre T1) de quatro moléculas de substrato em seus radicais correspondentes. Sub: molécula de substrato; Sub<sup>o</sup>: radical do substrato oxidado.



Fonte: Adaptado e traduzido de Riva et al. (2006).

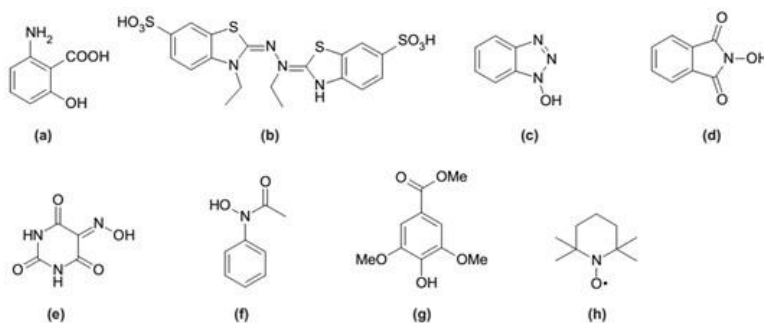
Alguns substratos podem não ser oxidados diretamente pela lacase, por possuírem um tamanho maior que o sítio ativo da enzima ou por apresentarem um potencial redox elevado. Dessa maneira, as lacases frequentemente combinam-se com mediadores (compostos de baixo peso molecular) para reagirem com unidades não fenólicas (ANDREU; VIDAL, 2013; MOROZOVA et al., 2007). Geralmente estes mediadores são compostos sintéticos do tipo N-OH (Figuras 13, 14 e 15). Todavia, esses compostos apresentam restrita aplicação industrial devido ao seu alto custo e toxicidade (ANDREU; VIDAL, 2013; RIVA; MOLECOLARE; BIANCO, 2006).

Figura 13. Mecanismo de ação da lacase em substratos não-fenólicos através de mediador.



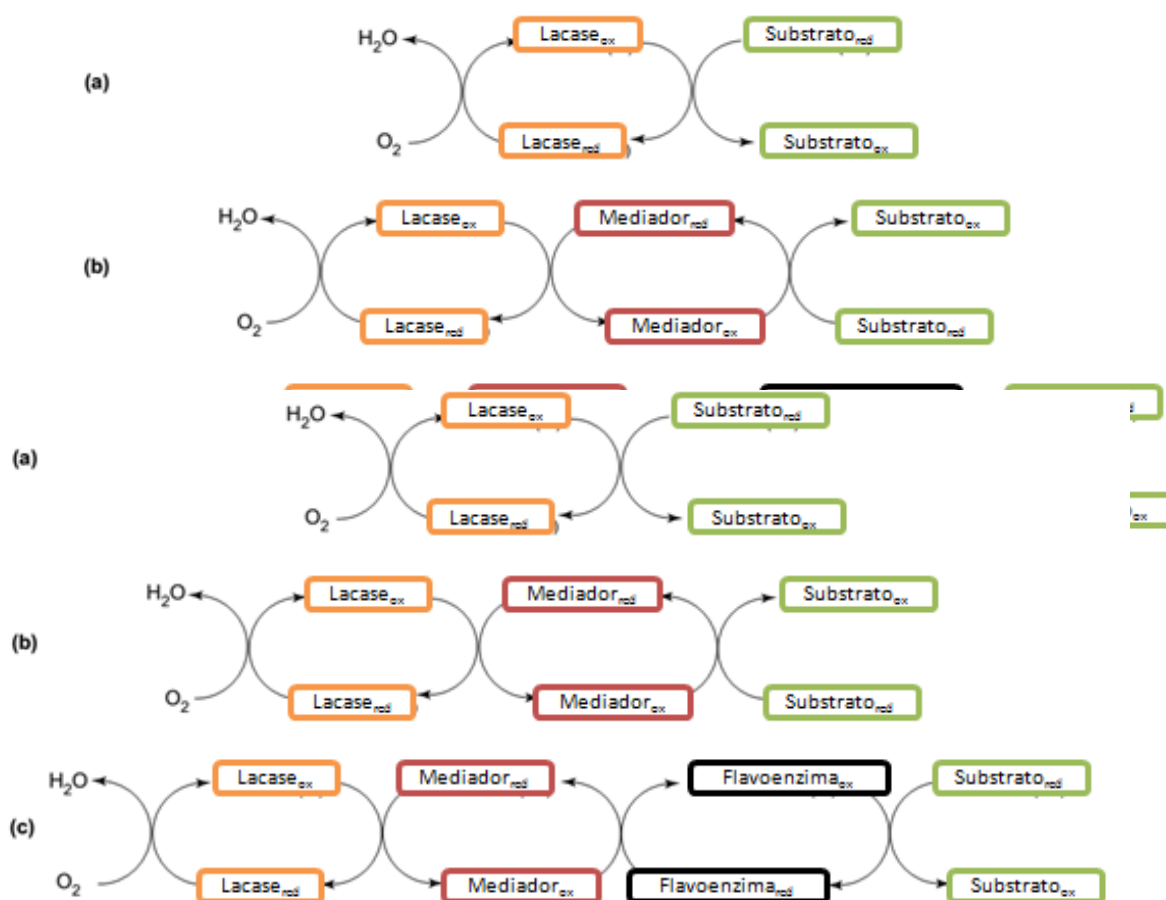
Fonte: Adaptado e traduzido de Brijwani et al. (2010).

Figura 14. Mediadores redox de lacase. Mediadores redox de lacase. (a) ácido 3-Hidroxi-antranílico (HAA); (b) ácido 2,2'-azino-bis-(3-etilbenzotiazolona-6-sulfônico) (ABTS); (c) N-hidroxi- benzotriazola (HBT); (d) N-hidroxiftaimida (HPI); (e) ácido violúrico (VLA); (f) N- hidroxiacetanilida (NHA); (g) metil-éster de ácido 4-hidroxi-3,5-dimetoxi- benzóico (ácido siríngico); (h) 2,2,6,6-tetrametilpiperidina-1-iloxi (TEMPO).



Fonte: Adaptado e traduzido de Riva et al.(2006).

Figura 15. Esquema do mecanismo de ação da lacase na ausência (a) e presença (b, c) de mediadores redox da lacase.



Fonte: Adaptado e traduzido de Riva et al. (2006).

Tendo em vista os aspectos econômicos e ambientais, torna-se importante a busca por mediadores de baixo custo e não tóxicos, como os compostos não-fenólicos naturais, particularmente aqueles advindos da degradação da lignina (CAÑAS; CAMARERO, 2010; ANDREU; VIDAL, 2013).

### **3. Resíduos lignocelulósicos como substratos para produção de enzimas lignocelulolíticas e como fontes de açúcares fermentescíveis**

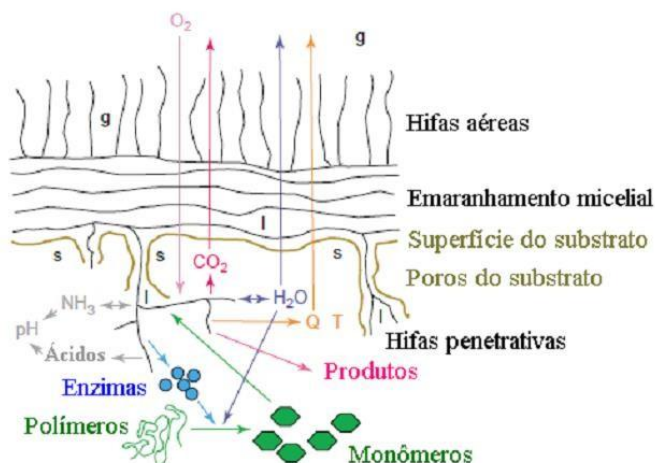
O interesse no aproveitamento de resíduos lignocelulósicos como substratos para o cultivo de microrganismos e produção de enzimas lignocelulolíticas tem crescido nas últimas décadas, dadas as diversas possibilidades de aplicações biotecnológicas destes catalisadores, especialmente na área de biocombustíveis (FARINAS 2015; PLÁCIDO; CAPAREDA, 2015). Materiais lignocelulósicos, incluindo os resíduos agroindustriais, são mais adequados para uso como substratos para crescimento microbiano quando empregados em processos de fermentação em estado sólido (FES) os quais, por sua vez, são mais adequados para o cultivo de fungos filamentosos (BEHERA; RAY, 2016).

#### ***3.1. Produção de enzimas por fermentação em estado sólido (FES)***

Fermentação em estado sólido (FES) ou Cultivo em estado sólido (CES) é um bioprocesso definido como o crescimento de microrganismos em um substrato sólido não solúvel, sem água excedente entre as partículas (BEHERA; RAY, 2015; GOWTHAMAN; KRISHNA; MOO-YOUNG, 2001; KRISHNA, 2005a). Além de ser fonte de carbono e outros nutrientes, o substrato neste processo é também utilizado como suporte para o crescimento microbiano, especialmente quando se trata do cultivo de fungos filamentosos, os microrganismos mais adaptados a este bioprocessos, já que apresentam maior tolerância a baixa atividade de água do meio quando comparados com leveduras e bactérias (PANDEY, 2003). Durante o cultivo de fungos por FES, o crescimento das hifas pode ocorrer sobre, intra e/ou entre as partículas do substrato sólido, pela secreção de enzimas como celulases e hemicelulases que, hidrolisam os respectivos substratos, fornecem açúcares mais prontamente assimiláveis para suportar o crescimento microbiano (KRISHNA, 2005a) (Figura 16).



Figura 16. Representação esquemática de alguns processos de micro-escala que ocorrem durante o crescimento fúngico em processos de FES. Gás (g); líquido (l); substrato (s); calor (Q); temperatura (T).



Fonte: Adaptado e traduzido de (HÖLKER; LENZ, 2005).

A Figura 16 mostra, além do crescimento das hifas, da secreção de enzimas (incluindo despolimerizantes) e da geração de produtos durante o cultivo fúngico por FES, a presença de água necessária para a catálise por hidrólise e para o metabolismo microbiano. O oxigênio é consumido, enquanto gás carbônico e calor são gerados como produtos do metabolismo, cujas atividades ocorrem principalmente na região mais próxima à superfície do substrato, nos poros. Os espaços vazios no “tapete” micelial, que se forma na superfície do substrato sólido, bem como aqueles entre as partículas do substrato são preenchidos por líquido. Os gases, por sua vez, preenchem principalmente os espaços entre as hifas aéreas. Formam-se, assim, gradientes no biofilme forçando a difusão do O<sub>2</sub> da fase gasosa para as regiões mais profundas e a difusão do CO<sub>2</sub> destas regiões para a superfície. O calor gerado eleva a temperatura, sendo removido do substrato por condução e também pela evaporação, a qual faz parte do complexo balanço de água do sistema (HÖLKER; LENZ, 2005).

Diversos parâmetros físico-químicos influenciam o desenvolvimento microbiano em processos de FES, como tipo de inóculo, umidade, pH,

temperatura, tipo de substrato e tamanho da partícula, aeração, agitação, fatores nutricionais, entre outros (KRISHNA, 2005).

Baixos teores de umidade em um sistema de FES tendem a reduzir a difusão dos nutrientes presentes no meio, a estabilidade das enzimas secretadas e o acoplamento destas enzimas ao substrato. Em contrapartida, o aumento da umidade leva à aglomeração das partículas do substrato diminuindo o a homogeneidade da colonização microbiana do material, além de propiciar o crescimento de bactérias contaminantes e desfavorecer a troca gasosa (ARZUMANOV; JENKINS; ROUSSOS, 2005; BARRANCO- FLORIDO et al., 2002)

A temperatura é um dos parâmetros mais importantes que influenciam o desenvolvimento microbiano em processos de FES. Temperaturas elevadas aumentam os índices de evaporação e diminuem a umidade do sistema, levando assim à desnaturação de proteínas, podendo levar à perda da atividade de algumas enzimas, e desorganização do sistema de membranas, influenciando negativamente no crescimento microbiano.

A facilidade ou dificuldade na aeração em processos de FES relaciona-se ao tipo de substrato utilizado e a difusão de gases, tanto na fração gasosa quanto na líquida, bem como a proporção destes gases durante o bioprocessamento também exercem influência no crescimento e desenvolvimento microbiano. Durante a FES, a fase gasosa, composta por  $O_2$  e  $CO_2$ , está próxima ao ponto de saturação do vapor de água. Entretanto, o tempo de equilíbrio entre a fase gasosa e a fase líquida absorvida pelo suporte sólido pode ser longo se comparado com as mudanças biofísicas provocadas durante o crescimento do microrganismo.

Além de carbono, outros nutrientes são importantes para o crescimento microbiano como nitrogênio, fósforo, enxofre, minerais, vitaminas, entre outros, os quais, além de presentes no substrato sólido empregado na FES, podem ser fornecidos pelo uso de soluções nutrientes utilizadas para umedecer o meio. Fontes de nitrogênio como tartarato, sulfato, nitrato, oxalato, cloreto de amônio, nitrato de sódio, uréia, peptona e aminoácidos estimulam a formação de conídios em fungos. Minerais como cálcio, magnésio, níquel, cobre, ferro, potássio, zinco, molibdênio, manganês, boro, entre outros, compostos orgânicos como tiamina, biotina, ácido fólico, esteróides também têm sido

reportados como estimuladores da esporulação fúngica (GOWTHAMAN; KRISHNA; MOO-YOUNG, 2001; KRISHNA, 2005b; WALISKO et al., 2012). O emprego destes nutrientes pode, portanto, ser interessante quando se objetiva uma maior esporulação.

Quanto ao sistema empregado para o processo de FES, diferentes biorreatores são utilizados para o processo (SOCCOL et al., 2017). Dentre eles estão frascos (AFRISHAM et al., 2016; BANSAL et al., 2012; CHEN; WU; XU, 2014; DHILLON; KAUR; BRAR, 2012; KAUR et al., 2012; LIU et al., 2011; PANDEY; EDGARD; NEGI, 2016), reatores cilíndricos (CERDA et al., 2016), do tipo bandeja (BHATTACHARYA; GARLAPATI; BANERJEE, 2011; DHILLON et al., 2012; GODOY et al., 2011), embalagens de polipropileno (de Cassia Pereira et al., 2015; Ferrarezi et al., 2014; Moretti et al., 2012), leite empacotado (BIZ et al., 2016), frasco estático (PEREIRA et al., 2014), bandeja de alumínio (KUMAR et al., 2011), tambor horizontal (BÜCK et al., 2015; CASCIATORI et al., 2016; RODRÍGUEZ-FERNÁNDEZ et al., 2011; UMSZA-GUEZ et al., 2011), placas de Petri (MELIKOGLU; LIN; WEBB, 2013), coluna (FREITAS et al., 2015; PIROTA et al., 2013), batelada (YAZID; BARRENA; SÁNCHEZ, 2016), entre outros. Cada reator apresenta particularidades diferentes quanto ao abastecimento de nutrientes, controle de gases, aeração, controle de pH, temperatura, entre outros fatores que influenciam no cultivo microbiano.

Na Tabela 1 são apresentadas algumas referências sobre o emprego FES para a produção de enzimas lignocelulolíticas por fungos filamentosos utilizando resíduos agroindustriais como substratos.

Tabela 1. Produção de enzimas celulolíticas e xilanolíticas por fungos utilizando-se resíduos lignocelulósicos.

<b>Microrganismo</b>	<b>Enzimas</b>	<b>Substratos</b>	<b>Referências</b>
<b><i>Schizophyllum commune</i> IBL-06</b>	MnP, LiP e lacase	Palha de arroz	Asgher et al. (2011) Asgher et al. (2016)
<b><i>Thermoascus aurantiacus</i> e <i>Aureobasidium pullulans</i></b>	$\beta$ -glicosidase	Farelo de trigo	Leite et al. (2008)
<b><i>Myceliophthora thermophila</i> JCP 1-4</b>	Endoglucanase, exoglucanase, $\beta$ -glicosidase, FPase, glicose-isomerase, xilanase, $\beta$ -xilosidase,	Bagaço de cana e farelo de soja	De Cassia Pereira et al. (2015); de Cassia Pereira et al. (2016)
<b><i>Myceliophthora thermophila</i> M7.7.</b>	Xilanase	Bagaço de cana e farelo de trigo	Moretti et al. (2012)
<b><i>Lichtheimia ramosa</i></b>	$\beta$ -glicosidase	Farelo de trigo	Garcia et al. (2015)
<b><i>Thermomucor indicae-seudaticae</i> N31</b>	$\beta$ -glicosidase	Farelo de soja	Pereira et al. (2014)
<b><i>Thermomucor indicae-seudaticae</i> N31</b>	lipase	Bagaço de cana	Ferrarezi et al. (2014)
<b><i>Thermomucor indicae-seudaticae</i> N31</b>	protease	Farelo de trigo	Merheb-Dini et al. (2012)
<b><i>Bjerkandera</i> sp. Y-HHM2, <i>Phanerochaete</i> sp. Y-RN1, <i>Pleurotus</i> sp. Y-RN3, <i>Hypocrea nigricans</i> SCT-4.4 and <i>Myrothecium</i> sp. S-3.20</b>	Polifenoloxidase, lacase, MnP e xilanase	Bagaço e palha de cana	Maza et al. (2014)

Entre os fungos filamentosos produtores de enzimas de degradação de material vegetal, os basidiomicetos, pertencentes ao Filo Basidiomycota, destacam-se pela capacidade de degradar enzimaticamente materiais de alta recalcitrância, como a lignina (GOMES et al., 2009). Foram inicialmente descritos taxonomicamente por Moore (1980) e podem ser designados como de podridão branca (*white-rot fungi*) ou marrom/parda (*brown-rot fungi*) (HERNÁNDEZ-ORTEGA et al., 2012; MARTINEZ et al., 2005). Fungos da podridão branca degradam celulose, hemicelulose e lignina a CO<sub>2</sub> e água. Por este motivo, são denominados de fungos lignocelulolíticos. O termo “podridão branca” relaciona-se à degradação da madeira por estes fungos, a qual adquire uma consistência esponjosa e fibrosa, resultando numa coloração branca. Já os fungos da “podridão marrom” (ou parda) degradam apenas celulose e hemicelulose e o termo é empregado pois, nos estágios finais de degradação, a madeira apresenta uma coloração escura (GIMENES; ROBERTO, 2010; HERNÁNDEZ-ORTEGA; FERREIRA; MARTÍNEZ, 2012; MARTÍNEZ et al., 2005).

O basidiomiceto *Pycnoporus sanguineus* (Figura 17) é um fungo de degradação branca pertencente da ordem Polyporales, também denominado *Polyporus sanguineus*, *Polystictus sanguineus*, *Trametes sanguinea* ou *Coriolus sanguineus* (GILBERTSON; RYVARDEN, 1987; NOBLES; FREW, 1962). *Pycnoporus sanguineus* participa ativamente do ciclo do carbono global, sendo a lignocelulose sua principal fonte de carbono (DOE-JGI). Quando se desenvolvendo na presença de material lignocelulósico, secreta enzimas hidrolíticas para a degradação gradual da celulose e hemicelulose, além de ligninases que mineralizam completamente a lignina (OTJEN; BLANCHETTE, 1986; RILEY et al., 2014).

Figura 17. *Pycnoporus sanguineus* em meio ágar com extrato de malte.



Fonte: Imagem do Laboratório de Bioquímica e Microbiologia Aplicada da UNESP/IBILCE, São José do Rio Preto.

*Pycnoporus sanguineus* é um eficiente produtor de fenoloxidasas que atuam em compostos aromáticos como doadores de hidrogênio e tem sido amplamente utilizado em bioprocessos que empregam agro-resíduos, na descoloração de efluente de polpa Kraft e corante (ONOFRE et al., 2015). Os mais recentes dados disponíveis na literatura sobre este fungo relacionam-se à produção de enzimas celulolíticas (AZMI; JALIL; KALIL, 2016), além da lacase já freqüentemente estudada (RAMÍREZ-CAVAZOS et al., 2014). A Tabela 2 apresenta alguns trabalhos que reportam ao emprego deste microrganismo na produção destas enzimas.

Tabela 2. Enzimas de degradação de lignocelulose produzidas por *Pycnoporus sanguineus*.

Cepa	Enzimas	Referências
<i>P. sanguineus</i>	Endoglucanase e exoglucanase	Azmi; Jali; Kalil (2016) Eugenio et al. (2009) Pointing; Jones; Vrijmoed (2000)
<i>P. sanguineus</i>	lacase	Ramírez-Cavazos et al. (2014) Valeriano et al. (2009) Vikineswary; Abdullah; Renuvathani (2006) Zimbardi et al. (2016)
<i>P. sanguineus</i> PF-2	FPase, endoglucanase, $\beta$ -glicosidase, xilanase, $\alpha$ -galactosidase, mananase, poligalacturonidase	Falkoski; Guimarães (2012)
<i>P. sanguineus</i>	MnP	Ferhan et al. (2012)
<i>P. sanguineus</i>	celulases	Onofre et al. (2015) Quiroz-Castañeda et al. (2009) Yoon et al. (2013) Yoon; Ngoh; Chua (2012)
<i>P. sanguineus</i>	xilanase	Quiroz-Castañeda et al. (2011)

### **3.2. Bagaço de cana-de-açúcar: substrato para cultivo fúngico e fonte de energia renovável**

Entre os resíduos lignocelulósicos mais utilizados como substratos para crescimento fúngico e produção de enzimas por fermentação em estado sólido está o bagaço de cana (REZENDE et al., 2002; BIZ et al., 2016; DE CASSIA PEREIRA et al., 2016). A cana-de-açúcar (*Saccharum officinarum* L.) é uma angiosperma, monocotiledônea, originada no sudoeste da Ásia e oeste da Índia. Cultivada no Brasil há cerca de 500 anos, foi de grande importância para a economia do país na produção de açúcar e etanol (CERQUEIRA-LEITE; LEAL, 2007; GOES; MARRA; SILVA, 2008).

No país, o bagaço tem sido muito utilizado para queima em caldeiras, gerando energia elétrica, e na composição de silagem empregada como alimento para animais de pastoreio, especialmente nos períodos de estiagem, tornando-se uma importante fonte de fibras juntamente com outros resíduos como farelo de trigo e soja, palha de milho, sabugo de milho (SOUZA; SANTOS, 2002).

O bagaço de cana-de-açúcar é um material fibroso resultante da extração do suco pela moagem da cana e sua composição varia de acordo com diversos fatores, como o processo de extração, a variedade da cana e a composição do solo. De modo geral, é composto por aproximadamente 50% de celulose, 25% de hemicelulose e 25% de lignina. Apresenta pequenas quantidades de outros compostos como pectinas, proteínas, minerais e compostos de baixo peso molecular, além de apresentar baixo teor de cinzas (em torno de 2,4%), o que representa uma grande vantagem para sua bioconversão por microrganismos quando comparado com outros resíduos, como a palha de arroz e de trigo, que contêm aproximadamente 17,5 e 11% de cinzas, respectivamente (PANDEY et al., 2000; PANDEY et al., 2000(1)). Em geral, o processamento de uma tonelada de cana gera em torno de 280 kg de bagaço.

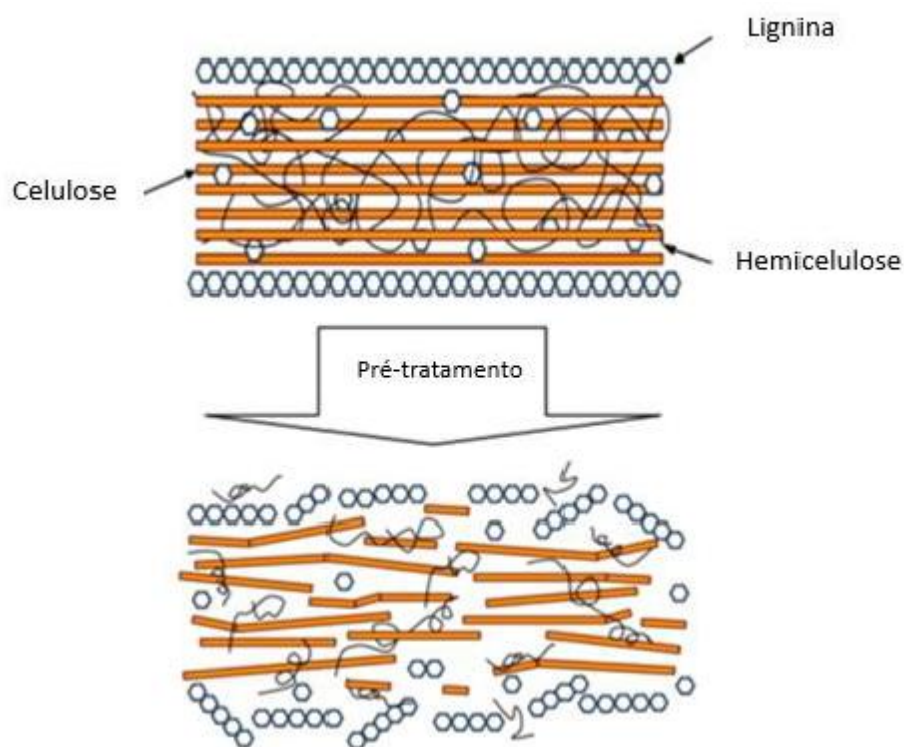
Embora boa parte deste bagaço seja utilizada para fins energéticos na própria usina, sendo queimado nas caldeiras, o restante ainda representa uma



grande oportunidade para agroindústria nacional. Desta forma, considera-se que o aproveitamento deste resíduo é uma necessidade nacional, com amplo espaço para o desenvolvimento de atividades mais nobres do que a geração direta de energia por combustão. Em termos biotecnológicos, além da utilização como substrato em processos de FES, segundo alguns autores o emprego do bagaço excedente no contexto de biorrefinaria viabilizaria economicamente o investimento necessário para adaptar as usinas de açúcar e álcool do país, as quais tradicionalmente produzem etanol de primeira geração (proveniente da sacarose presente no caldo da cana) à produção de etanol de segunda geração (produzido a partir dos açúcares fermentescíveis obtidos por hidrólise das frações de celulose e hemicelulose de materiais lignocelulósicos) (DIAS et al., 2013).

O acesso das celulasas e hemicelulasas aos seus substratos no material lignocelulósico é dificultado pela presença da lignina, cuja estrutura representa um obstáculo físico e químico à sacarificação enzimática. Desta forma, para a utilização do bagaço de cana, bem como de outros resíduos lignocelulósicos, como fontes de açúcares fermentescíveis para a obtenção de etanol 2G é necessária uma etapa de pré-tratamento a fim de desestruturar ou remover a lignina, bem como de diminuir o grau de cristalinidade da celulose (Figura 18).

Figura 18. Pré-tratamento do material lignocelulósico para uso com fins energéticos.

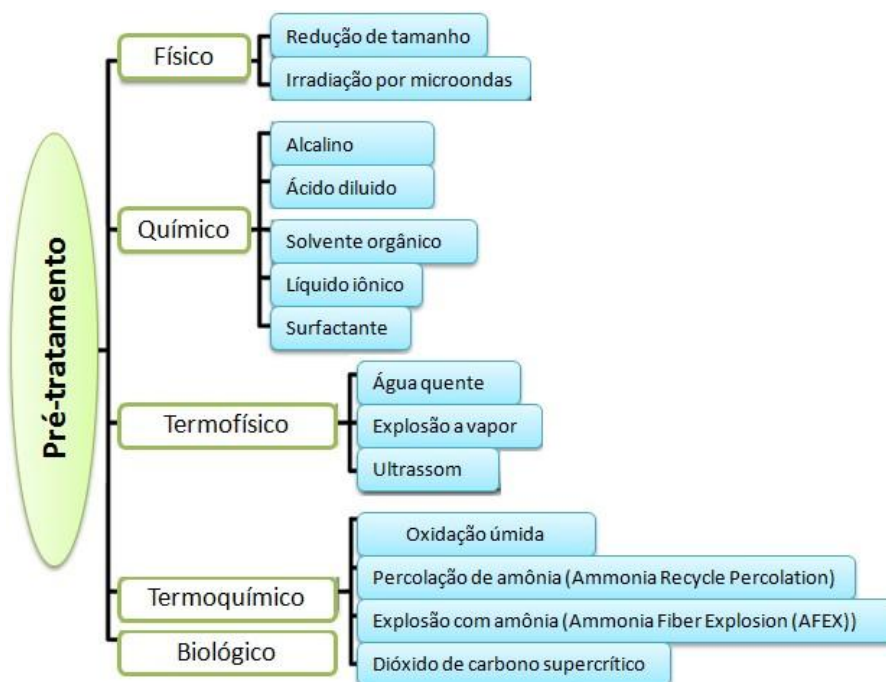


Fonte: Adaptado e traduzido de Mood et al. (2013).

#### 4. Pré-tratamentos da biomassa lignocelulósica

Vários métodos físicos e químicos (Figura 19) podem ser empregados como pré-tratamentos da biomassa lignocelulósica, visando sua utilização como fonte de açúcares fermentescíveis para a produção de etanol de segunda geração. Entre eles podem ser citados pirólise, explosão a vapor, radiação gama, tratamento hidrotérmico, alcalinos, organossolv e o uso de agentes oxidantes (CRESTINI et al., 2010; KIM; LEE; KIM, 2016; PARK et al., 2010; SANT'ANA DA SILVA et al., 2016; SITI AISYAH; UEMURA; YUSUP, 2014; SOUZA et al., 2013; SUN; CHEN, 2008; XIAOWEI; HONGZHANG, 2012).

Figura 19. Pré-tratamentos da biomassa lignocelulósica. Tratamentos físicos e químicos.



Fonte: Adaptado e traduzido de Madadi et al. (2017).

#### 4.1. Pré-tratamento hidrotérmico

O pré-tratamento hidrotérmico é um método físico-químico frequentemente utilizado para a desestruturação da biomassa lignocelulósica a ser utilizada para fins de obtenção de etanol 2G. Neste pré-tratamento, uma pressão é gerada sobre a água para mantê-la no estado líquido, sob elevada temperatura (TALEBNIA; KARAKASHEV; ANGELIDAKI, 2010). Sob alta pressão, a água penetra na biomassa, hidrata a celulose e remove parte da hemicelulose e lignina, promovendo um aumento na porosidade e na área de superfície do material, garantindo uma maior exposição da celulose (GAO et al., 2013; JÖNSSON; MARTÍN, 2016; TALEBNIA; KARAKASHEV; ANGELIDAKI, 2010). Devido às acetilações da hemicelulose, íons hidrônios são formados, resultando na solubilização da deste polímero (GÍRIO et al., 2010; JÖNSSON;

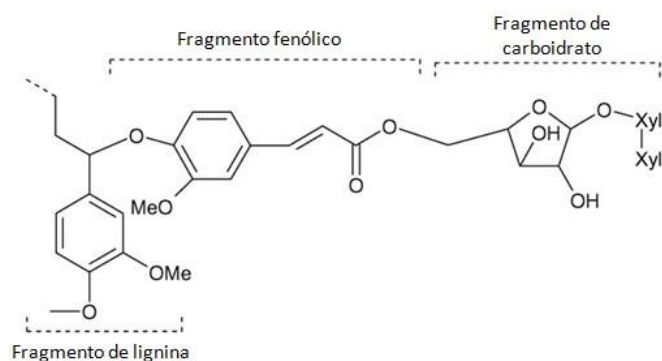
MARTÍN, 2016). Dessa maneira, o pré-tratamento hidrotérmico, sem a adição de químicos, também pode ser denominado autohidrólise, devido à sua natureza ácida (GÍRIO et al., 2010).

#### **4.1.1. Pré-tratamento hidrotérmico em meio alcalino**

O pré-tratamento hidrotérmico alcalino pode ser realizado utilizando-se hidróxido de sódio, sendo também denominado mercerização, por ter sido desenvolvido por John Mercer (1984). Este tratamento consiste em promover a turgescência da celulose nativa pelo emprego de soluções concentradas de NaOH, resultando em celulose do tipo II, menos cristalina, com baixo grau de polimerização e com maior área de superfície, o que favorece sua exposição e acessibilidade às celulasas (HABIBI; LUCIA; ROJAS, 2009; LEE; HAMID; ZAIN, 2014; SANTOS et al., 2012; TALEBNIA; KARAKASHEV; ANGELIDAKI, 2010). Além disso, a celulose sob mercerização apresenta maior solvatação (LASZKIEWICZ; WCISLO, 1990), o que favorece os processos de hidrólise enzimática.

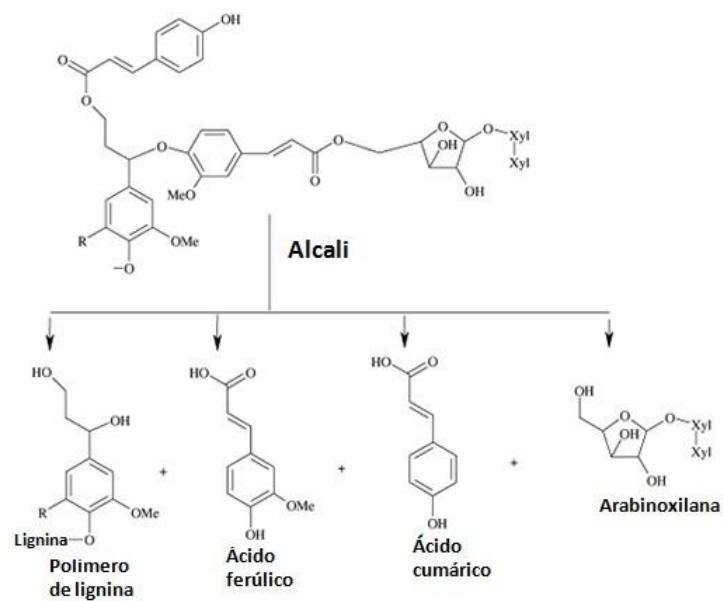
O pré-tratamento alcalino também promove a desestruturação/remoção da lignina pela hidrólise de ligações ésteres intermoleculares entre este polímero e a xilana, mineralizando-o. Além disso, a degradação alcalina da lignina envolve a quebra de dois tipos de ligações éter: (1) aquelas entre carbono alifático–O–carbono aromático e (2) aquelas entre carbono aromático–O–carbono aromático que liberam ácido ferúlico e cumárico (BURANOV; MAZZA, 2008; LEE; HAMID; ZAIN, 2014) (Figuras 20 a 23).

Figura 20. Fragmentos de lignina originados do pré-tratamento hidrotérmico alcalino.



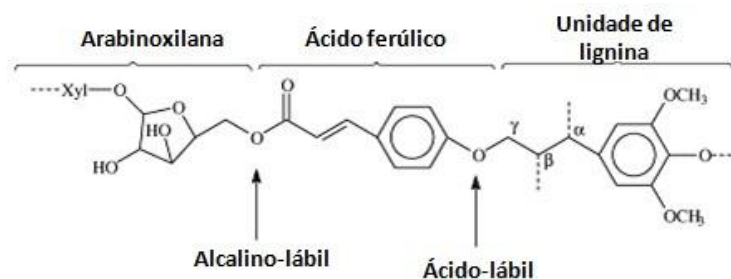
Fonte: Adaptado e traduzido de Buranov and Mazza (2008).

Figura 21. Fragmentos de lignina e ácidos da hemicelulose (cumárico e ferúlico) originados do pré-tratamento hidrotérmico alcalino.



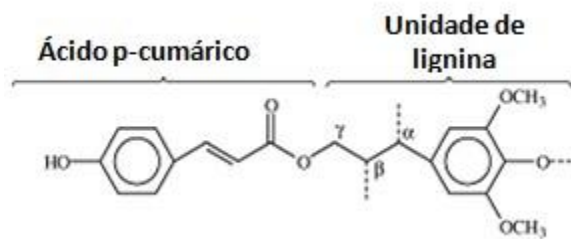
Fonte: Adaptado e traduzido de Buranov and Mazza (2008).

Figura 22. Porção da hemicelulose (arabinoxilana e ácido ferúlico) que mantêm ligação com a lignina.



Fonte: Adaptado e traduzido de Buranov and Mazza (2008).

Figura 23. Porção da hemicelulose (ácido cumárico) que mantêm ligação com a lignina.



Fonte: Adaptado e traduzido de Buranov and Mazza (2008).

#### 4.1.1.1 Pré-tratamento hidrotérmico na presença de agentes oxidantes

Agentes oxidantes tais como peróxido de hidrogênio, ozônio, permanganato de potássio, oxigênio e óxidos são utilizados para catalisar processos de deslignificação por meio do ataque do anel aromático da lignina (KIM; LEE; KIM, 2016). De acordo com Lee et al., (2014), normalmente agentes oxidantes são usados para aumentar os efeitos dos pré-tratamentos alcalinos. Sob pH básico, sobretudo acima de 12, o oxigênio é reduzido à superóxido, promovendo a abertura de anéis aromáticos da lignina por ataque nucleofílico (Sanchez et al. 2011). Este processo também promove oxidação de parte da hemicelulose à ácidos carboxílicos (ácido oxálico, fórmico, acético, etc).

Dentre os agentes oxidantes, permanganato de potássio ( $\text{KMnO}_4$ ) é um óxido metálico de fácil obtenção, sendo um forte oxidante solúvel em água, não tóxico e barato. Os resíduos de  $\text{KMnO}_4$  podem ser precipitados e reutilizados,

sendo portanto um agente ecológico (MA et al., 2015). Em fábricas de tecidos, é muito utilizado em tratamento de águas residuárias, sendo aplicado na oxidação de corantes que contém compostos tóxicos como tolueno, tricloroetileno, entre outros (WU et al., 2014; XU et al., 2005). Tendo em vista seu poder oxidante, o  $\text{KMnO}_4$  pode agir na lignina, rompendo cadeias laterais e reagindo com compostos fenólicos e não fenólicos (WELKER et al., 2015; ZHANG, 2013).

Assim como o  $\text{KMnO}_4$ , óxido de zinco e dióxido de titânio também são utilizados em tratamento de águas residuárias, por agirem em compostos alifáticos e aromáticos como benzeno, tolueno e xileno (BTEX). Dessa maneira,  $\text{ZnO}$  e  $\text{TiO}_2$  convertem compostos orgânicos em inorgânicos, mineralizando-os (KHAN; NAJEEB; TUIYEBAYEVA, 2014).

$\text{ZnO}$  e  $\text{TiO}_2$  também são comumente utilizados em processos fotocatalíticos, mineralizando compostos orgânicos de forma mais eficiente (YOO et al., 2013). Dessa forma, sendo os óxidos metálicos agentes oxidantes, podem contribuir na remoção da lignina. Em meio aquoso, podem quebrar ligações C-O e C-C (KANG et al., 2013) e em meio alcalino podem produzir radicais hidroxila e íons superóxido (YOO et al., 2013; RAMADOSS; MUTHUKUMAR, 2016).

Peróxido de hidrogênio também é um agente oxidante, contribuindo assim com a deslignificação de materiais lignocelulósico, promovendo seu fracionamento (DUSSAN et al., 2014). O  $\text{H}_2\text{O}_2$  é considerado um oxidante ecológico, sendo utilizado em tratamentos de águas residuárias, removendo compostos fenólicos (AGNEMO; GELLERSTEDT, 1979; CRESTINI et al., 2010; KHAN; NAJEEB; TUIYEBAYEVA, 2014; ROCHA-MARTIN et al., 2014). Em meio alcalino, pode decompor lignina em vanilina, seringaldeído e outros compostos aromáticos, dependendo do tipo do pré- tratamento (PANDEY; KIM, 2011), além de solubilizar a lignina e parte da hemicelulose (LU; RALPH, 2010; SU et al., 2014; YU et al., 2015). Na presença de  $\text{H}_2\text{O}_2$  no pré-tratamento, radicais oxigênio singleto e hidroxila são formados, bem como  $\text{CO}_2$ , água e ácidos carboxílicos (ZHU et al., 2012; KANG et al., 2011). Além da lignina,  $\text{H}_2\text{O}_2$  pode modificar e diminuir o grau de polimerização da hemicelulose, produzindo assim xilooligossacarídeos (BRAGATTO; SEGATO; SQUINA, 2013).

#### **4.2. Uso de hidrolisados enzimáticos para produção de etanol 2G**

Segundo alguns autores, a implantação do etanol de segunda geração como biocombustível favoreceria o abastecimento da indústria sucroalcooleira, oferecendo ainda vantagens sócio-ambientais e aumentando o rendimento econômico do processo (GÁMEZ et al.,2006). O Brasil apresentou diferentes estratégias para garantir uma estabilidade energética em relação ao cenário nacional dos biocombustíveis. Em 1975, o programa PROÁLCOOL propunha uma política de redução da dependência do petróleo de outros países, com a produção de etanol de cana em substituição à gasolina ou em adição a este combustível numa proporção de até 24% (HOFSETZ; SILVA, 2012).

Em 2015, durante a 21<sup>a</sup> Conferência das Partes (COP21), os governos de vários países, dentre eles o Brasil, assinaram um documento chamado de Acordo de Paris (UNFCCC, 2015), o qual aborda a importância da obtenção de biocombustíveis a partir da biomassa residual (biocombustíveis de segunda geração – 2G). Em 2017, o Ministério de Minas e Energia elaborou um plano chamado RENOVABIO visando garantir a aplicação dos biocombustíveis 2G no Brasil, com uma estimativa de implantação e execução em 2030 (MME, 2017).



A hidrólise ou sacarificação enzimática de resíduos lignocelulósicos como o bagaço de cana, para a obtenção de açúcares fermentescíveis e posterior obtenção de etanol, tem sido explorada por apresentar vantagens importantes em relação à hidrólise química, como condições moderadas de temperatura e pressão, o que diminui a demanda no consumo de energia, e pouca ou nenhuma produção de subprodutos tóxicos, devido à alta especificidade. (DELGENES et al., 1996; DE CASSIA PEREIRA et al., 2016; OLIVEIRA et al., 2018; MARQUES et al., 2018).

Além das celulasas e hemicelulasas, a presença de outras enzimas durante o processo de sacarificação é muitas vezes desejada, como por exemplo quando ainda existe lignina residual no material já pré-tratado. As ligninases removem esta lignina, facilitando o acesso das enzimas celulolíticas e hemicelulolíticas aos seus substratos. Além disso, tais enzimas podem ainda realizar um tratamento biológico no meio reacional, quando removem também compostos como os fenólicos, tóxicos para a levedura fermentadora (JURADO et al., 2009; JURADO et al., 2011; MORENO et al., 2016; ).

O hidrolisado obtido via sacarificação enzimática, contendo geralmente glicose e xilose, uma vez que as soluções enzimáticas microbianas empregadas para este fim contêm atividades tanto de celulasas quanto de hemicelulasas, pode então ser utilizado em processos de fermentação alcoólica obtendo-se o denominado de etanol de segunda geração ou etanol de segunda geração ou etanol 2G (GÍRIO et al., 2010).

*Saccharomyces cerevisiae*, tradicionalmente empregada nos processos de fermentação alcoólica (YANG; LIU; ZHANG, 2007) não é capaz de fermentar pentoses (VILELA et al., 2015). Desta forma, alguns trabalhos relatam a prospecção de leveduras que utilizem xilose para fermentação alcoólica (DELGENES et al, 1996; CHANDEL; KAPOOR; KUHAD, 2006; SUES et al., 2005; MARTINS et al., 2018) e outros têm utilizado a engenharia genética como recurso para a inserção de genes em *S. cerevisiae* para torná-la uma levedura fermentadora de xilose (VILELA et al., 2015; MOYSES et al. 2016).

## Referências Bibliográficas

ABBASI, T.; ABBASI, S. A. Biomass energy and the environmental impacts associated with its production and utilization. **Renewable and Sustainable Energy Reviews**, v. 14, p. 919–937, 2010.

ABDEL MONSSEF, R. A.; HASSAN, E. A.; RAMADAN, E. M. Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment. **Annals of Agricultural Sciences**, v. 61, n. 1, p. 145–154, 2016.

ABDEL-HAMID, A. M.; SOLBIATI, J. O.; CANN, I. K. O. Insights into Lignin Degradation and its Potential Industrial Applications. **Advances in Applied Microbiology**, v. 82, p. 1–28, 2013.

AFRISHAM, S. et al. Characterization of a thermostable, CaCl<sub>2</sub>-activated and raw-starch hydrolyzing alpha-amylase from *Bacillus licheniformis* AT70: Production under solid state fermentation by utilizing agricultural wastes. **Journal of Molecular Catalysis B: Enzymatic**, v. 132, n. November, p. 98–106, 2016.

AGNEMO, R. ; GELLERSTEDT, G. The Reactions of Lignin with Alkaline Hydrogen Peroxide. Part II.\* Factos Influencing the Decomposition of Phenolic Structures. **Acta Chemical Scandinavica B**, v. 33, p. 337–342, 1979.

AMIN, F. R. et al. Pretreatment methods of lignocellulosic biomass for anaerobic digestion. **AMB Express**, p. 2–12, 2017.

ANDREU, G.; VIDAL, T. Laccase from *Pycnoporus cinnabarinus* and phenolic compounds: Can the efficiency of an enzyme mediator for delignifying kenaf pulp be predicted? **Bioresource Technology**, v. 131, p. 536–540, 2013.

ARZUMANOV, T.; JENKINS, N.; ROUSSOS, S. Effect of aeration and substrate moisture content on sporulation of *Metarhizium anisopliae* var. *acidum*. **Process Biochemistry**, v. 40, n. 3–4, p. 1037–1042, 2005.

ASGHER, M. et al. Lignocellulose degradation and production of lignin modifying enzymes by *Schizophyllum commune* IBL-06 in solid-state fermentation. **Biocatalysis and Agricultural Biotechnology**, v. 6, p. 195–201, 2016.

ASGHER, M.; NASIR IQBAL, H. M. Characterization of a novel manganese peroxidase purified from solid state culture of *Trametes versicolor* IBL-04. **BioResources**, v. 6, n. 4, p. 4317–4330, 2011.

AZARPIRA, A.; RALPH, J.; LU, F. Catalytic Alkaline Oxidation of Lignin and its Model Compounds: A Pathway to Aromatic Biochemicals. **Bioenergy Research**, v. 7, n. 1, p. 78–86, 2014.

BANCI, L.; CIOFI-BAFFONI, S.; TIEN, M. Lignin and Mn peroxidase-catalyzed oxidation of phenolic lignin oligomers. **Biochemistry**, v. 38, p. 3205–3210, 1999.

BANSAL, N. et al. Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. **Waste management (New York, N.Y.)**, v. 32, n. 7, p. 1341–6, jul. 2012.

BARRANCO-FLORIDO, J. E. et al. Criteria for the selection of strains of entomopathogenic fungi *Verticillium lecanii* for solid state cultivation. **Enzyme and Microbial Technology**, v. 30, p. 910–915, 2002.

BEHERA, S. S.; RAY, R. C. Solid State Fermentation for Production of Microbial Cellulases: Recent Advances and Improvement Strategies. **International Journal of Biological Macromolecules**, v. 86, p. 656–669, 2015.

BENASSI, V. M. et al. Immobilization and biochemical properties of a  $\beta$ -xylosidase activated by glucose/xylose from *Aspergillus niger* USP-67 with transxylosylation activity. **Journal of Molecular Catalysis B: Enzymatic**, v. 89, p. 93–101, 2013.

BESTE, A. ReaxFF Study of the Oxidation of Softwood Lignin in View of Carbon Fiber Production. **Energy & Fuels**, v. 28, p. 7007–7013, 2014.

BHATTACHARYA, S. S.; GARLAPATI, V. K.; BANERJEE, R. Optimization of laccase production using response surface methodology coupled with differential evolution. **New Biotechnology**, v. 28, n. 1, p. 31–39, 2011.

BIELY, P. Microbial xylanolytic systems. **Trends in Biotechnology**, v. 3, n. 11, p. 286–290, 1985.

BIZ, A. et al. Production of pectinases by solid-state fermentation of a mixture of citrus waste and sugarcane bagasse in a pilot-scale packed-bed bioreactor. **Biochemical Engineering Journal**, v. 111, p. 54–62, 2016.

BLAND, D. E.; LOGAN, A F. The Properties of Syringyl, Guaiacyl and p-Hydroxyphenyl Artificial Lignins. **The Biochemical journal**, v. 95, p. 515–520, 1965.

BOISSET, C. et al. Imaging the enzymatic digestion of bacterial cellulose ribbons reveals the endo character of the cellobiohydrolase Cel6A from *Humicola insolens* and its mode of synergy with cellobiohydrolase Cel7A. **Applied and Environmental Microbiology**, v. 66, n. 4, p. 1444–1452, 2000.

BOISSET, C. et al. Optimized mixtures of recombinant *Humicola insolens* cellulases for the biodegradation of crystalline cellulose. **Biotechnology and Bioengineering**, v. 72, n. 3, p. 339–345, 2001.

BORASTON, A. B. et al. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. **The Biochemical journal**, v. 382, n. Pt 3, p. 769–81, 2004.

BRAGATTO, J.; SEGATO, F.; SQUINA, F. M. Production of xylooligosaccharides (XOS) from delignified sugarcane bagasse by peroxide-HAc process using recombinant xylanase from *Bacillus subtilis*. **Industrial Crops and Products**, v. 51, p. 123–129, 2013.

BRIJWANI, K.; RIGDON, A.; VADLANI, P. V. Fungal Laccases : Production , Function , and Applications in Food Processing. v. 2010, 2010.

BÜCK, A. et al. Model-based control of enzyme yield in solid-state fermentation. **Procedia Engineering**, v. 102, p. 362–371, 2015.

BUGG, T. D. H. et al. Pathways for degradation of lignin in bacteria and fungi. **Natural Products Reports**, v. 28, n. 12, p. 1883–1896, 2011.

BURANOV, A. U.; MAZZA, G. Lignin in straw of herbaceous crops. **Industrial Crops and Products**, v. 28, n. 3, p. 237–259, nov. 2008.

BUTT, M. S. et al. Xylanases and their application in baking industry. **Food Technology and Biotechnology**, v. 46, n. 1, p. 22–31, 2008.

CAÑAS, A. I.; CAMARERO, S. Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. **Biotechnology Advances**, v. 28, n. 6, p. 694–705, 2010.

CANILHA, L. et al. Bioconversion of Hemicellulose from Sugarcane Biomass Into Sustainable Products. **Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization**, p. 15–43, 2013.

CERDA, A. et al. Long term enhanced solid-state fermentation: Inoculation strategies for amylase production from soy and bread wastes by *Thermomyces* sp. in a sequential batch operation. **Journal of Environmental Chemical Engineering**, v. 4, n. 2, p. 2394–2401, 2016.

CERQUEIRA-LEITE, R. C.; LEAL, M. R. L. V. O biocombustível no brasil. **Novos estudos**, p. 15–21, 2007.

CHANDEL, A. K. et al. Economics and environmental impact of bioethanol production technologies : an appraisal. **Biotechnology and Molecular Biology Review**, v. 2, n. February, p. 14–32, 2007.

CHEN, B.; WU, Q.; XU, Y. Filamentous fungal diversity and community structure associated with the solid state fermentation of *Chinese maotai* - flavor liquor. **International Journal of Food Microbiology**, v. 179, p. 80–84, 2014.

CHEN, H. Chemical Composition and Structure of Natural Lignocellulose. In: **Biotechnology of Lignocellulose: Theory and Practice**. Beijing: Springer, 2014. p. 73–141.

COLLINS, T.; GERDAY, C.; FELLER, G. Xylanases, xylanase families and extremophilic xylanases. **FEMS Microbiology Reviews**, v. 29, n. 1, p. 3–23, 2005.

CRESTINI, C. et al. Oxidative strategies in lignin chemistry: A new environmental friendly approach for the functionalisation of lignin and lignocellulosic fibers. **Catalysis Today**, v. 156, p. 8–22, 2010.

DAOU, M.; FAULDS, C. B. Glyoxal oxidases: their nature and properties. **World Journal of Microbiology and Biotechnology**, v. 33, n. 5, p. 1–11, 2017.

DASHTBAN, M. et al. Fungal biodegradation and enzymatic modification of lignin. **International Journal of Biochemistry and Molecular Biology**, v. 1, n. 1, p. 36–50, 2010.

DE CASSIA PEREIRA, J. et al. Thermophilic fungi as new sources for production of cellulases and xylanases with potential use in sugarcane bagasse saccharification. **Journal of Applied Microbiology**, v. 118, n. 4, 2015.

DE CASSIA PEREIRA, J. et al. Saccharification of ozonated sugarcane bagasse using enzymes from *Myceliophthora thermophila* JCP 1-4 for sugars release and ethanol production. **Bioresource Technology**, v. 204, p. 122–129, 2016.

DELGENES, J. P.; MOLETTA, R.; NAVARRO, J. M. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehatae*. **Enzyme and Microbial Technology**, v. 19, n. 95, p. 220–225, 1996.

DHILLON, G. S. et al. Potential of apple pomace as a solid substrate for fungal cellulase and hemicellulase bioproduction through solid-state fermentation. **Industrial Crops and Products**, v. 38, p. 6–13, jul. 2012.

DIAS, M. O. S. et al. Biorefineries for the production of first and second generation ethanol and electricity from sugarcane. **Applied Energy**, v. 109, p. 72–78, 2013.

DONINI, Í. A. N. et al. Biossíntese e recentes avanços na produção de celulose bacteriana. **ECLÉTICA química**, v. 35, p. 165–178, 2010.

DUSSAN, K. et al. The effect of hydrogen peroxide concentration and solid loading on the fractionation of biomass in formic acid. **Carbohydrate Polymers**, v. 111, p. 374–384, 2014.

DWIVEDI, P. et al. Co-cultivation of mutant *Penicillium oxalicum* SAU<sub>E</sub>-3.510 and *Pleurotus ostreatus* for simultaneous biosynthesis of xylanase and laccase under solid-state fermentation. **New Biotechnology**, v. 28, n. 6, p. 616–626, 2011.

EBRINGEROVÁ, A. Structural diversity and application potential of hemicelluloses. **Macromolecular Symposia**, v. 232, n. 333, p. 1–12, 2006.

ERIKSSON, K.-E. L.; BERMEK, H. Lignin, Lignocellulose, Ligninase. **Encyclopedia of Microbiology**, p. 373–384, 2009.

ERTAN, H. et al. Kinetic and thermodynamic characterization of the functional properties of a hybrid versatile peroxidase using isothermal titration calorimetry: Insight into manganese peroxidase activation and lignin peroxidase inhibition. **Biochimie**, v. 94, n. 5, p. 1221–1231, 2012.

EUGENIO, M. E. et al. Laccase production by *Pycnoporus sanguineus* under different culture conditions. **Journal of Basic Microbiology**, v. 49, p. 433–440, 2009.

FALKOSKI, D. L.; GUIMARÃES, V. M. Characterization of Cellulolytic Extract from *Pycnoporus sanguineus* PF-2 and Its Application in Biomass Saccharification. **Applied Biochemistry and Biotechnology**, v. 166, p. 1586–1603, 2012.

FARINAS, C. S. Developments in solid-state fermentation for the production of biomass-degrading enzymes for the bioenergy sector. **Renewable and Sustainable Energy Reviews**, v. 52, p. 179–188, 2015.

FENG, Y. et al. Transformation and removal of tetrabromobisphenol a from water in the presence of natural organic matter via laccase-catalyzed reactions: Reaction rates, products, and pathways. **Environmental Science and Technology**, v. 47, n. 2, p. 1001–1008, 2013.

FERHAN, M. . et al. Ligninases Production and Partial Purification of Mnp from Brazilian Fungal Isolate in Submerged Fermentation. **Fermentation Technology**, v. 01, n. 05, p. 1–7, 2012.

FERRAREZI, A. L. et al. Production and characterization of lipases and immobilization of whole cell of the thermophilic *Thermomucor indicaseudaticae* N31 for transesterification reaction. **Journal of Molecular Catalysis B: Enzymatic**, v. 107, p. 106–113, 2014.

FROMMHAGEN, M. et al. Boosting LPMO - driven lignocellulose degradation by polyphenol oxidase - activated lignin building blocks. **Biotechnology for biofuels**, p. 1–16, 2017.

GÁMEZ, S. et al. Study of the hydrolysis of sugar cane bagasse using phosphoric acid. **Journal of Food Engineering**, v. 74, n. 1, p. 78–88, 2006.

GAO, Y. et al. Effects of different pretreatment methods on chemical composition of sugarcane bagasse and enzymatic hydrolysis. **Bioresource technology**, v. 144, p. 396–400, set. 2013.

GARCIA, N. F. L. et al. Production of  $\beta$ -glucosidase on solid-state fermentation by *Lichtheimia ramosa* in agroindustrial residues: Characterization and catalytic properties of the enzymatic extract. **Electronic Journal of Biotechnology**, v. 18, n. 4, p. 314–319, 2015.

GEORGE, S. J. et al. Reversible alkaline inactivation of lignin peroxidase involves the release of both the distal and proximal site calcium ions and bishistidine co-ordination of the haem. **Biochemical Journal**, v. 244, p. 237–244, 1999.

GILBERTSON, R.L.; RYVARDEN, L. North American Polypores, p. 1-885, 1987.

GIMENES, L. J.; ROBERTO, D. Fungos Basidiomicetos. **Apostila do Curso de Capacitação de Monitores e Educadores**, 2010.

GÍRIO, F. M. et al. Hemicelluloses for fuel ethanol: A review. **Bioresource technology**, v. 101, n. 13, p. 4775–800, jul. 2010.

GODOY, M. G. et al. Adding value to a toxic residue from the biodiesel industry: Production of two distinct pool of lipases from *Penicillium simplicissimum* in castor bean waste. **Journal of Industrial Microbiology and Biotechnology**, v. 38, n. 8, p. 945–953, 2011.

GOES, T.; MARRA, R.; SILVA, G. DE S. Setor sucroalcooleiro no Brasil Situação atual e perspectivas. **Revista da Política Agrícola**, p. 39–51, 2008.

GOMES, E. et al. Ligninases production by basidiomycetes strains on lignocellulosic agricultural residues and their application in the decolorization of synthetic dyes. **Brazilian Journal of Microbiology**, v. 40, p. 31–39, 2009.

GORJUP, B. et al. Solvent effects on ligninases. **Enzyme and Microbial Technology**, v. 25, n. 1–2, p. 15–22, 1999.

GÓRSKA, E. W. A. B. et al. Production of Ligninolytic Enzymes by Cultures of White Rot Fungi. **Polish Journal of Microbiology**, v. 63, n. 4, p. 461–465, 2014.

GOUVEIA, S.; SANROMÁN, M. A.; MOLDES, D. Polymerisation of Kraft lignin from black liquors by laccase from *Myceliophthora thermophila*: Effect of operational conditions and black liquor origin. **Bioresource Technology**, v. 131, p. 288–294, 2013.

GOWTHAMAN, M. K. .; KRISHNA, C. .; MOO-YOUNG, M. Fungal solid state fermentation- an overview. **Applied Mycology and Biotechnology**, v. 1, p. 305–352, 2001.

HABIBI, Y.; LUCIA, L. A; ROJAS, O. J. Cellulose Nanocrystals : Chemistry , Self-Assembly , and Applications. **Chemical Reviews**, v. d, p. 3479–3500, 2009.

HAMID, S. B. A.; ISLAM, M. M.; DAS, R. Cellulase biocatalysis: key influencing factors and mode of action. **Cellulose**, v. 22, n. 4, p. 2157–2182, 2015.



HARVEY, P. J. . et al. Catalytic mechanisms and regulation of lignin peroxidase. In: **Biochemical Society Transactions**. [s.l: s.n.]. v. 20p. 345–349.

HERNÁNDEZ-ORTEGA, A.; FERREIRA, P.; MARTÍNEZ, A. T. Fungal aryl-alcohol oxidase: A peroxide-producing flavoenzyme involved in lignin degradation. **Applied Microbiology and Biotechnology**, v. 93, n. 4, p. 1395–1410, 2012.

HIMMEL, M. E. et al. Biomass recalcitrance: engineering plants and enzymes for biofuels production. **Science (New York, N.Y.)**, v. 315, n. 5813, p. 804–7, 9 fev. 2007.

HOFSETZ, K.; SILVA, M. A. Brazilian sugarcane bagasse: Energy and non-energy consumption. **Biomass and Bioenergy**, v. 46, p. 564–573, nov. 2012.

HÖLKER, U.; LENZ, J. Solid-state fermentation--are there any biotechnological advantages? **Current opinion in microbiology**, v. 8, n. 3, p. 301–6, jun. 2005.

HSIEH, C.-W. C. et al. Cellobiohydrolase and endoglucanase respond differently to surfactants during the hydrolysis of cellulose. **Biotechnology for biofuels**, v. 8, n. 1, p. 52, 2015.

IEMBO, T. et al. Purification and partial characterization of a new  $\beta$ -xylosidase from *Humicola grisea* var. *thermoidea*. **World Journal of Microbiology and Biotechnology**, v. 22, n. 5, p. 475–479, 2006.

ISIKGOR, F. H.; BECER, C. R. Lignocellulosic Biomass: A Sustainable Platform for Production of Bio-Based Chemicals and Polymers. **Polymer Chemistry**, p. 1–61, 2015.

JÖNSSON, L. J.; MARTÍN, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. **Bioresource Technology**, v. 199, p. 103–112, 2016.

JOZALA, A. F. et al. Produção de celulose bacteriana: uma nova tendência. **ResearchGate**, v. 14, n. January, p. 14–17, 2011.

JURADO, M. et al. Laccase detoxification of steam-exploded wheat straw for second generation bioethanol. **Bioresource technology**, v. 100, n. 24, p. 6378–84, dez. 2009.

JURADO, M. et al. Application of White-Rot Fungi in Transformation, Detoxification, or Revalorization of Agriculture Wastes. **Comprehensive Biotechnology**, p. 595–603, 2011.

KANG, S. et al. Hydrothermal conversion of lignin: A review. **Renewable and Sustainable Energy Reviews**, v. 27, p. 546–558, 2013.

KATAPODIS, P. et al. Purification and characterization of a thermostable intracellular  $\beta$ -xylosidase from the thermophilic fungus *Sporotrichum thermophile*. **Process Biochemistry**, v. 41, n. 12, p. 2402–2409, 2006.

KAUR, S. et al. Carbohydrate degrading enzyme production by plant pathogenic mycelia and microsclerotia isolates of *Macrophomina phaseolina* through koji fermentation. **Industrial Crops and Products**, v. 36, n. 1, p. 140–148, 2012.

KHAN, W. Z.; NAJEEB, I.; TUIYEBAYEVA, M. Refinery wastewater degradation with titanium dioxide, zinc oxide and hydrogen peroxide in a photocatalytic reactor. **Process Safety and Environmental Protection**, v. 4, n. August 2013, p. 479–486, 2014.

KIM, J. S.; LEE, Y. Y.; KIM, T. H. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. **Bioresource Technology**, v. 199, p. 42–48, 2016.

KIRK, P. M. . et al. Dictionary of The Fungi. **Mycological Research**, v. 106, n. 4, p. 507–508, 2008.

KNOB, A.; TERRASAN, C. R. F.; CARMONA, E. C.  $\beta$ -Xylosidases from filamentous fungi: An overview. **World Journal of Microbiology and Biotechnology**, v. 26, n. 3, p. 389–407, 2010.

KRISHNA, C. Solid-state fermentation systems-an overview. **Critical reviews in biotechnology**, v. 25, n. 1–2, p. 1–30, 2005.

KUMAR, M. et al. Production of xylanase by *Promicromonospora* sp MARS with rice straw under non sterile conditions. **Process Biochemistry**, v. 46, n. 8, p. 1614–1618, 2011.

LANGE, H.; DECINA, S.; CRESTINI, C. Oxidative upgrade of lignin - Recent routes reviewed. **European Polymer Journal**, v. 49, p. 1151–1173, 2013.

LASZKIEWICZ, B.; WCISLO, P. Sodium cellulose formation by activation process. **Journal of Applied Polymer Science**, v. 39, n. 2, p. 415–425, 1990.

LEE, H. V.; HAMID, S. B. A.; ZAIN, S. K. Conversion of Lignocellulosic Biomass to Nanocellulose: Structure and Chemical Process Conversion of Lignocellulosic Biomass to Nanocellulose: **The Scientific World Journal**, v. 2014, n. July, p. 1–20, 2014.

LEITE, R. S. R. et al. Production and characteristics comparison of crude  $\beta$ -glucosidases produced by microorganisms *Thermoascus aurantiacus* e *Aureobasidium pullulans* in agricultural wastes. **Enzyme and Microbial Technology**, v. 43, n. 6, p. 391–395, nov. 2008.

LIAO, H. et al. Functional diversity and properties of multiple xylanases from *Penicillium oxalicum* GZ-2. **Scientific Reports**, v. 5, n. 1, p. 12631, 2015.

LIM, S. F.; MATU, S. U. Utilization of agro-wastes to produce biofertilizer. **International Journal of Energy and Environmental Engineering**, v. 6, n. 1, p. 31–35, 2015.

LIU, D. et al. Thermostable cellulase production of *Aspergillus fumigatus* Z5 under solid-state fermentation and its application in degradation of agricultural wastes. **International Biodeterioration and Biodegradation**, v. 65, n. 5, p. 717–725, 2011.

LU, F.; RALPH, J. Lignin. In: **Cereal Straw as a Resource for Sustainable Biomaterials and Biofuels**. First ed. Beijing, China: Elsevier B.V., 2010. p. 169–207.

LYND, L. R. et al. Microbial Cellulose Utilization: Fundamentals and Biotechnology. **Microbiology and Molecular Biology reviews**, v. 66, n. 3, p. 506–577, 2002.

MA, L. et al. Optimization and evaluation of alkaline potassium permanganate pretreatment of corncob. **Bioresource Technology**, v. 180, p. 1–6, 2015.

MADADI, M.; TU, Y.; ABBAS, A. Recent Status on Enzymatic Saccharification of Lignocellulosic Biomass for Bioethanol Production Recent Status on Enzymatic Saccharification of Lignocellulosic Biomass for Bioethanol Production. **Electronic Journal of Biology**, v. 13, n. April, p. 135–143, 2017.

MANAVALAN, T.; MANAVALAN, A.; HEESE, K. Characterization of Lignocellulolytic Enzymes from White-Rot Fungi. **Current Microbiology**, v. 70, n. 4, p. 485–498, 2015.

MARQUES, N. P. et al. Cellulases and xylanases production by endophytic fungi by solid state fermentation using lignocellulosic substrates and enzymatic saccharification of pretreated sugarcane bagasse. **Industrial Crops and Products**, v. 122, 2018.

MARTINEZ, A. T. et al. Biodegradation of lignocellulosics : microbial, chemical, and enzymatic aspects of the fungal attack of lignin. **International Microbiology**, v. 8, p. 195–204, 2005.

MARTINS, G. M. et al. The isolation of pentose-assimilating yeasts and their xylose fermentation potential. **Brazilian Journal of Microbiology**, v. 49, n. 1, p. 162–168, 2018.

MAYER, A. M.; STAPLES, R. C. New Functions for an Old Enzyme: **Phytochemistry** **60**, v. 60, p. 551–565, 2002.

MAZA, M. et al. Post-harvest sugarcane residue degradation by autochthonous fungi. **International Biodeterioration and Biodegradation**, v. 87, p. 18–25, 2014.

MELIKOGLU, M.; LIN, C. S. K.; WEBB, C. Kinetic studies on the multi-enzyme solution produced via solid state fermentation of waste bread by *Aspergillus awamori*. **Biochemical Engineering Journal**, v. 80, p. 76–82, 2013.

MERHEB-DINI, C. et al. Use of a new milk-clotting protease from *Thermomucor indiciae-seudaticae* N31 as coagulant and changes during ripening of Prato cheese. **Food Chemistry**, v. 130, n. 4, p. 859–865, 2012.

MME. RenovaBio: Biocombustíveis 2030. **Ministério de Minas e Energia (MME)**, 2017.

MOOD, S. H. et al. Lignocellulosic biomass to bioethanol ; a comprehensive review on pretreatment. **Renewable and Sustainable Energy Reviews**, v. 27, p. 76–93, 2013.

MORENO, A. D. et al. Exploring laccase and mediators behavior during saccharification and fermentation of steam-exploded wheat straw for bioethanol production. [s.d.].

MORETTI, M. M. S. et al. Selection of thermophilic and thermotolerant fungi for the production of cellulases and xylanases under solid-state fermentation. **Brazilian Journal of Microbiology**, p. 1062–1071, 2012.

MOROZOVA, O. V. et al. Laccase-mediator systems and their applications: A review. **Applied Biochemistry and Microbiology**, v. 43, n. 5, p. 523–535, 2007.

MOTTA, F.; ANDRADE, C.; SANTANA, M. A Review of Xylanase Production by the Fermentation of Xylan: Classification, Characterization and Applications. **Intech**, v. Chapter 10, p. 251–275, 2013.

MOYSÉS, D. N. et al. Xylose fermentation by *saccharomyces cerevisiae*: Challenges and prospects. **International Journal of Molecular Sciences**, v. 17, n. 3, p. 1–18, 2016.

NAVARRO, D. et al. Fast solubilization of recalcitrant cellulosic biomass by the basidiomycete fungus *Laetisaria arvalis* involves successive secretion of oxidative and hydrolytic enzymes. **Biotechnology for biofuels**, v. 7, p. 1–14, 2014.

NOBLES, M.K.; FREW, B.P. Studies in wood-inhabiting Hymenomyces V. The genus *Pycnoporus* Karst. **Canadian Journal of Botany**, p. 987-1016, 1962.

OLIVEIRA, P. DE; CÁSSIA, J. DE; QUEIROZ, D. Synergistic action of an *Aspergillus* (hemi-)cellulolytic consortium on sugarcane bagasse saccharification. **Industrial Crops and Products**, v. 109, n. July, p. 173–181, 2017.

ONOFRE, S. B. et al. Cellulases produced by the endophytic fungus *Pycnoporus sanguineus* (L.) Murrill. **African Journal of Agricultural Research**, v. 10, n. 13, p. 1557–1564, 2015.

OTJEN, L.; BLANCHETTE, R. A. A discussion of microstructural changes in wood during decomposition by white rot basidiomycetes. **Canadian Journal of Botany**, v. 64, n. 5, p. 905–911, 1986.

PANDEY, A. et al. Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. **Bioresource Technology**, v. 74, n. 1, p. 69–80, ago. 2000.

PANDEY, A. Solid-state fermentation. **Biochemical Engineering Journal**, v. 13, n. July 2002, p. 81–84, 2003.

PANDEY, A. K.; EDGARD, G.; NEGI, S. Optimization of concomitant production of cellulase and xylanase from *Rhizopus oryzae* SN5 through EVOP-factorial design technique and application in Sorghum Stover based bioethanol production. **Renewable Energy**, v. 98, p. 51–56, 2016.

PANDEY, A.; SOCCOL, C. R.; MITCHELL, D. New developments in solid state fermentation: I-bioprocesses and products. **Process Biochemistry**, v. 35, n. 10, p. 1153–1169, jul. 2000.

PANDEY, M. P.; KIM, C. S. Lignin Depolymerization and Conversion: A Review of Thermochemical Methods. **Chemical Engineering and Technology**, v. 34, n. 1, p. 29–41, 2011.

PARK, N. et al. Organosolv pretreatment with various catalysts for enhancing enzymatic hydrolysis of pitch pine (*Pinus rigida*). **Bioresource technology**, v. 101, n. 18, p. 7057–64, set. 2010.

PAYNE, C. M. et al. Fungal Cellulases. **Chemical Reviews**, 2015.

PEREIRA, J. C. et al. Production and Characterization of  $\beta$ - glucosidase Obtained by the Solid- State Cultivation of the Thermophilic Fungus *Thermomucor indicae-seudaticae* N31. p. 1–16, 2014.

PEREIRA, J. C. et al. Effect of Metal Ions , Chemical Agents and Organic Effect of Metal Ions , Chemical Agents and Organic Compounds on Lignocellulolytic Enzymes Activities. In: **Enzyme inhibitors and activators**. [s.l: s.n.].

PÉREZ, S.; SAMAIN, D. **Structure and engineering of celluloses**. [s.l: s.n.]. v. 64

PLÁCIDO, J.; CAPAREDA, S. Ligninolytic enzymes: a biotechnological alternative for bioethanol production. **Bioresources and Bioprocessing**, v. 2, n. 1, p. 23, 2015.

POINTING, S. B. .; JONES, E. B. G. .; VRIJMOED, L. L. P. Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. **Mycologia**, v. 92, n. 1, p. 139–144, 2000.

POLIZELI, M. L. T. M. et al. Xylanases from fungi: properties and industrial applications. **Applied microbiology and biotechnology**, v. 67, n. 5, p. 577–91, jun. 2005.

QI, M.; JUN, H. S.; FORSBERG, C. W. Characterization and synergistic interactions of *Fibrobacter succinogenes* glycoside hydrolases. **Applied and Environmental Microbiology**, v. 73, n. 19, p. 6098–6105, 2007.

QUIROZ-CASTAÑEDA, R. . et al. Evaluation of different lignocellulosic substrates for the production of cellulases and xylanases by the basidiomycete fungi *Bjerkandera adusta* and *Pycnoporus sanguineus*. **Biodegradation**, p. 565–572, 2011.

QUIROZ-CASTAÑEDA, R. E. et al. Characterization of cellulolytic activities of *Bjerkandera adusta* and *Pycnoporus sanguineus* on solid wheat straw medium. **Electronic Journal of Biotechnology**, v. 12, n. 4, p. 1–8, 2009.

RAGAUSKAS, A. J. . et al. The Path Forward for Biofuels and Biomaterials. **Science**, v. 311, n. 5760, p. 484–489, 2006.

RAMADOSS, G.; MUTHUKUMAR, K. Influence of dual salt on the pretreatment of sugarcane bagasse with hydrogen peroxide for bioethanol production. **Chemical Engineering Journal**, v. 260, p. 178–187, 2015.

RAMADOSS, G.; MUTHUKUMAR, K. Ultrasonics Sonochemistry Mechanistic study on ultrasound assisted pretreatment of sugarcane bagasse using metal salt with hydrogen peroxide for bioethanol production. **Ultrasonics Sonochemistry**, v. 28, p. 207–217, 2016.

RAMÍREZ-CAVAZOS, L. I. et al. Enhanced production of thermostable laccases from a native strain of *Pycnoporus sanguineus* using central composite design \*. **Journal of Zhejiang University-SCIENCE B**, v. 15, n. 4, p. 343–352, 2014.

REZENDE, M. I. et al. Xylanase production by *Trichoderma harzianum* Rifai by solid state fermentation on sugarcane bagasse. **Brazilian Journal of Microbiology**, v. 33, n. 1, p. 67–72, 2002.

RILEY, R. et al. Extensive sampling of basidiomycete genomes demonstrates inadequacy of the white-rot/brown-rot paradigm for wood decay fungi. **Proceedings of the National Academy of Sciences of the United States of America**, v. 111, n. 27, p. 9923–9928, 2014.

RIVA, S.; MOLECOLARE, R.; BIANCO, V. M. Laccases: blue enzymes for green chemistry. **Trends in Biotechnology**, v. 24, n. 5, p. 219–226, 2006.

ROCHA-MARTIN, J. et al. Oxidation of phenolic compounds catalyzed by immobilized multi-enzyme systems with integrated hydrogen peroxide production. **Green Chemistry**, v. 16, n. 1, p. 303, 2014.

RODRÍGUEZ-FERNÁNDEZ, D. E. et al. The behavior of kinetic parameters in production of pectinase and xylanase by solid-state fermentation. **Bioresource Technology**, v. 102, n. 22, p. 10657–10662, 2011.

SAINI, A. et al. Actinomycetes: A Source of Lignocellulolytic Enzymes. **Enzyme Research**, v. 2015, 2015.

SANCHEZ-AMAT, A.; SOLANO, F.; LUCAS-ELÍO, P. Finding new enzymes from bacterial physiology: A successful approach illustrated by the detection of novel oxidases in *Marinomonas mediterranea*. **Marine Drugs**, v. 8, n. 3, p. 519–541, 2010.

SANCHEZ, O.; SIERRA, R.; ALMÉCIGA-DIAZ, C. J. Delignification Process of Agro-Industrial Wastes an Alternative to Obtain Fermentable Carbohydrates for Producing Fuel. In: **Alternative Fuel**. [s.l: s.n.].

SANGHI, R. Enzymatic Formation of Gold Nanoparticles Using *Phanerochaete Chrysosporium*. **Advances in Chemical Engineering and Science**, v. 01, n. 03, p. 154–162, 2011.

SANT'ANA DA SILVA, A. et al. High-solids content enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse using a laboratory-made enzyme blend and commercial preparations. **Process Biochemistry**, v. 51, n. 10, p. 1561–1567, 2016.

SCHELLER, H. V.; ULVSKOV, P. Hemicelluloses. **Annual Review of Plant Biology**, v. 61, n. 1, p. 263–289, 2010.

SHALLOM, D.; SHOHAM, Y. Microbial hemicellulases. **Current Opinion in Microbiology**, v. 6, n. 3, p. 219–228, 2003.

SIGOILLOT, J. C. et al. Fungal Strategies for Lignin Degradation. **Advances in Botanical Research**, v. 61, n. December, p. 263–308, 2012.

SITI AISYAH, M. S.; UEMURA, Y.; YUSUP, S. The Effect of Alkaline Addition in Hydrothermal Pretreatment of Empty Fruit Bunches on Enzymatic Hydrolysis Efficiencies. **Procedia Chemistry**, v. 9, p. 151–157, 2014.

SOCCOL, C. R. et al. Recent developments and innovations in solid state fermentation. **Biotechnology Research and Innovation**, p. 1–20, 2017.



SØRENSEN, H. R. et al. Enzymatic hydrolysis of wheat arabinoxylan by a recombinant “minimal” enzyme cocktail containing  $\beta$ -xylosidase and novel endo-1,4- $\beta$ -xylanase and  $\alpha$ -L-arabinofuranosidase activities. **Biotechnology Progress**, v. 23, n. 1, p. 100–107, 2007.

SORIEUL, M. et al. **Plant fibre: Molecular structure and biomechanical properties, of a complex living material, influencing its deconstruction towards a biobased composite.** [s.l: s.n.]. v. 9

SOUZA, W. R. DE. Microbial Degradation of Lignocellulosic Biomass. In: **Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization.** [s.l: s.n.]. p. 207–248.

SOUZA, O.; SANTOS, I. E. O bagaço de cana-de-açúcar como alimento volumoso para ruminantes. **Comunicado Técnico, Ministério da Agricultura, Pecuária e Abastecimento.** Aracaju - SE: [s.n.].

SU, Y. et al. Fractional pretreatment of lignocellulose by alkaline hydrogen peroxide: Characterization of its major components. **Food and Bioproducts Processing**, v. 4, p. 322–330, 2014.

SUES, A. et al. Ethanol production from hexoses, pentoses, and dilute-acid hydrolyzate by *Mucor indicus*. **FEMS Yeast Research**, v. 5, n. 6–7, p. 669–676, 2005.

SUKUMARAN, R. K. et al. Lignocellulosic ethanol in India: Prospects, challenges and feedstock availability. **Bioresource Technology**, v. 101, p. 4826–4833, 2010.

SUN, F.; CHEN, H. Organosolv pretreatment by crude glycerol from oleochemicals industry for enzymatic hydrolysis of wheat straw. **Bioresource technology**, v. 99, n. 13, p. 5474–9, set. 2008.

SUNNA, A; ANTRANIKIAN, G. Xylanolytic enzymes from fungi and bacteria. **Critical reviews in biotechnology**, v. 17, n. 1, p. 39–67, 1997.

SUURNÄKKI, A et al. Hemicellulases in the bleaching of chemical pulps. **Advances in biochemical engineering/biotechnology**, v. 57, p. 261–87, 1997.

SWEENEY, M. D.; XU, F. Biomass Converting Enzymes as Industrial Biocatalysts for Fuels and Chemicals: Recent Developments. **Catalysts**, v. 2, n. 2, p. 244–263, 2012.

TAHERZADEH, M. J.; KARIMI, K. Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: A review. **BioResources**, v. 2, p. 707–738, 2007.

TALEBNIA, F.; KARAKASHEV, D.; ANGELIDAKI, I. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. **Bioresource technology**, v. 101, n. 13, p. 4744–53, jul. 2010.

TERRASAN, C. R. F. et al. Xylanase and  $\beta$ -Xylosidase from *Penicillium janczewskii*: Production, Physico-Chemical properties, and application of the crude extract to pulp biobleaching. **BioResources**, v. 8, n. 1, p. 1292–1305, 2013.

TOMÁS-PEJÓ, E. et al. Pretreatment Technologies for Lignocellulose-to-Bioethanol Conversion. In: **Biofuels: Alternative Feedstocks and Conversion Processes**. Madrid: Elsevier, 2011. p. 149–176.

UMSZA-GUEZ, M. A. et al. Effect of pectinolytic enzymes on the physical properties of caja-manga (*Spondias cytherea* Sonn.) pulp. **Ciência e Tecnologia de Alimentos**, v. 31, n. 2, p. 517–526, 2011.

UNFCCC. Adoption of The Paris Agreement: Proposal by the President to the United Nations Framework Convention on Climate Change. v. 21932, n. December, p. 1–32, 2015.

VALERIANO, V. S. et al. Production of laccase by *Pycnoporus sanguineus* using 2,5 - xylidine and ethanol. **Brazilian Journal of Microbiology**, v. 40, p. 790–794, 2009.

VAN DYK, J. S. .; PLETSCHKE, B. I. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes- Factors affecting enzymes, conversion and synergy. **Biotechnology Advances**, v. 30, n. 6, p. 1458–1480, 2012.

VÁRNAI, A. et al. Carbohydrate-Binding Modules of Fungal Cellulases. Occurrence in Nature, Function, and Relevance in Industrial Biomass Conversion. [s.l: s.n.]. v. 88

VIKINESWARY, S.; ABDULLAH, N.; RENUVATHANI, M. Productivity of laccase in solid substrate fermentation of selected agro-residues by *Pycnoporus sanguineus*. **Bioresource technology**, v. 97, p. 171–177, 2006.

VILELA, L. DE F. et al. Enhanced xylose fermentation and ethanol production by engineered *Saccharomyces cerevisiae* strain. **AMB Express**, v. 5, n. 1, 2015.

VLASENKO, E. et al. Substrate specificity of family 5, 6, 7, 9, 12, and 45 endoglucanases. **Bioresource Technology**, v. 101, n. 7, p. 2405–2411, 2010.

VOLYNETS, B.; EIN-MOZAFFARI, F.; DAHMAN, Y. Biomass processing into ethanol: pretreatment, enzymatic hydrolysis, fermentation, rheology, and mixing. **Green Process Synth**, p. 1–22, 2017.

VOUTILAINEN, S. P. et al. Heterologous expression of *Melanocarpus albomyces* cellobiohydrolase Cel7B, and random mutagenesis to improve its thermostability. **Enzyme and Microbial Technology**, v. 41, n. 3, p. 234–243, ago. 2007.

WALISKO, R. et al. Microparticle based morphology engineering of filamentous microorganisms for industrial bio-production. **Biotechnology Letters**, v. 34, n. 11, p. 1975–1982, 2012.

WELKER, C. et al. Engineering Plant Biomass Lignin Content and Composition for Biofuels and Bioproducts. **Energies**, v. 8, n. 8, p. 7654–7676, 2015.

WOODWARD, J. Synergism in cellulase systems. **Bioresource Technology**, v. 36, n. 1, p. 67–75, 1991.

WU, C. et al. Disintegration of excess activated sludge with potassium permanganate: Feasibility, mechanisms and parameter optimization. **Chemical Engineering Journal**, v. 240, p. 420–425, 2014.

XIAOWEI, P.; HONGZHANG, C. Hemicellulose sugar recovery from steam-exploded wheat straw for microbial oil production. **Process Biochemistry**, v. 47, p. 209–215, 2012.

XU, X. R. et al. Decolorization of dyes and textile wastewater by potassium permanganate. **Chemosphere**, v. 59, p. 893–898, 2005.

YAZID, N. A.; BARRENA, R.; SÁNCHEZ, A. Assessment of protease activity in hydrolysed extracts from SSF of hair waste by and indigenous consortium of microorganisms. **Waste Management**, v. 49, p. 420–426, 2016.

YEOMAN, C. J. et al. **Thermostable enzymes as biocatalysts in the biofuel industry**. 1. ed. [s.l.] Elsevier Inc., 2010. v. 70

YOO, C. G. et al. Enhancement of enzymatic hydrolysis and klason lignin removal of corn stover using photocatalyst-assisted ammonia pretreatment. **Applied Biochemistry and Biotechnology**, v. 169, p. 1648–1658, 2013.

YOON, L. W.; NGOH, G. C.; CHUA, A. S. M. Simultaneous production of cellulase and reducing sugar from alkali-pretreated sugarcane. **BioResources**, v. 7, p. 5319–5332, 2012.

YOON, L. W.; NGOH, G. C.; CHUA, A. S. M. Simultaneous production of cellulase and reducing sugar through modification of compositional and structural characteristic of sugarcane bagasse. **Enzyme and Microbial Technology**, v. 53, p. 250–256, 2013.

YU, H. et al. Comparative study of alkaline hydrogen peroxide and organosolv pretreatments of sugarcane bagasse to improve the overall sugar yield. **Bioresource Technology**, v. 187, p. 161–166, 2015.

ZHANG, J. Phenol Removal from Water with Potassium Permanganate Modified Granular Activated Carbon. **Journal of Environmental Protection**, v. 04, n. May, p. 411–417, 2013.

ZHANG, Y. H. P.; LYND, L. R. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. **Biotechnology and Bioengineering**, v. 88, n. 7, p. 797–824, 2004.

ZIMBARDI, A. L. R. L. et al. A High Redox Potential Laccase from *Pycnoporus sanguineus* RP15: Potential Application for Dye Decolorization. **International Journal of Molecular Sciences**, v. 17, p. 1–24, 2016.

#### **Outros acessos:**

Joint Genome Institute: <http://genome.jgi.doe.gov/PyCSA1/PyCSA1.home.html> - acessado dia 23 de junho de 2017

## Capítulo 1

### **Ligninases, cellulases and xylanases production by *Pycnoporus sanguineus* MCA 16 and enzymatic saccharification of sugarcane bagasse for ethanol production**

Josiani de Cassia Pereira<sup>1</sup>, Natália Paganini Marques<sup>2</sup>, Diego Alves Monteiro<sup>1</sup>, Gisele Marta Martins<sup>1</sup>, Ariela de Paula Veloso<sup>3</sup>, Maurício Boscolo<sup>1</sup>, Roberto da Silva<sup>1</sup>, Eleni Gomes<sup>1</sup>, Daniela Alonso Bocchini<sup>2</sup>

<sup>1</sup> Institute of Biosciences, Letters and Exact Sciences, São Paulo State University – IBILCE/UNESP, São José do Rio Preto, São Paulo State, Brazil

<sup>2</sup> Institute of Chemistry, São Paulo State University – IQ/UNESP, Araraquara, São Paulo State, Brazil

<sup>3</sup> Faculty of Pharmaceutical Science, São Paulo State University – FCFAR/UNESP, Araraquara, São Paulo State, Brazil.

#### **Abstract**

Research focused on obtaining both hydrolytic and oxidative enzymes by new fungal strains using low cost technologies are important for the effectiveness and feasibility of the process as a whole. In this context, in the present work *Pycnoporus sanguineus* MCA16 was cultivated by SSF on different mixtures (1:1 w/w) of lignocellulosic residues as substrates and the productions of cellulases, xylanases and ligninases were evaluated. Initially, the fungus was cultivated during 96 hours, at 40 °C and the highest endoglucanase, exoglucanase,  $\beta$ -glucosidase,  $\beta$ -xylosidase and manganese peroxidase activities (129.96, 44.26, 50.47, 1.81 and 8.92 U.g<sup>-1</sup>, respectively) were obtained when using the mixture of wheat bran and soybean meal (WB:SM). The activities of xylanase, lacase and lignin peroxidase (40.58, 22.04 and 9.16 U.g<sup>-1</sup>, respectively) in the enzymatic extract obtained under this condition were close to the highest obtained when using other substrates mixtures. The influence of time on the enzymes production was then evaluated and 96 hours was confirmed as the best time to obtain an enzymatic extract with the maximum activity of most of the tested enzymes. The enzymatic extract was used in saccharification experiments and statistical designs were used to evaluate the influence of pretreated sugarcane bagasse load, endoglucanase

load, time and temperature on glucose release. The highest glucose concentration in the hydrolysates ( $7.32 \text{ g.L}^{-1}$ ) was obtained when using sugarcane bagasse at 13.5 % (dry basis), endoglucanase load of 288.3 (total units/g of cellulose), 130 hours and  $57^{\circ}\text{C}$ . The hydrolysate obtained under these conditions was used as glucose source for cellulosic ethanol production by *Saccaromyces cerevisiae* CAT-1 and a yield of 73.5 % was obtained.

**Keywords:** cellulases, xylanases, ligninases, solid-state fermentation, enzymatic saccharification, second generation of ethanol.

## 1.Introduction

Lignocellulosic materials, constituted mainly by cellulose, hemicellulose and lignin, represent an important carbon source and are good substrates for filamentous fungi cultivation by solid-state fermentation (SSF), aiming the production of cellulases, xylanases, ligninases and also other plant degrading enzymes (GAO et al., 2008a, 2008b; LEITE et al., 2008). Sugarcane bagasse, wheat bran, corn cob and corn husk, soybean meal, rice husk, barley and others lignocellulosic materials have been used for this purpose (SAINI et al., 2015; SÁNCHEZ, 2009). These materials are abundant in Brazil, especially sugarcane bagasse which is generated in high quantity by the sugar and ethanol industries (SOARES et al., 2011).

Filamentous fungi are the adapted to SSF since this process simulates their natural environment in relation to low free water and to the insolubility of the substrates (BEHERA; RAY, 2015; BOCCHINI-MARTINS et al., 2011; PEREIRA et al., 2014; GOWTHAMAN et al., 2001; KRISHNA, 2005; MORETTI et al., 2012). Moreover, filamentous fungi stand out as the good producers of plant degrading enzymes by SSF (MTUI, 2012; SINGHANIA et al., 2010) and the search for new strains is interesting in order to obtain biocatalysts even more appropriate for industrial processes, besides of the exploration of microbial biodiversity (LETTERA et al., 2011; SØRENSEN et al., 2013; YOON et al., 2014). Microbial enzymes of lignocellulose degradation such as cellulases, xylanases and ligninases can be applied in several biotechnological processes (BONUGLI-SANTOS et al., 2015) and the use of these enzymes for

pretreated sugarcane bagasse saccharification has been extensively cited in recent years, in order to obtain fermentable sugars that can be converted into second generation ethanol (DE CASSIA PEREIRA et al., 2016; FARINAS, 2015).

Several physic and/or chemical methods can be used for sugarcane bagasse pretreatment (DE CASSIA PEREIRA et al., 2016; MENON; RAO, 2012; MORETTI et al., 2014; OLIVEIRA et al., 2017; PERRONE et al., 2017, 2016). Alkaline hydrothermal pretreatment using sodium hydroxide is interesting since it solubilizes and removes lignin and also promotes the swelling of native cellulose, resulting in Type II cellulose which is less crystalline, has a low degree of polymerization and a higher surface area and is, therefore, more accessible to cellulases (HABIBI et al., 2009; SANTOS et al., 2012; TALEBNIA et al., 2010).

In this context, the present work explored the production of cellulases, xylanases and ligninases by *Pycnoporus sanguineus* MCA16 by SSF using lignocellulosic residues as substrates and the use of the enzymatic crude solution in the saccharification of sugarcane bagasse submitted to alkaline hydrothermal pretreatment. The hydrolysate obtained was used as glucose source for ethanol production.

## **2. Material and methods**

### **2.1. Microorganism**

The basidiomycete *Pycnoporus sanguineus* MCA 16 and *Saccharomyces cerevisiae* CAT-1 belonging to Laboratory of Biochemistry and Applied Microbiology (work culture) was used to obtain cellulases, xylanases and ligninases (ABRAHÃO et al., 2009) and ethanol fermentation, respectively.

### **2.2. Influence of solid state fermentation (SSF) conditions on the enzymes productions**

Initially, *P. sanguineus* MCA 16 was cultivated by SSF using mixtures (1:1 w/w) of different agro-industrial wastes (sugarcane bagasse – donated by Usina Virgolino de Oliveira, Planalto, São Paulo, Brazil), wheat bran (Natural

Life ®), rice husk (donated by Patini® rice industry, São José do Rio Preto, São Paulo, Brazil) and soybean meal (donated by Trow Nutrition, Mirassol, São Paulo, Brazil) as substrates for 96 hours, at 40 °C, and the activities of endoglucanase, exoglucanase,  $\beta$ -glucosidase, xylanase,  $\beta$ -xylosidase, laccase, manganese peroxidase and lignin peroxidase were determined in the crude enzyme solution. The substrates were washed and dried at 40°C and stored. The mixture of soybean meal and wheat bran was chosen to be used in subsequent SSF experiments, performed in order to evaluate the influence of cultivation time on the enzymes productions. SSF were performed as duplicates, according to DE CASSIA PEREIRA et al. (2015).

### **2.3. Determination of enzymes activities**

Endoglucanase, exoglucanase,  $\beta$ -glucosidase, xylanase and  $\beta$ -xylosidase activities were assayed according to DE CASSIA PEREIRA et al. (2015) at 40 °C and were expressed as units per gram of substrate used in SSF (U/g).

Laccase activity was determined at 40°C, for 1 minute, using 0.9 ml of substrate solution (2,2-azino-bis-ethylbenzhihiaiazoline – ABTS (0.03%) in sodium acetate buffer solution, 1.0 mol L<sup>-1</sup>, pH 5.0) and 0.1 ml of the enzymatic extract (BUSWELL et al., 1995). The oxidation of ABTS was measured by monitoring the increase in absorbance at 420 nm. White assay was prepared by replacing the enzymatic extract with equal volume of distilled water, while controls assays were made by replacing ABTS with distilled water. A unit of enzymatic activity was defined as the amount of enzyme required to oxidize 1 mmol of ABTS per minute using the molar extinction coefficient ( $\epsilon$ ) 3.6 x 10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup> to 420 nm for the oxidized ABTS (BOURBONNAIS; PAICE, 1988).

The activity of manganese peroxidase (MnP) was determined at 40°C, for 10 minutes, in a reaction mixture composed of 0.25 ml of sodium acetate buffer (pH 4.5, 50 mmol L<sup>-1</sup>), 0.1 ml of manganese sulphate (0.4 mol L<sup>-1</sup>) and 0.1 ml of enzymatic extract. The reaction was started with the addition of hydrogen peroxide (40  $\mu$ mol L<sup>-1</sup>) and the absorbance was measured at 240 nm. White assay was prepared by substituting the crude extract for the same volume of distilled water, while controls assays were made by substituting distilled water with the MnSO<sub>4</sub> solution. A unit of enzymatic activity was defined



as the amount of enzyme required to form 1.0  $\mu\text{mol}$  of  $\text{Mn}^{+3}$  per minute using the molar extinction coefficient ( $\epsilon$ ) of  $8.1 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$  (GLENN et al., 1999).

The activity of lignin peroxidase (LiP) was determined at 40 °C, for 10 minutes, in a reaction mixture composed of sodium tartrate buffer (pH 5.0, 50  $\text{mmol L}^{-1}$ ), veratryl alcohol solution (40  $\text{mmol L}^{-1}$ ) and 0.1 ml of the enzymatic extract, with final volume of 1.0 ml. The reaction was initiated by the addition of hydrogen peroxide (0.2  $\text{mmol L}^{-1}$ ) and the absorbance was measured at 310 nm. White assay was prepared by replacing the enzymatic extract with equal volume of distilled water, while controls assays were made by replacing the veratryl alcohol substrate solution with distilled water. A unit of enzymatic activity was defined as the amount of enzyme required to form 1.0  $\mu\text{mol}$  veratraldehyde per minute, considering  $\epsilon_{310}$  is  $9.3 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$  for veratraldehyde (TIEN AND KIRK, 1988).

#### **2.4. Sugarcane bagasse hydrothermal alkaline pretreatment**

Pretreatment was performed in autoclave (solid load of 5.0 % w/v in 0.5  $\text{mol L}^{-1}$  NaOH solution) in a 250 mL Erlenmeyer flask, sealed with aluminum foil, for 1 hour, at 1 atm and 120 °C. After, the flask content was cooled in ice water bath and then kept under room temperature for stabilization. The material was filtered on nylon cloth and then the liquid phase was filtered on 0.22  $\mu\text{m}$  filters. The solid phase was washed with distilled water, the excess of water was removed with cotton cloth and the pretreated bagasse was stored at 4 °C.

#### **2.5. Enzymatic saccharification of pretreated sugarcane bagasse**

Saccharification experiments were performed using the crude enzyme solution obtained from *P. sanguineus* MCA 16 cultivation by SSF using the mixture (1:1 w/w) of wheat bran and soybean meal as substrates, during 96 hours, under 40 °C. Assays were performed, as duplicates, in 50 ml Erlenmeyer flasks sealed with latex caps using bagasse load at 3.0 % (dry weight basis), enzyme load of 100 total units of endoglucanase/g of cellulose on pretreated bagasse and citrate buffer solution (0.1  $\text{mol L}^{-1}$ , pH 5.0) to a final volume of 20 ml, at 50 °C, under 300 rpm, during 24 hours. After this time, the flasks contents were cooled in ice bath and filtered through nylon cloth. The solid was

discarded, the liquid phase was filtered again through 0.22  $\mu\text{m}$  filters and then analyzed by High Performance Liquid Chromatography (HPLC) to quantify glucose and xylose. Yields were calculated according the equations below:

$$(1) \text{Yield}_{\text{glucose}} (\%) = [\text{total glucose (g)} / \text{total cellulose (g) in pretreated bagasse}] \times 100$$

$$(2) \text{Yield}_{\text{xylose}} (\%) = [\text{total xylose (g)} / \text{total xylan (g) in pretreated bagasse}] \times 100$$

$$(3) \text{Yield}_{\text{glucose or xylose}} (\text{mg/g of pretreated sugarcane bagasse}) = [\text{total glucose or xylose (g)} / \text{bagasse dry mass (g)}] \times 1000$$

$$(4) \text{Yield}_{\text{glucose or xylose}} (\text{mg/g of cellulose or xylan in pretreated bagasse}) = [\text{total glucose or xylose (g)} / \text{total cellulose or xylan (g) in pretreated bagasse}] \times 1000$$

In order to determine the best saccharification condition, experimental designs were employed as described in items 2.5.1 and 2.5.2. Variables (biomass loading, enzyme loading, temperature and incubation time) were chosen based on the literature and variance analysis (ANOVA) was performed to estimate the effect of the variables and their interactions on glucose concentration in the hydrolysates. Hence,  $p < 0.05$  was considered statistically significant. Regression and graphical analysis were accomplished using the Statistica 10 software (StatSoft Inc., Tulsa, Oklahoma, USA).

### **2.5.1. Influence of enzyme and bagasse loads**

In order to determine the influence of endoglucanase and pretreated bagasse loads, a rotational central composite design (RCCD) ( $2^2$ , including 2 axial points and 4 repetitions of central points, totaling 12 trials) was used. The real and encoded values are presented in Table 1.

Table 1. Rotational central composite design (RCCD) ( $2^2$ ) used in saccharification of pretreated sugarcane bagasse.

Variable	Levels					Units
	-1,41	-1	0	+1	+1,41	
$x_1$ – pretreated sugarcane bagasse load	2.93	5.0	10.0	15.0	17.07	% (dry basis)
$x_2$ – endoglucanase load	203.43	220.0	260.0	300.0	316.57	Total units/g cellulose in pretreated bagasse

### 2.5.2. Influence of time and temperature

A Face centered central composite design (RCCD) ( $2^2$ , including 2 axial points and 4 repetitions of central points, totaling 12 trials) was performed to evaluate the influence of time and temperature in pretreated sugarcane bagasse saccharification. Pretreated sugarcane bagasse and enzyme loads were used at the best levels obtained from the experiments cited in item 2.5.1. The real and encoded values are presented in Table 2.

Table 2. Face centered central composite design (CCD) ( $2^2$ ) used in the saccharification of pretreated sugarcane bagasse.

Variable	Levels					Units
	-1,41	-1	0	+1	+1,41	
$x_3$ – Time	28.11	48.0	96.0	144.0	163.88	Hours
$x_4$ – Temperature	43	45	50	55	57	°C

### 2.6. Analytical methods

Glucose and xylose quantifications were performed by ion exchange chromatography using HPAEC-PAD (Thermo Scientific (Dionex) ICS-5000), CarboPac® PA-1 anion exchange column, at 25 °C. Diluents prepared with ultra pure water 18 MΩ and degassed with N<sub>2</sub>. The flow rate was 1.0 mL/min with solvents A (ultra pure water) and B (NaOH 500 mmol L<sup>-1</sup>), using isocratic elution with 4.8% B and 95.2% A for 25 minutes. Total phenolic compounds analysis

was performed using the Folin Ciocalteu method (Singleton et al., 1999) from the analytical curve of gallic acid. The quantification of organic acids (ferulic, syring and vanillic acids) and furan aldehydes (furfural and hydroxymethylfurfural) on the hydrolysates obtained from saccharification experiments was performed by high performance liquid chromatography (HPLC). Samples were centrifuged at 10000 xg during 15min and the supernatants were filtered using 22 µm membrane. Then, 20 µL were applied to the Agilent Tec. Model 1220 Infinity LC, with binary pump, automatic sampler, column oven and UV/VIS detector. The column used was C18 ZORBAX Eclipse Plus (4.6x250 mm), maintained at 25 °C. Chromatographic separation was performed using the mobile phase methanol:acetic:water (10:2:88 v/v) as the solvent A and methanol:acetic:water (90:2:8 v/v) as solvent B (Rodríguez-Delgado et al., 2001).

Ethanol concentration was determined by Gas Chromatograph (GC) Clarus 480 (PerkinElmer) with flame ionization detector (FID) and Agilent BD-EN14103 (30 m × 0.32 mm × 0.25 µm) column. Nitrogen was utilized as a carrier gas, at flow rate of 30 ml min<sup>-1</sup>, 100 of split, detector and injection temperature 250 °C. Oven temperature program was initial temperature at 40 °C, heating 15 °C min<sup>-1</sup> until 150 °C. Samples were prepared by a Headspace autosampler TurboMatrix 40 Trap (PerkinElmer). The incubation time was 10 min, at 60 °C, transfer line at 120 °C with a injection volume of 1.0 µL.

## **2.7. Alcoholic fermentation**

Alcoholic fermentation experiments were performed according de Cassia Pereira et al. (2016). *Saccharomyces cerevisiae* CAT-1 was precultured on YEPD solid medium composed by glucose (20 g L<sup>-1</sup>), agar (20 g L<sup>-1</sup>), yeast extract (5 g L<sup>-1</sup>) and peptone (10 g L<sup>-1</sup>). This medium was also used for culture stock, maintained at 4 °C. To prepare the inoculums, *S. cerevisiae* CAT-1T was grown in 250 mL Erlenmeyer flasks filled with 100 mL of nutrient media composed by glucose (30 g L<sup>-1</sup>), peptone (20 g L<sup>-1</sup>) and yeast extract (10 g L<sup>-1</sup>), in an orbital shaker (200 rpm), at 30 °C, for 24 h. Biomass was collected by centrifugation (9000 rpm, during 10 minutes, at 4 °C) and suspended in 0.9% NaCl solution to achieve a concentration of 200 mg L<sup>-1</sup> of fresh yeast. Alcoholic fermentation experiments were carried out in 100 mL Erlenmeyer flasks using

the hydrolysate obtained under the best saccharification condition (item 2.5.2.) and inoculated with 5 mg cells L<sup>-1</sup> (final volume of 40 mL), at 30 °C, under 150 rpm and anaerobic conditions, for 48 hours. All the experiments were performed as duplicates.

Ethanol yield was calculated according to de Cassia Pereira et al. (2016), based on the theoretical maximum:

$$(5) \text{ Yield (\%)} = [\text{ethanol content (g.L}^{-1}\text{)} / \text{glucose content (g.L}^{-1}\text{)} \times 0,511] \times 100$$

### 3. Results and discussion

#### 3.1. Enzymes production by solid-state fermentation

##### 3.1.1 Influence of lignocellulosic substrates

*P. sanguineus* MCA 16 was initially cultured by SSF on different mixtures of lignocellulosic materials as substrates and the activities of cellulases, xylanases and ligninases were determined in the crude enzyme solutions (Tables 3 and 4).

Table 3. Cellulases and xylanases activities in the crude enzyme solutions obtained by cultivation of *P. sanguineus* MCA 16 on different mixtures (1:1 w/w) of lignocellulosic substrates by SSF, for 96 hours, at 40 °C. SB: sugarcane bagasse; WB: wheat bran; RH: rice husk; SM: soybean meal.

Substrates (1:1 w/w)	Enzymatic activities (U g <sup>-1</sup> )				
	Endoglucanase	Exoglucanase	β- glucosidase	Xylanase	β- xylosidase
<b>SB+WB</b>	9.89±0.17	32.85±0.18	1.66±0.32	5.96±0.22	0.38±0.08
<b>SB+SM</b>	125.63±0.21	43.03±0.14	37.54±0.33	44.20±0.13	1.00±0.02
<b>SB</b>	5.85±0.32	30.04±0.23	2.39±0.21	7.18±0.19	0.41±0.03
<b>RH</b>	9.46±0.22	37.76±0.22	0.18±0.15	9.07±0.19	0.25±0.02
<b>RH+WB</b>	13.21±0.13	36.03±0.12	5.57±0.24	16.73±0.18	0.72±0.12
<b>RH+SM</b>	122.74±0.24	40.87±0.35	17.55±0.22	44.91±0.22	0.96±0.03
<b>WB+SM</b>	129.96±0.33	44.26±0.21	50.47±0.14	40.58±0.21	1.81±0.07
<b>SB+RH</b>	8.95±0.21	37.04±0.22	2.02±0.11	12.31±0.13	0.46±0.05

Table 4. Ligninases activities in the crude enzyme solutions obtained by cultivation of *P. sanguineus* MCA 16 different mixtures (1:1 w/w) of lignocellulosic substrates by SSF, for 96 hours, at 40 °C. SB: sugarcane bagasse; WB: wheat bran; RH: rice husk; SM: soybean meal; MnP: manganese peroxidase; LiP: lignin peroxidase; nd: not detected.

Substrates (1:1 w/w)	Enzymatic activities (U g <sup>-1</sup> )		
	Lacase	MnP	LiP
<b>SB+WB</b>	19.63±0.24	0.29±0.05	2.87±0.09
<b>SB+SM</b>	12.69±0.22	0.25±0.02	2.47±0.08
<b>SB</b>	20.15±0.31	0.81±0.02	8.15±0.08
<b>RH</b>	11.79±0.14	Nd	Nd
<b>RH+WB</b>	26.47±0.16	0.27±0.05	2.67±0.07
<b>RH+SM</b>	14.84±0.24	1.31±0.07	13.14±0.13
<b>WB+SM</b>	22.04±0.33	8.92±0.11	9.16±0.09
<b>SB+RH</b>	23.82±0.24	nd	Nd

Cellulases and xylanases activities were higher in the crude enzyme solutions obtained by fungus cultivation on mixtures containing soybean meal. The highest cellulases activities were obtained when the mixture of wheat bran and soybean meal was used as substrates for SSF. Cultivation on this substrates mixture also provided good xylanases activities in the enzymatic extracts (Table 4). *P. sanguineus* MCA 16 was versatile in relation to the production of laccase, since high activities were observed in the crude enzyme solutions obtained by the fungus cultivation on all the evaluated substrates mixtures. The highest laccase activity was obtained when the mixture of rice husk and wheat bran was used as substrate for SSF. Lignin peroxidase higher activities were observed in the crude enzyme solutions obtained from fungus cultivation on rice husk with soybean meal, wheat bran with soybean meal or on sugarcane bagasse as the sole lignocellulosic substrate. Regarding manganese peroxidase activity, only the mixture wheat bran with soybean meal induced the enzyme production (Table 4).

The production of cellulose, hemicellulose and/or lignin-depolymerizing enzymes by microorganisms has been frequently reported in the literature. However, there are a few works regarding to cellulases and hemicellulases

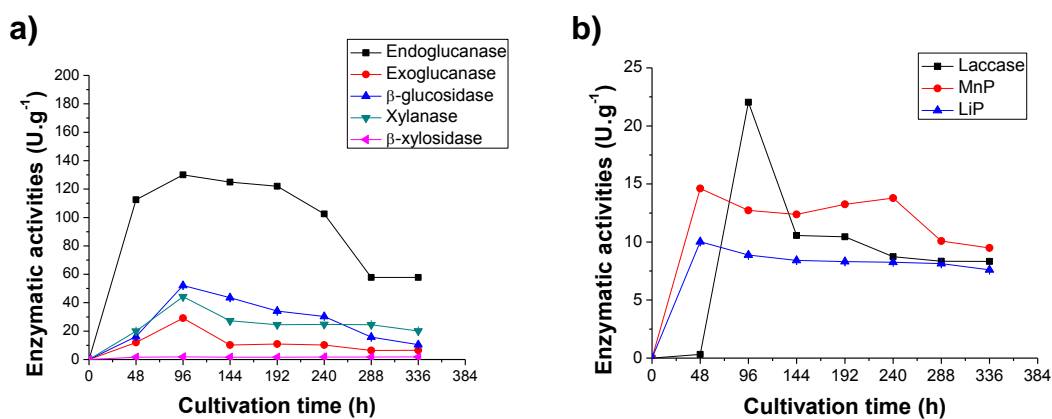
production by *P. sanguineus* (Falkoski and Guimarães, 2012; Yoon et al., 2013a). Works about this and other basidiomycetes have mainly focused on the study of ligninases (CAÑAS; CAMARERO, 2010; CHI et al., 2007; FALKOSKI; GUIMARÃES, 2012; GOMES et al., 2009; JAOUANI et al., 2005; KIRKPATRICK; PALMER, 1989; VIKINESWARY et al., 2006; YOON et al., 2013b; YOUN et al., 1995; ZIMBARDI et al., 2016).

Since the highest activities of the evaluated hydrolytic and oxidative enzymes were, in general, obtained when *P. sanguineus* MC 16 was cultivated on wheat bran and soybean meal, the enzymatic extract produced using this mixture as substrates was chosen for the subsequent experiments.

### 3.1.2. Influence of cultivation time on the enzymes production

The highest activities of endoglucanase (200 U/g), exoglucanase (29.1 U.g<sup>-1</sup>),  $\beta$ -glucosidase (52.1 U.g<sup>-1</sup>), xylanase (44.2 U.g<sup>-1</sup>),  $\beta$ -xylosidase (1.81 U.g<sup>-1</sup>) and laccase (22.04 U.g<sup>-1</sup>) were obtained after 96 hours of cultivation. Peroxidases higher activities were observed in 48 hours (14.61 and 10.02 U.g<sup>-1</sup> for manganese and lignin peroxidase, respectively). Based on these results, 96 hours was fixed as the cultivation time of SSF in the further experiments.

Figure 1. Time course of cellulases, xylanases (a) and ligninases (b) production by *P. sanguineus* MCA 16 cultivated by SSF on mixture (1:1 w/w) of wheat bran and soybean meal, at 40 °C.



### **3.2. Enzymatic saccharification of pretreated sugarcane bagasse**

*P. sanguineus* MCA 16 was cultivated, by SSF, using the mixture (1:1 w/w) of wheat bran and soybean meal, during 96 hours, at 40 °C and the enzymatic extract was used to saccharify pretreated sugarcane bagasse. Saccharification experiments were performed as described in item 2.5. Glucose and xylose concentrations in the hydrolysate were 3.19 g.L<sup>-1</sup> (yield of 17 %) and 2.12 g.L<sup>-1</sup> (yield of 47 %), respectively. The associated use of oxidative and hydrolytic microbial enzymes is still a novelty in processes of saccharification of lignocellulosic biomass for second generation ethanol production. Thus, the prospection of new strains producers of enzymatic cocktails whose components act in interaction to degrade lignin, cellulose and hemicellulose is necessary and interesting, since the removal of lignin facilitates the access of cellulases and xylanases to their substrates. In this context, basidiomycetes constitute an interesting group of fungi, since they produce ligninases, cellulases and xylanases (Kachlishvili et al., 2012; Navarro et al., 2014.). Although *P. sanguineus* strains have been extensively studied in relation to the production of ligninases (Petre et al., 2014; Quiroz-Castañeda et al., 2011; Vikineswary et al., 2006), the use of their enzymatic extracts for lignocellulosic substrates saccharification is still scarce in scientific literature. Falkoski and Guimarães, (2012) carried out saccharification of the sugarcane bagasse submitted to alkaline pre-treatment (NaOH 1.0%) using the enzymatic extract obtained from *P. sanguineus* PF-2 on submerged cultivation using corn cob as lignocellulosic substrate. Saccharification was performed with enzymatic loading of 10 FPU/g, 10% of dry solids, at 50 °C, for 72 hours, and a glucose yield of 22.6% was obtained.

#### **3.2.1. Influence of solid and enzyme loads**

The influence of pretreated sugarcane bagasse and endoglucanase loads was evaluated by a rotational central composite design (RCCD) (2<sup>2</sup>). Experimental runs and the response regarding glucose concentration on the hydrolysates are shown in Table 5. Xylose concentrations were determined as additional data.



Table 5. Rotational Central Composite Design (RCCD) model used in the enzymatic saccharification experiments of pre-treated sugarcane bagasse.

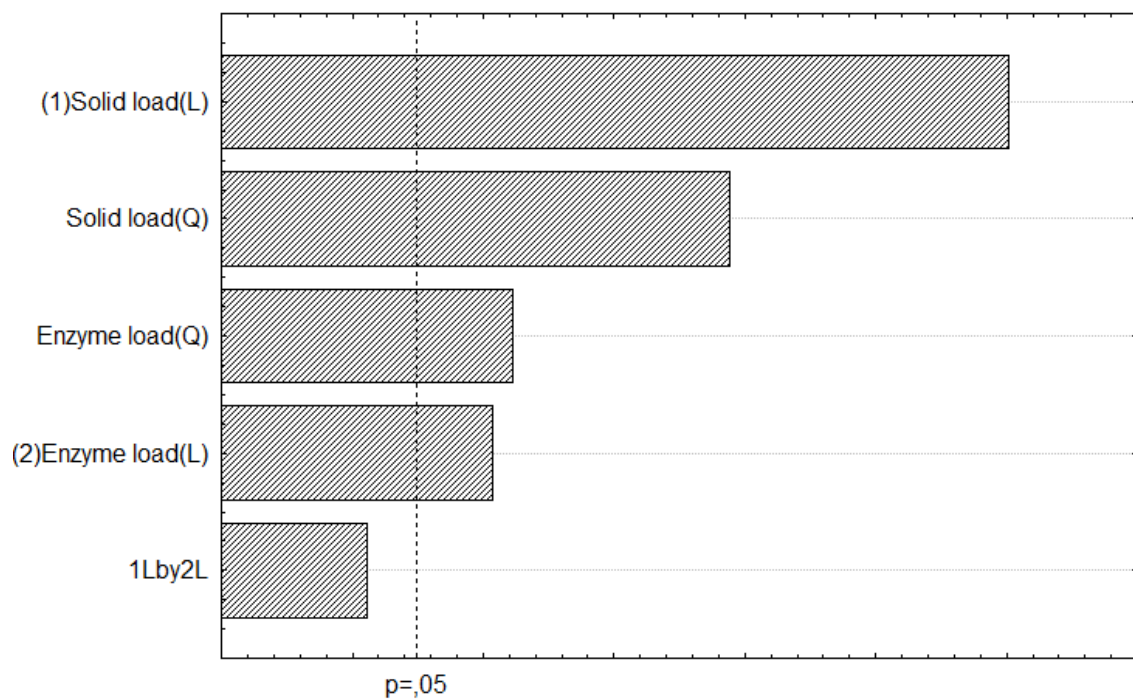
Assays	X <sub>1</sub>	X <sub>2</sub>	Y <sub>1</sub>	Y <sub>2</sub>
1	-1 (5)	-1 (220)	3.63	1.68
2	1 (15)	-1 (220)	5.67	4.66
3	-1 (5)	1 (300)	4.25	2.3
4	1 (15)	1 (300)	6.17	4.74
5	-1.41 (2.93)	0 (260)	2.98	1.55
6	1.41 (17.07)	0 (260)	6.2	4.25
7	0 (10)	-1.41 (203.43)	5.13	4.68
8	0 (10)	1.41 (316.57)	5.62	4.77
9	0 (10)	0 (260)	5.93	4.27
10	0 (10)	0 (260)	6	4.28
11	0 (10)	0 (260)	5.95	4.15
12	0 (10)	0 (260)	5.85	4.27

X<sub>1</sub>: sugarcane bagasse load (% of dry solid); x<sub>2</sub>: enzyme loading (units of endoglucanase per grama of cellulose); Y<sub>1</sub>: glucose (g.L<sup>-1</sup>); Y<sub>2</sub>: xylose (g.L<sup>-1</sup>).

Glucose and xylose concentrations in the hydrolysates varied from 2.98 to 6.20 g.L<sup>-1</sup> and 1.55 to 4.77 g.L<sup>-1</sup>, respectively. The highest glucose concentration (6.20 g.L<sup>-1</sup>) was obtained when 17.07 % and 260 total units of endoglucanase/g cellulose were used as solid and enzyme loads, respectively (assay 6, Table 5). This value corresponds to yields of 72.90 mg of glucose/g of cellulose and 45.93 mg of glucose/g of dry pretreated sugarcane bagasse.

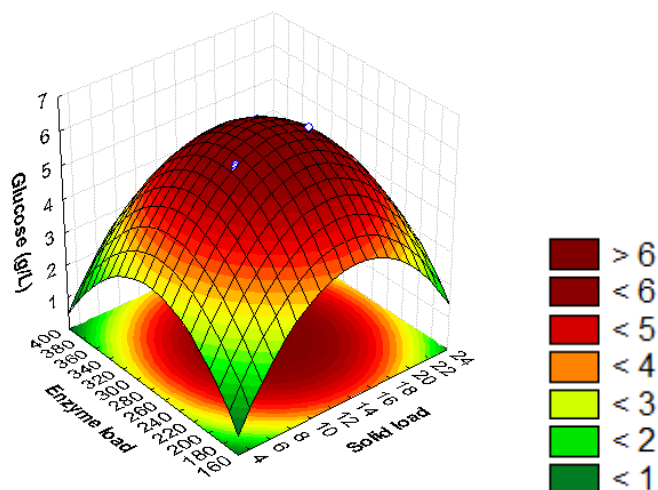
The estimated quantitative effect of each variable and its interactions on glucose concentration are presented by the Pareto Diagram (Figure 2). It can be observed that the variables evaluated were significant at the 95% confidence level. However, the interaction between them had no significant effect.

Figure 2. Pareto chart of effects of the variables solid and enzyme loads.



The response surface generated for glucose concentration (Figure 3) shows that the highest concentrations of this sugar are obtained when enzyme load is at the lower levels (below 260 units of endoglucanase) and pretreated bagasse load is between 8.0 and 16.0 %.

Figure 3 Response surface using pretreated bagasse and enzyme loads as variables in the enzymatic saccharification of sugarcane bagasse by the extract produced by *P. sanguineus fungus* MCA 16.



The desirability function given by the software indicated that higher glucose concentrations would be obtained using pretreated bagasse and endoglucanase loads of 13.5 % and 288.2 total units/g of cellulose, respectively. Under these conditions, the predicted maximum glucose concentration was 6.33 g.L<sup>-1</sup>. The validation experiment was performed in triplicate and the experimental result was 6.53 ± 0.22 g.L<sup>-1</sup>. Under these conditions, glucose yield obtained was 48.37 mg/g of pre-treated bagasse or 76.78 mg/g of cellulose. Xylose concentration in the hydrolysate was 5.48 ± 0.18 g.L<sup>-1</sup>, which represents yields of 40.59 mg/g of pre-treated sugarcane bagasse or 238.77 mg/g of xylan.

De Cassia Pereira et al. (2016) and Modenbach and Nokes (2013) reported that the increase of pre-treated sugarcane bagasse load in enzymatic saccharification experiments led to higher glucose concentrations. Qin et al. (2016) studied the influence of enzyme and substrate loads on enzymatic saccharification of avicel PH-101 (commercial substrate with 98% of glucan and 2% of xylan as a component) and demonstrated that high concentrations of solids load increased the interaction of the enzyme with substrate. According to these authors, the higher the enzyme-substrate complex formation, the lower the interaction of the enzyme with possible inhibitors present in the medium, which contributes to a more efficient saccharification.

### 3.2.2. Influence of time and temperature

A rotational central composite design (RCCD) ( $2^2$ ) was used to evaluate the influence of temperature and time on glucose concentration in the hydrolysates obtained from saccharification experiments. The real and encoded levels of the variables, the experimental runs and the response evaluated (glucose concentration) are shown in Table 6. Xylose concentrations in the hydrolysates were determined as additional data. Bagasse and enzyme loads used were 13.5 % and 288.3 U of endoglucanase/g cellulose, respectively.

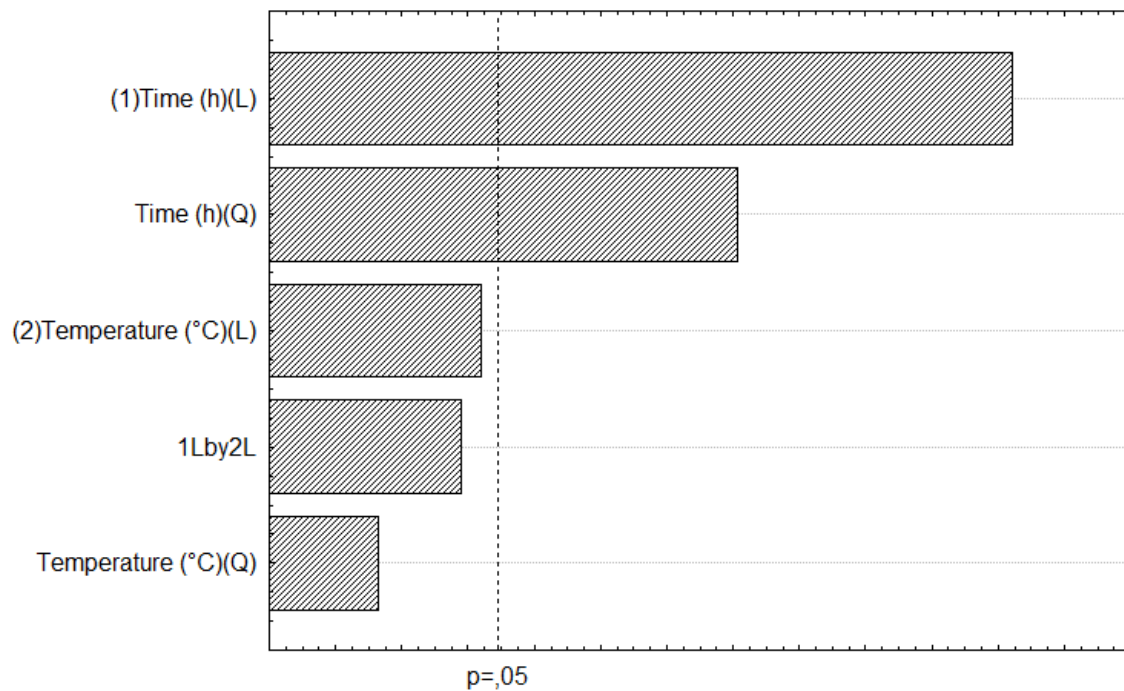
**Table 6.** Rotational Central Composite Design (RCDD) model used in the enzymatic saccharification experiments of pre-treated sugarcane bagasse. Enzymatic saccharification at 50 °C for 24 h using *P. sanguineus* MCA 16 enzymatic obtained by SSF at 40 °C during 96h.

Assays	X <sub>3</sub>	X <sub>4</sub>	Y <sub>1</sub>	Y <sub>2</sub>
1	-1 (24)	-1 (45)	3.42	2.87
2	1 (120)	-1 (45)	7.24	6.08
3	-1 (24)	1 (55)	5.23	4.61
4	1 (120)	1 (55)	7.23	6.74
5	-1.41 (4.12)	0 (50)	1.18	1.96
6	1.41 (139.88)	0 (50)	6.82	5.98
7	0 (72)	-1.41 (42.93)	6.12	4.12
8	0 (72)	1.41 (57.07)	6.94	5.56
9	0 (72)	0 (50)	6.80	6.17
10	0 (72)	0 (50)	6.43	5.88
11	0 (72)	0 (50)	6.71	6.24
12	0 (72)	0 (50)	6.23	6.68

X<sub>3</sub>: time (h) ; X<sub>4</sub>: temperature (°C); Y<sub>1</sub>: glucose (g/L); Y<sub>2</sub>: xylose (g/L).

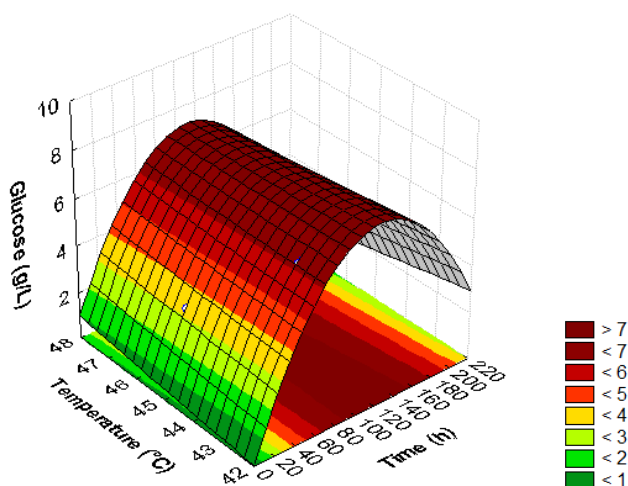
The highest glucose concentration (7.23 g.L<sup>-1</sup>) was obtained under saccharification condition of 144 hours and 55 °C (assay 4). According to the Pareto Diagram, only the variable time was significant at the 95% confidence level (p value <0.05) (Figure 4).

Figure 4. Pareto chart of effects of the variables time and temperature.



The response surface generated is presented in Figure 5 and indicates that the variable time was optimized, with the highest concentrations of glucose being obtained at 120 hours. On the other hand, it was not possible to optimize the temperature variable within the studied range. The response surface indicates that high temperatures led to increase in glucose concentration.

Figure 5. Response surface using time and temperature as variables in the enzymatic saccharification of sugarcane bagasse by the extract produced by *P. sanguineus fungus* MCA 16.



A desirability function was given by the Software and predicted that highest glucose concentration ( $7.49 \text{ g.L}^{-1}$ ) would be obtained at 130 hours and  $57.07 \text{ }^{\circ}\text{C}$ . The validation experiment, performed in triplicate at  $57 \text{ }^{\circ}\text{C}$ , resulted in a glucose concentration of  $7.32 \pm 0.36 \text{ g.L}^{-1}$ , corresponding to the yields of  $54.22 \text{ mg/g}$  pre-treated bagasse or  $86.07 \text{ mg/g}$  of cellulose. The concentration of xylose in this hydrolyzate was  $5.89 \pm 0.27 \text{ g.L}^{-1}$ , which represents yields of  $43.63 \text{ mg/g}$  of pre-treated sugarcane bagasse or  $256.64 \text{ mg/g}$  of xylan.

From the results obtained with the experimental designs it was possible to define the best saccharification conditions, considering glucose concentration as the analysed response: 13.5 % (dry basis) of sugarcane bagasse load, 288.3 units of endoglucanase/g cellulose, 130 hours, at  $57^{\circ}\text{C}$ .

Glucose yields obtained in the present work are higher than some reported in the literature which also use non-commercial enzymes. Moretti et al. (2014) reported a glucose concentration of  $23.4 \text{ mg/g}$  of pretreated bagasse when the enzymatic extract of *Myceliophthora thermophila* M7.7 was used to saccharify sugarcane bagasse submitted to a pretreatment with microwaves and glycerol.

### **3.3. Comparative enzymatic saccharifications using filter paper or xylan as substrates**

For comparative purposes, saccharification experiments were carried out under the best conditions (item 3.2.2) using filter paper or xylan as a substrate. The results obtained in terms of glucose and xylose concentrations in the hydrolysates, respectively, were considered as the maximum theoretical for saccharification by the cellulases or xylanases present in the enzymatic extract, since in these experiments there is no other component to which these enzymes could adsorb. Glucose and xylose concentrations obtained were 14.0 and 10.73 g.L<sup>-1</sup>, respectively. Considering these results as 100 % of saccharification, the highest glucose concentration obtained under optimized conditions (7.32 g.L<sup>-1</sup>) corresponds to 52.28 % of the maximum theoretical. Regarding xylose highest concentration obtained under optimized conditions (5.89 g.L<sup>-1</sup>) xylanases in the enzymatic extract led to 54.89 % of the maximum theoretical saccharification.

Most of the works about pretreated sugarcane bagasse (or other lignocellulosic biomass) available in the literature which aim at the use of hydrolysates for cellulosic ethanol report the use of commercial microbial enzymes (PERRONE et al., 2017, 2016; TOQUERO; BOLADO, 2014; TRAVAINI et al., 2015, 2013). The enzymes loads used in such studies are based on the activity of total cellulases (FPase). Therefore, it is difficult to establish comparisons between the literature data and the results obtained in the present work in terms of glucose concentration in the hydrolysates. Thus, studies involving enzymatic extracts obtained from new microbial strains are of great importance, since enzymes with interesting characteristics and as efficient as the commercial enzyme cocktails can be obtained at low cost (DE CASSIA PEREIRA et al., 2016).

### **3.3. Analysis of total phenolic compounds (TPC)**

Since the enzymatic extract obtained from *P. sanguineus* MCA 16 cultivation on wheat bran and soybean meal presented ligninases activities, the concentration of total phenolic compounds (TPC) generated by the action of these enzymes on the substrates was determined in this extract and it was 5.9 g.L<sup>-1</sup>.

TPC concentration was also determined in the hydrolysates obtained from saccharification of pretreated sugarcane bagasse performed under the best condition (item 3.2.2), since residual lignin present in the pretreated bagasse can be degraded in phenolic compounds by the action of ligninases in the enzymatic extract. TPC concentrations in the enzymatic extract and in the hydrolysate were 5.9 and 1.04 g L<sup>-1</sup>, respectively, indicating a reduction of 82.3% resulting from ligninases action during the saccharification experiments.

### **3.4. Analysis of fermentation inhibitors**

Under the established saccharification conditions (288.3 total units of endoglucanase/g cellulose in pretreated bagasse; 13.5% of pretreated sugarcane bagasse load, 57 °C and 130 hours), low amounts of organic acids derived from lignin were released as vanillic (0.16 mg.ml<sup>-1</sup> or 0.95 mmol.L<sup>-1</sup>), syringic (0.03 mg.ml<sup>-1</sup> or 0.16 mmol.L<sup>-1</sup>) and ferulic (0.046 mg.ml<sup>-1</sup> or 0.23 mmol.L<sup>-1</sup>). Regarding furan aldehydes derived from the degradation of glucose and xylose during saccharification, only 5-hydroxymethylfurfural (HMF) at 0.048 mg.ml<sup>-1</sup> (0.38 mmol.L<sup>-1</sup>) was detected. These concentrations of fermentation inhibitors in the hydrolysates are lower than those that interfere with the metabolism of yeast *Saccharomyces cerevisiae* in alcoholic fermentation processes. Ferulic acid at concentrations up to 1.8 mM acid does not interfere with fermentative metabolism, according to (Adeboye et al., 2015). Ando et al. (1986) reported that *S. cerevisiae* do not present toxicity of vanillic acid at concentrations below 1 mg ml<sup>-1</sup>. Furan aldehydes can be metabolized by *S. cerevisiae* decreasing the plasma membrane permeability and slowing the cell growth, thus extending the lag phase. However, this effect under anaerobic conditions is reduced (Palmqvist and Hahn-Hagerdal, 2000). Hawkins and Doran-Peterson (2011) reported that *S. cerevisiae* presented normal growth at concentrations of HMF until 2.15mg.ml<sup>-1</sup>.

### **3.5. Alcoholic fermentation**

Alcoholic fermentation experiments were performed using the hydrolysate obtained under the best saccharification conditions (item 3.2.2). The experiment provided 10 g ± 0.24 of ethanol from 8,8g of glucose



consumption (73.5 % of the maximum theoretical conversion). This result shows that the hydrolysate used in this study is viable for use in the production of cellulosic ethanol production. Wanderley et al. (2013) obtained 46.47% on ethanol efficiency using hydrolysates from commercial enzymes saccharification. The hydrolyzate from the crude enzyme extract of *Myceliophthora thermophila* JCP 1-4 obtained 63% of ethanol efficiency (De Cassia Pereira et al., 2016).

#### **4.Conclusions**

*P. sanguineus* MCA 16 is a versatile fungus on growth and enzymes production on relative short time by solid-state fermentation using agro residues as substrates. The enzymatic extract was efficient to saccharify sugarcane bagasse with alkaline hydrothermal pretreatment. The statistical experimental tools used to find out the physical and chemical conditions those are best for glucose release were efficient with 129% of increased compared to the first saccharification experiment. The hydrolyzed obtained in the last saccharification was suitable to ethanol production and no inhibitors compounds were presented with significant concentration.

#### **5.Aknowledgements**

The authors thank to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support, to the State University of São Paulo, to the Laboratory of Biochemistry and Applied Microbiology and to the Laboratory of Sucrochemistry and Analytical Chemistry of Institute of Biosciences, Letters and Exact Sciences, São José do Rio Preto and to the Laboratory of Microbial Enzymes of the Institute of Chemistry, Araraquara, São Paulo, Brazil.

## 6. References

ABRAHÃO, M.C., GUGLIOTTA, A.D.M., SILVA, R., JOANNE, R., FUJIEDA, Y. Ligninolytic activity from newly isolated basidiomycete strains and effect of these enzymes on the azo dye orange II decolourisation. **Ann. Microbiol.** 58, 427-432, 2008.

ADEBOYE, P.T., BETTIGA, M., ALDAEUS, F., LARSSON, P.T., OLSSON, L. Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by *Saccharomyces cerevisiae* yields less toxic products. **Microb. Cell Fact.** 1–14, 2015.

ANDO, S., ARAI, I., KIYOTO, K. Identification of Aromatic Monomers in Steam-Exploded Poplar and Their Influences on Ethanol Fermentation by *Saccharomyces cerevisiae*. **J. Ferment. Technol.** 64, 567–570, 1986.

BEHERA, S.S., RAY, R.C. Solid State Fermentation for Production of Microbial Cellulases: Recent Advances and Improvement Strategies. *Int. J. Biol. Macromol.* 86, 656–669, 2015.

BOCCHINI-MARTINS, D.A., ALVES-PRADO, H.F., LEITE, R.S.R., MORETTI, M.M.S., DA-SILVA, R., GOMES, E. Agroindustrial Wastes as Substrates for Microbial Enzymes Production and Source of Sugar for Bioethanol Production, in: Integrate Waste Management - Volume II. Mr Sunil Kumar. pp. 319–361, 2011.

BONUGLI-SANTOS, R.C., VASCONCELOS, M.R. DOS S., PASSARINI, M.R.Z., VIEIRA, G.A.L., LOPES, V.C.P., MAINARDI, P.H., SANTOS, J.A. DOS, DUARTE, L. DE A., OTERO, I.V.R., YOSHIDA, A.M. DA S., FEITOSA, V.A., PESSOA, A., SETTE, L.D. Marine-derived fungi: Diversity of enzymes and biotechnological applications. **Front. Microbiol.** 6, 2015.

BOURBONNAIS, R., PAICE, M.G. Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju*. **Biochem. J.** 255, 445–450, 1988.

BUSWELL, J. A, CAI, Y., CHANG, S. Effect of nutrient nitrogen and manganese on manganese peroxidase and lactase production by *Lentinula* (*Lentinus*) *edodes*. **FEMS Microbiol. Lett.** 128, 81–87, 1995.

CAÑAS, A.I., CAMARERO, S. Laccases and their natural mediators: Biotechnological tools for sustainable eco-friendly processes. **Biotechnol. Adv.** 28, 694–705, 2010.

CHI, Y., HATAKKA, A., MAIJALA, P. Can co-culturing of two white-rot fungi increase lignin degradation and the production of lignin-degrading enzymes? **Int. Biodeterior. Biodegradation** 59, 32–39, 2007.

CHRISTOV, L., SZAKACS, G., BALAKRISHNAN, H. Production, partial characterization and use of fungal cellulase-free xylanases in pulp bleaching. **Process Biochem.** 34, 511–517, 1999.

DE CASSIA PEREIRA, J., LEITE, R.S.R., DO PRADO, H.F.A., BOCCHINI MARTINS, D.A., GOMES, E., DA SILVA, R. Production and Characterization of  $\beta$ -glucosidase Obtained by the Solid-State Cultivation of the Thermophilic Fungus *Thermomucor indicae-seudaticae* N31. **Appl. Biochem. Biotechnol.** 175, 723–732, 2014

DE CASSIA PEREIRA, J., PAGANINI MARQUES, N., RODRIGUES, A., BRITO DE OLIVEIRA, T., BOSCOLO, M., DA SILVA, R., GOMES, E., BOCCHINI MARTINS, D.A. Thermophilic fungi as new sources for production of cellulases and xylanases with potential use in sugarcane bagasse saccharification. **J. Appl. Microbiol.** 1–12, 2015.

DE CASSIA PEREIRA, J., TRAVAINI, R., PAGANINI MARQUES, N., BOLADO-RODRÍGUEZ, S., BOCCHINI MARTINS, D.A. Saccharification of ozonated sugarcane bagasse using enzymes from *Myceliophthora thermophila* JCP 1-4 for sugars release and ethanol production. **Bioresour. Technol.** 204, 122–129, 2016.

FALKOSKI, D.L., GUIMARÃES, V.M. Characterization of Cellulolytic Extract from *Pycnoporus sanguineus* PF-2 and Its Application in Biomass Saccharification. **Appl. Biochem. Biotechnol.** 166, 1586–1603, 2012.

FARINAS, C.S. Developments in solid-state fermentation for the production of biomass-degrading enzymes for the bioenergy sector. Review. **Sustain. Energy Rev.** 52, 179–188, 2015.

GAO, J., WENG, H., XI, Y., Zhu, D., Han, S. Purification and characterization of a novel endo-beta-1,4-glucanase from the thermoacidophilic *Aspergillus terreus*. **Biotechnol. Lett.** 30, 323–7, 2008a

GAO, J., WENG, H., ZHU, D., YUAN, M., GUAN, F., XI, Y. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. **Bioresour. Technol.** 99, 7623–7629, 2008b.

GLENN, J.K., AKILESWARAN, L., GOLD, M.H. Mn (II) Oxidation Is the Principal Function of the Extracellular Mn-Peroxidase from *Phanerochaete chrysosporium*. **Arch. Biochem. Biophys.** 251, 688–696, 1999.

GOMES, E., AGUIAR, A.P., CARVALHO, C.C., BONFÁ, M.R.B., SILVA, R., BOSCOLO, M. Ligninases production by basidiomycetes strains on lignocellulosic agricultural residues and their application in the decolorization of synthetic dyes. **Brazilian J. Microbiol.** 40, 31–39, 2009.

GOWTHAMAN, M.K., KRISHNA, C., MOO-YOUNG, M. Fungal solid state fermentation- an overview. **Appl. Mycol. Biotechnol.** 1, 305–352, 2001.

HABIBI, Y., LUCIA, L.A., ROJAS, O.J. Cellulose Nanocrystals: Chemistry, Self-Assembly and Applications. **Chem. Rev.**, 3479–3500, 2009.

HAWKINS, G.M., DORAN-PETERSON, J. A strain of *Saccharomyces cerevisiae* evolved for fermentation of lignocellulosic biomass displays improved growth and fermentative ability in high solids concentrations and in the presence of inhibitory compounds. **Biotechnol. Biofuels** 1–14, 2011.

JAOUANI, A., GUILLÉN, F., PENNINGCKX, M.J., MARTÍNEZ, A.T., MARTÍNEZ, M.J. Role of *Pycnoporus coccineus* laccase in the degradation of aromatic compounds in olive oil mill wastewater. **Enzyme Microb. Technol.** 36, 478–486, 2005.

KACHLISHVILI, E., KHARDZIANI, T., METREVELI, E., KOBAKHIDZE, A., ELISASHVILI, V. Screening of Novel Basidiomycetes for the Production of Lignocellulolytic Enzymes During Fermentation of Food Wastes. **Journal of Waste Conversion, Bioproducts and Biotechnology** 1, 9–15, 2012.

KIRKPATRICK, N., PALMER, J.M. A natural inhibitor of lignin peroxidase activity from *Phanerochaete chrysosporium*, active at low pH and inactivated by divalent metal ions. **Appl. Microbiol. Biotechnol.** 30, 305–311, 1989.

KRISHNA, C. Solid-State Fermentation Systems—An Overview. **Crit. Rev. Biotechnol.** 25, 1–30, 2005.

LEITE, R.S.R., ALVES-PRADO, H.F., CABRAL, H., PAGNOCCA, F.C., GOMES, E., DA-SILVA, R. Production and characteristics comparison of crude  $\beta$ -glucosidases produced by microorganisms *Thermoascus aurantiacus* e *Aureobasidium pullulans* in agricultural wastes. **Enzyme Microb. Technol.** 43, 391–395, 2008.

LETTERA, V., DEL VECCHIO, C., PISCITELLI, A., SANNIA, G. Low impact strategies to improve ligninolytic enzyme production in filamentous fungi: The case of laccase in *Pleurotus ostreatus*. **Comptes Rendus - Biol.** 334, 781–788, 2011.

MENON, V., RAO, M. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. **Prog. Energy Combust. Sci.** 38, 522–550, 2012.

MODENBACH, A. A., NOKES, S.E. Enzymatic hydrolysis of biomass at high-solids loadings - A review. **Biomass and Bioenergy** 56, 526–544, 2013.

MORETTI, M.M. DE S., BOCCHINI-MARTINS, D.A., NUNES, C. DA C.C., VILLENA, M.A., PERRONE, O.M., SILVA, R. DA, BOSCOLO, M., GOMES, E. Pretreatment of sugarcane bagasse with microwaves irradiation and its effects on the structure and on enzymatic hydrolysis. **Appl. Energy** 122, 189–195, 2014.

MORETTI, M.M.S., BOCCHINI-MARTINS, D.A., DA-SILVA, R., RODRIGUES, A., SETTE, L.D., GOMES, E. Selection of thermophilic and thermotolerant fungi for the production of cellulases and xylanases under solid-state fermentation. **Brazilian J. Microbiol.** 1062–1071, 2012.

MTUI, G.Y.S. Lignocellulolytic enzymes from tropical fungi: Types, substrates and applications. **Sci. Res. Essays** 7, 1544–1555, 2012.

NAVARRO, D., ROSSO, M., HAON, M., OLIVÉ, C., BONNIN, E., LESAGE-MEESSEN, L., CHEVRET, D., COUTINHO, P.M., HENRISSAT, B., BERRIN, J. Fast solubilization of recalcitrant cellulosic biomass by the basidiomycete fungus *Laetisaria arvalis* involves successive secretion of oxidative and hydrolytic enzymes. **Biotechnol. Biofuels** 7, 1–14, 2014.

RODRIGUES, P. O., PEREIRA, J.C., SANTOS, D.Q., GURGEL, L.V.A., PASQUINI, D., BAFFI, M.A. Synergistic action of an *Aspergillus* (hemi-)cellulolytic consortium on sugarcane bagasse saccharification. **Ind. Crop. Prod.** 109, 173–181, 2017.

PALMQVIST, E., HAHN-HAGERDAL, B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. **Bioresour. Technol.** 74, 25–33, 2000.

PERRONE, O.M., COLOMBARI, F.M., ROSSI, J.S., MARIA, M., MORETTI, S., BORDIGNON, S.E., CARREIRA, C., GOMES, E., BOSCOLO, M., DA-SILVA, R. Ozonolysis combined with ultrasound as a pretreatment of sugarcane bagasse: Effect on the enzymatic saccharification and the physical and chemical characteristics of the substrate. **Bioresour. Technol.** 218, 69–76, 2016.

PERRONE, O.M., ROSSI, J.S., MARIA, M., MORETTI, D.S., CARREIRA, C., BORDIGNON, S.E., GOMES, E., DA-SILVA, R., BOSCOLO, M. Influence of ozonolysis time during sugarcane pretreatment: Effects on the fiber and enzymatic saccharification. **Bioresour. Technol.** 224, 733–737, 2017.

PETRE, C.-V., BALAES, T., TANASE, C. Lignicolous basidiomycetes as valuable biotechnological agents. **Mem. Sci. Sect. Rom. Acad.** Tome XXXVII, 2014.

QIN, L., LI, W.-C., LIU, L., ZHU, J.-Q., LI, X., LI, B.-Z., YUAN, Y.-J. Inhibition of lignin-derived phenolic compounds to cellulase. **Biotechnol. Biofuels** 9, 70, 2016

QUIROZ-CASTAÑEDA, R., PÉREZ-MEJÍA, N., MARTÍNEZ-ANAYA, C., ACOSTA-URDAPILLETA, L., FOLCH-MALLOL, J. Evaluation of different lignocellulosic substrates for the production of cellulases and xylanases by the basidiomycete fungi *Bjerkandera adusta* and *Pycnoporus sanguineus*. **Biodegradation** 565–572, 2011.

RODRÍGUEZ-DELGADO, M.A., MALOVANÁ, S., PÉREZ, J.P., BORGES, T., GARCÍA MONTELONGO, F.J. Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. **J. Chromatogr. A** 912, 249–257, 2011.

ROMANÍ, A., PEREIRA, F., JOHANSSON, B., DOMINGUES, L. Metabolic engineering of *Saccharomyces cerevisiae* ethanol strains PE-2 and CAT-1 for efficient lignocellulosic fermentation. **Bioresour. Technol.** 179, 150–158, 2015.

SAINI, A., AGGARWAL, N.K., SHARMA, A., YADAV, A. Actinomycetes: A Source of Lignocellulolytic Enzymes. **Enzyme Res.** 2015, 2015.

SÁNCHEZ, C. Lignocellulosic residues: biodegradation and bioconversion by fungi. **Biotechnol. Adv.** 27, 185–94, 2009.

SANTOS, C.R., PAIVA, J.H., SFORÇA, M.L., NEVES, J.L., NAVARRO, R.Z., COTA, J., AKAO, P.K., HOFFMAM, Z.B., MEZA, A.N., SMETANA, J.H., NOGUEIRA, M.L., POLIKARPOV, I., XAVIER- NETO, J., SQUINA, F.M., WARD, R.J., RULLER, R., ZERI, A.C., MURAKAMI, M.T., 2012. Dissecting structure-function-stability relationships of a thermostable GH5-CBM3 cellulase from *Bacillus subtilis* 168. *Biochem. J.* 441, 95–104. doi:10.1042/BJ20110869

SINGHANIA, R.R., SUKUMARAN, R.K., PATEL, A.K., LARROCHE, C., PANDEY, A. Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. **Enzyme Microb. Technol.** 46, 541–549, 2010.

SOARES, I.B., TRAVASSOS, J. A., BAUDEL, H.M., BENACHOUR, M., ABREU, C. A. M. Effects of washing, milling and loading enzymes on the enzymatic hydrolysis of a steam pretreated sugarcane bagasse. **Ind. Crops Prod.** 33, 670–675, 2011.

SØRENSEN, A., LÜBECK, M., LÜBECK, P.S., AHRING, B.K. Fungal Beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials. **Biomolecules** 3, 612–31, 2013.

TALEBNIA, F., KARAKASHEV, D., ANGELIDAKI, I. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. **Bioresour. Technol.** 101, 4744–53, 2010.

TIEN, M., KIRK, T.K. *Methods in enzymology*, vol. 161 Copyright © 1988 by Academic Press, Inc. All rights of reproduction in any form reserved. **Methods** 161, 6080–6080, 1980.

TOQUERO, C., BOLADO, S. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. **Bioresour. Technol.** 157, 68–76, 2014



TRAVAINI, R., DERLY, M., OTERO, M., COCA, M., DA-SILVA, R., BOLADO, S. Sugarcane bagasse ozonolysis pretreatment: Effect on enzymatic digestibility and inhibitory compound formation. **Bioresour. Technol.** 133, 332–339, 2013.

TRAVAINI, R., MARTÍN-JUÁREZ, J., LORENZO-HERNANDO, A., BOLADO-RODRÍGUEZ, S. Ozonolysis: an advantageous pretreatment for lignocellulosic biomass revisited. **Bioresour. Technol.** 199, 2-12, 2015.

VIKINESWARY, S., ABDULLAH, N., RENUVATHANI, M., SEKARAN, M., PANDEY, A., JONES, E.B.G. Productivity of laccase in solid substrate fermentation of selected agro-residues by *Pycnoporus sanguineus*. **Bioresour. Technol.** 97, 171–177, 2006.

WANDERLEY, M.C.D.A., MARTÍN, C., ROCHA, G.J.D.M., GOUVEIA, E.R. Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. **Bioresour. Technol.** 128, 448–53, 2013.

YOON, L.W., ANG, T.N., NGOH, G.C., CHUA, A.S.M. Fungal solid-state fermentation and various methods of enhancement in cellulase production. **Biomass and Bioenergy** 67, 319–338, 2014.

YOON, L.W., NGOH, G.C., CHUA, A.S.M. Simultaneous production of cellulase and reducing sugar through modification of compositional and structural characteristic of sugarcane bagasse. **Enzyme Microb. Technol.** 53, 250–256, 2013a.

YOON, L.W., NGOH, G.C., SEAK, A., CHUA, M. Simultaneous production of cellulase and reducing sugar through modification of compositional and structural characteristic of sugarcane bagasse. **Enzyme Microb. Technol.** 53, 250–256, 2013b.

YOUN, H., HAH, Y.C., KANG, S. Role of lactase in lignin degradation by white-rot fungi. **FEMS Microbiol. Lett.** 132, 183–188, 1995.

ZIMBARDI, A.L.R.L., CAMARGO, P.F., CARLI, S., NETO, S.A., MELEIRO, L.P., ROSA, J.C., ANDRADE, A.R. DE, JORGE, J.A., Furriel, R.P.M. A High Redox Potential Laccase from *Pycnoporus sanguineus* RP15: Potential Application for Dye Decolorization. **Int. J. Mol. Sci.** 17, 1–24, 2016.

## Capítulo 2

### Use of oxidizing agents in the hydrothermal alkaline pretreatment of sugarcane bagasse, enzymatic saccharification and cellulosic ethanol production

Josiani de Cassia Pereira<sup>1</sup>, Natália Paganini Marques<sup>2</sup>, Diego Alves Monteiro<sup>1</sup>, Ariela Veloso de Paula<sup>3</sup>, Maurício Boscolo<sup>1</sup>, Roberto da Silva<sup>1</sup>, Eleni Gomes<sup>1</sup>, Daniela Alonso Bocchini<sup>2</sup>

<sup>1</sup> Institute of Biosciences, Letters and Exact Sciences, São Paulo State University – IBILCE/UNESP, São José do Rio Preto, São Paulo State, Brazil

<sup>2</sup> Institute of Chemistry, São Paulo State University – IQ/UNESP, Araraquara, São Paulo State, Brazil

<sup>3</sup> Faculty of Pharmaceutical Science, São Paulo State University – FCFAR/UNESP, Araraquara, São Paulo State, Brazil.

#### Abstract

The use of chemical agents in hydrothermal pretreatment of sugarcane bagasse can influence lignocellulose structure and facilitate cellulases accessibility to cellulose. Some chemical agents such as  $\text{KMnO}_4$ ,  $\text{ZnO}$ ,  $\text{TiO}_2$  and  $\text{H}_2\text{O}_2$  can react with lignin, depolymerizing it by redox reactions. In the present work, these chemical agents were added to the hydrothermal pretreatment of sugarcane bagasse in alkaline and non-alkaline medium. The analysis of lignocellulose fractions showed that all the chemical agents, when used in alkaline medium, provided the reduction of lignin content, with consequent increase in cellulose content. Bagasse samples submitted to alkaline pretreatment in the presence of oxidizing agents were used in saccharification experiments, employing the enzymatic solution produced by *Pycnoporus sanguineus* MCA 16. The highest glucose concentration was observed in hydrolysates obtained when bagasse submitted to pretreatment in the presence of hydrogen peroxide was used. A statistical design was used to evaluate the influence of hydrogen peroxide concentration used on pretreatment and also of saccharification parameters (sugarcane bagasse and enzymes loads, time and temperature) on glucose releasing in enzymatic hydrolysates. The best condition for the enzymatic saccharification is 11% of pre-treated sugarcane bagasse, 250 total units of endoglucanase per gram of

cellulose in pretreated bagasse, during 106 hours, at 50 °C, resulting in a glucose concentration of 8.05 g/L.

**Keywords:** oxidizing agents, hydrogen peroxide, sugarcane bagasse pretreatment, enzymatic saccharification

## 1. Introduction

In 2015, the Agreement of Paris highlighted the importance of biofuels production from plant biomass residues generated in some industrial processes due to the possibility of replacing fossil fuels and, consequently, of reducing the emission of harmful gases (UNFCCC, 2015). Brazil expects that the biofuels second generation, ethanol and biodiesel, will contribute with 18% of the national energy matrix over the next 14 years (UNICA, 2016).

Cellulosic ethanol is a second-generation biofuel produced by the fermentation of glucose obtained from hydrolysis of cellulose present in lignocellulosic residues. It represents a complement to the first generation ethanol traditionally produced in many countries from different raw materials, such as the sugarcane juice in Brasil. In this context, the use of lignocellulosic biomass from industrial wastes becomes an important alternative to increase ethanol production through the fermentation of glucose released by enzymatic saccharification. However, the access to cellulose fiber is hampered by lignin, an amorphous phenylpropanoid polymer highly recalcitrant. Lignin removal is, therefore, extremely important for enzymatic saccharification and it can be performed by different pre-treatment strategies (TENGERDY; SZAKACS, 2003).

Lignocellulosic biomass pre-treatments can be physical, chemical and/or biological and the choice of method should be based on its efficiency in removing lignin, at low cost, with the lower release of harmful compounds to the environment. Chemical agents such as ozone, potassium permanganate, zinc oxide, titanium dioxide and hydrogen peroxide have been used in wastewater treatment processes to remove dyes and other toxic compounds by oxidation (ANANDGAONKER et al., 2014; DASH; PATEL; MISHRA, 2009; KADLA; CHANG, 2001; KHAN; NAJEEB; TUIYBAYEVA, 2014; MA et al., 2015; XU et al., 2005; ZHANG, 2011, YU et al., 2015, 2013). Thus, these compounds may

contribute to the delignification of lignocellulosic residues since they can react with lignin promoting its oxidation and disruption. The use of potassium permanganate, zinc oxide and titanium dioxide in pre-treatment of sugarcane bagasse is still a novelty and, therefore, studies using these agents are important to evaluate its effectiveness for lignin removal. Hydrothermal pretreatment of lignocellulosic biomass is a physical-chemical method where a steam pressure is applied at high temperature (TALEBNIA; KARAKASHEV; ANGELIDAKI, 2010). Under these conditions, steam penetrates in the biomass, hydrates cellulose and removes part of the hemicellulose and lignin, increasing the surface area, porosity and accessibility of cellulose (GAO et al., 2013, JÖNSSON; MARTÍN, 2016, TALEBNIA; KARAKASHEV; ANGELIDAKI, 2010). Hemicellulose is solubilized since its acetylation promotes medium acidification (GÍRIO et al., 2010; JÖNSSON; MARTÍN, 2016). Thus, hydrothermal pretreatment without any chemicals or additives is referred as autohydrolysis (GÍRIO et al., 2010).

In this work, sugarcane bagasse was subjected to alkaline hydrothermal pretreatment with or without oxidizing agents such as potassium permanganate, zinc oxide, titanium dioxide and hydrogen peroxide, in order to identify the most efficient method for delignification, based on glucose yield in hydrolysates obtained by enzymatic saccharification of pretreated bagasse. The enzymes crude solution used was produced by the basidiomycete *Pycnoporus sanguineus* MCA 16, showing cellulases and ligninases activities. The hydrolyzate obtained by enzymatic saccharification of bagasse submitted to the selected pretreatment was used as source of glucose to obtain cellulosic ethanol using *Saccharomyces cerevisiae* CAT-1 as fermenting microorganism.

## **2. Material and methods**

### **2.1. Sugarcane bagasse**

Sugarcane bagasse (donated by Usina Virgolino de Oliveira S/A, José Bonifácio - São Paulo, Brazil) was washed with distilled water to remove sugar residues and particulate material, dried in a ventilated oven at 40 °C and ground in an agricultural crusher (Trapp Model TRF- 400) to a size of 3 mm.

## **2.2. Hydrothermal Pretreatment**

Pretreatment was performed in autoclave, according to the methodology described by Zhuang et al. (2009). Sugarcane bagasse, previously washed, dried and milled, was autoclaved in 250 ml Erlenmeyer flask sealed with aluminum foil (5% of solid load w/v in 0.5 mol.L<sup>-1</sup> of NaOH solution) at 120 °C, for 1 hour at 1 atm. After, the flask content was cooled using ice water bath and then kept under room temperature for stabilization. The material was filtered on nylon cloth and then the liquid phase was filtered on 0.22 µm filters. The solid phase was washed with water for detoxication according to Travaini et al. (2013) and Torquero et al. (2014) and stored at 4 °C for subsequent use in saccharification experiments. The liquid phase was filtered in 0.22 µm filters and then analyzed regarding alcohol fermentation inhibitors (ferulic, syringic, p-coumaric and vanilic acids), phenolic compounds and reducing sugars.

### **2.2.1. Use of oxidizing agents**

Oxidizing agents were added to the hydrothermal pretreatment in order to evaluate the influence in the efficacy of the pretreatment, based on the composition of pretreated bagasse (recovery of cellulose and hemicellulose, reduction of lignin content) and also on glucose concentration in the hydrolysates obtained by enzymatic saccharification. Potassium permanganate, hydrogen peroxide, titanium dioxide and zinc oxide were used separately as oxidizing agents at 5.0 g.L<sup>-1</sup>.

## **2.3. Microorganism**

The basidiomycete *Pycnoporus sanguineus* MCA 16 belonging to the Laboratory of Biochemistry and Applied Microbiology (work culture) from IBILCE/UNESP, São José do Rio Preto, São Paulo, Brazil was used in this work to obtain the crude enzymes solution.

### **2.4. Enzymes production by solid-state fermentation (SSF)**

To obtain the crude enzymes solution, *P. sanguineus* MCA 16 was precultivated on malt agar plates, for 120 hours, at 40 °C. Six micelial disks of 0.9 cm diameter were then used as inoculum for each polypropylene bag containing 5 g of substrates mixture (1:1 w/w) composed by wheat bran (Natural

Life®) and soybean meal (donated by Trow Nutrition®, Mirassol, São Paulo, Brazil). Substrates were previously washed and dried at 40 °C and, for SSF, an initial moisture content of 70% was achieved by adding 11 mL of nutrient solution as described by Toyama and Ogawa (1978). The polypropylene bags were then sterilized in autoclave (120 °C, 1 atm, 20 minutes), their content were cooled, inoculated and incubated at 40 °C, for 96 hours, to obtain the crude enzymes solution.

### **2.5. Endoglucanase activity**

Endoglucanase activity was assayed in reaction mixture containing 25 µL of crude enzymes solution and 225 µL of substrate solution (sodium acetate buffer 0.1 mol.L<sup>-1</sup>, pH5.0, containing carboxymethyl cellulose 4% – CMC C5768 from Sigma, St Louis, Missouri, USA), incubated at 50°C, for 10 min. The quantification of reducing sugars was carried out by the DNS (3,5-dinitrosalicylic acid) method (Miller 1959), using glucose standard curve. One unit of enzymatic activity (U) was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute, under the assay conditions.

### **2.6. Enzymatic saccharification of pretreated sugarcane bagasse**

Enzymatic saccharification preliminary experiments were carried out in 250 ml Erlenmeyer flasks using 3% of sugarcane bagasse submitted to hydrothermal alkaline pretreatment in the presence and absence of oxidizing agents, using 100 units of endoglucanase per gram of cellulose in pretreated bagasse and citrate buffer solution (0.1 mol.L<sup>-1</sup>, pH 5.0), to a final volume of 20 ml. An amount of endoglucanase required for calculation of cellulose content obtained from the compositional analysis experiment, according to NREL (HAMES et al., 2008; SLUITER et al., 2008a, 2008b). The Erlenmeyer flasks were sealed with rubber stopper, incubated in an orbital shaker at 300 rpm, for 24 hours, at 50 °C. After this period, the contents were filtered in nylon tissue, the solid phase was discarded and the liquid fase was filtered in microfilters of 0.22µm and analyzed regarding glucose and xylose contents by High Performance Liquid Chromatography (HPLC). The assays were performed in duplicate, the best hydrothermal pretreatment condition was selected based on the highest glucose concentration obtained in the hydrolysates, and used in the

subsequent saccharification experiments involving experimental designs. Yields were calculated according to the equations below:

$$(1) \text{Yield}_{\text{glucose}} (\%) = [\text{total glucose (g)} / \text{total cellulose (g) in pretreated bagasse}] \times 100$$

$$(2) \text{Yield}_{\text{xylose}} (\%) = [\text{total xylose (g)} / \text{total xylan (g) in pretreated bagasse}] \times 100$$

$$(3) \text{Yield}_{\text{glucose or xylose}} (\text{mg/g of pretreated sugarcane bagasse}) = [\text{total glucose or xylose (g)} / \text{bagasse dry mass (g)}] \times 1000$$

$$(4) \text{Yield}_{\text{glucose or xylose}} (\text{mg/g of cellulose or xylan in pretreated bagasse}) = [\text{total glucose or xylose (g)} / \text{total cellulose or xylan (g) in pretreated bagasse}] \times 1000$$

### **2.6.1 Optimization of enzymatic saccharification conditions**

Experimental designs were used aiming the optimization of saccharification conditions using sugarcane bagasse submitted to alkaline hydrothermal pretreatment in the presence of hydrogen peroxide. Initially, the influence of hydrogen peroxide concentration used in sugarcane bagasse pretreatment (%), sugarcane bagasse load (%) and enzyme load (totals units of endoglucanase per gram of pretreated sugarcane bagasse) was evaluated using a face-centered design was performed, including the 8 factorial points, 4 axial points and 4 central points. Then, the influence of temperature (°C) and time (hours) was evaluated using a rotational central composite design (RCCD) including the 4 factorial points, 4 axial points and 4 central points. The variables and their levels were selected based on data reported in scientific literature (DE CASSIA PEREIRA et al., 2016; GAO et al., 2014; MORETTI et al., 2014). The real and encoded values of each variable are presented in Tables 6 and 7.

Statistical software (Statsoft 7.0) was used to analyze the results, which were submitted to analysis of variance (ANOVA).



Table 6. Coded and real values of the face-centered design design ( $2^3$ ) used in saccharification of sugarcane bagasse submitted to alkaline hydrothermal pretreatment in the presence of hydrogen peroxide. SB: sugarcane bagasse.

Variable	Level			Unit
	-1	0	+1	
* $X_1 - H_2O_2$	1	2	3	%
$X_2 - SB$	5	8	11	%
$X_3 -$ Endoglucanase	150	200	250	Total units per gram of cellulose

\*different percentages used on the pretreatment.

Table 7. Coded and real values of the rotational central composite design (RCCD) ( $2^2$ ) used in the enzymatic saccharification of the sugarcane bagasse.

Variable	Level					Unit
	-1.41	-1	0	+1	+1.41	
$X_3 -$ time	4.12	24	72	120	139.88	hours
$X_4 -$ temperature	42.93	45	50	55	57.07	°C

## 2.7. Analytical methods

*In natura* and pretreated bagasse were characterized regarding lignocellulose fractions according the protocol established by NREL (National Renewable Energy Laboratory (Sluiter et al., 2004)). Fourier Transformed Infra Red (FT-IR) spectroscopy was used to evaluate the lignin content by the pretreatments for analysis of amorphous and crystalline cellulose.

Glucose and xylose quantifications were performed by ion exchange chromatography using HPAEC-PAD (Thermo Scientific (Dionex) ICS-5000), CarboPac® PA-1 anion exchange column, at 25 °C, and diluents prepared with water ultra pure deionized 18 M $\Omega$  and degassed with N<sub>2</sub>. The flow rate was 1.0

mL/min with solvents A (ultra pure water) and B (NaOH 500 mmol L<sup>-1</sup>), using isocratic elution with 4.8% B and 95.2% A for 25 minutes. The analysis of total phenolic compounds was performed using the Folin Ciocalteu method (SINGLETON et al., 1999) from the analytical curve of gallic acid.

The quantification of alcoholic fermentation inhibitors such as organic acids (ferulic, syring and vanillic acids) and furan aldehydes (furfural and hydroxymethylfurfural) on the hydrolysates obtained from saccharification experiments was performed by high performance liquid chromatography (HPLC). The samples were centrifuged at 10000 xg during 15min and the supernatant was filtered using 22 µm membrane. Then, 20 µL were applied to the Agilent Tec. Model 1220 Infinity LC, with binary pump, automatic sampler, column oven and UV/VIS detector. The column used was C18 ZORBAX Eclipse Plus (4.6x250 mm), maintained at 25 °C. Chromatographic separation was performed using the mobile phase methanol:acetic:water (10:2:88 v/v) as the solvent (A) and methanol:acetic:water (90:2:8 v/v) as solvent (B) (RODRÍGUEZ-DELGADO et al., 2001).

### **2.8. Scanning electron microscopy**

Scanning electron microscopy (SEM) was performed using a FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) with an accelerating voltage of 12.5 kV. The bagasse was fixed using a solution of glutaraldehyde 2.5% in 0.1mol.L<sup>-1</sup> phosphate buffer (pH 7.3), for 48 h, at room temperature. After this period, the material was washed with distilled water and post fixed in 1% osmium tetroxide aqueous solution, for 30 min, at room temperature. After fixation, the bagasse was dehydrated in an ethanol series, critical point-dried with CO<sub>2</sub>, and sputter coated with gold (Bal-Tec SCD 050).

### 3. Results and discussion

#### 3.1. Analysis of sugarcane bagasse lignocellulosic fractions

The analysis of the lignocellulosic fractions was performed for *in natura* and pretreated bagasse (Table 8). All the pretreatments led to a decrease in xylan and lignin content and consequently increased the proportion of cellulose in the material. This was most markedly observed for the alkaline treatment, probably due to the reaction of sodium hydroxide with ether and ester bonds in lignin, promoting the solubilization of this polymer (BURANOV; MAZZA, 2008).

Table 8. Effect of pretreatments on chemical composition of sugarcane bagasse.

Pretreatment	Oxidizing agents	Composition (%)			
		Cellulose	Xylan	Total lignin	Ashes
Untreated	-	47.3 ± 0.18	22 ± 0.25	28 ± 0.18	1.55 ± 0.22
Hydrothermal	-	48.54 ± 0.21	21.7 ± 0.16	27 ± 0.2	1.54 ± 0.11
	KMnO <sub>4</sub>	50 ± 0.22	22 ± 0.14	22.9 ± 0.13	1.1 ± 0.12
	H <sub>2</sub> O <sub>2</sub>	51 ± 0.16	22.23 ± 0.13	13.43 ± 0.1	1.43 ± 0.24
	ZnO	51 ± 0.11	22.11 ± 0.11	25.5 ± 0.13	1.3 ± 0.13
	TiO <sub>2</sub>	49.6 ± 0.22	12.6 ± 0.14	26.7 ± 0.14	3.12 ± 0.14
Alkaline hydrothermal	-	63.5 ± 0.32	17 ± 0.12	13 ± 0.23	1.9 ± 0.12
	KMnO <sub>4</sub>	61.2 ± 0.14	14.5 ± 0.17	20 ± 0.11	1.5 ± 0.16
	H <sub>2</sub> O <sub>2</sub>	59 ± 0.11	18 ± 0.13	11 ± 0.24	1.7 ± 0.19
	ZnO	62.7 ± 0.24	17 ± 0.23	19.3 ± 0.23	1.3 ± 0.14
	TiO <sub>2</sub>	51.7 ± 0.14	18 ± 0.11	18.5 ± 0.13	0.4 ± 0.21

In general, alkaline hydrothermal pretreatments using oxidizing agents were more efficient for lignin reduction. The lowest lignin content was observed in sugarcane bagasse submitted to pretreatment alkaline hydrothermal with hydrogen peroxide. On the other hand, the presence of oxidizing agents, in general, also reduced xylan contents, which is not desirable when the objective is to obtain xylose rich enzymatic hydrolysates. The highest cellulose content

(63.5 %) was observed when hydrothermal alkaline pretreatment without the addition of chemicals was used. It represents an increase of 34.25 % in this carbohydrate content, taking into account decreases of 22.7 and 53.57 % in xylan and lignin contents, respectively.

Scientific literature reports the application of different oxidizing agents for lignocellulosic material modification (KHAN; NAJEEB; TUIYEBAYEVA, 2014; MA et al., 2015; YOO et al., 2013). The use of potassium permanganate was cited by Bland; Logan (1965); Bose et al. (2009); Lin et al. (2013); Ma et al. (2015); Miron; Ben-Ghedalia (1982) in pretreatments of corncobs, grasses, woods and wheat bran. In these works the oxidation of lignin and consequent formation of organic acids and other components were observed. Zinc oxide and titanium dioxide were used in the present work since metallic oxides are commonly used in photochemical pretreatments of corn straw under ultraviolet light, which helps in the degradation of organic matter (YOO et al., 2013). However, hydrothermal pretreatment was employed in this work at lower cost and risk, being more feasible especially for industrial applications.

In the presence of sodium hydroxide and zinc oxide, there is the formation of sodium zinc ions ( $\text{Zn}(\text{OH})_4^{-2}$ ) which lead to the establishment of intramolecular hydrogen bonds in cellulose, while intermolecular hydrogen bonds are breaking down. These ions form stronger hydrogen bonds with cellulose than with water in a process that has not yet been fully elucidated (KIHLMAN et al., 2013; YANG et al., 2011; YANG; QIN; ZHANG, 2011). At high temperatures and in the presence of sodium hydroxide, zinc oxide makes the cellulose less polar and more stable (YANG et al., 2011). This makes the pretreatment used in the present work interesting, since it does not favor losses in the cellulose fraction.

Hydrogen peroxide, in turn, has an effect on lignin. When using hydrogen peroxide associated to hydrothermal pre-treatment of corn cob and wheat bran, Su et al. (2014) and Toquero and Bolado (2014), respectively, observed a high reaction with lignin, mainly under alkaline conditions. As previously reported by Sun et al. (2004) and Sun et al. (2003), the treatment with hydrogen peroxide in alkaline media promotes the breakdown of the covalent bonds between lignin and hemicelluloses and also saponifies hydroxycinnamic esters, such as ferulic and p-coumaric acids.

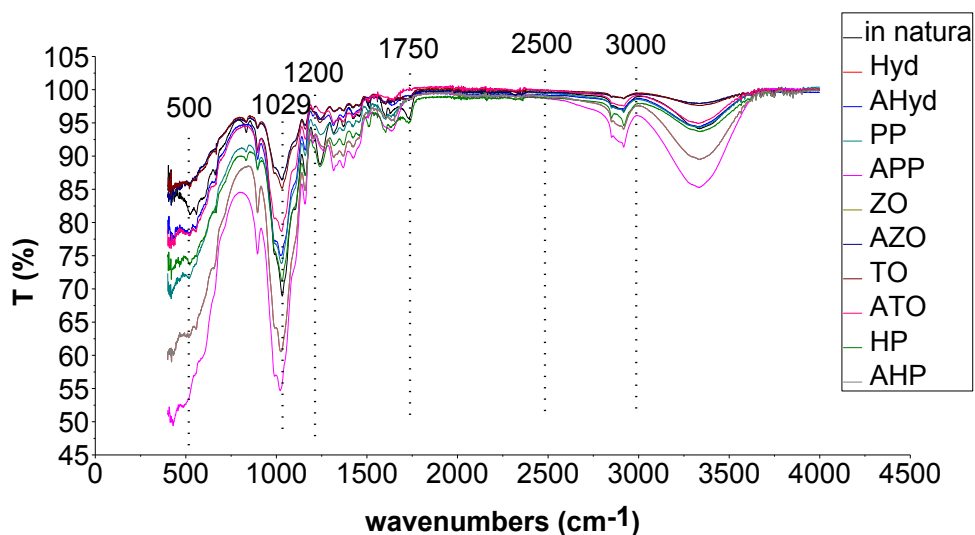
According to Xiang and Lee (2000) and Kadla and Chang (2001) the reaction of hydrogen peroxide with the lignin under alkaline conditions and high temperatures results in nucleophilic peroxide ( $\text{HOO}^-$  or  $\text{ROO}^-$ ). Then, the nucleophilic attack to the lignin bonds occurs, especially at the aryl-ether bonds. Consequently, lignin is gradually unstructured resulting in fragments of lower molecular weight.

Regarding FT-IR analysis (Figure 6) reductions in the vibrations bands of  $500\text{-}1200\text{ cm}^{-1}$  were observed for sugarcane bagasse samples submitted to alkaline or non-alkaline hydrothermal pretreatment associated with the use of oxidizing agents. This fact is related to the reduction of ether and CH bonds, indicating lignin degradation and it was observed when alkaline potassium permanganate, alkaline hydrogen peroxide, alkaline zinc oxide, zinc oxide and titanium dioxide were used as oxidizing agents on hydrothermal pretreatment.

The decrease in these bands is also related to the breakdown of  $\beta$ -bonds of hemicellulose such as observed of the  $1200\text{-}1750\text{ cm}^{-1}$  spectra in bagasse samples submitted to pretreatments using alkaline potassium permanganate, alkali hydrogen peroxide, titanium dioxide and zinc oxide. This reduction corresponds to the degradation of non-cellulosic polysaccharides, guaiacyl and syringyl portions, lignin aromatic rings, disruption of lignin, hemicellulose and cellulose interaction, disruption of hemicellulose and soluble lignin association, degradation of alkali-soluble lignin, C=C and C=O of lignin .

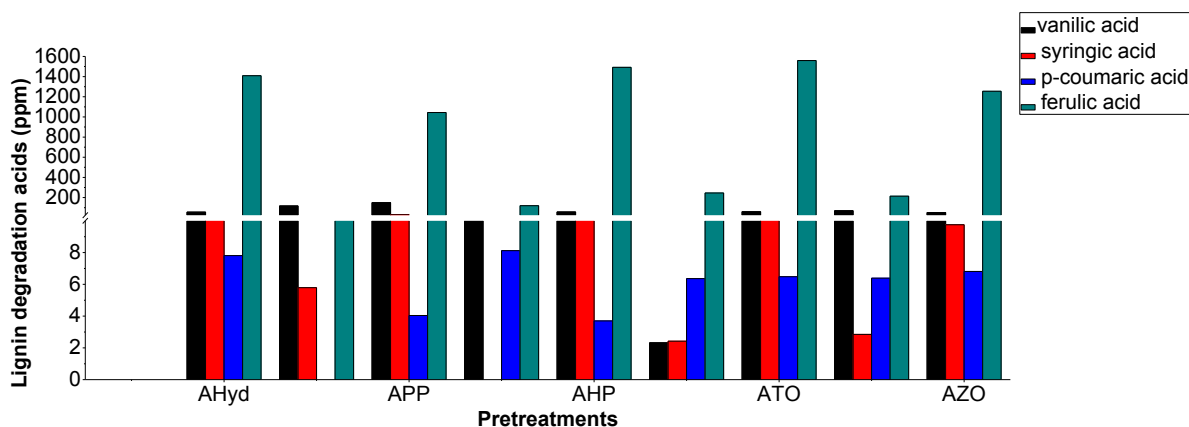
Finally, alkaline potassium permanganate was the chemical agent that promoted the decrease in  $2500\text{-}3000\text{ cm}^{-1}$  bands, which is related to the removal of methyl and methylene groups from lignin. Phenols and alcohols, represented by  $3000\text{-}3500\text{ cm}^{-1}$  bands, decreased when alkaline potassium permanganate and alkaline hydrogen peroxide were used (COLOM et al., 2003).

Figure 6. Fourier Transformed Infra Red (FT-IR) spectroscopies of sugarcane bagasse under different pretreatments. Hyd: hydrothermal; AHyd: alkaline hydrothermal; PP: potassium permanganate; APP: alkaline potassium permanganate; ZO: zinc oxide; AZO: alkaline zinc oxide; TO: titanium dioxide; ATO: alkaline titanium dioxide; HP: hydrogen peroxide; AHP: alkaline hydrogen peroxide.



Phenolic compounds such as syringic, p-coumaric and ferulic acids can be generated in acid and alkaline pretreatments of lignocellulosic biomass, by the breaking down of bonds between lignin and hemicellulose and also between lignin monomers (BURANOV; MAZZA, 2008; UYAMA et al., 2000). Vanillic acid, in turn, is generated by breaking double bond between two carbon atoms of ferulic acid during alkaline pretreatment (MATHEW; ABRAHAM, 2006; ROSAZZA et al., 1995). The effects of the pretreatments using oxidizing agents on delignification/disruption of sugarcane bagasse were also evaluated based on the quantification of phenolic compounds in the liquid phase resulting from the pretreatments (Figure 7). In all pretreatments, ferulic acid was the compound generated highest amount, followed by vanillic acid.

Figure 7. Phenolic compounds present in the liquor obtained after alkaline and non-alkaline hydrothermal pretreatment, in the presence of oxidizing agents. Hyd: hydrothermal; AHyd: alkaline hydrothermal; PP: potassium permanganate; APP: alkaline potassium permanganate; ZO: zinc oxide; AZO: alkaline zinc oxide; TO: titanium dioxide; ATO: alkaline titanium dioxide; HP: hydrogen peroxide; AHP: alkaline hydrogen peroxide.



The highest amounts of vanillic acid were observed in the liquid phases obtained from the pre-treatments with potassium permanganate, under alkaline and non-alkaline conditions ( $150 \pm 0.33$  and  $117.6 \pm 0.46$  ppm, respectively). The alkaline pre-treatment in the presence of potassium permanganate led to the highest amount of syringic acid ( $29.7 \pm 0.63$  ppm). In general, p-coumaric acid was the minor phenolic compound generated with the highest concentration observed in the non-alkaline pretreatment with the use of hydrogen peroxide ( $8.12 \pm 0.55$  ppm). The low concentration of syringic acid released from all pre-treatments shows the possibility of ether-bond breaks present in the molecule (confirmed by bands  $1029 \text{ cm}^{-1}$  of Figure 6), which is converted into gallic acid, its correspondent with absence of the methyl groups. Glucose and xylose were detected at very low concentrations in the liquid phase from all the pretreatments, indicating that there was no significant degradation of cellulose and xylan, respectively.

The use of the oxidizing agents with the aim of removing lignin from sugarcane bagasse by pre-treatment processes is a relevant aspect of the

present work since it is underexplored. Studies regarding the action of these agents on lignocellulose disruption are relevant for second generation ethanol production since they lead to a decrease in the lignin content and, consequently, a higher exposure of cellulose and hemicellulose to the hydrolytic enzymes. In addition, the use of these catalysts in pretreatments of lignocellulosic materials is interesting for industrial applications, since it generates of biorefining products such as vanillic acid, vanillin, among others (ABDELAZIZ et al., 2015; AMIN et al., 2017; CARDONA; SÁNCHEZ, 2007; CELEBI et al., 2017; Daou; Faulds, 2017; González-García et al., 2016; GRAY; ZHAO; EMPTAGE, 2012; HERNÁNDEZ-ORTEGA; FERREIRA; MARTÍNEZ, 2012; MENON; RAO, 2012; VIVEK et al., 2017; VIVEK; PANDEY; BINOD, 2016).

### ***3.2. Enzymatic saccharification of pretreated sugarcane bagasse***

#### ***3.2.1. Influence of the oxidizing agents used in the hydrothermal pretreatment***

Saccharification experiments were performed as an initial screening in order to evaluate the influence of the different oxidizing agents used in the hydrothermal pre-treatments, based on the glucose concentration in the hydrolysates obtained. The highest concentration of this sugar was obtained when bagasse was submitted to alkaline pre-treatment in the presence of hydrogen peroxide (Table 9), and this condition was selected for the subsequent experiments. As additional information, the concentration of xylose released in the hydrolysates was evaluated.



Table 9. Enzymatic saccharification of sugarcane bagasse submitted to different pre-treatments. Assays conducted at 50 °C for 24 hours with bagasse and endoglucanase loads at 3.0% and 100 total units/g cellulose, respectively.

Pretreatment	Oxidizing agents	Glucose (g/L)	Xylose (g/L)
<b>Untreated</b>	-	0.02 ± 0.22	0.16 ± 0.12
<b>Hydrothermal</b>	KMnO <sub>4</sub>	0.26 ± 0.12	0.33 ± 0.24
	ZnO	0.37 ± 0.22	0.12 ± 0.26
	TiO <sub>2</sub>	0.03 ± 0.22	0.03 ± 0.18
	H <sub>2</sub> O <sub>2</sub>	0.27 ± 0.27	0.12 ± 0.13
<b>Alkaline hydrothermal</b>	KMnO <sub>4</sub>	0.15 ± 0.24	0.03 ± 0.22
	ZnO	1.46 ± 0.15	0.9 ± 0.21
	TiO <sub>2</sub>	1.45 ± 0.11	0.62 ± 0.17
	H <sub>2</sub> O <sub>2</sub>	2.01 ± 0.14	1.04 ± 0.16

Cao et al. (2012) employed hydrogen peroxide in the hydrothermal pretreatment of sorghum and carried out saccharification of the pretreated material using commercial enzymes for 72 hours, obtaining glucose and xylose at 1.46 and 0.62 g/L in the hydrolysates. Toquero and Bolado (2014) submitted the wheat meal to different pre-treatments and considered the alkaline in the presence of hydrogen peroxide (at 50 °C, for 1 hour) as the best, when compared to the other evaluated (hydrothermal, acid hydrothermal, hydrothermal in alkaline medium in the presence of hydrogen peroxide). Such consideration was based on the absence of inhibitors in the hydrolyzate well in the higher sugar yield in 72 hours.

### **3.2.2. Influence of hydrogen peroxide concentration, sugarcane bagasse and enzyme loading**

The sugarcane bagasse submitted to the alkaline hydrothermal pretreatment in the presence of hydrogen peroxide was selected to continue saccharification experiments. A face-centered design was then carried out. The coded and actual levels of the variables studied and the response obtained in terms of glucose concentration in the hydrolysates are shown in Table 10. The

xylose concentrations were also determined, as additional information, one as this sugar can be converted to ethanol by some microorganisms.

For this experiment, hydrogen peroxide was introduced to the pretreatment in different percentages, and the pre-treated bagasse was used in the statistical planning of the enzymatic saccharification, using as a response the released glucose (g/L). The analyzes of the lignocellulosic fractions of the pre-treated bagasse at different concentrations of hydrogen peroxide are contained in Table 11. This information was used for the addition of enzyme extract based on units of endoglucanase/g of cellulose.

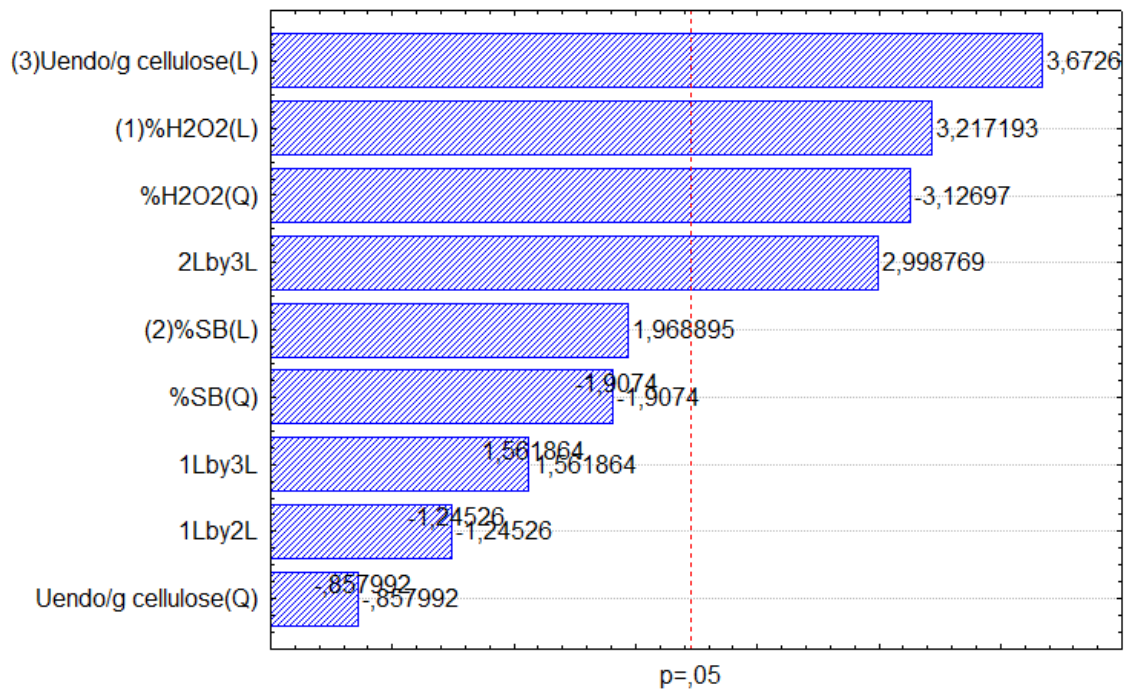
In relation to glucose, the presence of this sugar in some hydrolysates was not detected. The concentrations detected varied from 0.25 to 5.66 g/L, the maximum obtained in test 8, where all variables were employed at their maximum levels (Table 10).

The effects of each of the variables and their interactions on enzymatic saccharification are presented by the Pareto Diagram (Figure 8). As can be observed by the Pareto diagram, the percent variables of hydrogen peroxide and endoglucanase units were significant at the 95% confidence level (p-value <0.05), as well as the interaction between the variables percentage of sugarcane bagasse and endoglucanase units.

Table 10. Factorial design of the face-centered type used for the evaluation of the influence, in the saccharification, of the bagasse and hydrogen peroxide loads used in the pretreatment, as well as the endoglucanase loading used in saccharification. An enzymatic extract produced by *Pycnoporus sanguineus* MCA 16 was used for cultivation on wheat bran and soybean meal (2:3 w/w) at 40 °C for 96 hours. The tests were kept at 50 °C for 24 hours under 300 rpm.

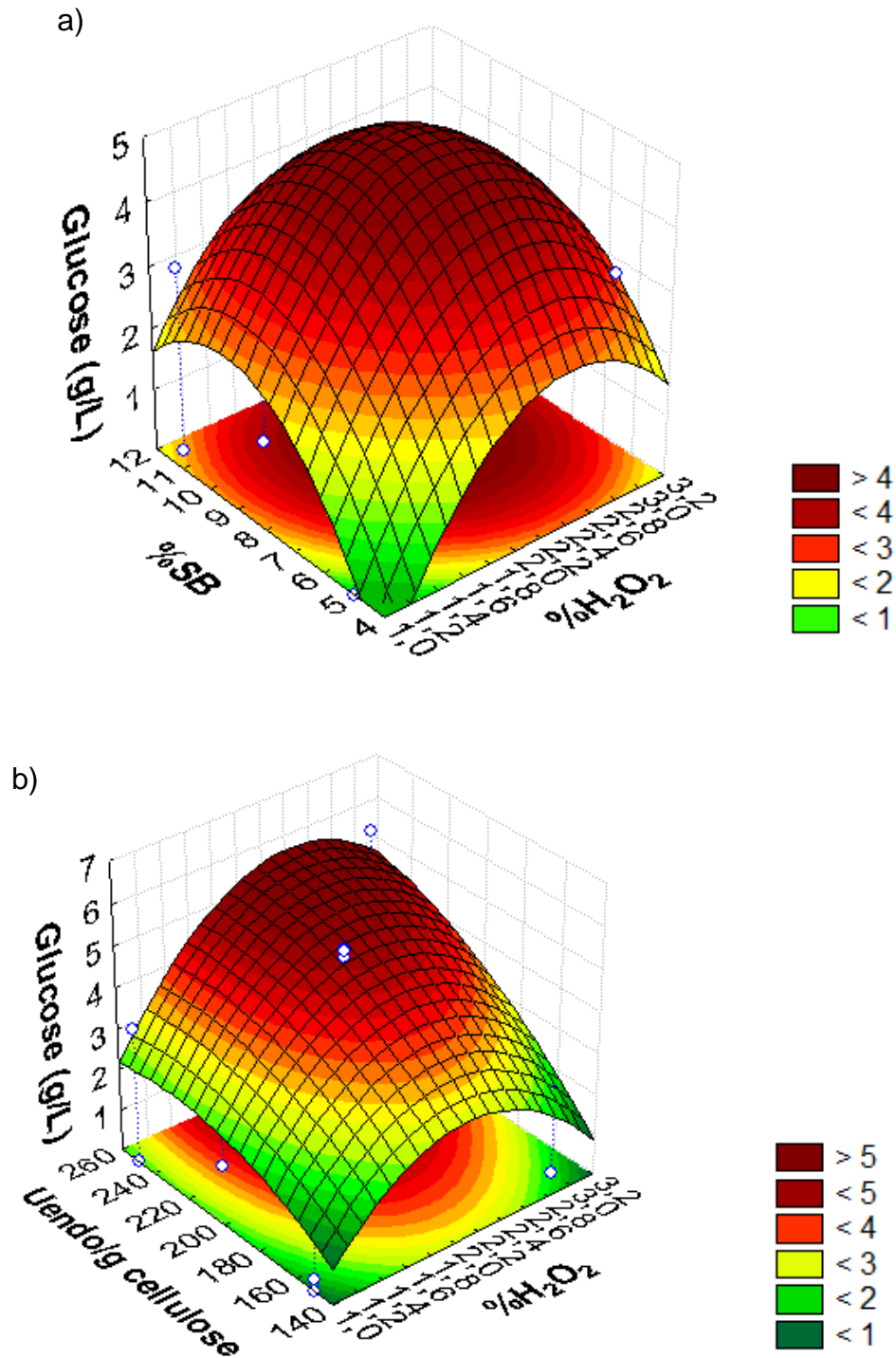
Runs	%H <sub>2</sub> O <sub>2</sub>	%Biomass	Uendo/g	Glucose (g/L)	Xylose (g/L)
1	-1 (1)	-1 (5)	-1 (150)	0	0
2	1 (3)	-1 (5)	-1 (150)	1.99	0.73
3	-1 (1)	1 (11)	-1 (150)	0.31	0
4	1 (3)	1 (11)	-1 (150)	0	0
5	-1 (1)	-1 (5)	1 (250)	0.25	0.33
6	1 (3)	-1 (5)	1 (250)	3.17	1.19
7	-1 (1)	1 (11)	1 (250)	3.27	2.21
8	1 (3)	1 (11)	1 (250)	5.66	3.23
9	-1 (1)	0 (8)	0 (200)	1.44	0.52
10	1 (3)	0 (8)	0 (200)	3.13	1.18
11	0 (2)	-1 (5)	0 (200)	2.24	1.03
12	0 (2)	1 (11)	0 (200)	3.62	2.17
13	0 (2)	0 (8)	-1 (150)	3.29	1.98
14	0 (2)	0 (8)	1 (250)	3.68	1.55
15	0 (2)	0 (8)	0 (200)	5.33	3.21
16	0 (2)	0 (8)	0 (200)	5.11	3.66
17	0 (2)	0 (8)	0 (200)	5.14	3.37
18	0 (2)	0 (8)	0 (200)	5.29	3.12
19	0 (2)	0 (8)	0 (200)	5.34	3.19
20	0 (2)	0 (8)	0 (200)	5.23	3.18

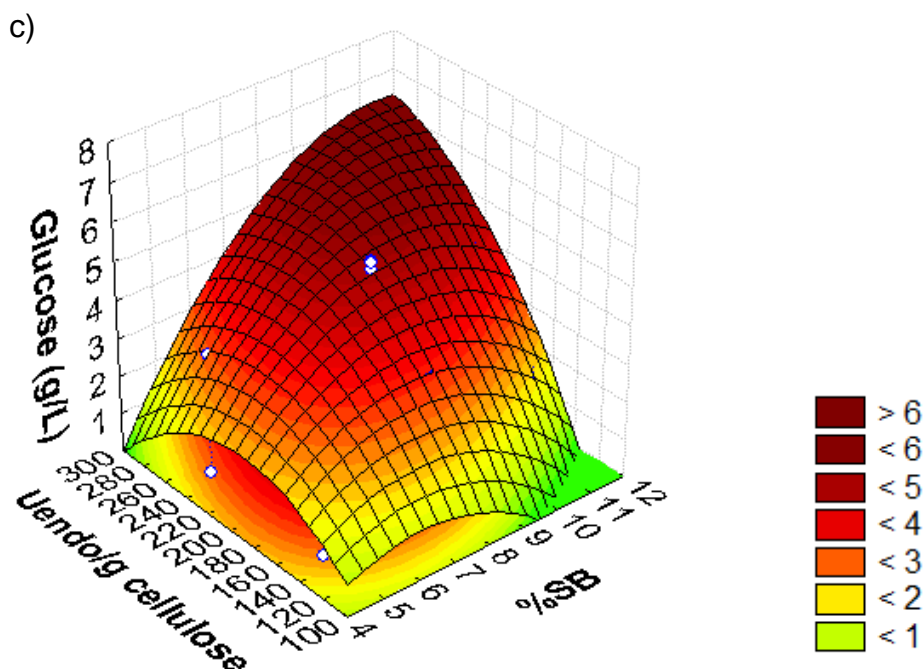
Figure 8. Pareto diagram of the estimated effects of the hydrogen peroxide concentration and enzyme loading variables on saccharification of pre-treated sugarcane bagasse.



The response surfaces generated by the model (Figures 9) show that the highest concentrations of glucose are obtained when the variables studied are used at levels close to the maximum: hydrogen peroxide between 2.0 and 2.8%, upper 220 endoglucanase units/g cellulose and sugarcane bagasse loading between 9.0 and 11.0%.

Figure 9. Response surfaces generated to the glucose concentration in function of hydrogen peroxide concentration and endoglucanase load (a); hydrogen peroxide concentration and sugarcane bagasse load (b); endoglucanase load and sugarcane bagasse load (c).





Using the desirability function of the STATISTICA 10.0 software, it was possible to predict the optimum values of the independent variables analyzed, namely 2.5% hydrogen peroxide, endoglucanase loading at 250 units/g cellulose and pre-treated at 11.0%. Under these conditions, the expected released glucose concentration was 5.83 g/L. Experimental validation was performed with enzymatic saccharification assays in triplicate, obtaining glucose at 5.91 g/L  $\pm$  0,46, being within the desirability parameter. Under these conditions, the xylose concentration obtained was 4.21 g/L  $\pm$  0,51.

### ***3.2.3. Influence of hydrogen peroxide concentration on pretreatment on the composition of sugarcane bagasse***

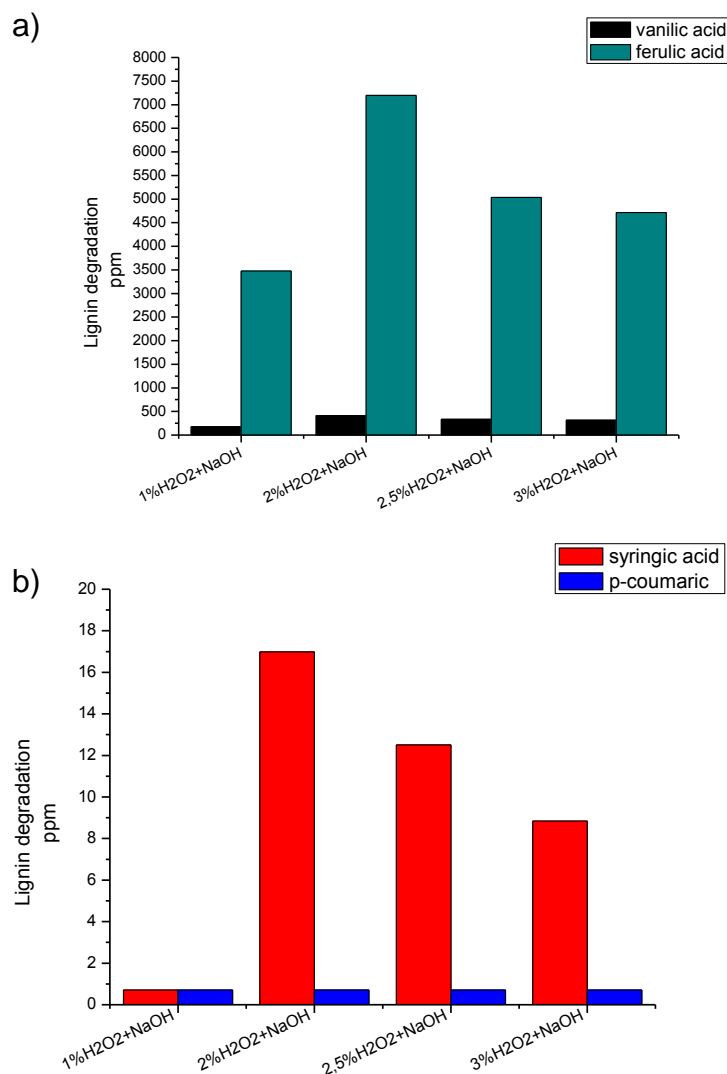
In order to better analyze the influence of the percentage of hydrogen peroxide used in the pretreatment on the release of glucose in saccharification, a compositional analysis of the pretreated bagasse used in experimental design trials was carried out, according to the methodology developed by NREL (National Renewable Energy Laboratory - USA) (item 2.3). Table 11 shows that 2.5% of hydrogen peroxide in the alkaline hydrothermal pretreatment led to the highest percentage of cellulose (69.4%).

Table 11. Analysis of the lignocellulosic fractions of sugarcane bagasse subjected to alkaline pre-treatment in the presence of different percentages of hydrogen peroxide.

<b>H<sub>2</sub>O<sub>2</sub> (%)</b>	<b>Cellulose (%)</b>	<b>Xylan (%)</b>	<b>Total lignin (%)</b>
1.0	62 ± 0.14	16.3 ± 0.18	13.5 ± 0.06
2.0	63 ± 0.11	17 ± 0.08	9.6 ± 0.16
2.5	69.4 ± 0.23	19.5 ± 0.17	10.2 ± 0.11
3.0	67.4 ± 0.24	19 ± 0.09	11 ± 0.08

The liquid fractions obtained from the alkaline hydrothermal pre-treatments performed in the presence of different concentrations of hydrogen peroxide were also analyzed for the presence of organic acids (Figure 10). This analysis was carried out in order to infer the influence of the concentrations of the catalyst on the degradation of the lignin by the quantification of the organic acids formed. Figure 10 shows a higher release of ferulic and syringic acids, especially when using 2.0% hydrogen peroxide in the pretreatment stage (7197.4 and 17 ppm, respectively).

Figure 10. Organic acids present in the liquid fractions obtained from alkaline hydrothermal pretreatments in the presence of different hydrogen peroxide concentrations. (a) Vanillin and ferulic acids concentrations and (b) syringic and p-coumaric acids concentrations.



According to the Table 11 the increase of cellulose and hemicellulose fractions indicate a removal of lignin. Some works using hydrogen peroxide as an oxidizing agent approach this behavior on the lignocelluloses composition. Ayeni et al. (2015) reported 56.7, 12.3 and 22.9% of cellulose, lignin and hemicelluloses contents, respectively, in the pre-treated bagasse with 1% of hydrogen peroxide during 3 hours at 90°C. Brienzo et al. (2009) using the sugarcane bagasse pretreated with 6.0% hydrogen peroxide for 4 hours at 20 °C reported 5.9% of lignin, representing a decrease of 88.0% over untreated



material. Cheng et al. (2008) when working with pre-treatment of sugarcane bagasse using 0.6% hydrogen peroxide in alkaline media reported 55.8 and 36.5% of cellulose and hemicellulose, respectively, in addition to removal of 70% of lignin.

#### **3.2.4. Influence of time and temperature**

A second statistical planning was carried out in order to evaluate the influence of time and temperature variables on the enzymatic saccharification of pretreated sugarcane bagasse, as a result of the glucose concentration in the hydrolysates (Table 12). The xylose concentration was also evaluated as additional information to the experiment. In these tests, hydrogen peroxide, endoglucanase loading and pre-treated bagasse load were maintained at their optimal levels determined in the previous planning (2.5%, 250 units/g cellulose and 11.0% dry mass, respectively). The tests were conducted for 24 hours under agitation of 300 rpm.

The glucose concentrations detected varied from 1.28 to 8.18 g/L, the maximum obtained in test 2, where all variables were employed at their maximum levels. The effects of each of the variables and their interactions on enzymatic saccharification are presented by the Pareto Diagram (Figure 11) and can be confirmed by analysis of variance (ANOVA) presented in Table 8. As can be observed by the Pareto diagram, only time was significant at the 95% confidence level ( $p$ -value  $<0.05$ ). The response surfaces generated by the model (Figure 12) show that the highest concentrations of glucose are obtained when the saccharification is maintained for 80-120 hours.

Table 12. Rotational Central Composite Design (RCCD) used in the enzymatic saccharification experiments of pre-treated sugarcane bagasse.

Runs	Time (h)	Temperature (°C)	Glucose (g.L <sup>-1</sup> )	Xylose (g.L <sup>-1</sup> )
1	-1 (24)	-1 (45)	4.88	3.94
2	+1 (120)	-1 (45)	8.18	6.54
3	-1 (24)	+1 (55)	6.61	4.84
4	+1 (120)	+1 (55)	7.93	7.22
5	-1.41 (4.12)	0 (50)	1.28	2.3
6	+1.41 (139.88)	0 (50)	6.98	5.6
7	0 (72)	-1.41 (42.93)	6.73	5.3
8	0 (72)	+1.41 (57.07)	6.46	5.98
9	0 (72)	0 (50)	8.03	6.04
10	0 (72)	0 (50)	8.12	6.12
11	0 (72)	0 (50)	8.05	5.99
12	0 (72)	0 (50)	8.04	6.03

Figure 11. Pareto diagram of time and temperature influence on the enzymatic saccharification.

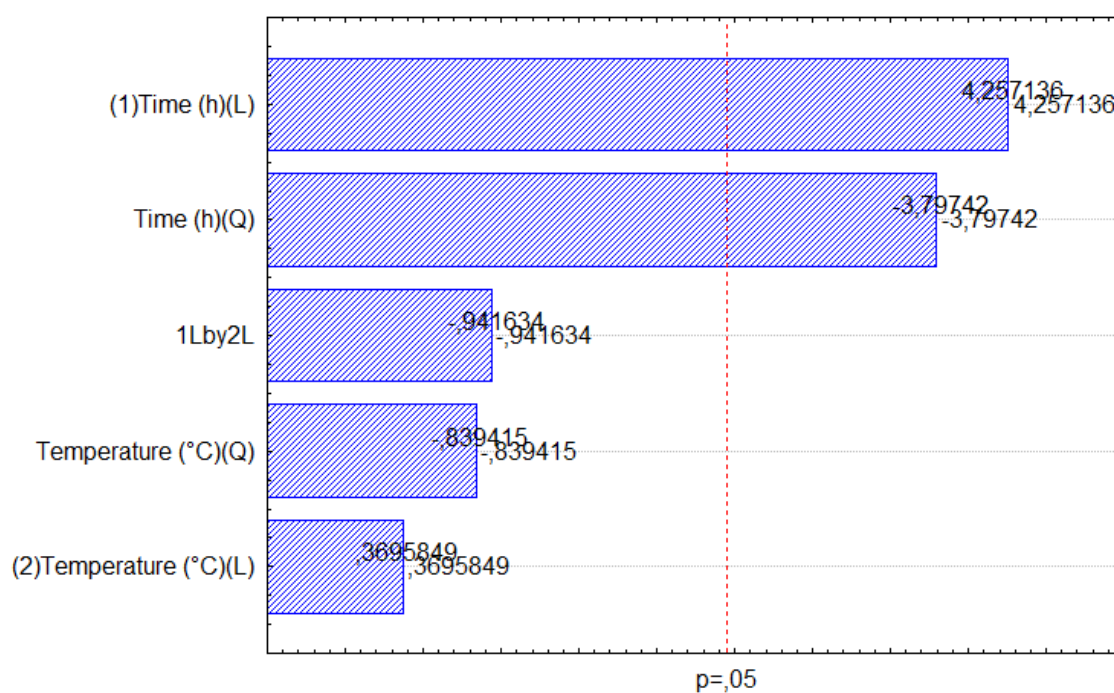
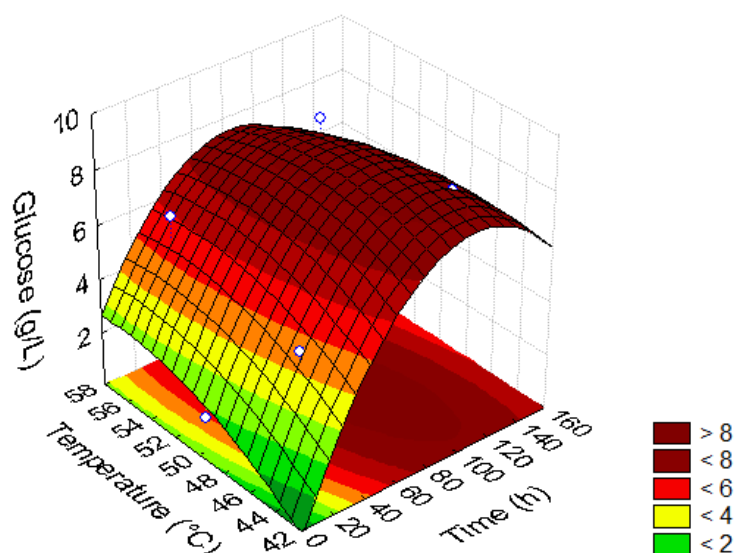


Table 13. Analyzes of variance (ANOVA) to enzymatic saccharification of sugarcane bagasse.

Variables	F	p-value
Time (L)	18.12	0.005
Time (Q)	14.42	0.008
Temperature (L)	0.15	0.72
Temperature (Q)	0.70	0.43
1L by 2L	0.88	0.38

Figure 12. Response surface generated to the glucose concentration obtained after enzymatic saccharification using time and temperature as variable in the statistical planning.



Using the desirability function of the STATISTICA 10.0 software, it was possible to predict the optimum values of the independent variables analyzed. For this analysis, the best conditions expected for the enzymatic saccharification were 106 hours at 50°C with 8.39 g/L of glucose concentration. Experimental validation was performed with enzymatic saccharification assays in triplicate, obtaining glucose at 8.05 g/L  $\pm$  0,22, being within the desirability parameter. Under these conditions, the xylose concentration obtained was 5.75 g/L  $\pm$  0,34. Reducing sugars were analyzed and the validation presented 29.1 g/L that corresponding to the yield of the 63 mg glucose/g bagasse or 85.05mg glucose/g cellulose.

Zhu et al. (2012) carried out saccharification of sugarcane bagasse submitted to alkaline pre-treatment using ammonium hydroxide in the presence of hydrogen peroxide for 20 hours of pre-treatment at 60 ° C using commercial enzyme Celluclast 1.5L (Novozymes). The enzyme loading employed was 20 FPU/g cellulose and the assay was maintained at 40 ° C for 36 hours. The authors cite a concentration of 7.0 g/L of reducing sugars in the hydrolysates, lower than that obtained in the present study. Cheng et al. (2008) cited the concentration of reducing sugars of 30.0 g/L in the hydrolysates obtained by the saccharification of cane bagasse pretreated in alkaline medium (NaOH) in the presence of 0.6% hydrogen peroxide using the commercial enzyme Cellulase ZC- 1700 (CTATEX Chemical Co. LTD) (loading 20 FPU/g cellulose into 4% sugarcane bagasse).

### **3.3. Alcoholic fermentation inhibitors**

The alcoholic fermentation inhibitors were evaluated from the optimized hydrolyzate according to the fixed statistical design with 250 units of endoglucanase/g cellulose, 11% of sugarcane bagasse pretreated with sodium hydroxide under hydrothermal conditions, at 50° C for 106 hours of enzymatic saccharification. According to Table 9, the amounts of organic acids such as vanillic, sirgenic and ferulic, derived from lignin during the saccharification process, are insignificant, with about 0.03 mg/ml (vanillic acid), 0.05 mg/ml (syringic acid) and 0.04 mg/ml (ferulic acid).

According to the literature, concentrations of up to 1.8mM of ferulic acid and 1mg/ml of vanillic acid do not interfere with the fermentation metabolism of yeast *Saccharomyces cerevisiae* (Adeboye et al., 2015; Anda; Arai; Kyoto, 1986). Other inhibitors such as hydroxymethylfurfural and furfural that destabilize the growth rate of yeast, increasing the lag phase and decreasing the permeability of the cell membrane (PALMQVIST; HAHNHÄGERDAL, 2000), did not present significant concentrations. In up to 2.15 mg/ml HMF, studies by Hawkins and Doran-Peterson (2011) show that *S. cerevisiae* presented growth without any change.

The concentrations of alcoholic fermentation inhibitors generated along the enzymatic saccharification with enzymatic extract of the *P. sanguineus* MCA 16 are lower. Thus, the respective hydrolyzate resulting from the enzymatic

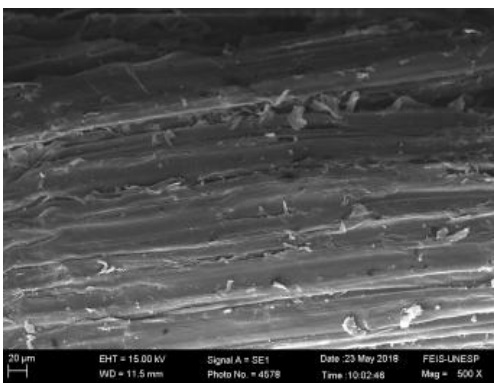
saccharification of sugarcane bagasse under hydrothermal pretreatment using 2.5% hydrogen peroxide under alkaline conditions with sodium hydroxide did not present relevant characteristics that may confer toxicity to a subsequent alcoholic fermentation.

### **3.2.5. Scanning electron microscopy of sugarcane bagasse**

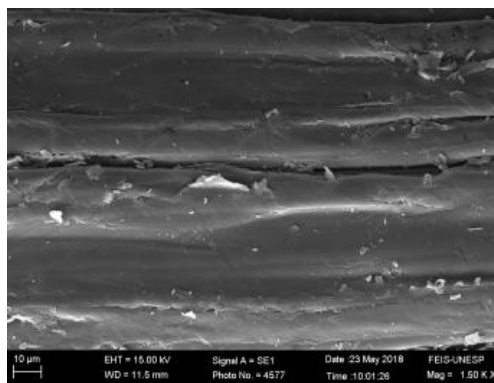
Samples of *in natura* and pretreated (alkaline hydrothermal and alkaline hydrothermal with hydrogen peroxide at 2.5%) sugarcane bagasse were analyzed by scanning electron. Figure 13 (a and b) shows that *in natura* bagasse surface is smooth and continuous while pretreated bagasses have more rough surfaces (Figure 13c – f). These results are according to the works reported by (Lima et al., 2014; Ramadoss and Muthukumar, 2015; Rezende et al., 2011; Zhu et al., 2016). It can be observed that, after pretreatments, the fibers were bundled probably due to lignin removal from the inner cell wall which in turn resulted in increased cellulose network exposure (Figure 13d – f) . The addition of 2.5% of hydrogen peroxide promoted more bundled fibers separated in the surface and the lignin removal.

Figure 13. Scanning electron microscopy of sugarcane bagasse. (a) *In natura* 500x, (b) *in natura* 1500x, (c and d) submitted to hydrothermal alkaline pretreatment 500x and 1500x, respectively, (e and f) submitted to hydrothermal alkaline pretreatment with hydrogen peroxide (2.5%) 500x and 1500x, respectively.

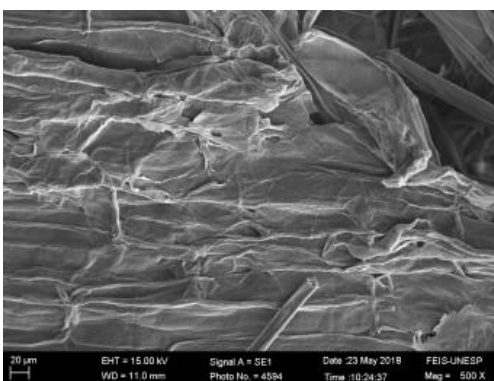
a)



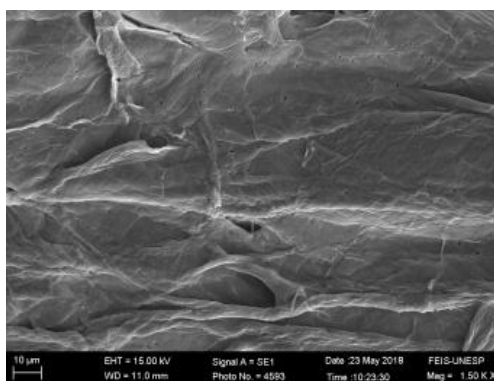
b)



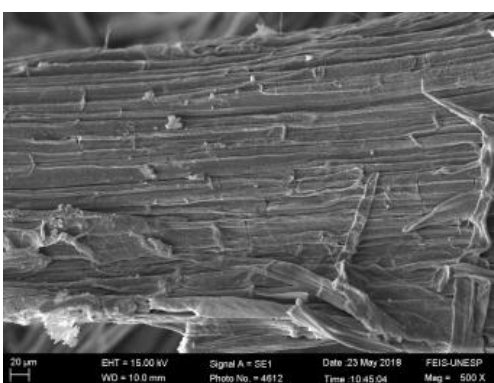
c)



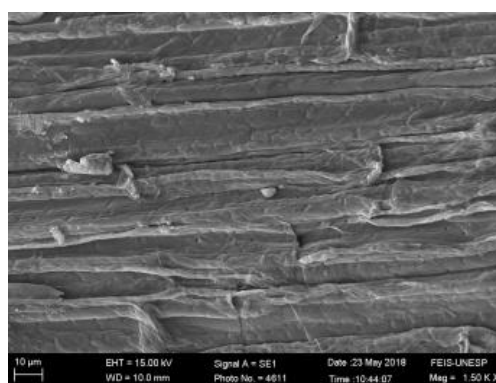
d)



e)



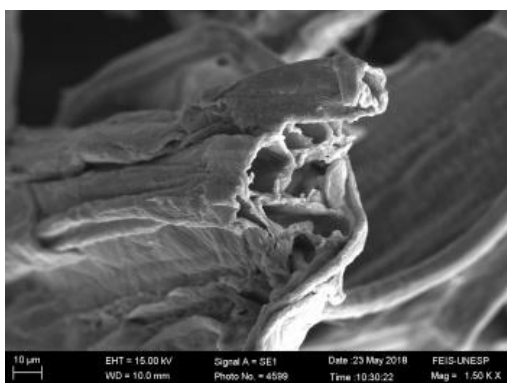
f)



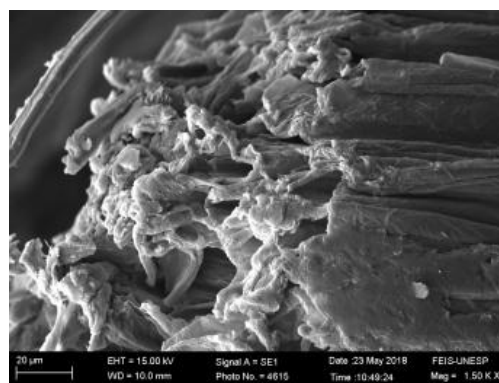
When analyzing the conducting vessels surrounded by cell bundles, it can be noted that they are more damaged in bagasse submitted to alkaline pretreatment with hydrogen peroxide at 2.5% (Figure 14b) when compared to the bagasse submitted to the same pretreatment without the oxidizing agent (Figure 14a).

Figure 14. Scanning electron microscopy of sugarcane bagasse submitted to alkaline hydrothermal pretreatment 1500x (a) and alkaline hydrothermal pretreatment with hydrogen peroxide at 2.5% 1500x (b).

a)



b)



## 6. Conclusions

The use of oxidizing agents in the pretreatment process of hydrothermal treatment of sugarcane bagasse decreased the lignin content, with a highlighted effect in alkaline medium. The glucose concentration after the saccharification of the sugarcane bagasse pretreated with 2.5% of  $H_2O_2$  was greater compared to those released using only alkaline medium, with an increase of 10% (compared with Chapter 1).

## 7. Acknowledgements

The authors thank to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support, to the State University of São Paulo, to the Laboratory of Biochemistry and Applied Microbiology and to the Laboratory of Sucrochemistry and Analytical Chemistry of Institute of Biosciences, Letters and Exact Sciences, São José do Rio Preto and to the Laboratory of Microbial Enzymes of the Institute of Chemistry, Araraquara, São Paulo, Brazil.

## 8. References

- ABDELAZIZ, O.Y., GADALLA, M.A., EL-HALWAGI, M.M., ASHOUR, F.H. A hierarchical approach for the design improvements of an Organocat biorefinery. **Bioresour. Technol.** 181, 321–329, 2015.
- ADEBOYE, P.T., BETTIGA, M., ALDAEUS, F., LARSSON, P.T., OLSSON, L. Catabolism of coniferyl aldehyde, ferulic acid and p-coumaric acid by *Saccharomyces cerevisiae* yields less toxic products. **Microb. Cell Fact.** 1–14, 2015.
- AMIN, F.R., KHALID, H., ZHANG, H., RAHMAN, S., ZHANG, R., LIU, G., CHEN, C. Pretreatment methods of lignocellulosic biomass for anaerobic digestion. **AMB Express** 2–12, 2017.
- ANANDGAONKER, P., KULKARNI, G., GAIKWAD, S., RAJBHOJ, A. Nanocrystalline titanium dioxide catalyst for the synthesis of azlactones. **Chinese J. Catal.** 35, 196–200, 2014.
- AYENI, A.O., ADEEYO, O.A., AYOOLA, A. Effective Gravimetric Characterization for Lignocellulosic Biomass: Comparison of NaOH-H<sub>2</sub>O<sub>2</sub> and Ca(OH)<sub>2</sub>-H<sub>2</sub>O<sub>2</sub> Oxidation Pretreated Sugarcane Bagasse. **Int. J. Sci. Eng. Technol.** 9, 5–9, 2015.
- BLAND, D.E., LOGAN, A F. The Properties of Syringyl, Guaiacyl and p-Hydroxyphenyl Artificial Lignins. **Biochem. J.** 95, 515–520, 1965.
- BOSE, S.K., FRANCIS, R.C., GOVENDER, M., BUSH, T., SPARK, A. Lignin content versus syringyl to guaiacyl ratio amongst poplars. **Bioresour. Technol.** 100, 1628–1633, 2009.



- BRIENZO, M., SIQUEIRA, A.F., MILAGRES, A.M.F. Search for optimum conditions of sugarcane bagasse hemicellulose extraction. **Biochem. Eng. J.** 46, 199–204, 2009.
- BURANOV, A.U., MAZZA, G. Lignin in straw of herbaceous crops. **Ind. Crops Prod.** 28, 237–259, 2008.
- CAO, W., SUN, C., LIU, R., YIN, R., WU, X. Comparison of the effects of five pretreatment methods on enhancing the enzymatic digestibility and ethanol production from sweet sorghum bagasse. **Bioresour. Technol.** 111, 215–221, 2012.
- CARDONA, C. A, SÁNCHEZ, O.J. Fuel ethanol production: process design trends and integration opportunities. **Bioresour. Technol.** 98, 2415–57, 2007.
- CELEBI, A.D., ENSINAS, A.V., SHARMA, S., MARÉCHAL, F. Early-stage decision making approach for the selection of optimally integrated biorefinery processes. **Energy** 137, 1–9, 2017
- CHENG, K., ZHANG, J., PING, W., GE, J.-P., ZHOU, Y.-J., LING, H.-Z., XU, J.-M. Sugarcane Bagasse Mild Alkaline/Oxidative Pretreatment for Ethanol Production by Alkaline Recycle Process. **Appl. Biochem. Biotechnol.** 151, 43–50, 2008.
- COLOM, X., CARRILLO, F., NOGUÉS, F., GARRIGA, P. Structural analysis of photodegraded wood by means of FTIR spectroscopy. **Polym. Degrad. Stab.** 80, 543–549, 2003.
- DAOU, M., FAULDS, C.B. Glyoxal oxidases: their nature and properties. **World J. Microbiol. Biotechnol.** 33, 1–11, 2017.
- DASH, S., PATEL, S., MISHRA, B.K. Oxidation by permanganate: synthetic and mechanistic aspects. **Tetrahedron** 65, 707–739, 2009.
- DE CASSIA PEREIRA, J., TRAVAINI, R., PAGANINI MARQUES, N., BOLADO-RODRÍGUEZ, S., BOCCHINI MARTINS, D.A. Saccharification of ozonated sugarcane bagasse using enzymes from *Myceliophthora thermophila* JCP 1-4 for sugars release and ethanol production. **Bioresour. Technol.** 204, 122–129, 2016.

- GAO, D., CHUNDAWAT, S.P.S., SETHI, A., BALAN, V., GNANAKARAN, S., DALE, B.E. Increased enzyme binding to substrate is not necessary for more efficient cellulose hydrolysis. **Proc. Natl. Acad. Sci.** 110, 10922–10927, 2013.
- GÍRIO, F.M., FONSECA, C., CARVALHEIRO, F., DUARTE, L.C., MARQUES, S., BOGEL-ŁUKASIK, R. Hemicelluloses for fuel ethanol: A review. **Bioresour. Technol.** 101, 4775–800, 2010.
- GONZÁLEZ-GARCÍA, S., GULLÓN, B., RIVAS, S., FEIJOO, G., MOREIRA, M.T. Environmental performance of biomass re fi ning into high-added value compounds. **J. Clean. Prod.** 120, 170–180, 2016.
- GRAY, K.A., ZHAO, L., EMPTAGE, M. Bioethanol. **Curr. Opin. Chem. Biol.** 141–146, 2012.
- HAWKINS, G.M., DORAN-PETERSON, J. A strain of *Saccharomyces cerevisiae* evolved for fermentation of lignocellulosic biomass displays improved growth and fermentative ability in high solids concentrations and in the presence of inhibitory compounds. **Biotechnol. Biofuels** 1–14, 2011.
- HERNÁNDEZ-ORTEGA, A., FERREIRA, P., MARTÍNEZ, A.T. Fungal aryl-alcohol oxidase: A peroxide-producing flavoenzyme involved in lignin degradation. **Appl. Microbiol. Biotechnol.** 93, 1395–1410, 2012.
- JÖNSSON, L.J., MARTÍN, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. **Bioresour. Technol.** 199, 103–112, 2016.
- KADLA, J.F., CHANG, H. The Reactions of Peroxides with Lignin and Lignin Model Compounds, in: Argyropoulos, D. (Ed.), In *Oxidative Delignification Chemistry*. American Chemical Society, Washington, DC, pp. 108–129, 2001.
- KHAN, W.Z., NAJEEB, I., TUIYEBAYEVA, M. Refinery wastewater degradation with titanium dioxide , zinc oxide , and hydrogen peroxide in a photocatalytic reactor. **Process Saf. Environ. Prot.** 4, 479–486, 2014.
- KIHLMAN, M., MEDRONHO, B.F., ROMANO, A.L., GERMGÅRD, U., LINDMAN, B. Cellulose dissolution in an alkali based solvent: Influence of additives and pretreatments. **J. Braz. Chem. Soc.** 24, 295–303, 2013.

- LIMA, M.A., GOMEZ, L.D., STEELE-KING, C.G., SIMISTER, R., BERNARDINELLI, O.D., CARVALHO, M.A., REZENDE, C.A., LABATE, C.A., EDUARDO, R., MCQUEEN-MASON, S.J., POLIKARPOV, I. Evaluating the composition and processing potential of novel sources of Brazilian biomass for sustainable biorenewables production. **Biotech. Biofuels**, 2–19, 2014.
- LIN, T., PAN, S., CHEN, W., BIN, S. Role of pre-oxidation, using potassium permanganate, for mitigating membrane fouling by natural organic matter in an ultrafiltration system. **Chem. Eng. J.** 223, 487–496, 2013.
- LV, W., YU, Z. Isolation and characterization of two thermophilic cellulolytic strains of *Clostridium thermocellum* from a compost sample. **J. Appl. Microbiol.** 114, 1001–1007, 2013.
- MA, L., CUI, Y., CAI, R., LIU, X., ZHANG, C., XIAO, D. Optimization and evaluation of alkaline potassium permanganate pretreatment of corncob. **Bioresour. Technol.** 180, 1–6, 2015.
- MATHEW, S., ABRAHAM, T.E. Bioconversions of Ferulic Acid, an Hydroxycinnamic Acid. **Crit. Rev. Microbiol.** 32, 115–125., 2006.
- MILLER, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. **Anal. Chem.** 426–428, 1959.
- MIRON, J., BEN-GHEDALIA, D. Effect of hydrolysing and oxidizing agents on the composition and degradation of wheat straw monosaccharides. **Eur. J. Appl. Microbiol. Biotechnol.** 15, 83–87, 1982.
- MORETTI, M.M. DE S., BOCCHINI-MARTINS, D.A., NUNES, C. DA C.C., VILLENA, M.A., PERRONE, O.M., SILVA, R. DA, BOSCOLO, M., GOMES, E. Pretreatment of sugarcane bagasse with microwaves irradiation and its effects on the structure and on enzymatic hydrolysis. **Appl. Energy** 122, 189–195, 2014.
- PALMQVIST, E., HAHN-HAGERDAL, B. Fermentation of lignocellulosic hydrolysates . II: inhibitors and mechanisms of inhibition. **Bioresour. Technol.** 74, 25–33, 2000.
- RABELO, S.C., ANDRADE, R.R., FILHO, R.M., COSTA, A.C. Alkaline hydrogen peroxide pretreatment , enzymatic hydrolysis and fermentation of sugarcane bagasse to ethanol. **Fuel** 136, 349–357, 2014.

- RAMADOSS, G., MUTHUKUMAR, K. Influence of dual salt on the pretreatment of sugarcane bagasse with hydrogen peroxide for bioethanol production. **Chem. Eng. J.** 260, 178–187, 2015.
- REZENDE, C.A., LIMA, M.A. DE, MAZIERO, P., RIBEIRO, E. Chemical and morphological characterization of sugarcane bagasse submitted to a delignification process for enhanced enzymatic digestibility. **Biotech. Biofuels**, 2-18, 2011.
- RODRÍGUEZ-DELGADO, M.A., MALOVANÁ, S., PÉREZ, J.P., BORGES, T., GARCÍA MONTELONGO, F.J. Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. **J. Chromatogr A** 912, 249–257, 2001.
- ROSAZZA, J.P.N., HUANG, Z., DOSTAL, L., VOLM, T., Rousseau, B. Biocatalytic transformations of ferulic acid: an abundant aromatic natural product. **J. Ind. Microbiol.** 15, 457–471, 1995.
- SINGLETON, V.L., ORTHOFER, R., LAMUELA-RAVENTÓS, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin Ciocalteu reagent. **Methods Enzymol.** 299, 152–178, 1999.
- SLUITER, A., HAMES, B., RUIZ, R., SCARLATA, C., SLUITER, J., TEMPLETON, D. Determination of Sugars, Byproducts and Degradation Products in Liquid Fraction Process Samples Laboratory Analytical Procedure (LAP). **Anal. Technol. Team Lab. Anal. Proced.** 2008, 1–4. Technical Report NREL/TP-510-42623, 2008a.
- SLUITER, A., HAMES, B., RUIZ, R., SCARLATA, C., SLUITER, J., TEMPLETON, D. Determination of Ash in Biomass Laboratory Analytical Procedure (LAP). **Biomass Anal. Technol. Team Lab. Anal. Proced.** 2008, 1–8. Technical Report NREL/TP-510-42622, 2008b.
- SLUITER, A., HAMES, B., RUIZ, R.O., SCARLATA, C., SLUITER, J., TEMPLETON, D., Energy, D. Determination of Structural Carbohydrates and Lignin in Biomass. **Biomass Anal. Technol. Team Lab. Anal. Proced.** 2011, 1–14. Technical Report NREL/TP-510-42618, 2004.
- SU, Y., DU, R., GUO, H., CAO, M., WU, Q. Fractional pretreatment of lignocellulose by alkaline hydrogen peroxide : Characterization of its major components. **Food Bioprod. Process.** 4, 322–330, 2014.

- SUN, J.X., SUN, X.F., SUN, R.C., FOWLER, P., BAIRD, M.S. Inhomogeneities in the Chemical Structure of Sugarcane Bagasse Lignin. **J. Agric. Food Chem.** 51, 6719–6725, 2003.
- SUN, J.X., SUN, X.F., ZHAO, H., SUN, R.C. Isolation and characterization of cellulose from sugarcane bagasse. **Polym. Degrad. Stab.** 84, 331–339, 2004.
- TALEBNIA, F., KARAKASHEV, D., ANGELIDAKI, I. Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation. **Bioresour. Technol.** 101, 4744–53, 2010.
- TENGERDY, R.P., SZAKACS, G. Bioconversion of lignocellulose in solid substrate fermentation. **Biochem. Eng. J.** 13, 169–179, 2003.
- TOQUERO, C., BOLADO, S. Effect of four pretreatments on enzymatic hydrolysis and ethanol fermentation of wheat straw. Influence of inhibitors and washing. **Bioresour. Technol.** 157, 68–76, 2014.
- TRAVAINI, R., OTERO, M.D.M., COCA, M., DA-SILVA, R., BOLADO, S. Sugarcane bagasse ozonolysis pretreatment: effect on enzymatic digestibility and inhibitory compound formation. **Bioresour. Technol.** 133, 332–9, 2013.
- UNITED NATIONS CLIMATE CHANGE CONFERENCE (UNFCCC). Adoption of The Paris Agreement: Proposal by the President to the United Nations Framework Convention on Climate Change 21932, 1–32, 2015.
- UNIÃO DA INDÚSTRIA DE CANA-DE-AÇÚCAR (UNICA). Relatório final da safra de 2013/2014, região centro-sul, 2014.
- UYAMA, H., IKEDA, R., YAGUCHI, S., KOBAYASHI, S. Enzymatic polymerization of natural phenol derivatives and enzymatic synthesis of polyesters from vinyl esters. **ACS Symp. Ser.** 764, 113–127, 2000.
- VIVEK, N., CHRISTOPHER, M., KUMAR, M.K., CASTRO, E., BINOD, P., PANDEY, A. Pentose rich acid pretreated liquor as co-substrate for 1,3-propanediol production. **Review. Energy** 1–6, 2017.
- VIVEK, N., PANDEY, A., BINOD, P. Biological valorization of pure and crude glycerol into 1,3-propanediol using a novel isolate *Lactobacillus brevis* N1E9.3.3. **Bioresour. Technol.** 213, 222–230, 2016.

- XIANG, Q., LEE, Y.Y. Oxidative cracking of precipitated hardwood lignin by hydrogen peroxide. **Appl. Biochem. Biotechnol.** 84–86, 153–162, 2000.
- XU, X.R., LI, H. BIN, WANG, W.H., GU, J.D. Decolorization of dyes and textile wastewater by potassium permanganate. **Chemosphere** 59, 893–898, 2005.
- YANG, Q., QIN, X., ZHANG, L. Properties of cellulose films prepared from NaOH/urea/zincate aqueous solution at low temperature. **Cellulose** 18, 681–688, 2011.
- YOO, C.G., WANG, C., YU, C., KIM, T.H. Enhancement of enzymatic hydrolysis and klason lignin removal of corn stover using photocatalyst-assisted ammonia pretreatment. **Appl. Biochem. Biotechnol.** 169, 1648–1658, 2013.
- YU, H., YOU, Y., LEI, F., LIU, Z., ZHANG, W., JIANG, J. Comparative study of alkaline hydrogen peroxide and organosolv pretreatments of sugarcane bagasse to improve the overall sugar yield. **Bioresour. Technol.** 187, 161–166, 2015.
- ZHANG, J., MA, X., YU, J., ZHANG, X., TAN, T. The effects of four different pretreatments on enzymatic hydrolysis of sweet sorghum bagasse. **Bioresour. Technol.** 102, 4585–4589, 2011.
- ZHU, Z., ZHU, M., WU, Z. Pretreatment of sugarcane bagasse with NH<sub>4</sub>OH–H<sub>2</sub>O<sub>2</sub> and ionic liquid for efficient hydrolysis and bioethanol production. **Bioresour. Technol.** 119, 199–207, 2012.
- ZHU, Z., ALVES, C., SIMISTER, R., MCQUEEN-MASON, S.J., MACQUARRIE, D.J., POLIKARPOV, I., GOMEZ, L.D. Efficient sugar production from sugarcane bagasse by microwave assisted acid and alkali pretreatment. **Biomass and Bioenergy** 93, 269–278, 2016.
- ZHUANG, X., YUAN, Z., MA, L., WU, C., XU, M., XU, J., ZHU, S., Qi, W. Kinetic study of hydrolysis of xylan and agricultural wastes with hot liquid water. **Biotechnol. Adv.** 27, 578–582, 2009.

## Capítulo 3

### Degradation of phenolic compounds by ligninases from *Pycnoporus sanguineus* MCA 16 during enzymatic saccharification of pretreated sugarcane bagasse

Josiani de Cassia Pereira<sup>1</sup>, Natália Paganini Marques<sup>2</sup>, Ariela Veloso de Paula<sup>3</sup>, Maurício Boscolo<sup>1</sup>, Roberto da Silva<sup>1</sup>, Eleni Gomes<sup>1</sup>, Daniela Alonso Bocchini<sup>2</sup>

<sup>1</sup> Institute of Biosciences, Letters and Exact Sciences, São Paulo State University – IBILCE/UNESP, São José do Rio Preto, São Paulo State, Brazil

<sup>2</sup> Institute of Chemistry, São Paulo State University – IQ/UNESP, Araraquara, São Paulo State, Brazil

<sup>3</sup> Faculty of Pharmaceutical Science, São Paulo State University – FCFAR/UNESP, Araraquara, São Paulo State, Brazil.

#### Abstract

In enzymatic saccharification of lignocellulosic biomass, phenolic compounds may be an obstacle by inhibiting cellulolytic enzymes activities and hindering the releasing of glucose. This problem may be solved by the action of ligninolytic enzymes such as laccase, manganese peroxidase and lignin peroxidase which can degrade the phenolic compounds. In the present work, the activity of ligninases present in the enzymatic solution produced by *Pycnoporus sanguineus* MC 16 was evaluated after incubation at different temperatures and also during enzymatic saccharification of pretreated sugarcane bagasse. In both experiments, the concentration of phenolic compounds was also monitored. After 48h of incubation at 50 and 70 °C, the total phenolic compounds (TPC) concentration decreased approximately 66 %. Regarding enzymes thermal stability under these conditions, laccase was more sensitive, totally losing its activity after 12 or 4 hours of incubation, at 50 and 70°C, respectively. Regarding bagasse enzymatic saccharification, after 48 h a reduction of 45% in TPC was observed. It is worth mentioning that laccase and LiP activities were improved during the period of 12 and 48 hours, respectively. These results serve as a basis on how these ligninolytic enzymes act in the absence and presence of sugarcane bagasse, which can act as a substrate, since it has lignin in its structure, besides the phenolic compounds present in

the medium. As soon as they find their substrates, these enzymes contribute to the enzymatic pretreatment of both the enzymatic extract and the hydrolyzed medium.

## 1.Introduction

Phenolic compounds are molecules with antioxidant properties, of great interest for food, pharmaceutical and fine chemicals industries. Besides the importance of phenolic compounds for human health, some hydroxyl, carbonyl and methyl groups of these molecules can react with cellulases and cause their inactivation (QIN et al., 2016; XIMENES et al., 2011). This fact can represent an obstacle to the action of these enzymes when they are used in biotechnological processes, such as saccharification of plant biomass to obtain glucose for cellulosic ethanol production (DE CASSIA PEREIRA et al., 2016).

Cellulosic ethanol is produced by the fermentation of glucose obtained from chemical or enzymatic saccharification of lignocellulosic biomass such as sugarcane bagasse, wheat bran, corn straw, among others. For the efficient bioconversion of this material, cellulose degrading enzymes such as endoglucanase, exoglucanase and  $\beta$ -glucosidase, are required. These enzymes depolymerize the polysaccharide releasing celooligosaccharides, cellobiose and glucose. Ligninases, such as laccases and peroxidases, offer enzymatic pretreatment and remove recalcitrant material that remains even after pretreatment.

Cellulases are produced by several microorganisms, especially filamentous fungi that can be cultivated using lignocellulosic residues as substrates by solid-state fermentation. This type of bioprocessing is advantageous not only from the economic point of view, since the residues used are easily obtainable and widely available at low cost that it provides when compared to submerged fermentation.

In solid-state fermentation the substrates provide support for hyphae growth and are also used as carbon, nitrogen and minerals sources. Under these conditions, in addition to cellulases and other enzymes, secondary metabolites are also secreted, including phenolic compounds (isoflavones, flavonoids, flavanols, flavonols, antocyanines, flavones e flavanones) (BALA et



al., 2014; MARTINS et al., 2011; RASHAD et al., 2011; SALAR et al., 2012; SINGH et al., 2010; ZHENG; SHETTY, 2000). Besides, during the cultivation on lignocellulosic substrates, phenolic compounds can be generated from lignin degradation and they include hydroxycinnamic and hydroxybenzoic acids such as ferulic, vanillic, vanillic acid, caffeic, gallic acid, among others (VASILE et al., 2016). Thus, enzymes and phenolic compounds resulting from fungal cultivation remain in the liquid phase obtained after the extraction process, commonly called crude enzyme solution.

In order to avoid cellulases inhibition by phenolic compounds present in the extract obtained from the solid-state fermentation, a biological treatment can be carried out by the action of ligninases (Hernández-Ortega et al., 2012; Tapia-Tussell et al., 2015). If the cellulase-producing fungus itself is also a producer of ligninases, there is an advantage in the process.

Among the lignin-degrading enzymes there are the peroxidases (lignin peroxidase and manganese peroxidase) and laccases. Lignin peroxidase (LiP) and manganese peroxidase (MnP) (E.C.1.11.14 and E.C. 1.11.1.13, respectively) present iron in their molecular structures, which promote redox reactions. LiP can degrade both lignin and phenolic compounds present in the medium while MnP acts only on phenolic compounds. Laccases (E.C. 1.10.3.2.) oxidize phenolic and non-phenolic substrates including those in lignin structure. They are also called “blue” proteins because they have copper in their active site (ERIKSSON; BERMEK, 2009; FALADE et al., 2017; FUJIAN et al., 2001; RIVA et al., 2006).

There are several studies about the use of peroxidases and laccases to degrade lignin and also phenolic compounds, including those present in different industrial wastewater (FENG et al., 2013a, 2013b; KOLB et al., 2012; TROVASLET et al., 2007; VERMA; MADAMWAR, 2002). However, data related to the action of these enzymes on the phenolic compounds present in microbial enzymatic extracts with cellulases activities, obtained by solid-state fermentation and destined to the saccharification of lignocellulosic materials, are still absent in the literature. In addition, there is also no data available about the performance of these enzymes on phenolic compounds and/or lignin during the enzymatic saccharification of lignocellulosic materials.

Therefore, this work aimed to evaluate the degradation of phenolic compounds present in the enzymatic extract obtained from the cultivation of *Pycnoporus sanguineus* MCA 16 by solid-state fermentation on wheat and soybean meal as substrates, by the action of ligninases components of this extract. In addition, the enzymatic extract was used to saccharify pre-treated sugarcane bagasse and the ligninases activities, as well as their actions on the phenolic compounds, were monitored during these process.

## **2. Material and methods**

### **2.1. Lignocellulosic substrates**

The sugarcane bagasse was donated by Virgolino de Oliveira S/A Sugar and Alcohol, José Bonifácio-SP, Brazil, washed with distilled water to remove residues of sugars and particulate matter, dried at 37 °C under air circulation and crushed (3 mm mesh), in Trapp Forage Crusher model TRF-400. Soybean meal was donated by Trow Nutrition, from Mirassol - São Paulo, Brazil, wheat bran was purchased in the Natural Life® market and both were used without washing or crushing.

### **2.2. Ligninases activities**

Laccase activity was determined at 40 °C for 1 minute using 0.9 mL of substrate solution (2,2'-azino-bis-ethylbenziaholine - ABTS - 0.03% in sodium acetate buffer solution 0.1 mol.L<sup>-1</sup>, pH 5.0) and 0.1 mL of the enzymes solution (BUSWELL et al., 1995). The oxidation of ABTS was measured by monitoring the increase in absorbance at 420 nm. Blank assay was prepared by replacing the enzymes solution with equal volume of distilled water, while controls were made replacing the ABTS by distilled water. A unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 mmol of ABTS per minute using  $\epsilon_{420} = 3.6 \times 10^4 \text{ M}^{-1}.\text{cm}^{-1}$  for the oxidized ABTS (BOURBONNAIS; PAICE, 1988)

The activity of manganese peroxidase (MnP) was determined at 40 °C for 10 minutes in a reaction mixture composed of 0.25 mL of 50 mmol/L sodium acetate buffer (pH 4.5), 0.1 mL of manganese sulphate (0.4 mol/L) and 0.1 mL of enzyme solution. The reaction was started with the addition of hydrogen peroxide (40 µmol/L) and the absorbance measured in a spectrophotometer at 240 nm. Blank assay was prepared substituting the crude extract by the same volume of distilled water, while controls were made substituting distilled water by MnSO<sub>4</sub>. A unit of enzyme activity was defined as the amount of enzyme required to form 1.0 µmol of Mn<sup>+3</sup> per minute using the molar extinction coefficient ( $\epsilon$ ) of  $8.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  (GLENN et al., 1999).

The activity of lignin peroxidase (LiP) was determined at 40 °C for 10 minutes in a reaction mixture composed of 50 mmol.L<sup>-1</sup> sodium acetate buffer (pH 5.0), 40 mmol.L<sup>-1</sup> veratryl alcohol solution and 0.1 mL of the enzyme extract, with final volume of 1.0 mL. The reaction was started by the addition of hydrogen peroxide (0.2 mmol.L<sup>-1</sup>) and the increase in absorbance, due to the oxidation of veratryl alcohol, was measured in a spectrophotometer at 310 nm. White was prepared replacing the enzymatic extract by equal volume of distilled water, while controls were made replacing the veratryl alcohol substrate by distilled water. A unit of enzyme activity was defined as the amount of enzyme required to form 1.0 µmol veratraldehyde per minute, considering  $\epsilon_{310} = 9.3 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$  for veratraldehyde (TIEN; KIRK, 1988).

### ***2.3. Ligninases physico-chemical characterization***

#### ***2.3.1 Effect of pH and temperature on enzyme activity and stability***

A 2<sup>2</sup> type face centered design was used to determine the optimum conditions of pH and temperature for laccase, LiP and MnP activities including 4 replicates at the central point the enzymes activities according to Rodrigues and lemma (2009). Statistical software (Statsoft 7.0) was used to analyze the results, which were submitted to analysis of variance (ANOVA). Table 14 presents the coded and real values of the studied variables. The buffers used to determine the enzymatic activities were: McIlvaine (0.2 mol.L<sup>-1</sup> sodium phosphate, 0.1 mol.L<sup>-1</sup> citric acid, pH 3.0 and 4.0), MES (Sigma-Aldrich M8250, pH 5.0) and MOPS (Sigma-Aldrich M1254, pH 6.0 and 7.0).

Enzymes stability regarding pH variations was evaluated by incubating the enzymes during 24h, at 25 °C and different pH values. The buffers used (0.1 mol.L<sup>-1</sup>) were McIlvaine (pH 3.0 - 8.0), Tris–aminomethane (pH 8.0 - 8.5) and Glycine-NaOH (pH 8.5 - 10.5). Thermal stability was studied by incubating the enzymes for 1h at temperature values ranging from 30 to 80 °C. The residual activities were determined under optimal conditions of pH and temperature of the enzyme. The highest enzymes activities, considered as 100% were 2.37, 1.02 and 1.6 U/mL for laccase, lignin peroxidase and manganese peroxidase, respectively.

Table 14. Coded and real values used in the the 2<sup>2</sup> type face-centered performed to study the effect of pH and temperature (°C) on enzymes activities.

Enzyme	Variable	Level		
		-1	0	+1
Laccase and LiP	X <sub>1</sub>	4.0	4.5	5.0
	X <sub>2</sub>	45	50	55
MnP	X <sub>1</sub>	3.0	5.0	7.0
	X <sub>2</sub>	65	70	75

X<sub>1</sub>: pH; X<sub>2</sub>: temperature

### 2.3.2. Effect of glucose, ions, organic and inorganic compounds

The effect of glucose on enzymes activities was evaluated by adding it at different concentrations (0 - 100 mmol.L<sup>-1</sup>) to the reaction mixture . The assays were carried out under optimum conditions of pH and temperature for each enzyme (item 2.3.1.). The effects of ions, organic and inorganic compounds on the enzymes activities were determined by adding them to the enzymes solution (final concentration of 10 mmol.L<sup>-1</sup>) and determining, after 15 minutes of incubation at 25 °C, the residuals activities under optimum conditions of pH and temperature (item 2.3.1.). The enzymes activities under optimal conditions without any treatment were considered as 100%.

#### **2.4. Sugarcane bagasse hydrothermal alkaline pretreatment**

Pretreatment was performed in autoclave (solid load of 5.0 % w/v in 0.5 mol L<sup>-1</sup> NaOH solution), in a 250 mL Erlenmeyer flask sealed with aluminum foil, for 1 hour, at 1 atm and 120 °C. After, the flask content was cooled using ice water bath and then kept under room temperature for stabilization. The material was filtered on nylon cloth and then the liquid phase was filtered on 0.22 µm filters. The solid phase was washed with distilled water, the excess of water was removed with cotton cloth and the pretreated bagasse was stored at 4 °C.

#### **2.5. Enzymes production**

In order to obtain the enzymes solution containing cellulases and ligninases, *P. sanguineus* MCA 16 was cultivated, by solid state fermentation, in a mixture (1:1 w/w) of wheat and soybean meal and 11.0 mL of nutrient solution described by Toyama, Ogawa (1978) (initial substrates moisture of 70%) in 250 mL Erlenmeyer flasks. The material was autoclaved at 121 °C, 1 atm, for 20 minutes, cooled and then inoculated with 6 mycelial discs of 1.0 cm diameter taken from a preculture in Petri dishes containing Malt Agar maintained at 40 °C, for 96 hours. Solid-state fermentations were maintained at 40°C, for 96 hours. After this period, 50 mL of distilled water were added to the Erlenmeyer flask and the mixture was homogenized in an orbital shaker, at 100 rpm, for 1h. The extract obtained was filtered on nylon cloth and centrifuged at 10.000 xg, for 15 minutes, at 4 °C. The supernatant was collected and used as crude enzymes solution for the determination of cellulase and ligninase activities, as well as for enzymatic saccharification. Solid state fermentations were performed as duplicates.

#### **2.6. Degradation of phenolic compounds by ligninases in the enzymatic extract**

To evaluate the action of ligninases on the phenolic compounds present in the enzyme extract, it was incubated, at 50 and 70 °C, for up to 48 hours. Samples were taken at 4, 24 and 48 hours, laccase, lignin peroxidase and manganese peroxidase activities were determined and total phenolic

compounds were quantified according to the Folin Ciocalteu method (SINGLETON et al., 1999) from analytical curve of gallic acid. All the samples were centrifuged at 10000 xg and 4°C for 15 minutes and the analysis were performed in duplicate. The enzymatic activities were presented as relative, considering 100% the activities before the incubation.

### ***2.7. Enzymatic saccharification of pretreated sugarcane bagasse***

Saccharification experiments were performed, in duplicate, in 250 mL Erlenmeyer flasks sealed with rubbers stoppers, using 3.0% of pretreated bagasse, 100 units of endoglucanase/g cellulose as enzyme load and citrate buffer solution (0.1 mol/L, pH 5.0) to a final volume of 20 mL, at 50 °C, under agitation of 300 rpm, for up to 48 hours. At each determined time interval a vial was taken, its contents was centrifuged at 10000 xg and 4°C for 15 minutes and filtered on 0.22 µm membranes. The solid phase was discarded and the liquid phase was used to determinelaccase, LiP and MnP activities, under optimal conditions of pH and temperature for each enzyme (item 2.3.), and also to quantify total phenolic compounds (TPC) (item 2.5.).

### ***2.8. Action of ligninases on phenolic compounds during enzymatic saccharification***

To evaluate the action of ligninases on phenolic compounds during enzymatic saccharification, aliquots were taken at 4, 24, and 48 hours and laccase, LiP and MnP activities were determined. In these samples, the concentration of total phenolic compounds was also determined according to the method described in item 2.5. The enzymatic activities were presented as relative, considering 100% the activities before the incubation.

### ***2.9. Analytical Methods***

Organic acids such as ferulic, *p*-coumaric, vanillic and syringic acids were quantified by high performance liquid chromatography (HPLC). The samples were centrifuged at 10000 xg, for 15 minutes, the supernatants were

filtered on 22  $\mu\text{m}$  and 20  $\mu\text{l}$  of each sample were applied to the Agilent Tec., Model 1220 Infinity LC, with binary pump, automatic sampler, column oven and a UV/VIS detector. The column used was C18 ZORBAX Eclipse Plus (4.6x250 mm), maintained at 25 °C. Chromatographic separation was performed using mobile phase methanol:acetic acid:water (10:2:88 v/v) as the solvent "A" and methanol:acetic:water (90:2:8 v/v) as solvent "B" (RODRIGUES-DELGADO et al., 2001).

### **2.10. Scanning electron microscopy**

Scanning electron microscopy (SEM) was performed using a FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) with an accelerating voltage of 12.5 kV. The bagasse was fixed using a solution of glutaraldehyde 2.5% in 0.1 mol.L<sup>-1</sup> phosphate buffer (pH 7.3), for 48 h, at room temperature. After this period, the material was washed with distilled water and post fixed in 1% osmium tetroxide aqueous solution, for 30 min, at room temperature. After fixation, the bagasse was dehydrated in an ethanol series, critical point-dried with CO<sub>2</sub>, and sputter coated with gold (Bal-Tec SCD 050).

## **3. Results and discussion**

### **3.1. Ligninases physico-chemical characterization**

Optimum conditions of pH and temperature for ligninases activities were determined through statistical design according to Rodrigues and lemma (2009). Tables 15 and 16 show the coded and real values employed in the 2<sup>2</sup> type face centered and central composite designs, respectively, and also the evaluated responses (relative activities).

Laccase, manganese peroxidase and lignin peroxidase highest activities (2.37, 1.59 and 0.08 U/mL) were obtained at pH 4.5/50 °C, pH 5.0/70 °C and pH 5.0/50 °C, respectively.

Table 15. Coded and real values of the face centered design ( $2^2$ ) used to evaluate the effect of pH and temperature on laccase and lignin peroxidase activities.

Runs	$X_1$	$X_2$	$Y_1$	$Y_2$
1	-1 (4.0)	-1 (45)	35.31	42.64
2	1 (5.0)	-1 (55)	48.25	26
3	-1 (4.0)	1 (45)	24.71	24.8
4	1 (5.0)	1 (55)	37.51	57
5	-1 (4.0)	0 (50)	55.45	55.53
6	1 (5.0)	0 (50)	62.16	100
7	0 (4.5)	-1 (45)	50.58	24.24
8	0 (4.5)	1 (55)	54.47	13.64
9	0 (4.5)	0 (50)	100	68.69
10	0 (4.5)	0 (50)	94.94	78.27
11	0 (4.5)	0 (50)	95.72	71.64
12	0 (4.5)	0 (50)	99.22	69.89

$x_1$ - pH;  $x_2$ - Temperature ( $^{\circ}$ C);  $Y_1$  and  $Y_2$  - Laccase and Lignin peroxidase relative activities (%), respectively.



Table 16. Coded and real values of the central composite design (RCCD) used to evaluate the effect of pH and temperature on manganese peroxidase activity.

Ensaio	$x_1$	$x_2$	$Y_3$
1	-1 (3.0)	-1 (65)	84.5
2	1 (7.0)	-1 (65)	39.92
3	-1 (3.0)	1 (75)	68.73
4	1 (7.0)	1 (75)	32.56
5	-1.41 (2.17)	0 (70)	43.93
6	1.41 (7.83)	0 (70)	30.62
7	0 (5.0)	-1.41 (62.93)	63.37
8	0 (5.0)	1.41 (77.07)	66.67
9	0 (5.0)	0 (70)	87.86
10	0 (5.0)	0 (70)	100
11	0 (5.0)	0 (70)	84.5
12	0 (5.0)	0 (70)	88.89

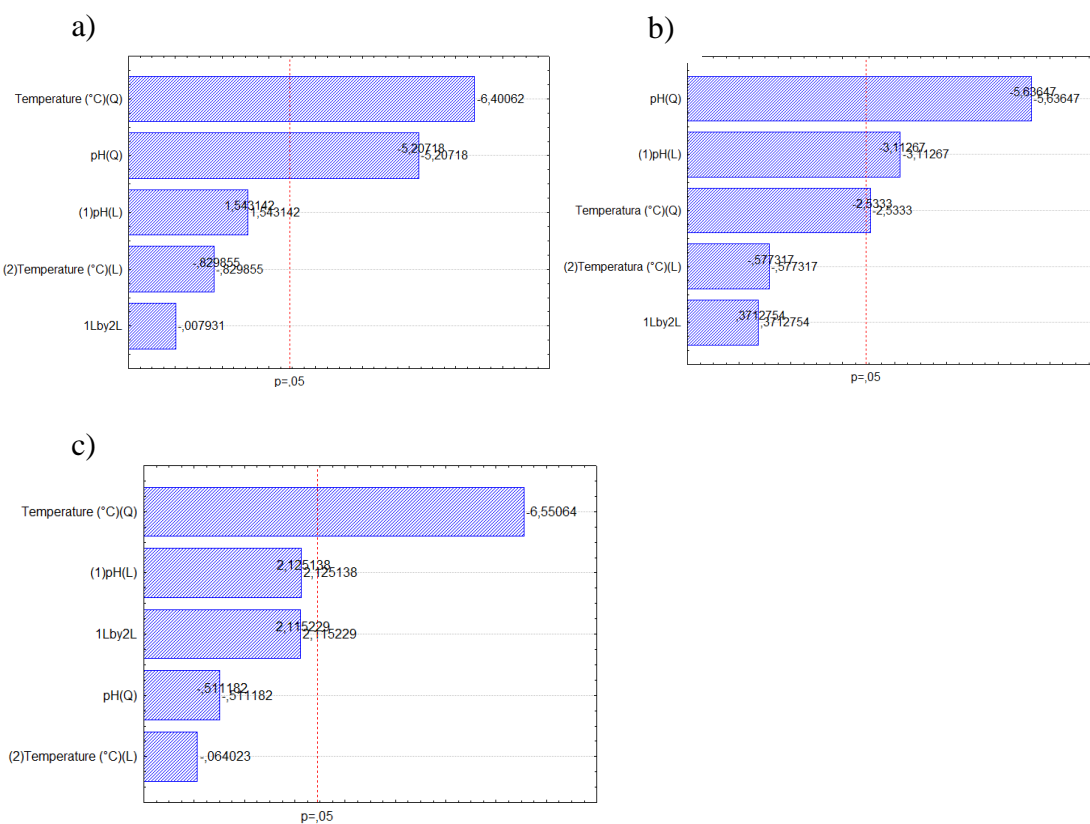
$x_1$ - pH;  $x_2$ - Temperature ( $^{\circ}$ C);  $Y_3$  - Manganese peroxidase relative activity (%).

The effects that each variable and their interactions exerted on the enzymes activities are presented by Pareto Diagram (Figure 15) and are confirmed by the analysis of variance (Table 17). It can be observed that pH and temperature were significant at the 95% confidence level ( $p$ -value<0.05) for laccase and manganese peroxidase (Table 17 and Figures 15 a and b). For lignin peroxidase only temperature was significant at this confidence level (Table 17 and Figure 15c). The interaction among the variables was not significant for all the enzymes.

Table 17. Analysis of variance ANOVA of the experimental design for ligninases activities.

Variable	Laccase		MnP		LiP	
	F	p	F	p	F	p
pH (L)	2.38	0,17	9.69	0.02	4.51	0.78
pH (Q)	27.11	0.002	31.77	0.001	0.26	0.63
Temperature (°C) (L)	0.69	0.44	0.33	0.58	0.004	0.95
Temperature (°C) (Q)	40.96	0.0007	6.41	0.044	42.91	0.0006
1L by 2L	0.00006	0.99	0,14	0,72	4.47	0.08

Figure 15. Estimated effects by Pareto Diagram of the variables in the laccase (a), MnP (b) and LiP (c) activities.



The models coefficients of determination ( $R^2$ ) were 0.94, 0.89 and 0.89 for laccase, MnP and LiP, respectively, which indicate fine adjustments to the experimental data. The equations of the models generated for laccase, LiP and MnP activities are represented by the respective equations 1, 2 and 3 below:

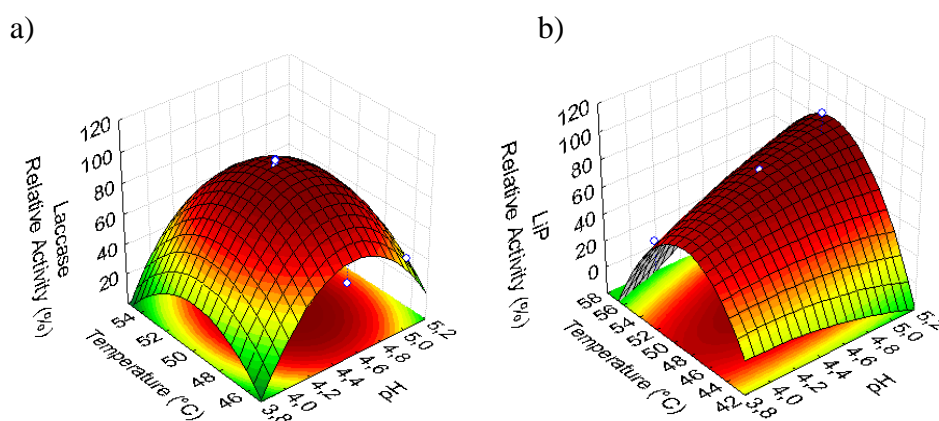
$$\text{(Eq. 1): } Y_1 = -5511 + 997.03*x_1 - 109.5*x_2 - 1.35*x_2^2 - 0.01*x_1*x_2;$$

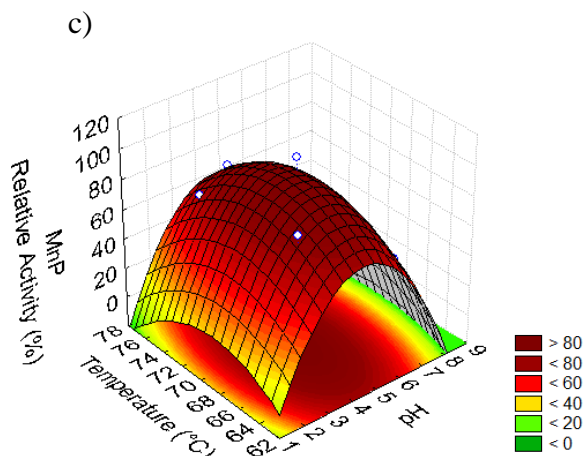
$$\text{(Eq. 2): } Y_2 = -2117.8 - 13.63*x_1 + 95.03*x_2 - 1.17*x_2^2 + 4.88*x_1*x_2;$$

$$\text{(Eq. 3): } Y_3 = -2149.98 + 42.08*x_1 + 61.91*x_2 - 0.45*x_2^2 + 0.21*x_1*x_2.$$

As observed in the response surfaces generated by the models the maximum enzymatic activities are obtained when both variables are used at their central levels (Figure 16).

Figure 16. Response surface obtained for pH and temperature effects on ligninases activities.

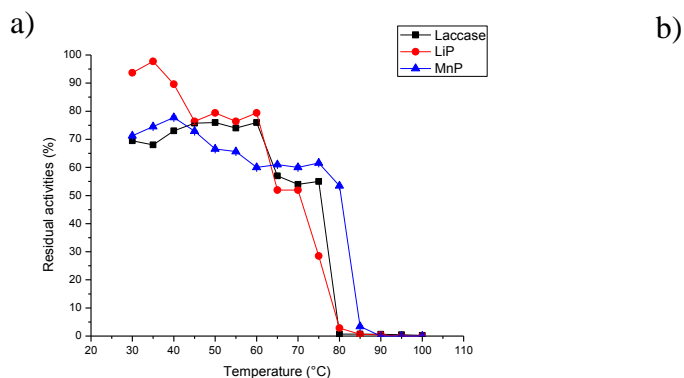




The best conditions of pH and temperature for laccase, manganese peroxidase and lignin peroxidase activities predicted by the Software were pH 4.5 and 50 °C pH 5.0 and 70 °C, pH 5.0 and 50 °C, respectively. Under these conditions, the predicted residual activities were of 93.7, 90.3 and 80.5 %, respectively. The validation experiments, performed in triplicate, resulted in  $92,52 \pm 0,14$ ,  $90,41 \pm 0,22$  e  $80,2 \pm 0,11$  % for laccase, manganese peroxidase and lignin peroxidase residual activities, respectively, indicating reproducibility of the data and confirming model validation

The enzymes stabilities to pH and temperature variations are shown in Figure 17. Laccase, LiP and MnP remained stable (70, 80 and 85% of the original activities, respectively) in the ranges of pH 3.5-12.5, 4.0-6.0 and 4.5-6.0, respectively. Regarding thermostability, these enzymes retained 75, 85 and 70% of the original activities at 40-60 °C, 30-65 °C and 30-80°C, respectively.

Figure 17. Effects of pH (a) and temperature (b) on ligninases stability.



### 3.2. Effect of chemical compounds on ligninases activities

The effect of some metal ions and other chemical compounds on ligninases activities were evaluated (Table 18). Positive effect was observed only for laccase activity, in the presence of copper. In general, laccase was the enzyme less affected by the compounds evaluated. Regarding lignin peroxidase activity, most ions had a moderately negative effect and the other compounds, such as detergents, markedly decreased the enzyme activity. Manganese peroxidase was less sensitive to the effect of detergents and chelating when compared do lignin peroxidase.

Table 18. Influence of metal ions, organic and inorganic compounds on ligninases activities.

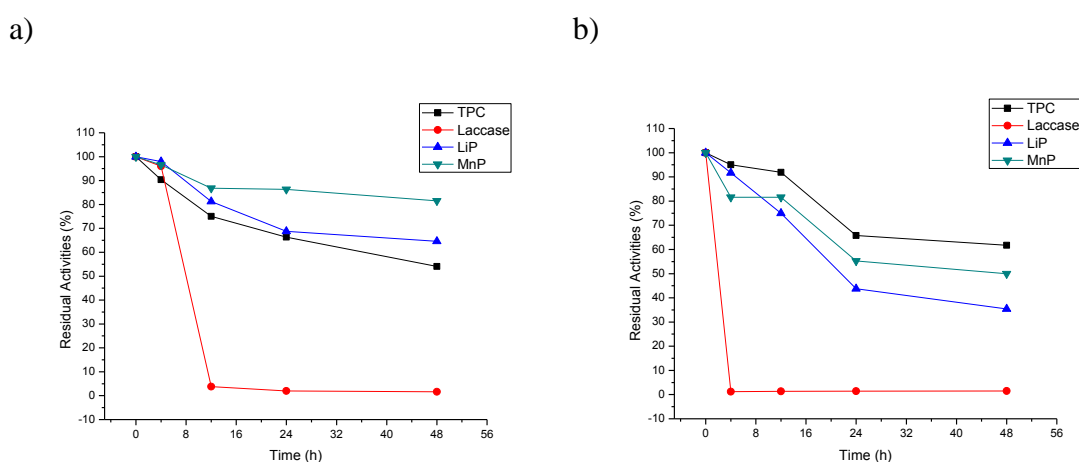
Compound	Residual activity (%)		
	Laccase	LiP	MnP
Cu <sup>+2</sup>	109	68.3	71
Ca <sup>+2</sup>	84.2	87.55	66
Fe <sup>+2</sup>	82.3	93.4	78
Tween 20	70.8	7	12
Mg <sup>+2</sup>	61.2	84.16	77
Cd <sup>+2</sup>	59.3	0.24	63
Mn <sup>+2</sup>	55.5	84.3	98
DMSO	53.6	48	22
Co <sup>+2</sup>	51.6	0.19	55
K <sup>+</sup>	44	84.46	69
Tween 80	41	3.5	11
EDTA	39.2	0	6
SDS	22	7.6	14
B-mercaptoethanol	21.04	0	17
Triton	18.2	0	9
Ag <sup>+</sup>	14	2.2	0.2
Hg <sup>+2</sup>	13.4	0	0.1

### 3.3. Degradation of phenolic compounds by the ligninases in the enzyme solution

Since phenolic compounds can inhibit cellulases activities, the degradation of these compounds present in the enzyme solution produced by *P. sanguineus* MCA 16 by the ligninases of the extract itself was evaluated by incubating it at different temperatures, up to 48 hours. Figure 18 shows that

total phenolics compounds (TPC) degradation was more pronounced at 50 °C, in the first 12h of incubation. After 48 hours, at this temperature, it was observed a reduction of 46 % in TPC concentration (final concentration of 2.18 g/L). However, after 24 hours, the residual percentage of these compounds was practically the same for the temperatures evaluated, 50 and 70 °C, with a reduction of approximately 66 %, corresponding to a TPC concentration of 2.67 g/L.

Figure 18. Total phenolic compounds concentration and ligninases activities profiles during the incubation of enzymes solution at 50 °C (a) and 70 °C (b). Enzyme solution was obtained from *P. sanguineus* MCA 16 cultivation in the mixture of soybean meal and wheat bran (1:1 w/v) for 96 hours at 40 °C. All the enzymatic activities were determined under optimum conditions of pH and temperature.



Ligninases activities were monitored during the experiments and it was observed that lacase was the most temperature sensitive enzyme, especially when the assays were conducted at 70 °C. MnP was the most stable enzyme and maintained about 85 % of its initial activity when incubated at 50 °C for 12 to 48 hours. After 12 hours of incubation at 70 °C, the residual activity of this enzyme was around 50-55%. Finally, LiP retained about 65 and 35% of its initial activity after 48 hours of incubation at 50 and 70 °C, respectively.

Several studies about the use of lignolytic enzymes to detoxify solutions containing phenolic compounds are reported in the literature (BRIJWANI et al.,

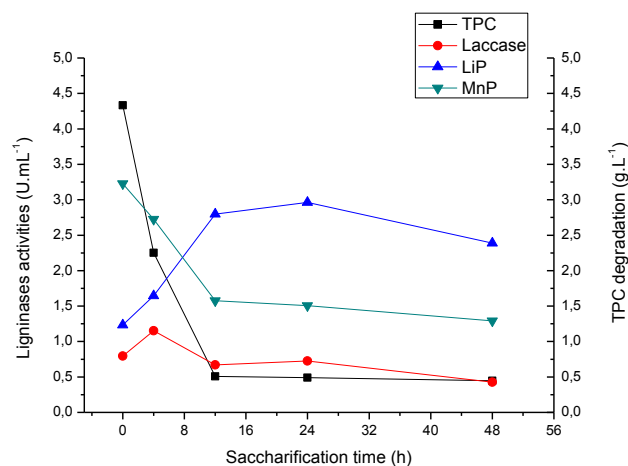
2010; MARTÍN-SAMPEDRO et al., 2011; MCCUE et al., 2004; TROVASLET et al., 2007). In the present work, it was observed that the ligninases of *P. sanguineus* MCA 16 were efficient in the degradation of total phenolic compounds. Tapia-Tussell et al. (2015), using laccase from by *Trametes hirsuta* BM-2 to treat vinasse, obtained a reduction of 20 % in the concentration of TPC after 192 hours of treatment, at 28 °C.

#### **3.4. Degradation of phenolic compounds by the ligninases during pretreated sugarcane bagasse enzymatic saccharification**

The TPC concentration was monitored during the enzymatic saccharification of pretreated sugarcane bagasse since these compounds, besides being present in the enzymatic extract itself, may also be formed during process. Ligninases activities were also monitored during saccharification, which was conducted as described in item 2.3. According to the data presented in Figure 19, lignin peroxidase was the enzyme most activated during the process. There was a very evident increase during the first 12 hours of saccharification and, at the end of this period, the activity was 2.96 U/mL, representing an increase of 126 % in relation to the original activity. Then, there was a slight decrease and LiP activity at the end of saccharification process was 2.8 U/mL.

Laccase was activated during the first hours of saccharification, reaching 1.15 U/mL at 4h (representing an increase of 45 %) and after this time this enzyme activity declined, remaining around 0.43 U/mL. In contrast, manganese peroxidase activity decreased significantly during the first 12 hours, remaining around 49 % of the original activity (1.58 U/mL) at the end of saccharification. In relation to TPC, the concentration decreased markedly in the initial 12 hours, then remained around 0.45 g.L<sup>-1</sup> until the end of the saccharification process (representing a decrease of, approximately 90 % in relation to the original concentration).

Figure 19. Profiles of TPC concentration and ligninases activities during enzymatic saccharification of sugarcane bagasse submitted to alkaline hydrothermal pretreatment. Saccharification was conducted at 50 °C and the enzymes activities were determined under optimum conditions.



The low molecular weight compounds released from lignin degradation, such as the phenolic compounds, can act as natural mediators of these enzymes, since they have a greater redox potential than lignin (DASHTBAN et al., 2010; WESENBERG et al., 2003). Thus, the oxirredution mechanism occurs by the phenolic compounds themselves (ABDEL-HAMID et al., 2013). To corroborate this affirmation, ligninases activities were determined in the presence of some phenolic compounds resulting from the breakdown of lignin (Table 19). Lignin peroxidase activity was improved in the presence of all evaluated compounds. Laccase was activated only by *p*-hydroxybenzoic acid and vanillin. In contrast, manganese peroxidase activity decreased in the presence of all the evaluated organic acids.

According to the Table 19 lignin peroxidase was activated by all the organic acid evaluated, with emphasis on *p*-coumaric acid. On the other hand, the activity of manganese peroxidase was negatively influenced by all these compounds, with the lowest activity observed in the presence of gallic acid. In relation to laccase, the phenolic compounds evaluated exerted different effects on the activity of this enzyme, highlighting the complete inactivation in the presence of gallic acid.



Table 19. Effect of organic acids on ligninases activities. All the enzymes activities were determined under optimum conditions of pH and temperature.

Organic acid	Relative enzymatic activity (%)		
	Laccase	LiP	MnP
Vanilic acid	99.5	107.95	88
<i>p</i> -hydroxybenzoic acid	114.8	106.2	91
Ferulic acid	68.8	113.76	93
<i>p</i> -coumaric acid	50	137.8	73
Vanillin	111	136.8	77
Galic acid	0	125.5	62

\*100% of original activities were considered as 2.37, 1.02 and 1.6 U/mL for laccase, LiP and MnP, respectively.

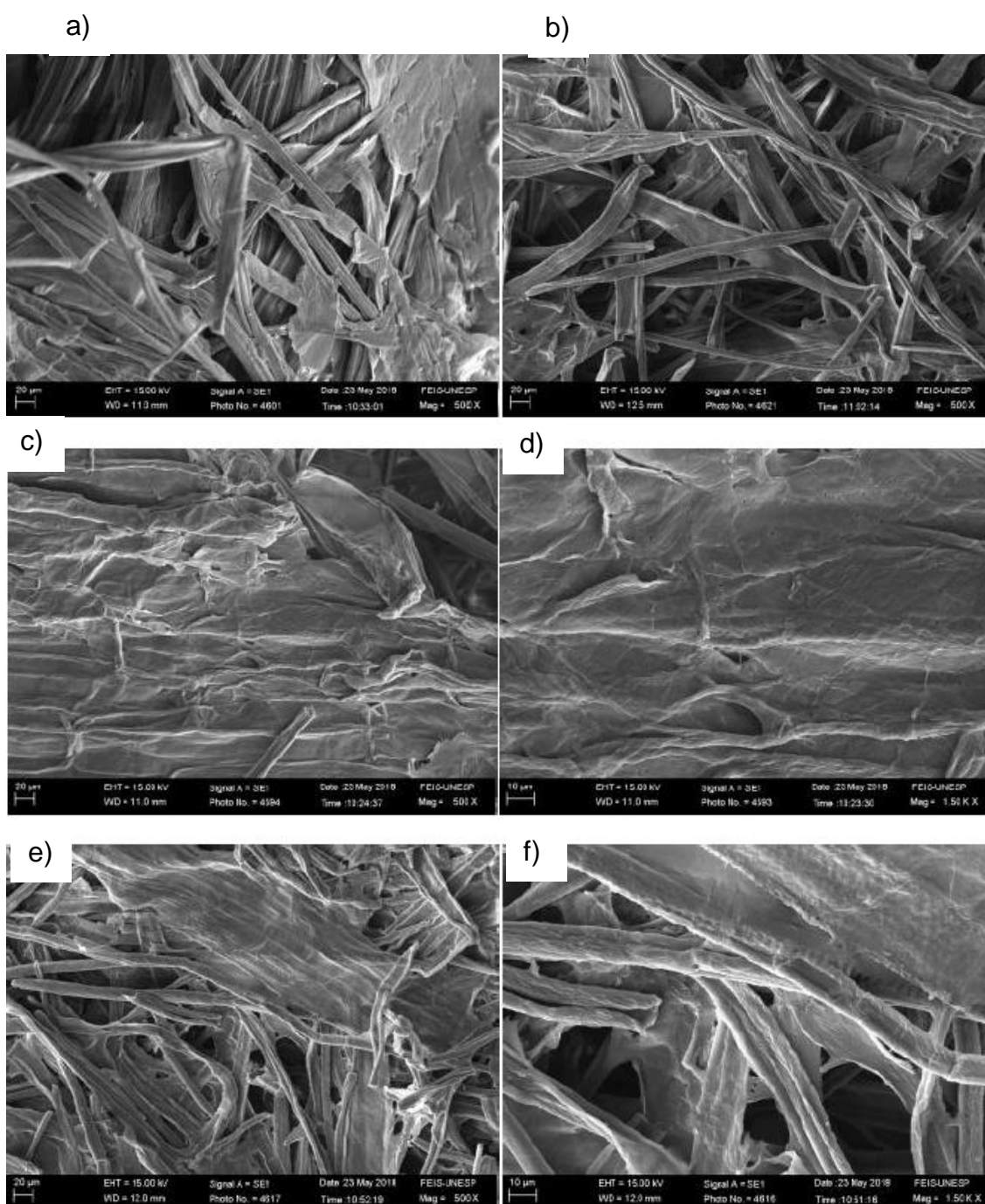
Tapia-Tussel et al. (2015) reported that lacase produced by *Trametes hirsute* BM-2 was activated by vanillin, guaiacol and ferulic acid during the growth on submerged fermentation. However, studies about phenolic compounds degradation by ligninases present in the enzymes solution itself and/or during the enzymatic saccharification is a novelty on the scientific literature.

The results obtained in the present work regarding to the degradation of phenolic compounds by the ligninases present in the enzymatic extract, as well as regarding to these enzymes activations (specially LiP and MnP) during saccharification, encourage the use of enzymatic cocktails containing both cellulases and ligninases activities in lignocellulosic materials saccharification processes. While cellulases act on cellulose degradation, releasing glucose as the final product, ligninases can reduce the concentration of phenolic compounds in the reaction medium, avoiding the inhibition of cellulases and reducing the toxicity of the hydrolysates to be used in alcoholic fermentation processes.

### ***3.5. Morphological analysis of sugarcane bagasse after enzymatic saccharification***

After enzymatic saccharification, a morphological analysis of sugarcane bagasse fibers was performed by scanning electronic micrography. Despite pretreatment effect, there were morphological changes after the saccharification. After saccharification (Fig 20b) looser fibers are observed when compared to the aspect of non-saccharified pretreated bagasse (Fig 20a), probably due to the enzymes action. The presence of pores before and after the saccharification step was also noted in Figures 8c. These pores, however, are more frequently observed in the fibers after the enzymatic hydrolysis bioprocess (Figure 20). This was also observed by (FERHAN et al., 2012) who reported that cellulases in the lignocellulosic material open the fiber while ligninases promote the formation of pores, besides separate the fibers, favoring the action of cellulases along the saccharification (ARANTES; SADDLER, 2010).

Figure 20. Scanning electronic micrography (SEM) of pretreated sugarcane bagasse before enzymatic saccharification with 500x (a), after enzymatic saccharification with 500x (b), before enzymatic saccharification with 500x (c), before enzymatic saccharification with 1500x (d), after enzymatic saccharification with 500x (e) and after enzymatic saccharification with 1500x (f).



## 4. Conclusions

Ligninases of enzyme solution from *P. sanguineus* MCA 16 cultivation were able to degrade total phenolic compounds after enzymatic saccharification step. Lacase and LiP showed activation during the enzymatic saccharification, demonstrating that the degradation of total phenolic compounds is probably exerted by these enzymes. Another important aspect was the morphological structure of sugarcane bagasse after the bioprocess. As enzymatic pretreatment, the enzymes present in the enzymes solution promoted a greater disorganization of the lignocellulosic fiber and pores were generated and facilitating the access of the enzymes on the sugarcane bagasse.

## 5. Acknowledgements

The authors thank to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support, to the State University of São Paulo, to the Laboratory of Biochemistry and Applied Microbiology and to the Laboratory of Sucochemistry and Analytical Chemistry of Institute of Biosciences, Letters and Exact Sciences, São José do Rio Preto and to the Laboratory of Microbial Enzymes of the Institute of Chemistry, Araraquara, São Paulo, Brazil.

## 6. References

- ABDEL-HAMID, A.M., SOLBIATI, J.O., CANN, I.K.O. Insights into Lignin Degradation and its Potential Industrial Applications. **Adv. in App. Microbiol.** 82, 1-28, 2013.
- ARANTES, V., SADDLER, J.N. Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates. **Biotechnol. Biofuels** 4, 1-17, 2011.
- BALA, A., SAPNA, JAIN, J., KUMARI, A., SINGH, B. Production of an extracellular phytase from a thermophilic mould *Humicola nigrescens* in solid state fermentation and its application in dephytinization. **Biocatal. Agric. Biotechnol.** 3, 259–264, 2014.

- BRIJWANI, K., RIGDON, A., VADLANI, P. V. Fungal Laccases : Production , Function , and Applications in Food Processing. **Enzyme Research** 2010, 1-11, 2010.
- BUSWELL, J. A, CAI, Y., CHANG, S. Effect of nutrient nitrogen and manganese on manganese peroxidase and lactase production by *Lentinula (Lentinus) edodes*. **FEMS Microbiol. Lett.** 128, 81–87, 1995.
- DASHTBAN, M., SCHRAFT, H., SYED, T.A., QIN, W. Fungal biodegradation and enzymatic modification of lignin. **Int. J. Biochem. Mol. Biol.** 1, 36–50, 2010.
- DULF, F.V., VODNAR, D.C., SOCACIU, C. Effects of solid-state fermentation with two filamentous fungi on the total phenolic contents, flavonoids, antioxidant activities and lipid fractions of plum fruit (*Prunus domestica* L.) by-products. **Food Chem.** 209, 27–36, 2016.
- ERIKSSON, K.-E.L., BERMEK, H. Lignin, Lignocellulose, Ligninase. **Encycl. Microbiol.** 373–384, 2009.
- FALADE, A.O., NWODO, U.U., IWERIEBOR, B.C., GREEN, E., MABINYA, L. V., OKOH, A.I. Lignin peroxidase functionalities and prospective applications. **Microbiologyopen** 6, 1–14, 2017.
- FENG, Y., COLOSI, L.M., GAO, S., HUANG, Q., MAO, L. Transformation and removal of tetrabromobisphenol from water in the presence of natural organic matter via laccase-catalyzed reactions: Reaction rates, products, and pathways. **Environ. Sci. Technol.** 47, 1001–1008, 2013a.
- FENG, Y., MAO, L., CHEN, Y., GAO, S. Ligninase-mediated transformation of 4,4-dibromodiphenyl ether (BDE 15). **Environ. Sci. Pollut. Res.** 20, 6667–6675, 2013b.
- FERHAN, M., LEAO, A.L., MELO, I.S., YAN, N., SAIN, M. Ligninases Production and Partial Purification of Mnp from Brazilian Fungal Isolate in Submerged Fermentation. **Ferment. Technol.** 01, 1–7, 2012.
- FUJIAN, X., HONGZHANG, C., ZUOHU, L. Solid-state production of lignin peroxidase (lip) and manganese peroxidase (mnp) by *Phanerochaete chrysosporium* using steam-exploded straw as substrate. **Bioresour. Technol.** 80, 149–151, 2001.

- HERNÁNDEZ-ORTEGA, A., FERREIRA, P., MARTÍNEZ, A.T. Fungal aryl-alcohol oxidase: A peroxide-producing flavoenzyme involved in lignin degradation. **Appl. Microbiol. Biotechnol.** 93, 1395–1410, 2012.
- KOLB, M., SIEBER, V., AMANN, M., FAULSTICH, M., SCHIEDER, D. Removal of monomer delignification products by laccase from *Trametes versicolor*. **Bioresour. Technol.** 104, 298–304, 2012.
- MARTÍN-SAMPEDRO, R., EUGENIO, M.E., CARBAJO, J.M., VILLAR, J.C. Combination of steam explosion and laccase-mediator treatments prior to *Eucalyptus globulus* kraft pulping. **Bioresour. Technol.** 102, 7183–9, 2011.
- MARTINS, S., MUSSATTO, S.I., MARTÍNEZ-AVILA, G., MONTAÑEZ-SAENZ, J., AGUILAR, C.N., TEIXEIRA, J.A. Bioactive phenolic compounds: Production and extraction by solid-state fermentation. A review. **Biotechnol. Adv.** 29, 365–373, 2011.
- MCCUE, P., HORII, A., SHETTY, K. Mobilization of phenolic antioxidants from defatted soybean powders by *Lentinus edodes* during solid-state bioprocessing<sup>1</sup> is associated with enhanced production of laccase. **Innov. Food Sci. Emerg. Technol.** 5, 385–392, 2004.
- QIN, L., LI, W.-C., LIU, L., ZHU, J.-Q., LI, X., LI, B.-Z., YUAN, Y.-J. Inhibition of lignin-derived phenolic compounds to cellulase. *Biotechnol. Biofuels* 9, 1–10, 2016.
- RASHAD, M.M., MAHMOUD, A.E., ABDOU, H.M., NOOMAN, M.U. Improvement of nutritional quality and antioxidant activities of yeast fermented soybean curd residue. **African J. Biotechnol.** 10, 5504–5513, 2011.
- RIVA, S., MOLECOLARE, R., BIANCO, V.M. Laccases: blue enzymes for green chemistry. **Trends Biotechnol.** 24, 219–226, 2006.
- RODRIGUES, M.I.; IEMMA, A.F. Planejamento de experimentos e otimização de processos. 3ª. Ed. Campinas: Ed. Cárita, 2014.

- RODRÍGUEZ-DELGADO, M.A., MALOVANÁ, S., PÉREZ, J.P., BORGES, T., GARCÍA MONTELONGO, F.J. Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. **J. Chromatogr. A** 912, 249–257, 2001.
- SALAR, R.K., CERTIK, M., BREZOVA, V. Modulation of phenolic content and antioxidant activity of maize by solid state fermentation with *thamnidium elegans* CCF 1456. **Biotechnol. Bioprocess Eng.** 17, 109–116, 2012.
- SINGH, A., PANT, D., KORRES, N.E., NIZAMI, A.-S., PRASAD, S., Murphy, J.D. Key issues in life cycle assessment of ethanol production from lignocellulosic biomass: Challenges and perspectives. **Bioresour. Technol.** 101, 5003–5012, 2010.
- SINGLETON, V.L., ORTHOFER, R., LAMUELA-RAVENTÓS, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of FolinCiocalteau reagent. **Methods Enzymol.** 299, 152–178, 1999.
- TAPIA-TUSSELL, R., PÉREZ-BRITO, D., TORRES-CALZADA, C., CORTÉS-VELÁZQUEZ, A., ALZATE-GAVIRIA, L., CHABLÉ-VILLACÍS, R., SOLÍS-PEREIRA, S. Laccase gene expression and vinasse biodegradation by *Trametes hirsuta* strain Bm-2. **Molecules** 20, 15147–15157, 2015.
- TIEN, M., KIRK, T.K. METHODS IN ENZYMOLOGY, VOL. 161 Copyright © 1988 by Academic Press, Inc. All rights of reproduction in any form reserved. **Methods** 161, 6080–6080, 1988.
- TOYAMA, N., OGAWA, K., 1978. Cellulase production of *Trichoderma viride* in solid and submerged culture methods, in: Ghose, T. (Ed.), *Bioconversions of Cellulosic Substrates for Energy, Chemicals and Microbial Protein*. New Delhi.
- TROVASLET, M., ENAUD, E., GUIAVARC'H, Y., CORBISIER, A.-M., VANHULLE, S., 2007. Potential of a *Pycnoporus sanguineus* laccase in bioremediation of wastewater and kinetic activation in the presence of an anthraquinonic acid dye. **Enzyme Microb. Technol.** 41, 368–376. doi:10.1016/j.enzmictec.2007.03.007
- VERMA, P., MADAMWAR, D. Production of ligninolytic enzymes for dye decolorization by cocultivation of white-rot fungi *Pleurotus ostreatus* and *Phanerochaete chrysosporium* under solid-state fermentation. **Appl. Biochem. Biotechnol.** 102–103, 109–118, 2002.

WESENBERG, D., KYRIAKIDES, I., AGATHOS, S.N. White-rot fungi and their enzymes for the treatment of industrial dye effluents. **Biotechnol. Adv.** 22, 161–187, 2003.

XIMENES, E., KIM, Y., MOSIER, N., DIEN, B., LADISCH, M. Deactivation of cellulases by phenols. **Enzyme Microb. Technol.** 48, 54–602011.

ZHENG, Z., SHETTY, K. Solid-state bioconversion of phenolics from cranberry pomace and role of *Lentinus edodes*  $\beta$ -glucosidase. **J. Agric. Food Chem.** 48, 895–900, 2000.



**TERMO DE REPRODUÇÃO XEROGRÁFICA**

Autorizo a reprodução xerográfica do presente Trabalho de Conclusão, na íntegra ou em partes, para fins de pesquisa.

São José do Rio Preto, \_\_\_\_/\_\_\_\_/\_\_\_\_

---

Assinatura do autor