**UNIVERSITY OF SÃO PAULO
LORENA SCHOOL OF ENGINEERING**

BRUNA CAROLINE MARQUES GONÇALVES

**Evaluation of microorganisms' performance in hemicellulosic hydrolyzate
detoxification and sugarcane bagasse lignin removal for ethanol production**

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BRUNA CAROLINE MARQUES GONÇALVES

Avaliação do desempenho de microorganismos na destoxicação de hidrolisado hemicelulósico e na remoção de lignina de bagaço de cana-de-açúcar para a produção de etanol

Tese apresentada à Escola de Engenharia de Lorena – Universidade de São Paulo para a obtenção do título de Doutor em Ciências do Programa de Biotecnologia industrial na área de Microbiologia Aplicada.

Orientador: Prof. Dr. Silvio Silvério da Silva

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*This work is dedicated to my uncle
Mauro Veronezi Gonçalves.*

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*“Everyone who has never made a mistake never
tried anything new”*

Albert Einstein

ABSTRACT

Marques-Gonçalves, B. C. **Evaluation of microorganisms' performance in hemicellulose hydrolyzate detoxification and sugarcane bagasse lignin removal for ethanol production** 2015. 167 p. Thesis (Doctoral Science) – Lorena School of Engineering, University of São Paulo, Lorena, 2016.

Environmental concerns have driven the development of green technologies towards second-generation ethanol (2G) production in order to increase the global production of ethanol and to minimize usage of fossil fuels. Furthermore, the use of biological processes through the steps required to obtain 2G bioethanol reduces the generation of residues, some of which are toxic to the environment, and the need for energy usage, reducing the overall costs of the process. The objective of this study was to evaluate the influence of the biodelignification of the sugarcane bagasse (SCB) (step 1) and the sugarcane bagasse hemicellulosic hydrolyzate (SCBHH) biodegradation (step 2) for the production of cellulosic and hemicellulosic ethanol. Firstly, six fungal strains (JLS6-A6, JLS6-A7, JLS6-A12, JLS6-F6, JLS6-G4 and JLS6-H9) and one *Streptomyces* strain (JLS3-C3), were explored according to their potential for lignin removal from SCB during a biological pretreatment in order to increase the accessibility of fermentable sugars. Fractional factorial design 2^{4-1} and Taguchi L_8 orthogonal array were utilized to assess the impact of physical and nutritional parameters in the hydrolytic and ligninolytic enzymes activity and lignin transformation over 21 days of incubation. Secondly, SCB was subjected to diluted-acid pretreatment to obtain the SCBHH, rich in pentoses sugar. Nutritional parameters for biodegradation of SCBHH were evaluated utilizing the Box and Behnken 2^3 design. The strains JLS6-A6, JLS6-A7, JLS6-F6 and JLS6-A12 showed a high capacity for synthesis of xylanase enzyme. The xylanase activity was significantly reduced (from a maximum of 30 U/mg to 5 U/mg) in the optimized condition (moistened SCB milled to 20 mesh size, pH 8.5, agitation 100 rpm for 14 days) determined with the fractional factorial design. The strain JLS6-A6 showed high potential for synthesis of manganese peroxidase enzyme and efficiency during SCB biodelignification, standing out among the other strains. The optimum saccharification conditions identified by Taguchi orthogonal array was moistened SCB and pH 8.5. The value of $Y_{P/S}$ obtained after fermentation of the sugars released from pretreated milled SCB ($Y_{P/S} = 0.45$ g cellulosic ethanol/g) was 2.14 times higher than that obtained for pretreated unmilled SCB and 1.29 times higher than untreated milled SCB after the simultaneous saccharification and fermentation process. The yeast *I. occidentalis* Y1'a was able to grow on unsupplemented SCBHH using acetic acid as an energy source during the biodegradation process. The fermentation step (30°C, 150 rpm, 24 h) was successful with a volumetric yield ($Y_{P/S}$) of 0.26 g hemicellulosic ethanol/g and productivity (Q_p) of 0.14 g/Lh by the yeast *Candida shehatae* UFMG 52.2. These results confirm the potential of biological processes related to the production of ethanol 2G. Furthermore, JLS6-A6 and *I. occidentalis* Y1'a are promising strains for SCB biological pretreatment and SCBHH biological detoxification, respectively. Thus, this work will contribute to the development of cellulosic and hemicellulosic ethanol production, providing the integration of the production of the first and 2G ethanol. **Keywords:** Sugarcane bagasse. Biodegradation. Biodelignification. Ethanol. Biofuel

RESUMO

Marques-Gonçalves, B. C. **Avaliação do desempenho de microorganismos na destoxicação de hidrolisado hemicelulósico e na remoção de lignina de bagaço de cana-de-açúcar para a produção de etanol** 2015. 167 p. Tese (Doutorado em Ciências) – Escola de Engenharia de Lorena, Universidade de São Paulo, Lorena, 2016.

As preocupações com o meio ambiente têm impulsionado o desenvolvimento de tecnologias verde para a produção de etanol de segunda geração (2G), com o intuito de aumentar a produção mundial de etanol e reduzir o uso de combustíveis fósseis. Além disso, o uso de processos biológicos para a obtenção de etanol 2G minimizam a geração de resíduos tóxicos e o uso de energia, reduzindo os custos do processo. O objetivo deste trabalho foi avaliar os processos de biodeslignificação do bagaço de cana-de-açúcar (BCA) (etapa 1) e biodestoxificação do hidrolisado hemicelulósico de bagaço de cana-de-açúcar (HHBCA) (etapa 2) na produção de etanol celulósico e hemicelulósico. Inicialmente foi explorado o potencial de remoção de lignina presente no BCA por seis isolados fúngicos (JLS6-A6, JLS6-A7, JLS6-A12, JLS6-F6, JLS6-G4 and JLS6-H9) e uma cepa de *Streptomyces* (JLS3-C3), visando o aumento da acessibilidade aos açúcares fermentescíveis. Foram utilizados o planejamento fatorial fracionário 2^{4-1} e o arranjo ortogonal de Taguchi L_8 para avaliar o impacto de parâmetros físicos e nutricionais na atividade das enzimas hidrolíticas e ligninolíticas e transformação da lignina durante 21 dias. Como segundo passo foi utilizado o pré-tratamento com ácido diluído para a obtenção do HHBCA, rico em pentoses. Os parâmetros nutricionais para a biodestoxificação do HHBCA foram avaliados utilizando o modelo Box and Behnken 2^3 . As cepas JLS6-A6, JLS6-A7, JLS6-F6 and JLS6-A12 apresentaram alta capacidade de síntese da enzima xilanase, que foi reduzida (de 30 U/mg para 5 U/mg) com o auxílio do planejamento fatorial fracionário 2^{4-1} na condição otimizada (BCA moído e umedecido, pH 8,5, agitação de 100 rpm durante 14 dias). A cepa JLS6-A6 apresentou alto potencial de síntese da enzima manganês peroxidase, destacando-se entre as outras estirpes. A condição de sacarificação ótima identificada por meio do arranjo ortogonal de Taguchi foi pH 8,5 e BCA umedecido. O valor de $Y_{P/S}$ obtido após a fermentação dos açúcares liberados a partir do BCA moído e pré-tratado ($Y_{P/S} = 0,45$ g de etanol celulósico/g) foi 2,14 vezes mais elevado do que o obtido para o BCA não moído e pré-tratado, e 1,29 vezes mais elevado do que o BCA moído e não pré-tratado durante o processo de sacarificação e fermentação simultâneas. A levedura *Issatchenkia occidentalis* Y1'a foi capaz de crescer no HHBCA não suplementado, utilizando ácido acético como fonte de energia. A etapa de fermentação (30°C, 150 rpm, 24 h) foi bem sucedida com uma produção volumétrica ($Y_{P/S}$) de 0,26 g de etanol hemicelulósico/g e produtividade (Q_P) de 0,14 g/Lh pela levedura *Candida shehatae* UFMG 52.2. Estes resultados confirmam o potencial de processos biológicos relacionados com a produção de etanol 2G. Além disso, JLS6-A6 e *I. occidentalis* Y1'a são cepas promissoras no pré-tratamento biológico do BCA e destoxificação biológica do HHBCA, respectivamente. Desta forma, este trabalho contribuirá com o desenvolvimento da produção de etanol celulósico e hemicelulósico proporcionando a integração da produção do etanol de primeira e 2G. **Palavras-chave:** Bagaço de cana-de-açúcar. Biodestoxificação. Biodeslignificação. Etanol. Biocombustível

LIST OF FIGURES

CHAPTER 1

Figure 1-1: Flowchart illustrating the research developed steps for Chapter 3 and Chapter 4.	28
Figure 1-2: Flowchart illustrating the research developed steps for Chapter 5.	29

CHAPTER 2

Figure 2-1: Composition and structure of plant cell wall.	36
Figure 2-2: (a) Scheme of the primary structure of cellulose. (b) Scheme of the structure of a cellulose fibril.	37
Figure 2-3: Types of hemicellulose structure.	39
Figure 2-4: The three fundamental basic precursors of lignin and their respective phenylpropanoids (in blue): <i>p</i> -coumaryl alcohol and <i>p</i> -hydroxyphenyl; coniferyl alcohol and guaiacyl; sinapyl alcohol and syringyl.	40
Figure 2-5: Schematic representation of the lignin molecule, the types of bonds and phenylpropanoid monomers.	41
Figure 2-6: Scheme for first generation bioethanol production from sugarcane broth. ...	42
Figure 2-7: Simplified scheme of corn-ethanol process obtaining.	44
Figure 2-8: Simplified scheme of first generation sugar and ethanol production from sugarcane	46
Figure 2-9: Second generation bioethanol production from sugarcane waste.	47
Figure 2-10: Acid-catalyzed hydrolysis of glucopyranosides (a) and disaccharides structures (b).	51
Figure 2-11: Average breakdown of lignocellulosic biomass and the main products derived from hydrolysis.	53
Figure 2-12: Oxidation of veratryl alcohol catalyzed by lignin peroxidases generating veratraldehyde (a) and proposed mechanism of the aromatic ring veratryl alcohol opening (b) through H ₂ O (A) attack or O ₂ attack (B).	62
Figure 2-13: Modifications in lignin structure caused during action of laccase.	64

Figure 2-14: Enzyme systems involved in the degradation of cellulose (a) and xylan (b).66

Figure 2-15: D-xylose and L-arabinose metabolic pathway in fungi and bacteria.....69

Figure 2-16: Integration of first and second generation ethanol production.70

CHAPTER 3

Figure 3-1: APPL concentration after 7, 14 and 21 days of biological pretreatment of sugarcane bagasse by the strains (a) JLS6-A6, (b) JLS6-A7, (c) JLS6-A12 and (d) JLS6-F6.94

Figure 3-2: Xylanase activity after 7, 14 and 21 days of biological pretreatment in of sugarcane bagasse by the strains (a) JLS6-A6, (b) JLS6-A7, (c) JLS6-A12 and (d) JLS6-F6.98

Figure 3-3: Pareto chart showing the effect of the variables (a) and response surface showing the impact of the significant variables (b) on xylanase activity after 14 days of biodelignification by JLS6-A6.103

Figure 3-4: Pareto chart showing the effect of the variables (a) and response surface showing the impact of the significant variables (b) on xylanase activity after 14 days of biodelignification by JLS6-A7.104

Figure 3-5: Pareto chart showing the effect of the variables (a) and response surface showing the impact of the significant variables (b) on xylanase activity after 14 days of biodelignification by JLS6-A12.....105

Figure 3-6: Pareto chart showing the effect of the variables (a) and response surface showing the impact of the significant variables (b) on xylanase activity after 14 days of biodelignification by JLS6-F6.106

Figure 3-7: Natural and biological pre-treated SCB weight (a) and APPL concentration (b) after 14 days of biological pretreatment in confirmation experiment.....108

Figure 3-8: Reducing sugars concentration (a) and APPL concentration (b) after 14 days of biological pretreatment in scale-up experiment.....109

Figure 3-9: Untreated and biological pre-treated SCB weight after 14 days of biological pretreatment in scale-up experiment.110

CHAPTER 4

- Figure 4-1:** The effect of variables on the saccharification of the biologically pretreated SCB bagasse by JLS6-A6. 126
- Figure 4-2:** Xylanase activity and reducing sugars concentration during the confirmation experiment of biological pretreatment by JLS6-A6 for milled and unmilled SCB. 127
- Figure 4-3:** MnP activity during the confirmation experiment of biological pretreatment by JLS6-A6 for milled and unmilled SCB. 129
- Figure 4-4:** Reducing sugars concentration during simultaneous saccharification and fermentation of biological pretreated milled SCB by JLS6-A6 after 7 (a), 14 (b) and 21 (c) days and controls. 130
- Figure 4-5:** Reducing sugars concentration during simultaneous saccharification and fermentation of biological pretreated unmilled SCB by JLS6-A6 after 7 (a), 14 (b) and 21 (c) days and controls. 131
- Figure 4-6:** Reducing sugars released after 24 h of saccharification (blue) of: pretreated SCB by JLS6-A6 according to Taguchi design (a), pretreated SCB by JLS6-A6 according to Taguchi design after acid wash to remove fungal mycelia (b) natural milled and unmilled SCB (c) and natural milled and unmilled SCB after acid wash (d). The control (red) is related to the SCB without enzyme addition. 134

CHAPTER 5

- Figure 5-1:** Scanning electron microscopy (SEM) with 500 x magnification of (a) natural SCB and (a) sulfuric acid pretreated. 146
- Figure 5-2:** Pareto chart indicating the effect of the variables and interactions between the variables after 48 h of hemicellulosic hydrolyzate detoxification (a). Response of surface related to acetic acid consumption and (b) glucose concentration versus yeast extract concentration (c) glucose concentration and peptone concentration, after 48h of hemicellulosic hydrolyzate detoxification by the yeast *I. occidentalis* Y1'a. 150
- Figure 5-3:** Central point average of sugars consumption from the hemicellulosic hydrolyzate supplemented with 10 g/L of glucose and their conversion into ethanol and xylitol during 96 h of biodetoxification by the yeast *I. occidentalis* Y1'a. 152
- Figure 5-4:** Sugars consumption from the no-glucose supplemented hemicellulosic hydrolyzate and their conversion into ethanol and xylitol during 96 h of biodetoxification by the yeast *I. occidentalis* Y1'a in the run 5 (a), 6 (b), 9 (c) and 10 (d). 153

Figure 5-5: Sugars consumption from the hemicellulosic hydrolyzate supplemented with 10 g/L of glucose and their conversion into ethanol and xylitol during 96 h of biodegradation by the yeast *I. occidentalis* Y1'a in the run 1 (a), 2 (b), 3 (c) and 4 (d).154

Figure 5-6: Sugars consumption from the hemicellulosic hydrolyzate supplemented with 20 g/L of glucose and their conversion into ethanol and xylitol during 96 h of biodegradation by the yeast *I. occidentalis* Y1'a in the run 7 (a), 8 (b), 11 (c) and 12 (d).
.....155

Figure 5-7: Acetic acid removal from the hemicellulosic hydrolyzate during 96 h of biodegradation by the yeast *I. occidentalis* Y1'a from the hemicellulosic hydrolyzate supplemented with 10 g/L of glucose (a) no supplemented with glucose (b) and supplemented with 20 g/L of glucose (c).157

Figure 5-8: Phenolic compounds metabolism from the hemicellulosic hydrolyzate supplemented with 10 g/L of glucose (a) no-glucose supplemented hemicellulosic hydrolyzate (b) and supplemented with 20 g/L of glucose (c) during 96 h of biodegradation by the yeast *I. occidentalis* Y1'a estimated using Folin-Ciocalteu Reagent.....159

Figure 5-9: Ethanol production by the yeast *I. occidentalis* Y1'a during the biodegradation process of the no supplemented hemicellulosic hydrolyzate and by the yeast *C. shehatae* UFMG 52.2 during the fermentation process from the detoxified hemicellulosic hydrolyzate. The initial glucose content regards the glucose released from hemicellulose and cellulose during the diluted-acid pretreatment.....161

LIST OF TABLES

CHAPTER 2

Table 2-1: Composition of plant cell wall of several lignocellulosic materials.	35
---	----

CHAPTER 3

Table 3-1: Fractionary design 2^{4-1} investigating effects of agitation (X_1), SCB mesh size (X_2), solid:liquid ratio (X_3) and medium pH (X_4) on xylanase activity after 14 days of biodelignification by fungal isolates JLS6-A6, JLS6-A7, JLS6-A12, JLS6-F6.	100
--	-----

Table 3-2: ANOVA for xylanase activity results after 14 days of biodelignification by JLS6-A6.	101
--	-----

Table 3-3: ANOVA for xylanase activity after 14 days of biodelignification by JLS6-A7.	101
--	-----

Table 3-4: ANOVA for xylanase activity after 14 days of biodelignification by JLS6-A12.	101
---	-----

Table 3-5: ANOVA for xylanase activity after 14 days of biodelignification by JLS6-F6.	102
--	-----

CHAPTER 4

Table 4-1: Orthogonal array Taguchi L_8 experimental design. Factors used for biological pretreatment of SCB by JLS6-A6.	124
--	-----

Table 4-2: ANOVA for effects of factors after saccharification of SCB biodelignification by JLS6-A6.	126
--	-----

Table 4-3: Reducing sugars release after the saccharification of the un-washed and acid washed biological pretreated SCB by JLS6-A6.	133
--	-----

CHAPTER 5

Table 5-1: Chemical composition of sugarcane bagasse.	146
---	-----

Table 5-2: Partial composition of the hemicellulosic hydrolyzate before and after the concentration step.	147
---	-----

Table 5-3: Supplementation of the hemicellulosic hydrolyzate and acetic acid removal (%) after 48h of biodegradation with the yeast *I. occidentalis* Y1'a.149

Table 5-4: Analysis of variance (ANOVA) for the effect of the variables yeast extract, peptone and glucose concentration on the removal of acetic acid from the hemicellulosic hydrolyzate by the yeast *I. occidentalis* Y1'a after a 48 h-degradation.149

Table 5-5: Initial concentration of sugars in the cultivation medium.152

LIST OF ABBREVIATIONS AND ACRONYMS

(L)	Linear term
(Q)	Quadratic term
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ANOVA	Analysis of variance
APPL:	Acid-precipitable polymeric lignin
ATP:	Adenosine triphosphate
CO₂:	Carbon dioxide
CoA:	Coenzyme A
CONAB:	Companhia Nacional de Abastecimento
EL:	Yest extract
EMP:	<i>Embden-Meyerhof-Parnas</i> pathway
FAD⁺⁺:	Flavin adenine dinucleotide
FADH₂:	Flavin adenine dinucleotide in reduced form
CF:	Concentration factor
G:	Glucose
df:	Degree of freedom
HH:	Hemicellulosic hydrolyzate
HMF:	5-Hydroxymethyl-furfural
LiP:	Lignin peroxidase
MnP:	Manganese peroxidase
MS:	Mean of squares
NAD⁺:	Nicotinamide adenine dinucleotide
NADH:	Nicotinamide adenine dinucleotide in reduced form
NADP⁺:	Nicotinamide adenine dinucleotide phosphate
NADPH:	Nicotinamide adenine dinucleotide phosphate in reduced form
O₂:	Oxygen
P:	Peptone
PDH:	Pyruvate dehydrogenase
PPP:	Pentose phosphate pathway
SM:	Medium square
rpm:	Revolution per minute

SCB:	Sugarcane bagasse
SHF:	Separated hydrolysis and fermentation
SS:	Sum of squares
SSF:	Simultaneous saccharification and fermentation
YNB:	Yeast Nitrogen Base
YPD:	Yeast Peptone Dextrose

SUMÁRIO

CHAPTER 1	25
1. Introduction and Research Overview.....	25
1.1. Introduction.....	25
1.2. Research overview	28
1.3. Objectives	30
1.3.1. Main Objective.....	30
1.3.2. Specific Objectives.....	30
References.....	31
CHAPTER 2	34
2. An Overview of Sugarcane First and Second Generation Bioethanol Production based on Biological Processes	34
Abstract	34
2.1. Vegetal biomass as feedstock for bioethanol production	35
2.1.1. Cellulose	36
2.1.2. Hemicellulose	38
2.1.3. Lignin	40
2.2. First generation bioethanol.....	41
2.2.1. Bioethanol produced from corn starch	43
2.2.2. Bioethanol produced from sugarcane.....	45
2.3. Second generation bioethanol	46
2.3.1. Pretreatments of biomass.....	48
2.3.1.1. Acid-catalyzed hydrolysis	50
2.3.1.1.1. Inhibitor compounds	52
2.3.1.1.1.1. Furan derivatives	53
2.3.1.1.1.2. Weak acids.....	57
2.3.1.1.1.3. Phenolic derivatives	58
2.3.1.1.2. Detoxification of hemicellulosic hydrolyzate.....	59
2.3.1.2. Biological pretreatment.....	60
2.3.2. Enzymatic hydrolysis of lignocellulosic biomass	65
2.3.3. Fermentation of the sugars recovered from lignocellulosic biomass	67
2.4. Integration of first and second generation bioethanol production	69

2.5. Future perspectives	71
---------------------------------------	-----------

References	72
-------------------------	-----------

CHAPTER 3.....	88
-----------------------	-----------

3. The Impact of Short Duration Fungal Pretreatment of Sugarcane Bagasse on Sugar Accessibility	88
--	-----------

Abstract	88
-----------------------	-----------

3.1. Introduction	88
--------------------------------	-----------

3.2. Material.....	89
---------------------------	-----------

3.3. Methods.....	90
--------------------------	-----------

3.3.1. Microbial strains and inoculation	90
--	----

3.3.2. Screening of significant factors using fractionary factorial design	90
--	----

3.3.3. Solid state fermentation and biodelignification.....	90
---	----

3.3.4. Acid-precipitable polymeric lignin (APPL)	91
--	----

3.3.5. Ligninase enzyme assays	91
--------------------------------------	----

3.3.5.1. Lignin peroxidase	91
----------------------------------	----

3.3.5.2. Laccase	91
------------------------	----

3.3.6. Hydrolytic enzyme assays	92
---------------------------------------	----

3.3.6.1. Xylanase	92
-------------------------	----

3.3.6.2. Cellulase	92
--------------------------	----

3.3.7. Determination of Protein Content.....	92
--	----

3.4. Results	92
---------------------------	-----------

3.4.1. Acid-Precipitable Polymeric Lignin	93
---	----

3.4.2. Enzyme assays	95
----------------------------	----

3.4.3. Statistical analysis.....	99
----------------------------------	----

3.4.4. Confirmation experiment	107
--------------------------------------	-----

3.4.5. Scale-up.....	109
----------------------	-----

3.5. Conclusion	110
------------------------------	------------

4.6. Acknowledgements	110
------------------------------------	------------

References	111
-------------------------	------------

CHAPTER 4.....	114
-----------------------	------------

4. Taguchi method-optimized fungal pretreatment increases the release of reducing sugars from sugarcane bagasse during enzymatic hydrolysis	114
--	------------

Abstract	114
4.1. Introduction.....	114
4.2. Material	117
4.2.1. Sugarcane bagasse.....	117
4.2.2. Microbial strains and inoculation preparation	117
4.3. Methods	117
4.3.1. Screening of significant factors for SSF applying Taguchi Design	117
4.3.2. Statistical analysis	118
4.3.3. Bidelignification and solid state fermentation	118
4.3.4. Mycelia removal	119
4.3.5. Saccharification of pretreated sugarcane bagasse	119
4.3.6. Simultaneous Saccharification and Fermentation (SSF).....	119
4.3.7. Assays.....	119
4.3.7.1. Acid-precipitable polymeric lignin (APPL).....	119
4.3.7.2. Reducing sugars.....	120
4.3.7.3. Enzyme assays.....	120
4.3.7.3.1. Ligninolytic enzymes	120
4.3.7.3.2. Hydrolytic enzymes	121
4.3.7.4. Quantification of sugars, ethanol and organic acids.....	121
4.3.8. Calculations	121
4.1 Results and Discussion	122
4.4.1. Biological pretreatment effect on reducing sugars released during the saccharification process.....	124
4.4.2. Optimization of saccharification parameters involving the biologically pretreated SCB by Taguchi method.....	125
4.4.2.1. Statistical optimization using Taguchi Orthogonal Array Design	125
4.4.2.2. Confirmation Experiment and Scale-up.....	126
4.4.3. Simultaneous Saccharification and Fermentation.....	129
4.4.3.1. Confirmation experiment	129
4.4.3.2. Scale-up experiment	132
4.4.4. Acid wash of sugarcane bagasse for mycelia removal.....	132
4.5. Conclusion	135
4.6. Acknowledgements.....	135
References.....	136
CHAPTER 5	139

5.	Acetic acid removal from hemicellulosic hydrolyzate by the yeast <i>Issatchenkia occidentalis</i> Y1'a is increased in absence of glucose	139
	Abstract	139
5.1.	Introduction	139
5.2.	Material	142
5.3.	Methods	142
5.3.1.	Inoculum preparation	142
5.3.2.	Hemicellulosic hydrolyzate	143
5.3.3.	Experimental design	143
5.3.4.	Supplementation of biotetoxified hemicellulosic hydrolyzate	144
5.3.5.	Analytical methods	144
5.3.5.1.	Phenolic compounds	144
5.3.5.2.	Sugars, ethanol and acetic acid	144
5.3.5.3.	Furan compounds	144
5.3.6.	Calculations	144
5.4.	Results	145
5.4.1.	Chemical composition of sugarcane bagasse	145
5.4.2.	Physico-chemical characterization of hemicellulosic hydrolyzate	146
5.4.3.	Evaluation of hemicellulosic hydrolyzate supplementation using the factorial design Box and Behnken 2 ³ during the biotetoxification process	148
5.4.4.	Evaluation of sugars consumption and toxic compounds removal from hemicellulosic hydrolyzate during biotetoxification process	151
5.4.5.	Confirmation experiment	160
5.4.6.	Ethanol production from the hemicellulosic hydrolyzate biologically detoxified	160
5.5.	Conclusions	162
	Acknowledgments	162
	References	163
6.	General conclusion	167

CHAPTER 1

1. Introduction and Research Overview

1.1. Introduction

Global demand for energy is basically dependent on fossil fuel resources. As long as the economy is based on consumption, the energy required for industrial processes has been growing significantly, due to the industrial demand. The burning of fossil fuels generates gases, such as CO₂, which contributes to increasing the concentration of greenhouse gases in the atmosphere, resulting in global warming. The search for alternatives to fossil fuels is increasing researches related to the production of biofuels. The increased use of biomass in first and second generation biofuels production can play a significant role in replacing conventional fossil fuels and reducing greenhouse gases emissions.

Biofuels are basically fuels derived from either vegetal or biological material. According to Nigam and Singh (2011) biofuels may be classified as primary and secondary biofuels. Primary biofuels are derived from unprocessed wood for heating, cooking or electricity production (FULLER; MCKEON; BILLS, 1996), whereas secondary biofuels are produced from the processing of plant biomass and/or microorganisms. Secondary biofuels are subdivided into first, second, third and fourth generation. First and second generations derive from vegetal biomass (NAIK et al., 2010), whereas third generation is based on microalgae processing (ALASWAD et al., 2015). The fourth generation corresponds to the biofuel production from genetically engineered crops. The bases of this generation is to obtain crops with higher CO₂ consumption when compared to the amount of CO₂ released into the atmosphere after the biofuel combustion (DUTTA; DAVEREY; LIN, 2014).

First generation biofuels are the most common biofuel-type produced in the world and include bioethanol, biodiesel and biogas. Currently, first generation of bioethanol is produced from the easily extracted sugars from sugar-containing or starchy biomass, such as sugarcane (CARDONA; QUINTERO; PAZ, 2010; COELHO et al., 2006; DIAS et al., 2015; MARTINELLI; FILOSO, 2008) and corn (BIAŁAS; SZYMANOWSKA; GRAJEK, 2010; BOTHAST; SCHLICHER, 2005; SHIGECHI et al., 2004). First generation biofuels present several challenges, such as food and fuel competition, change in land-use and potential increased greenhouse gas emissions, due to fossil fuel are utilized in the upstream

processes. Besides, the land that was firstly designated to food crops, is being converted to biofuel production, affecting the food prices. The economic aspects of this generation largely depends upon the type of feedstock and region where the feedstock has been cultivated to contribute with the reduction of greenhouse gases release (FARGIONE et al., 2008; SANTOS; NUSSIO; MOURÃO, 2009; WOHLT; CORCIONE; ZAJAC, 1998).

Second generation biofuels production is based on recovery of sugars from the plant cell wall. Second generation bioethanol represents both an alternative over first generation ethanol or a way of improving the overall ethanol yield by means of converting the sugars contained in plant cell walls into ethanol. Lignocellulosic biomass, such as woods and agricultural residues, is an attractive feedstock for bioethanol production because of its large amount of fermentable sugars. The main structural components of lignocellulosic biomass are cellulose, hemicellulose, and lignin. Among them, only cellulose and hemicellulose can be used to produce ethanol by fermentation of carbohydrates obtained by chemical or enzymatic hydrolysis (saccharification). The central issue of this technology is to overcome the recalcitrance of cellulosic biomass (LYND et al., 2008).

Several pretreatments may be utilized to disrupt and unconfigure the lignocellulosic matrix. Among the four types of pretreatments, physical, physico-chemical, chemical and biological, the diluted-acid hydrolysis, classified as a chemical pretreatment, and the biological pretreatment, also known as biodelignification, are worth mentioning.

Diluted-acid pretreatment requires special equipment due to the corrosive effect of the acid. It is performed under high temperatures (112.5°C – 190°C) (AVCI et al., 2013; CANILHA et al., 2011; JUNG et al., 2013; MOHANRAM et al., 2015; SUN; CHENG, 2005) and may be performed under short time (3-5 min) (AVCI et al., 2013; CANILHA et al., 2011; JUNG et al., 2013) or long time (30 - 48 min) (CANILHA et al., 2011; MOHANRAM et al., 2015; SUN; CHENG, 2005) of incubation, depending on the feedstock. As a result, a hemicellulosic hydrolyzate rich in pentose sugars, mostly D-xylose, is generated. As long as these sugars can be recovered with no need of enzymes, this pretreatment is an alternative to the enzymatic hydrolysis, therefore a less expensive way of recovering hemicellulosic sugars.

The major inconvenience of acid pretreatments is the formation of undesired compounds from the degradation of lignin and sugars, such as phenolic and furanic derivatives, due to the low pH and high temperature applied during the process. Besides, weak acid may be formed either from the degradation of sugars or release of acetyl radicals. These compounds are toxic and act synergistically inhibiting the metabolism of sugars by

the fermenting strains, thus they must be removed from the hydrolyzate prior to the fermentation step to assure high ethanol yields (ZHANG et al., 2010). Several physical and physico-chemical methods may be utilized for the removal of toxic compounds, such as evaporation, membrane extraction, solvent extraction, overliming, etc (PALMQVIST; HAHN-HÄGERDAL, 2000a). However, these processes usually require high energy input and generally result in waste generation. As an alternative biological detoxification can be utilized over those methods. In this case, microorganisms which are capable of metabolizing degradation compounds from both lignin and sugars are utilized, conferring less energy input and no hazardous waste generation (PARAWIRA; TEKERE, 2011).

Biological pretreatment is an environmentally friendly process in which microorganisms are utilized to remove lignin from the lignocellulosic biomass due to its ability to produce lignolytic enzymes, such as manganese peroxidase, lignin peroxidase and laccase (MORENO et al., 2014). However, hydrolytic enzymes are also produced in the very beginning of the process, reducing the sugars availability at the end of the process, therefore the ethanol yield may be compromised (SINGHAL; JAISWAL; THAKUR, 2015; WANG et al., 2013). The amount of enzymes synthesized during the biological pretreatment and the time-course depends on the feedstock type and size, the strain and the availability of nutrients for the microorganism. Therefore, the process must be prospected under certain conditions leading to low production of hydrolytic enzymes.

First generation bioethanol presents a very consolidated industrial process all over the world. In addition, high ethanol yields are obtained after the feedstock processing. On the other hand, second generation ethanol offers the possibility of increasing the overall ethanol yields and the possibility of the full utilization of the vegetal biomass. In this way, this work was developed to evaluate the efficiency of the hemicellulosic and cellulosic second generation ethanol production aiming the utilization of the carbohydrates from sugarcane bagasse. Environmentally friendly process based on the utilization of microorganisms, such as biological detoxification of the hemicellulosic hydrolyzate and biological pretreatment were applied, taking into account the environmental issues and its preservation.

1.2. Research overview

The objective of this research is to investigate the production of cellulosic bioethanol from biodelignified sugarcane bagasse and the production of hemicellulose ethanol from hemicellulosic hydrolyzate generated after diluted-acid pretreatment of sugarcane bagasse. This research is divided into 2 phases, one developed in the Laboratory of Bioprocess at School of Engineering of Lorena/University of São Paulo in Brazil and other developed in the Molecular Microbial Biochemistry Laboratory at the University of Ontario and Institute of Technology (UOIT) in Canada, which are discussed in Chapter 3 to Chapter 5 (Figure 1-1 and Figure 1-2) and are outlined as follow.

Figure 1-1: Flowchart illustrating the research developed steps for Chapter 3 and Chapter 4.

Source: Personal file

Seven potential lignin-degrader strains from the microorganism collection from the Molecular Microbial Biochemistry Laboratory (UOIT) were utilized in the studies described in Chapters 3 and 4. From the seven strains, four showed high undesired potential for production of hydrolytic enzymes. In Chapter 3, the nutritional and physical parameters of the biodelignification process are evaluated towards the reduction of xylanase activity. In Chapter 4, the nutritional and physical parameters of the biodelignification process are

evaluated towards the increase of simultaneous saccharification and fermentation yield. Chapter 5 is focused on biodetoxification of hemicellulosic hydrolyzate and shows the relation between the nutritional parameters and the consumption of toxic compounds by the yeast *I. occidentalis* Y1'a and hemicellulosic ethanol production from the biodetoxified hydrolyzate by the yeast *Candida shehatae* UFMG 52.2. The yeasts *I. occidentalis* Y1'a and *Candida shehatae* UFMG 52.2 are both from the microorganism collection at the Laboratory of Bioprocesses (EEL/USP).

Figure 1-2: Flowchart illustrating the research developed steps for Chapter 5.

Source: Personal file

1.3. Objectives

1.3.1. Main Objective

Evaluate the production of cellulosic and hemicellulosic ethanol from biologically pretreated and diluted-sulphuric acid pretreated sugarcane bagasse, respectively.

1.3.2. Specific Objectives

➤ Biodelignification

- ✓ Evaluate the production of ligninolytic and hydrolytic enzymes by seven microbial strains during the biodelignification of sugarcane bagasse;
- ✓ Evaluate the physical and nutritional parameters which reduce the synthesis of hydrolytic enzymes and increase the synthesis of ligninolytic enzymes by the microbial strains;
- ✓ Evaluate the physical and nutritional parameters which increase the enzymatic hydrolysis by the cellulase enzymes from the biodelignified sugarcane bagasse;
- ✓ Evaluate the efficiency of cellulosic ethanol production by the yeast *Saccharomyces cerevisiae* after enzymatic hydrolysis of biologically pretreated sugarcane bagasse.

➤ Biodetoxification

- ✓ Obtaining a hemicellulosic hydrolyzate from sugarcane bagasse using diluted-sulfuric acid pretreatment and concentrate the hydrolyzate under vacuum;
- ✓ Evaluate the supplementation of the hemicellulosic hydrolyzate during the biodetoxification process by the yeast *I. occidentalis* Y1'a;
- ✓ Evaluate the hemicellulosic ethanol production from the biodetoxified hemicellulosic hydrolyzate by the yeast *C. shehatae* UFMG HM 52.2.

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CHAPTER 2

2. An Overview of Sugarcane First and Second Generation Bioethanol Production based on Biological Processes

Abstract

Search for alternatives to fossil fuels is increasing due to the increase in the release of gases that contribute directly to the greenhouse effect. First generation biofuels are the most common biofuel-type produced in the world. Even though bioethanol production from sugar-containing or starchy feedstocks, such as sugarcane and corn, is a widespread technology, the utilization of the feedstock may be optimized. The residues generated after the sugar extraction are rich in carbohydrates, which can be depolymerized and converted into ethanol. In this case, second generation ethanol is an alternative to improve the overall ethanol yield in an integrated process where pentoses and hexoses may be converted into fuel. However, due to its recalcitrance, lignocellulosic materials must be pretreated in order to release carbohydrates prior to enzymatic hydrolysis. Diluted-acid and biological pretreatments are promising alternatives for sugars exposure. Besides cellulose exposition, diluted-acid pretreatment results in a hemicellulosic hydrolyzate rich in pentose sugars with no need of enzymatic hydrolysis, reducing process costs. On the other hand, a detoxification step is required to remove the undesired compounds generated by the degradation of lignin and sugars. In this case, specific microorganisms capable of metabolizing such compounds may be employed in a detoxification process, free of waste generation. During biological pretreatments lignin structures are disrupted during a solid state fermentation by the action of lignocellulosic enzymes resulting in carbohydrates exposure at the end of the process. However, hydrolytic enzymes are also synthesized by the microorganism during the biodelignification, reducing the amount of sugars which would be converted into ethanol. Even though biological processes usually require long time to be completed due to the microorganism adaptation period and enzyme apparatus synthesis, the major advantage of this procedure is that they represent a green technology and they are environmentally friendly. They also avoid the use of chemicals or hazardous products, making the process less costly. However, issues related to second generation bioethanol production and the integration of its process for use with first generation plants must be resolved, attracting many researchers to this area.

2.1. Vegetal biomass as feedstock for bioethanol production

Besides the well-known food applications of vegetal biomass, its composition rich in sugars allows for nonfood applications such as feed, and feedstock for fuels and fine chemicals production (FULLER; MCKEON; BILLS, 1996). These alternative applications are related to both the low cost of biomass feedstocks and the availability on a large scale, which have a great impact on energy and sustainability challenges (LYND et al., 2008). Agricultural, agro-industrial and forestry residues etc. are the most abundant organic compounds in the biosphere. They are classified as lignocellulosic materials due to the cell wall composition based on cellulose, hemicellulose and lignin (Figure 2-1) (HON, 1996). The amount of these three fractions may fluctuate depending on the specie of the plant (Table 2-1) or within the same plant species, according to its age and stage of growth (PÉREZ et al., 2002). Furthermore, ashes and waxes are also found but at lower concentrations (Table 2-1).

Table 2-1: Composition of plant cell wall of several lignocellulosic materials.

Source		Components (%)				Reference
		Cellulose	Hemicellulose	Lignin	Others	
Bagasse	Sorghum	40.40	35.50	3.90	0.20	Dogaris et al., 2009
	Sugarcane	42.80	25.90	22.10	7.50	Da Silva et al., 2010
		43.10	25.20	22.90	7.10	Rocha et al., 2011
Hull	Rice	35.00	12.00	16.00	20.00	Saha; Cotta, 2008
		35.00	13.00	15.20	20.00	Singh et al., 2011
Straw	Corn	43.30	26.30	13.60	6.70	Sun et al., 2011
	Rice	38.00	26.00	7.00	15.00	Singh et al., 2011
	Sorghum	35.10	24.00	25.40	NA	Téllez-Luis; Ramírez; Vázquez, 2002
	Sugarcane	33.60	28.90	31.80	5.70	Da Silva et al., 2010
	Wheat	43.80	28.20	8.30	NA	Tuyen et al., 2012

* NA: information not available

Cell wall provides mechanical strength, cell shape maintenance, cell expansion control, transport regulation of substances and protection. It also confers a protective coating to the plant cell, conferring resistance to mechanical, chemical and microbial attacks to the cell. The basic model of the cell wall structure is shown in Figure 2-1. Middle lamella (M.L.) connects the cells together and is followed by the primary wall (P) that can be divided into an outer and an inner surface. The primary wall consists of an untidy aggregation of microfibrils oriented to the cell axis on the outer surface. Primary wall covers the secondary wall, which comprises of first (S1), second (S2) and third (S3) layers. In first layer

microfibrils are oriented in a cross-helical middle with crossed structure, whereas in the second layer, the thickest one, microfibrils show a consistent orientation. In contrast, the microfibrils in the third layer may arrange in two or more orientations and is characterized as a flat helix. In some cases, there is a warty layer on the inner surface of the cell wall (FUJITA; HARADA, 2000; KIRK; CULLEN, 1998).

Figure 2-1: Composition and structure of plant cell wall.

Source: modified from Ratanakhanokchai et al. (2013) and Kirk; Cullen (1998)

2.2.1. Cellulose

Cellulose is the principal constituent of the plant cell wall. Depending on the feedstock, cellulose represents about 35% to 43% of the plant cell wall composition (Table 2-1). It is an unbranched, linear homopolymer formed by the polymerization of D-glucose units (hexose sugar) linked by β -1,4 glycosidic bonds (Figure 2-2a). Once depolymerized, D-glucose may be converted into cellulosic ethanol (WIRAWAN et al., 2012). Each individual cellulose chain is rigid and ribbon-shaped due to the covalent and hydrogen bonds and intrachain Van der Waal's force. The rigidity of the whole structure is conferred by the arrangement of the individual cellulose chains, which are arranged into microfibrils by

interchain hydrogen bonds. Microfibrils are arranged into fibrils by the same bonds (Figure 2-2b) (DESVAUX, 2005; MESHITSUKA; ISOGAI, 1996).

The structure of the cellulose can be classified into three organizational levels. The first is defined by the sequence of β -D-glucopyranoside residues joined by covalent bonds, forming the homopolymer of anhydroglucose with β -D (1 $\rightarrow$ 4) glycoside linkages (Figure 2-2a). The second level describes the spatial arrangement of the repeating units, which is characterized by the distances between the linkages and their respective angles and also by intramolecular hydrogen bonds (Figure 2-2b). The third level defines the association of the molecules forming aggregates showing a certain level of crystalline structure. Therefore, cellulose shows two different structural organization types (Figure 2-2b). A crystalline region, characterized by a cohesive structure completely insoluble in water and most solvents and an amorphous region, which is less organized and more susceptible to external attacks (ZHOU; WU, 2012). The crystalline region is a result of hydrogen bonding and Vander Waals forces, which attach cellulose molecules and align them together in a more highly ordered fashion than the noncrystalline region (LAI, 1996).

Figure 2-2: (a) Scheme of the primary structure of cellulose. (b) Scheme of the structure of a cellulose fibril.

Source: Desvaux (2005)

The primary, secondary and tertiary walls of plants are composed of cellulose (Figure 2-2). Although the tertiary cell wall is comprised of only a small amount of cellulose, the primary and secondary cell walls are rich in this homopolymer; the structure of these walls differ according to the arrangement of cellulose chains. While the primary wall chains are

arranged in a disorderly manner, secondary wall chains are arranged in parallel to each other forming microfibrils that provide a dense structure. Thus, the primary cell wall is more susceptible to external attacks (O'SULLIVAN, 1997).

2.1.2. Hemicellulose

Hemicellulose is a branched heteropolymer which represents 13-35% of plant biomass (Table 2-1), which main chain is composed of pentoses (β -D- α -xylose and L-arabinose), and in some cases hexoses (β -D-mannose, β D-glucose, α -D-galactose). Uronic acids (α -D-glucuronic acid, 4-O-methyl- α -D-glucuronic acid and β -D-galacturonic acid) and small amounts of sugars (α -L-rhamnose and α -L-fucose) which may have some of its hydroxyl radicals replaced by acetyl groups. In addition, sugars are typically joined by glycosidic β (1 $\rightarrow$ 4) and occasionally β (1 $\rightarrow$ 3) bonds (GÍRIO et al., 2010; MESHITSUKA; ISOGAI, 1996). Three types of hemicellulose polymers can be found in vegetal biomass: xylan, mannans and glucomannans, and xyloglucan.

The backbone of xylan is formed by β -D-(1,4)-xylose residues linked to side chains of α -(1,2)-glucuronic acid and 4-O-methyl glucuronic acid residues (Figure 2-3a), where L-arabinose residues are usually attached. It is a major hemicellulose type found in softwoods (KIRK; CULLEN, 1998; OCHOA-VILLARREAL et al., 2012). They are major hemicelluloses in the primary cell wall of monocots and are found in smaller amounts in the primary cell wall of dicots. Xylans having a high degree of side chains are more water soluble and bind less tightly to cellulose whilst molecules with fewer side chains are less water soluble and bind to cellulose tightly (ISHII; SHIMIZU, 2000).

Mannans are β -(1,4)-linked polysaccharides rich in mannose (Figure 2-3b), whereas the structure of glucomannans (Figure 2-3c) consist of both mannose and D-glucose in a nonrepeating pattern. These polymers are the major hemicellulose-type found in conifers (gymnosperms), whereas minor amounts are found in secondary walls of angiosperms (hardwood). Besides, mannans and glucomannans are not frequently identified in the primary cell wall (ISHII; SHIMIZU, 2000; KIRK; CULLEN, 1998; OCHOA-VILLARREAL et al., 2012).

Xyloglucans are formed by a β -D-glucan main chain, which is branched with α -D-xylose linked to carbon 6 of the backbone (Figure 2-3d) (OCHOA-VILLARREAL et al., 2012) They are the hemicellulose-type of the primary cell wall of dicotyledonous plants and are hydrogen bonded to cellulose (ISHII; SHIMIZU, 2000).

Figure 2-3: Types of hemicellulose structure.

Source: modified from Jordan et al. (2012)

2.1.3. Lignin

Lignin is the second most abundant macromolecule among the lignocellulosic biomass and has an entirely different structure when compared to cellulose and hemicellulose. It generally plays a negative role in the chemical utilization of lignocellulosic materials and must be modified, partially degraded or completely removed, depending on the final application of the material (LAI, 1996). Lignin structure appears to be amorphous and is basically formed by *p*-coumaryl, coniferyl and sinapyl alcohols (Figure 2-4), which form a polyphenolic cohesive structure (Figure 2-5). Lignin in softwoods is largely composed of guaiacyl units and is called “guaiacyl lignin”, whereas in hardwood lignin is found as a copolymer of guaiacyl and syringyl units in different ratio and a small amount of *p*-hydroxyphenyl and is called “guaiacyl syringyl lignin” (MESHITSUKA; ISOGAI, 1996). Lignin plays a structural role, providing hardness and impermeability to the plant and protecting it against microbial attacks (PINKERT et al., 2011; ZHU et al., 2014).

Figure 2-4: The three fundamental basic precursors of lignin and their respective phenylpropanoids (in blue): *p*-coumaryl alcohol and *p*-hydroxyphenyl; coniferyl alcohol and guaiacyl; sinapyl alcohol and syringyl.

Source: Pinkert et al. (2011)

The composition and organization of the lignin constituents depends on the specie and the cellulose-hemicellulose matrix. The intimate association between these three recalcitrant components hampers the release of monomeric sugars, therefore it acts as a physical barrier during the enzymatic hydrolysis. In addition, enzymes can be irreversibly captured by the lignin and therefore influence the enzyme loading required for hydrolysis, causing loss in the recovery of the enzymes after hydrolysis (ZENG et al., 2014).

Figure 2-5: Schematic representation of the lignin molecule, the types of bonds and phenylpropanoid monomers.

Source: Zhu et al. (2014)

2.2. First generation bioethanol

Produced from raw materials cultivated on land, first generation biofuels are well known by the low technological complexity requirement. Sugars or oil may be extracted from plants and converted into bioethanol and biodiesel, respectively. Bioethanol is mainly obtained through the fermentation of sugars released from corn starch, (MONTESINOS; NAVARRO, 2000; SHIHADDEH et al., 2014) or from sugars contained in sugarcane broth (juice) or molasses (LIMTONG; SRINGIEW; YONGMANITCHAI, 2007). Other feedstocks such as sugar beet molasses (ERGUN; FERDA MUTLU, 2000) can also be utilized in this process. The simplified scheme of bioethanol obtained from sugarcane broth is shown in Figure 2-6. Corn-based and sugarcane-based ethanol production will be discussed in item 2.2.1 and item 2.2.2, respectively.

Sugarcane (*Saccharum officinarum*) is generally grown as a perennial crop in tropical and subtropical areas. It has been used as feedstock for production of ethanol on a large scale basis in Brazil for over three decades. Apart from ethanol, most of the sugarcane mills produce sugar and electricity (DIAS et al., 2015). Sugarcane trash, such as bagasse and leaves fuels the cane processing plants, providing electricity to the grids through cogeneration. They can also be used as source of fiber for papers and boards and animal feed, most successfully for dairy cattle (CLARKE; EDYE, 1996). Processing of sugarcane engenders extraction of sucrose (main product), residual fibers and molasses, which corresponds to the final concentrated sugarcane juice, after several crystallizations.

Figure 2-6: Scheme for first generation bioethanol production from sugarcane broth.

Source: modified from BUGG et al. (2011)

Unlike corn bioethanol, sugarcane bioethanol is obtained from a simpler and less costly process, since the sugars are more easily extracted from sugarcane. In addition, liquefaction and gelatinization steps are not required. Moreover, sugarcane ethanol delivers substantially more energy than corn ethanol (CRAGO et al., 2010). Sugarcane crops also show a best net energy yields per hectare when compared to corn crops, resulting in good nitrogen and pesticide use efficiency besides water productivity, implying an efficient use of land (DE VRIES et al., 2010). According to Chum et al. (2014) sugarcane systems achieve double the greenhouse gases emission reduction per unit of harvested land in comparison to corn ethanol. This considerable difference is explained based on the type of the crop and the climatic zone in which the crop is grown. While sugarcane is grown as perennial crop in tropical climatic zone, corn is grown as an annual crop in temperate areas.

In spite of sugarcane being recognized as a more promising feedstock, according to García et al. (2011), the Brazilians ethanol production process achieves lower greenhouse gas emissions and higher energy ratios when compared to Mexican ethanol production from sugarcane. This situation is explained by the lower amount of fertilizers utilized in Brazilian crops, the shorter distance of sugarcane transport from the cultivation area to the processing

plant and to the smaller proportion of sugarcane areas that were burned to facilitate manual harvesting. In addition, a mill based on the biorefinery system of mechanized farming, where green sugarcane is utilized instead of burned sugarcane, coupled with sugarcane trash utilization for power generation is a great alternative to reduce the overall cost of the ethanol production process, leading lower greenhouse gas emissions of the biorefinery system (SILALERTRUKSA; GHEEWALA; PONGPAT, 2015).

In contrast to the advantages related to the easy extraction of sugars, first generation bioethanol has several challenges such as food competition, change in land-use and potential increased greenhouse gas emissions since fossil fuels are used in the upstream processes. The land, which was firstly designated for food crops, is being converted to accommodate biofuel production, affecting the food prices. Besides, the economic aspects of this generation largely depends upon the type of feedstocks and region where the feedstock have been cultivated. To avoid these drawbacks, degraded and abandoned agricultural lands are promising alternatives to be explored towards second generation biofuel production, which could lay aside the destruction of native ecosystems (FARGIONE et al., 2008).

Even though first generation Brazilian ethanol has been demonstrated to be one of the best substitutes for fossil fuels, environmental and social impacts must be considered. Matters such as atmospheric pollution caused by the burning of sugarcane residues for manual harvesting, degradation of soils due to compaction promoted by the machinery traffic, erosion and degradation of aquatic systems owing to the sediments generated by the erosion, must be prevented (MARTINELLI; FILOSO, 2008).

2.2.1. Bioethanol produced from corn starch

Corn is essentially composed by starch, which works as a storage compound for plants. Starches are found as insoluble and partially crystalline granules in the endosperm of the corn kernel. Corn starch is made up of D-glucose units linked via α -1,4 (linear chain) and α -1,6 (branches) glycosidic linkages, forming amylose, and amylopectin, respectively. Corn-to-ethanol is a fairly mature technology mainly developed in the United States due to the high production of this grain. However, the deflected use of corn crops for fuel production instead of food have been generating competition and impacts in the corn prices (CONDON; KLEMICK; WOLVERTON, 2015). According to WANG et al. (2015), biorefinery would be an alternative to overcome the overall cost of corn bioethanol, enabling the obtaining of a wide range of hydrocarbons such as aromatics, olefins and synthetic gasoline and diesel along with bioethanol. However, even though these co-products could

be used as ‘drop in’ transportation fuels and value-added petrochemicals, the impact on corn prices would not be eliminated.

Corn-to-ethanol conversion process involves multiple steps and requires enzymes to liquefy the starch and release D-glucose. Ethanol may be produced from corn by either dry grind or wet mill process (Figure 2-7). The difference between these processes is that the first focuses on maximizing the capital return per gallon of ethanol, whereas in the second, the capital investments allow for the separation of other valuable components in the grain before fermentation to ethanol (BOTHAST; SCHLICHER, 2005). Independent of the type of milling process utilized, the next steps are liquefaction of the starch, saccharification, fermentation of released D-glucose, distillation and dehydration of the ethanol.

Figure 2-7: Simplified scheme of corn-ethanol process obtaining.

Source: modified from McAloon et al. (2000)

Liquefaction of the starch is the bottleneck of the process and accomplishes the gelatinization of the starch and oligosaccharides release. This step promotes the conversion of the semi-crystalline starch granules into its amorphous conformation by means of high temperature (up to 100°C) and the addition of thermostable α -amylase and alkalis (ammonia and lime for pH adjustment to 6.0). Further conversion of the oligosaccharides to D-glucose is performed by glucoamylase enzymes during the saccharification step. Sulphuric acid is added to the reaction mixture to lower the pH (4.5) and the process runs at a temperature of 61°C. As long as glucoamylase continues to be active, this enzyme can continue the hydrolysis during the fermentation whether any unhydrolysed dextrin remains. Following

the saccharification reaction, the slurry is transferred to the fermentation vessel with heat being recovered from the outlet stream, and cooled to 32°C prior to fermentation, where yeast is added. Fermentation medium is supplemented with nitrogen source (ammonium sulfate or urea) for the growth of yeast and is performed during up to 72 h accompanied pH declining (below pH 4), due to CO₂ formed during the ethanol fermentation. The decrease in pH is important for increasing the activity of glucoamylase and inhibiting the growth of contaminating bacteria (BOTHAST; SCHLICHER, 2005; KWIATKOWSKI et al., 2006).

As long as high temperatures are a requirement during the liquefaction step, the overall cost of the process is increased (KNUTSON et al., 1982). According to Masiero et al., (2014), noncooked starch hydrolysis is more advantageous than gelatinization. In this process, granular starch degrading enzymes are added to the feedstock, acting directly on raw starch granules under lower temperature than that required for gelatinization process, reducing energy input. Besides, simultaneous saccharification and fermentation (SSF) can be performed, saving time. In addition, low temperature also avoids Maillard reactions. The bioconversion of starch to ethanol may also be carried out in a single-step process by co-culture of microorganism (VERMA et al., 2000) or utilizing genetically modified strains capable of producing amylolytic enzymes and fermenting ethanol simultaneously (SHIGECHI et al., 2004).

2.2.2. Bioethanol produced from sugarcane

Ethanol and sugar production from sugarcane may be performed in the same plant, but different processes are required, as shown in Figure 2-8. According to Dias et al. (2015, 2011), sugarcane is transported from the field to the plant and prepared for juice extraction by milling. After extraction, impurities (minerals, salts, organic acids, dirt and fine fiber particles) are removed from sugarcane juice in the juice treatment system by a physicochemical treatment. Fibers and sand are separated by screens, the juice is pre-heated from 30 to 70°C and after the addition of lime its heated to 105°C. Air is removed and a flocculent polymer is added. The rest of impurities are removed through a clarification process. Mud obtained in the clarifier is filtrated to improve sugars recovery. Clarified sugarcane juice is blended with molasses, a byproduct of the sugar production process, and concentrated. Sugars are converted into ethanol during the fermentation step by *Saccharomyces cerevisiae* and the final culture medium is sent for distillation and dehydration to obtain the ethanol. All the energy (steam and electricity) is produced by the mill using sugarcane bagasse and trash, producing high pressure steam as fuel. Some mills

have been recovering a fraction of the sugarcane straw (sugarcane tops and leaves) and using it as fuel as well, avoiding the burn.

Figure 2-8: Simplified scheme of first generation sugar and ethanol production from sugarcane

Source: modified from Dias et al. (2015)

2.3. Second generation bioethanol

Plants such as Raízen, located in São Miguel dos Campos, state of Alagoas (VIEIRA, 2014) and GranBio – Bioflex I located in Piracicaba, state of São Paulo (SCARAMUZZO, 2015), have been producing second generation cellulosic bioethanol in commercial scale since 2014 in Brazil. According to Milanez et al. (2015) the costs regarding the production of this generation will be economically feasible by 2020. On the other hand, there is no information about second generation hemicellulosic bioethanol production in such scale.

Second generation biofuels eliminate the apparent competition for land between fuel and food crops or even crops designated for food or fuel production (COELHO et al., 2006), overcoming one of the most worrying drawbacks of first generation of biofuels. Instead of only using easily extractable sugars and starch for ethanol production, second generation biofuels involve lignocellulosic biomass processing (Figure 2-9). In addition, it shows the best perspective regarding the greenhouse gases emission when compared to the first generation. There are several potential feedstocks for second generation biofuel production,

nevertheless waste biomass and by-products should be preferred to principal products. Regarding the area, abandoned land should be preferred instead of those utilized for crops or preservation of native species of plants, assuring erosion protection and biodiversity conservation. Deforestation must be avoided, as well (HAVLÍK et al., 2011).

The process comprises four stages (i) pretreatment process, responsible for lignocellulosic matrix disruption (ii) enzymatic hydrolysis, depolymerizing cellulose and hemicellulose into D-glucose and D-xylose by hydrolytic enzymes (iii) fermentation for the conversion of sugars into ethanol, and (iv) distillation, rectification and dehydration, for purification of the final product (MARGEOT et al., 2009). In this way, all the sugars present in lignocellulosic biomass can be availed.

Figure 2-9: Second generation bioethanol production from sugarcane waste.

Source: modified from Dashtban; Schraft; Qin (2009)

According to Dias et al., (2012, 2013) and Macrelli; Galbe; Wallberg (2014), second generation ethanol from sugarcane has not been thought as a stand-alone technology but in association to first generation. The viability of this biorefinery concept application will only

be possible when bioethanol produced from sugarcane broth is optimized1.2.4. The overall process for second generation bioethanol obtained from sugarcane waste is shown in Figure 2-9. Pretreatment of biomass is a mandatory step to overcome the recalcitrance of the lignocellulosic biomass. There are several types of pretreatments and the choice is based on the vegetal feedstock and the type of sugar of interest. Pretreated biomass must be subjected to enzymatic hydrolysis by either cellulase or hemicellulase enzymes, or both before the fermentation step where recovered sugars are converted to ethanol by microorganisms.

2.3.1. Pretreatments of biomass

The recalcitrance of the plant cell walls hampers the recovery of fermentable sugars from lignocellulosic materials and a pretreatment is a mandatory step prior the enzymatic hydrolysis to release the sugars. Pretreatment disrupts the plant cell wall and improves enzymatic access to the polysaccharides. Several physical, chemical and physico-chemical pretreatments can be used to unconfigure the lignocellulosic matrix to make the sugars accessible to enzymes, or release them from the biomass. These pretreatments have been extensively studied and reviewed by several authors (AGBOR et al., 2011; CANILHA et al., 2012; HENDRIKS; ZEEMAN, 2009; KUMAR et al., 2009a; MOSIER et al., 2005; SATHITSUKSANO; GEORGE; ZHANG, 2013).

The most commonly utilized physical pretreatments to reduce the size of the plant fibers and increase the contact surface are grinding (DA SILVA et al., 2010; JIN; CHEN, 2006), pyrolysis (PISKORZ et al., 1989; YU; ZHANG, 2003) and extrusion (KARUNANITHY; MUTHUKUMARAPPAN; GIBBONS, 2012). After this step, the particulate material may be subjected to a second most effective method (JIN; CHEN, 2006). Due to the high energy input necessary for these pretreatments, they undesirably increase the overall cost of the process.

Chemical pre-treatments range from acidic to alkaline, showing different effects upon the constituents in biomass. Some examples are acid hydrolysis (BONDESSON; GALBE; ZACCHI, 2013; CANILHA et al., 2011; ROCHA et al., 2011; SAHA et al., 2005; SUN; CHENG, 2005; TAN et al., 2010) and alkaline hydrolysis (SINGH; BAJAR; BISHNOI, 2014), ozonolysis (LI et al., 2015; TRAVAINI et al., 2013), ionic liquids (DADI; VARANASI; SCHALL, 2006; GEORGE et al., 2015), organosolv (ARAQUE et al., 2008; OSTOVAREH; KARIMI; ZAMANI, 2015) etc. These pretreatments involve the use of chemicals and differ from each other according to the mechanism responsible for the structural and chemical modifications of the cell wall. Alkaline pretreatment preserves

cellulose and hemicellulose, removing lignin from the lignocellulosic matrix whereas acid pretreatments are utilized to hydrolyze the hemicellulose fraction due to the weakness of the hemicellulose caused by the branches. On the other hand, cellulose and lignin remain intact in the residual solids (XIN et al., 2015). According to Van der Pol et al. (2015) enzymatic hydrolysis of cellulignin from sugarcane bagasse pretreated with diluted sulphuric acid (0.75%) was more efficient (81% of sugars release) when compared to autohydrolysis (77%) and alkaline pretreatment (57%). These results indicate that acid pretreatment is a promising pretreatment for both, pentoses sugar release from hemicellulose and D-glucose recovery after the saccharification step. Acid pretreatment has been widely utilized to recover pentose sugars from vegetal biomass without the need of enzymes; the steps are detailed discussed in topic 2.3.2.1.

Physico-chemical pre-treatments are usually more effective because they combine physical processes that act directly on the biomass structure and chemical reactions, promoted by acids or bases. Although these pretreatments show greater efficiency when compared to physical or chemical ones, they are more expensive. Some examples of physico-chemical pre-treatments are steam explosion (JIN; CHEN, 2006; PALMQVIST et al., 1997; PEREIRA RAMOS, 2003), hydrothermal (DA CRUZ et al., 2012; DOGARIS et al., 2009), wet oxidation (JI et al., 2015; SCHMIDT; THOMSEN, 1998) microwaves (SINGH; BAJAR; BISHNOI, 2014; XU et al., 2011), ammonia fiber expansion (MURNEN et al., 2007) etc. In some cases, acid or alkali can be used as a catalyst agent, increasing the efficiency (BONDESSON; GALBE; ZACCHI, 2013; SUN et al., 2015). The choice of the pretreatment is generally based on the overall cost of the process, taking into account the scale-up, the efficiency and the final product.

As an alternative to the chemical, physico-chemical or physical pretreatments, the biological pretreatment is an environmentally friendly process in which microorganisms are utilized to remove the lignin from the lignocellulosic biomass by the production of certain enzymes that are able to unconfigure the lignin structure in a process called biodelignification. It comprises microorganisms that are capable of producing ligninolytic enzymes to remove lignin from lignocellulosic biomass and cellulase and/or xylanase enzymes which are able to break cellulose and hemicellulose into sugars. Pure ligninolytic enzymes can also be used, but their high cost increases the overall cost of the process.

2.3.2.1. Acid-catalyzed hydrolysis

The polysaccharide component of lignocellulosic biomass is a major source of fermentable carbohydrate. However, the polysaccharide fractions must be broken down into available monosaccharides, which can be converted to ethanol. Hemicellulose fraction is effectively removed by acid hydrolysis after a short duration of hot dilute acid pretreatment (PINGALI et al., 2010) and 85-95% D-xylose and a few percent of L-arabinose and D-glucose are released (CLARKE; EDYE, 1996). However, although lignin is redistributed, it remains in the fiber along with cellulose fraction. Acid pretreatment disorganizes the lignocellulose structure promotes significant morphological changes, which are manifested at certain specific length scales and with a clear dependence on temperature and pretreatment time. In addition, a synergism might be related to lignin redistribution and hemicellulose removal and dissolution into the solvent due to these two processes occurring in the early stages of pretreatment (PINGALI et al., 2010). As reported by several authors, a hydrolyzate rich in pentose sugars with a small amount of D-glucose and other degradation compounds along with a solid fraction are the product of the acid hydrolysis (GARCÍA et al., 2014; JUNG et al., 2013; MARTIN et al., 2007; ROCHA et al., 2011; SAHA et al., 2005; SUN; CHENG, 2005; TAN et al., 2010). Therefore, hemicellulose is susceptible to acid hydrolysis due to its branched structure, accompanied by amorphous region of cellulose. Cellulose and lignin are in compacted form after dilute acid hydrolysis. Removal of the hemicellulosic fraction from the material is responsible for pore formation which allows cellulase enzyme access to cellulose during enzymatic hydrolysis, releasing D-glucose which is used for ethanol production (DEMIRBAS, 2005).

The same mechanism for acid-catalyzed hydrolysis is followed by glycopyranosides and xylopyranosides. The general mechanism of glycopyranosides is presented in Figure 2-10a. The glycoside is activated by the rapid and reversible protonation of the glycosidic oxygen forming a cation, after a slowly unimolecular heterolysis. A water molecule attacks at C1 position, forming a protonated hemiacetal that results in a neutral reducing sugar after losing a proton. Hydrolysis of mono-, di- and oligosaccharides are conducted under the same mechanism, however under different constant rates (k). Xylobiose ($k = 69.8 \times 10^6 \text{ k}$), a disaccharide composed of two units of D-xylose, is the most readily hydrolyzed when compared to cellobiose ($k = 9.63 \times 10^6 \text{ k}$), maltose ($k = 23.6 \times 10^6 \text{ k}$), mannobiose ($k = 16.8 \times 10^6 \text{ k}$) and lactose ($k = 22.5 \times 10^6 \text{ k}$) (PENNER et al., 1996), which may justify the high

susceptibility of hemicellulose fractions to acid hydrolysis when compared to cellulose. The structure of the disaccharides above mentioned are shown in Figure 2-10b.

Diluted-sulfuric acid is the most popular acid pretreatment utilized to improve the biomass hydrolysis even though with some important limitation such as the corrosion of the vessel, which mandates expensive materials of construction and generation of toxic compounds (MOSIER et al., 2005). Moreover, the acid must be neutralized before the sugars proceed to fermentation. The conditions applied to the pretreatment may reduce the amount of degradation compounds generated. Lower concentration of acid (0.75% v/v) combined with lower holding time (5 min) reduced the generation of toxic compounds in corn stover hydrolyzate, even under higher temperatures (160°C). Non-detoxified corn stover hydrolyzate was efficiently fermented by the recombinant *Escherichia coli* strain in concentrations of furfural below to 0.5 g/L (AVCI et al., 2013). Similar conditions were utilized for palm empty fruit bunches pretreatment (acid sulfuric 1% (w/v), 190°C, 3 min) in a microwave digester and 52.5% of theoretical ethanol yield was obtained after SSF of the slurry using cellulase and *Saccharomyces cerevisiae*. In contrast, the SSF furnished 87.5% ethanol yield when the slurry was treated with activated carbon before subjecting to SSF, (JUNG et al., 2013), showing that toxic compounds generations depends on the set condition of the pretreatment and the feedstock composition.

Figure 2-10: Acid-catalyzed hydrolysis of glucopyranosides (a) and disaccharides structures (b).

Source: modified from Penner et al. (1996)

Degradation compounds are a result of the oxidation of monomeric sugars, due to the high temperature and low pH of the reaction mixture. These compounds are also called toxic compounds or inhibitor compounds due to their negative effect during the fermentation step.

Other toxic compounds are generated during the acid hydrolysis as phenolic compounds from lignin depolymerization or weak acids (PALMQVIST; HAHN-HÄGERDAL, 2000b). The conditions applied to the pretreatment may reduce the amount of degradation compounds generated. Lower concentration of acid (0.75%) combined with lower holding time (5 min) reduced the generation of toxic compounds in corn stover hydrolyzate, even under high temperatures (160°C).

2.3.2.1.1. Inhibitor compounds

The pretreatment for fermentable sugars obtaining is an essential step in converting lignocellulosic materials into value-added products from the microbial fermentation. The D-glucose is obtained especially after the hydrolysis of cellulose, while hexose sugars (D-galactose, D-mannose and D-rhamnose), pentose sugars (D-xylose and L-arabinose) and uronic acids (D-glucuronic acid and 4-O-methylglucuronic acid) are obtained from the hemicellulose fraction. Some pretreatments, such as those catalyzed by acids, may result in degradation of lignin and few monomeric sugars. The degradation compounds are divided into three groups: furan derivatives (furfural and 5-hydroxymethyl-furfural (HMF)), weak acids (especially acetic acid, formic, ferulic and levulinic), and phenolic compounds (vanillin and 4-hydroxybenzaldehyde) (Figure 2-11) (ALMEIDA et al., 2007, 2009). When isolated, these compounds are probably not the most responsible for the inhibition of microbial metabolism, but the sum of all the inhibitors present in the hydrolyzate can produce inhibitory effects by the synergism among them (IBRAHEEM; NDIMBA, 2013; PARAWIRA; TEKERE, 2011). In addition, the degree of inhibition of the toxic compounds greatly depends on both nature and concentration of inhibitors and the nature of the microorganisms (DELGENES; MOLETTA; NAVARRO, 1996).

Some inorganic ions can also affect the growth of microorganisms. These ions may come from the decomposition of the chemical reagents added to the lignocellulosic biomass during the process of pretreatment or originated by equipment corrosion by acids, which causes liberation of metal such as aluminum, copper, nickel and iron (FRAZER; MCCASKEY, 1989; LIU, 2011). Besides, plant material can contain large amounts of metals, especially when grown in an area with metal rich soil. Moreover, the chemical pretreatment also adds to the amounts of sulphur and sodium present in the bagasse after pretreatment (VAN DER POL et al., 2015). In some cases, the inorganic ions present in the culture medium in moderate concentrations, can increase ethanol production, since they promote, in this case increased demand for ATP. This extra ATP is acquired by increased

ethanol production at the expense of formation of biomass (JÖNSSON; ALRIKSSON; NILVEBRANT, 2013). In addition, some extractives released during the pretreatment of biomass, characterized by resins (fatty acids, terpenes, sterols and waxes) and phenolic compounds (flavonoid, tannin, etc.) may also act as inhibitors of microbial metabolism during the fermentation of lignocellulosic hydrolyzate although they are found in low concentrations (LARSSON et al., 1999; SAKA, 2000)

Figure 2-11: Average breakdown of lignocellulosic biomass and the main products derived from hydrolysis.

Source: Ibraheem; Ndimba (2013)

2.3.2.1.1.1. Furan derivatives

Furans are organic compounds, heterocyclic and aromatic. Similar to benzene, their aromaticity results from the interaction between one of the pairs of electrons with oxygen double bond in the ring, which generates a charge of relocation. They are transparent compounds, flammable and have low solubility in water. Chemical functional groups such as aldehydes, ketones, esters, alcohols, ethers, acids and thiols may be present in the structure (SOLOMONS, 2001)

Furans are formed by dehydration of monomeric sugar during the pretreatment process. Furfural and 5-hydroxymethylfurfural (HMF) are the two major furanic compounds, generated by the dehydration of pentose and hexose sugars, respectively (Figure 2-11). The amount of furan formed during the pretreatment of the biomass varies according to the method and the raw material utilized due to the amount and the type of sugars available in the plant structure and the temperature applied to the process. Usually after alkaline pretreatment, only small amounts of furans compounds are observed due to an absence of monomeric sugars combined with a high pH and high temperature. In autohydrolysis and acid pretreatment, furans compounds are found in larger quantity. The formation of these compounds is related to the decrease in sugars concentration in the hydrolysate, which could be converted into ethanol. In sugarcane bagasse hydrolyzate, furfural is usually more abundant than HMF, due to the higher presence of monomeric pentoses, such as D-xylose, during the initial pretreatment (VAN DER POL et al., 2015). These degradation products are considered by Dong and Bao (2010) to be the most toxic to microorganisms, due to the high concentration present in the hydrolyzate when compared to other inhibitors.

Similar to other aldehydes, the furan aldehydes are highly reactive molecules capable of contributing to the formation of reactive oxygen species, causing damage to proteins, nucleic acids and cell organelles. As a consequence, reduced enzymatic and biological activities of yeasts, DNA breaks and inhibition of protein synthesis and RNA are found (ALLEN et al., 2010; ALMEIDA et al., 2009; LIU et al., 2004; MODIG; LIDÉN; TAHERZADEH, 2002). Studies by Taherzadeh et al. (1999) indicate that although furfural in a concentration of 4 g/L and HMF may contribute to enzyme inhibition, the other by-products generated during the fermentation process, while at high concentrations, also contribute to strengthen this inhibition.

Several mechanisms may explain the inhibitory effect of furans during ethanol fermentation. *In vitro* assessments showed that furfural and HMF are responsible for inhibition of glycolytic enzymes: alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALDH) (MODIG; LIDÉN; TAHERZADEH, 2002), resulting in accumulation of acetaldehyde and induction of lag phase which becomes longer (PALMQVIST; ALMEIDA; HAHN-HÄGERDAL, 1999). Thus, the microbial growth is inhibited and the volumetric rate and the ethanol productivity is reduced (ALMEIDA et al., 2007). The crude extract of cells grown in the presence of furfural also showed a reduction in the activity of ADH and glycolytic enzymes hexokinase and glyceraldehyde-3-phosphate dehydrogenase (BANERJEE; BHATNAGAR; VISWANATHAN, 1981). The reduction of

furans by yeasts can also result in the depletion of NAD(P)H, which is suggested by the fact that high levels of acetaldehyde were excreted when the furfural was added to the culture medium (PALMQVIST et al., 1999). In addition to the metabolic flux analysis showed that furfural affect the flow in the glycolytic pathway and tricarboacetic acid (TCA), which are involved in energy metabolism (HORVÁTH et al., 2003).

In order to alleviate the toxic effect of these inhibitors, some microorganisms are able to reduce (equation 1) or oxidize (equation 2) the furan aldehydes and turn them into alcohols or carboxylic acids through the action of the enzymes alcohol dehydrogenase and aldehyde dehydrogenase, respectively (ALMEIDA et al., 2007; MODIG; LIDÉN; TAHERZADEH, 2002; WIERCKX et al., 2011). Furfural is reduced to furfuryl alcohol, which in turn is re-oxidized to furfural that is oxidized to furoic acid (TAHERZADEH et al., 1999). Although the first reaction of oxidation-reduction is apparently unnecessary, the cell necessity in getting rid of the toxic compounds justifies such a procedure. Therefore, considering the high toxicity of furan aldehydes, initial preference for reducing the furfural instead of oxidizing it can be interpreted as a non-specific detoxification mechanism to ensure that the concentrations of furfural are maintained below the inhibition levels (WIERCKX et al., 2011).

The reduction of furfural to furfuryl alcohol affects the redox balance of the cell, resulting in reduction of glycerol and intracellular accumulation of acetaldehyde, which occurs probably due to oxidation of ethanol in an attempt to increase the concentration of NADH. The reduced electrons employed in the furfural reduction reaction catalyzed by the enzyme alcohol dehydrogenase. Since this enzyme is also responsible for the conversion of acetaldehyde into ethanol, it can be inferred that there is a competition between the substrates for the same enzyme. The use of the reduced carrier in the reduction of furfural competes with reduction of dihydroxyacetone phosphate, since the enzymes that catalyze these reactions also require NADH. Some studies indicate that alcohol dehydrogenase competes with the enzyme glycerol-3-phosphate dehydrogenase, involved in the glycerol synthesis, for the coenzyme NADH, which leads to reduction of furfural in the absence of glycerol in the fermentation medium. The competition between these two enzymes for the coenzyme in

its reduced form decreases the intracellular levels of NADH (PALMQVIST; ALMEIDA; HAHN-HÄGERDAL, 1999; PALMQVIST et al., 1999; TAHERZADEH et al., 2000).

The large amount of NADH required for the reduction of furfural leads to insufficient levels of this coenzyme for cellular respiration, resulting in a small concentration of ATP insufficient to sustain cell growth. Therefore, the effects of furfural on the inhibition of cell growth can be associated in part by the high demand of NADH and partly by enzymatic inhibition level (TAHERZADEH et al., 2000). Thus, although it is possible to convert the toxic aldehyde through redox reactions, into compounds of lower toxicity, the use of cofactors required for such conversions (NAD/NADH) causes an increase of lag phase during the growth of the microorganism (ALMEIDA et al., 2007; LIN; QIAO; YUAN, 2009; LIU, 2006). The conversion of furan aldehydes by oxidation-reduction reactions should not be confused with their degradation. The metabolism of these compounds by the cell is still not fully elucidated. In some studies it is only mentioned the disappearance of furan compound, without specifying the metabolic fate of alcohol or corresponding carboxylic acid (WIERCKX et al., 2011).

Van Niel et al. (2012) studied the effect of furfural (1 g/L) on the metabolism of *Lactobacillus reuteri* ATCC 55730 in culture medium supplemented with D-glucose. The author reported that the exposure of the bacteria to furfural immediately after the inoculation of the culture medium increased the lag phase and reduced the growth rate, confirming the inhibitory effect of this compound. In contrast, when furfural was added at the beginning of the exponential phase, the growth of the microorganism was increased. According to the study, furfural was used as the electron acceptor and increased the microbial growth rate by 25% of and the biomass production by 15%. Although the stoichiometric reduction between furfural and furfuryl alcohol was reduced, this fact did not interfere with the integration of phosphoketolase enzyme activity and Embden-Meyerhof pathway. Moreover, there was an increase in production of acetate. Furthermore, the furfural and HMF were able to reoxidize NADH and/or NADPH.

It is important to highlight that the degradation of furfural and HMF is possible only if these compounds are converted into 2-furoic acid and 5-hydroxymethyl-2-furoic acid, respectively. Specific aldehyde dehydrogenase enzymes were not identified among the essential enzymes for catabolism of furanic derivatives, although the oxidoreductase present in HMF degradation pathway is also capable of oxidizing furfural and HMF to their alcohol and aldehyde form (KOOPMAN et al., 2010). Generally speaking, the enzymes involved in

the degradation pathways defend the cells against toxic aldehydes and seems to be generic dehydrogenases, presenting a broad specificity spectrum (WIERCKX et al., 2011).

Roberto et al. (1991) studied the production of ethanol by *Pichia (Scheffersomyces) stipitis* CBS 5773 and observed a complete inhibition of cell growth under 2.0 g/L of furfural concentration. On the other hand, at lower concentrations (less than 0.5 g / L), furfural served as carbon source, contributing to the cell growth. Likewise, studies of ethanol production by *Pichia (Scheffersomyces) stipitis* NRRL Y-7124 showed that furfural negatively influenced the cellular respiration when present in concentrations above 1.5 g/L. However, there was no significant interference in the yield and process productivity at a concentration of 0.25 g/L. In addition, furfural was immediately reduced to furfuryl alcohol at high concentrations, negatively affecting the cellular growth rate and the specific rate of ethanol production (NIGAM, 2002)

2.3.2.1.1.2. Weak acids

Small organic acids can be originated from several sources. The acids can already be present in the structure of the lignocellulosic material for instance acetyl radicals, which are linked to the backbone of hemicellulosic xylans, and are released as acetic acid during the acid pretreatment. Levulinic acid and formic acid, can be formed when furans are dehydrated. While levulinic acid is formed by the degradation of HMF, formic acid is a degradation product of both furfural and HMF (Figure 2-11). Furthermore, microorganisms can contaminate the substrate and produce a range of different acids, for example lactic acid. Usually, the most abundant organic acid in acid and alkaline pretreated material is acetic acid. In an environment with a low pH and high temperature, HMF can easily dehydrate to levulinic acid and formic acid (Figure 2-11). The presence of glycolic acid in substantial concentrations is typical for sugar rich plants like sugarcane or sugar beets (ULBRICHT; NORTHUP; THOMAS, 1984; VAN DER POL et al., 2015). Weak acids, in general, inhibit the fermentative capacity of the yeast for reducing the formation of biomass and reduced yield of ethanol.

The toxic effects of weak acids are related to the value of pK. At pH values below the weak acid pK_a value, the acid is mainly in their undissociated form. In this case, acids are soluble and can reach the interior of the microbial cell by diffusion through the plasma membrane (PAMPULHA; LOUREIRO-DIAS, 1989). Intracellular pH is near neutrality (pH around 7.4) and promotes the dissociation of these acids, which is followed by the

intracellular pH reduction to values lower than the physiological limits, causing cell death and a reduction in ethanol production rate (LOHMEIER-VOGEL; SOPHER; LEE, 1998).

Although a small amount of formic acid ($pK_a = 3.75$ at 25°C), remains in the non-dissociated form in the fermentation medium, this acid is considered more toxic than levulinic acid ($pK_a = 4.64$ at 25°C) and acetic acid ($pK_a = 4.76$ at 25°C) due to the difference in their pK_a values. Moreover, formic acid has a small molecular structure in relation to the other inhibitors acids, facilitating its transportation through the cell wall. The inhibitory effect on yeast metabolism has been observed at concentrations above 100 mM of formic acid. However, high levels of ethanol have been obtained in the presence of concentrations below 100 mM (LARSSON et al., 1999; TAHERZADEH; NIKLASSON; LIDÉN, 1997).

The inhibitory effect of weak acids has been described as a decoupling reaction of ATP production by the ATPase enzyme located at the plasma membrane. This reaction is followed by intracellular accumulation of anions. In an attempt to maintain a constant internal pH, the enzyme ATPase is stimulated to pump protons across the plasma membrane to outside the cell, resulting in a consumption of ATP followed by the non-reduction of electrons carriers (NAD^+) due to the protons had been eliminated (VERDUYN et al., 1990, 1992; VIEGAS; SÁ-CORREIA, 1991). As a result, there is the intracellular accumulation of anions. Thus, while the protons are excreted and anions are captured and accumulated, causing cell death (TAHERZADEH et al., 1999).

Some uncoupler compounds such as amphiphilic molecules have a mechanism of action similar to weak acids. However, these compounds do not cross the plasma membrane and dissociate within the cell, but they install in the mitochondrial membrane where proton transfer occurs hampering the respiratory chain reactions and oxidative phosphorylation reactions both responsible for the generation of ATP from ADP (NORMAN et al., 2004).

2.3.2.1.1.3. Phenolic derivatives

Derivatives of phenol, also known as phenolic compounds, are molecules that have one or more hydroxyl groups attached to an aromatic ring, and may have substituent groups like carbon, methoxyl and other non-aromatic cyclic structures (Figure 2-11). Various phenolic and aromatic compounds, e.g. syringaldehyde, 4- hydroxybenzaldehyde and vanillin are formed from the degradation of the molecular structure of lignin during the lignocellulosic biomass pretreatment, especially acid pretreatment or acid as a catalyst agent under high temperature is employed (CANTARELLA et al., 2004).

Phenolic and aromatic compounds can inhibit both microbial growth and reduce product yield. The mechanism of action is variable and can be related to specific functional groups (ANDO et al., 1986). Although the mechanism of toxicity has not been elucidated yet, it is believed

that these inhibitors are able to interact with the cell membrane, influencing its function and changing the protein-lipid ratio present in the composition (HERIBERT; GERD; HANS-JÜRGEN, 1990).

2.3.2.1.2. Detoxification of hemicellulosic hydrolyzate

Several alternatives have been applied to remove the cellulosic hydrolyzate inhibitors, avoiding problems related to the low yield of product and the microbial growth rate in the fermentation process. The concentration of inhibitors and sugars present in the hydrolyzate depends on both the raw material and the pretreatment utilized (JÖNSSON; ALRIKSSON; NILVEBRANT, 2013; PALMQVIST; HAHN-HÄGERDAL, 2000b).

Various physical methods and physico-chemical have been developed to remove the degradation products of the lignocellulosic hydrolyzate (CANILHA et al., 2012; ZHANG et al., 2010). The effectiveness of a detoxification method depends on both type of hemicellulose hydrolyzate and specie of microorganism used, since each microorganism has a different degree of tolerance to inhibitors (LARSSON et al., 1999). However, many of detoxification methods used are costly, and generate additional waste to the process of obtaining ethanol (JÖNSSON; ALRIKSSON; NILVEBRANT, 2013; PALMQVIST; HAHN-HÄGERDAL, 2000a). The detoxification of the pre-treated hydrolysate using different kinds of alkali agents for removal of specific inhibitors generates the loss of fermentable sugars and volume (PERSSON et al., 2002). Similarly, using methods such as washing with water, overliming, vaporization and absorption of ion exchange may involve drawbacks such as the massive use of water and waste generation, in addition to loss of particles of material, losses of fermentable sugars and incomplete removal of inhibitors (DONG; BAO, 2010).

An alternative methods of physical and chemical detoxification is the use of microorganisms and/or their enzymes for the removal of toxic compounds from lignocellulosic hydrolyzate. The biological detoxification (biodetoxification) has advantages over the other methods, mainly by reducing the amount of waste generated at the end of the process. Microorganisms such as *Issatchenkia occidentalis* (FONSECA et al., 2011), *Saccharomyces cerevisiae* (FONSECA et al., 2013) and *Enterobacter* sp. (ZHANG et al.,

2013) *Aspergillus nidulans* (YU et al., 2011) and *Coniochaeta ligniaria* (CAO et al., 2013) or their enzymes such as laccase (VITHANAGE et al., 2015) are capable of acting on specific toxic compounds by changing their composition or structure, thus reducing their toxicity. Advantages such as mild reaction conditions, avoiding the use of toxic and corrosive chemicals, generation of small amount of toxic side products and lower energy input requirements make this method worth potential industrial applications. Moreover, biodegradation can be performed in the same bioreactor where the fermentation was carried out, after the fermentation or in co-culture, saving space and time (DONG; BAO, 2010; LÓPEZ et al., 2004). On the other hand, limitations such as long incubation periods and eventual consumption of sugars by the microorganism during the detoxification process still hinder its implementation and reinforce the need for additional studies (YANG; WYMAN, 2008). In addition, cells may be recycled, improving the detoxification capability due to the gradual adaptation to the toxic compounds, leading to a more efficient biodegradation, reducing costs in industrial application (ZHANG et al., 2013).

Ligninolytic enzymes synthesized by fungi, such as laccases and peroxidases, can also be used for the biological detoxification of hydrolyzates. Jönsson et al. (1998) utilized *Trametes versicolor* enzymes derived from fungal metabolism for complete elimination of selective phenolic monomers present in willow hydrolyzate. Based on the absorption spectra, the enzymes reduced the toxic effect of these inhibitors by oxidative polymerization of phenolic compounds to low molecular weight aromatic compounds of high molecular mass, less toxic.

2.3.2.2. Biological pretreatment

Biodelignification of lignocellulosic biomass consists of the partial or total removal of lignin from the lignocellulosic biomass by enzymes synthesized by microorganisms. The advantage of this pretreatment is that there are no harsh chemicals or strict conditions required and microorganisms are grown on substrate moistened with a nutrients solution during solid state fermentation process over semi-solid or submerged cultivations. In addition, it is an eco-friendly process with no generation of fermentation inhibitors, toxic compounds nor effluent (SINDHU; BINOD; PANDEY, 2016). Microbial pretreatment has been described as a process which requires a long time due to the slow rate of lignin degradation. Also losses of carbohydrate fraction along with lignin removal have been reported (DINIS et al., 2009; GARG et al., 2011; SINGH; HARMS; SCHLOSSER, 2014; WANG et al., 2013; YANG; WYMAN, 2004). However, recent studies reported that the biodelignification of rice straw

by the fungi *Trametes hirsuta* and *Myrothecium roridum* resulted in 19 and 70% enrichment of its holocellulose content, respectively after seven days of incubation characterizing a short-time pretreatment (MOHANRAM et al., 2015).

White rot fungi are the most widely studied microorganisms for microbial delignification. Several white rot fungi have been utilized for lignin removal from vegetal biomass, such as *Phanerochaete chrysosporium* (RUTTIMANN-JOHNSON; CULLEN; LAMAR, 1994; ZHAO et al., 2015), *Ceriporiopsis subvermispora* (BAKER; CHARLTON; HALE, 2015; YELLE et al., 2014), *Physisporinus rivulosus* (HAKALA et al., 2005; MAIJALA et al., 2008), *Pleurotus ostreatus* (PALMIERI et al., 2000; TANIGUCHI et al., 2005; VERMA; MADAMWAR, 2002), *Trametes versicolor* (BARI et al., 2015) among other species. These fungi can be grown isolated or in co-culturing (CHI; HATAKKA; MAIJALA, 2007; DONG et al., 2013). They provide special advantages due to their selective lignin degradation ability and their minimal effect on carbohydrates. These characteristics are owing to their potential of producing high amounts of ligninolytic enzyme (oxidases and peroxidases) and low amounts of hydrolytic enzymes. The action of oxidases and peroxidases results in the generation of non-specific and highly reactive free radicals that participate in lignin degradation (BUGG et al., 2011). The main enzymes involved in lignin degradation by fungi and bacteria are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. The oxidative reactions catalyzed by these enzymes have no common steps or similarity unless the need of a redox mediator, which is particular to each enzyme (HATTORI; SHIMADA, 2000).

Ligninolytic enzymes can be activated or deactivated according to the composition of the culture medium. Besides lower living *Phanerochaete chrysosporium* biomass and low LiP and MnP activities (0.34 and 5.21U/g) were observed after culture exposure to cadmium, reactive oxygen species, such as hydroxyl radicals (OH), superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) were found under cadmium stress cultivation (ZHAO et al., 2015). Moreover, the pressure under which the cultivation is developed may also influence the biodelignification process. According to Baker; Charlton and Hale (2015), the pressure applied to the system confers a higher water uptake avoiding the reduction on gas diffusion that would prevent the fungal growth. Great delignification rate was observed by these authors after cultivation of *C. subvermispora* in refined *Miscanthus* under pressure (6 and 8 bar).

LiP is a H_2O_2 -dependent heme-containing enzyme, which has the ability to degrade lignin by oxidizing its phenolic and non-phenolic substructures (RENGANATHAN; GOLD, 1986). This enzyme appears to be a more effective delignifying agent than laccase (KEILIN; MANN, 1939). Lignin-degrading system may be activated by veratryl alcohol. This alcohol is a natural secondary metabolite of some fungi and works as a redox mediator in ligninolytic system, attacking lignin polymer at a distance instead of attacking directly the insoluble lignin polymer, increasing hydrogen peroxide production and D-glucose oxidation rates (Figure 2-12) (LEISOLA et al., 1984).

MnP is a heme protein that catalyzes the oxidation of Mn^{2+} in the presence of hydrogen peroxide to form Mn^{3+} as a direct oxidant. As veratryl alcohol in LiP system, manganese acts as a redox mediator in MnP system, however Mn^{3+} is not strong enough to oxidize the nonphenolic lignin moiety (HATTORI; SHIMADA, 2000). Even though the overall structure of MnP is similar to LiP, with two structural calcium ions, this enzyme is unique with respect to reducing the substrate Mn^{+2} to Mn^{+3} (SUNDARAMOORTHY et al., 1994). MnP seems to be more widespread than LiP among white rot fungi and plays a key role in the initial stages of lignin catabolism (HATTORI; SHIMADA, 2000).

Figure 2-12: Oxidation of veratryl alcohol catalyzed by lignin peroxidases generating veratraldehyde (a) and proposed mechanism of the aromatic ring veratryl alcohol opening (b) through H_2O (A) attack or O_2 attack (B).

Source: modified from Hattori; Shimada (2000)

Laccase is a phenol oxidase and belongs to a large and diverse family of blue multi-copper enzymes. This enzyme catalyzes the oxidation of a *p*-diphenol to *p*-benzoquinone using molecular oxygen as a terminal electron acceptor. It is capable of oxidizing both phenolic and non-phenolic moieties of lignin by one-electron abstraction, resulting in radicals, which can bind each other. The non-phenolic oxidation is dependent on the co-presence of primary laccase substrates (HATTORI; SHIMADA, 2000).

Munk et al. (2015) reviewed the complete mechanism of laccase, standardizing the different nomenclatures utilized so far and describing the changes in phenylpropanoid units of lignin as follows. As a first step the lignin surface structure is activated by a single electron extraction from a phenylpropanoid subunit (Figure 2-13a). In a second step, the reactive radical can be cleaved, modified by changes in its functional group or be attached to other phenylpropanoids or mediator (Figure 2-13b). Bond cleavage causes the depolymerization of the lignin molecule whereas the coupling can cause both, grafting followed by changes in lignin properties such as solubility, and polymerization, which increases the average molecular weight of the molecule (Figure 2-13c).

Not all of the ligninolytic enzymes are produced by a specific fungal culture. Two different studies utilizing corn stover as feedstock for two different white rot fungi demonstrated that laccase was the major ligninolytic enzyme produced. Sun et al. (2011) did not detect neither LiP nor MnP during the 42 days of pretreatment of corn stover with *Trametes hirsuta* yj9 and laccase played a main role in delignification. In line with these results, Ma and Ruan (2015) reported that laccase was the main enzyme synthesized by *Coprinus comatus* when incubated with corn stover with two strong activity peaks (1160 U/ml and 1520 U/ml) appeared after 3 days and 6 days of incubation and no MnP and LiP activities were detected. On the other hand, in a study of Verma and Madamwar (2002) the three ligninolytic enzymes were produced for *Pleurotus ostreatus* and *Phanerochaete chrysosporium* growing in neem hull, sugarcane bagasse and wheat bran, but different levels of activity were observed. *P. ostreatus* showed high levels of laccase (up to 156 U/g, 112 U/g and 142 U/g) and MnP (up to 254 U/g, 212 U/g, 217 U/g) and very low LiP (up to 20.2 U/g, 21.2 U/g and 19.2 U/g) activities on neem hull waste, sugarcane bagasse and wheat bran, respectively. In contrast, high levels of LiP (up to 192 U/g, 119 U/g and 167 U/g) activity and very low levels of laccase (up to 28.2 U/g, 25.2 U/g and 21.2 U/g) and MnP (up to 54.1 U/g, 45.1 U/g, 34.2 U/g) activities were observed when *P. chrysosporium* was incubated with neem hull waste, sugarcane bagasse and wheat bran, respectively. Therefore,

the type of enzyme to be produced is mainly dependent on the fungal culture, but the enzymatic activity levels relies on the substrate.

Figure 2-13: Modifications in lignin structure caused during action of laccase.

Source: modified from Munk et al. (2015)

Hydrolytic enzymes may be produced or not during the biodelignification process. These enzymes are usually produced at the initial stage of fermentation due to sugars metabolism by the fungi (SUN et al., 2011). As long as the sugars are utilized for ethanol production, hydrolytic enzymes are undesirable during the biodelignification process. The amount of xylanase and cellulase may differ among the cultivations, according to the fungi and the substrate. Besides, the activity may fluctuate according to the period of incubation.

High xylanase activity results in hemicellulose loss, whereas high cellulase activity leads to cellulose loss. Sun et al. (2011) detected cellulase (up to 2.86 U/g solid) and large amounts of xylanase (up to 11.22 U/g solid) during the 42-days incubation of corn stover with *T. hirsuta* yj9. On the other hand, according to Ma and Ruan (2015) very little hydrolytic enzymes were secreted during corn stover biodelignification, as long as cellulase and xylanase production exhibited considerable low activity. Aligned, El-Nasser; Helmy; El-Gammal (1997) observed very low xylanase and cellulase activity after incubation of *P. chrysosporium* NRRL 6359 during 7 days with sugarcane bagasse (6.6 U/mL and 1.2 U/mL) and rice husks (6.0 U/mL and 0.6 U/mL) and higher when incubated with wheat straw (27.6 U/mL and 4.2 U/mL), corn cobs (13.8 U/mL and 2.4 U/mL) and peanut shells (10.2 U/mL and 3.6 U/mL). After 14 days of incubation, xylanase activity was in between 3.0 U/mL and 7.8 U/mL for the five tested substrate and only wheat straw and corn cobs cultivations presented cellulase activity (2.4 U/mL and 1.2 U/mL, respectively). Besides xylanase activity was barely altered, cellulase activity increased in all of the cultivations, ranging from 1.8 U/mL to 3.0 U/mL, except for wheat straw and corn cobs, which remained constant.

2.3.2. Enzymatic hydrolysis of lignocellulosic biomass

Prior to fermentation the polymerized carbohydrates must be broken down during the enzymatic hydrolysis. This step is also known as saccharification and consists of cellulose and/or hemicellulase depolymerization by a group of enzymes (Figure 2-9). As long as lignocellulosic biomass presents two different types of source of sugar, two different group of enzymes are utilized. Cellulose is converted into D-glucose by means of a cellulase enzymes as follows. Endoglucanases (EC 3.2.1.4) cleave internal β -1,4-glucosidic bonds, producing oligomers with new chain ends; they act preferentially in the amorphous region of the fibrils. Cellobiohydrolases (endoglucanases) (EC 3.2.1.91) act on the reducing (CBHs I) and non-reducing (CBHs II) ends of cellulose chains, releasing cellobiose molecules. β -glucosidases (EC 3.2.1.21) hydrolyze soluble cellobiose to two D-glucose molecules (KIRK;

CULLEN, 1998; ORTEGA; BUSTO; PEREZ-MATEOS, 2001; RATANAKHANOKCHAI et al., 2013).

Due to the heteropolymeric and branched hemicellulose structure, its hydrolysis requires a more complex group of enzymes. Therefore, hemicellulase complex include specific enzymes responsible for the cleavage for both the main chain and the branches. Xylanases (EC 3.2.1.8) and β -xylosidases (EC 3.2.1.37) are responsible for the break down of β -1,4- xylan bonds mainly found in the backbone, whereas the various side chains are cleaved by α -L-arabinofuranosidases (EC 3.2.1.55), α -glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), feruloyl esterases (EC 3.1.1.73), and galactosidases (EC 3.2.1.22) (KIRK; CULLEN, 1998; RATANAKHANOKCHAI et al., 2013) as shown in Figure 2-14a. Due to the extensive use of dilute-acid pretreatment, where hemicellulose is removed before saccharification, cellulase enzymes have been more explored than hemicellulosic enzymes (GRAY; ZHAO; EMPTAGE, 2006).

Figure 2-14: Enzyme systems involved in the degradation of cellulose (a) and xylan (b).

Source: Ratanakhanokchai et al. (2013)

2.3.3. Fermentation of the sugars recovered from lignocellulosic biomass

Simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) are two different processes utilized for the conversion of the released sugars into ethanol (Figure 2-9). SSF has been extensively explored for cellulosic ethanol production (BIAŁAS; SZYMANOWSKA; GRAJEK, 2010; CANTARELLA et al., 2004; DAS NEVES et al., 2006; SANTOS et al., 2012b; WIRAWAN et al., 2012). The major advantage with SSF over SHF is the reduction of the process time by combining hydrolysis and fermentation (OLOFSSON et al., 2008). In addition, SSF reduces the chances of end-product inhibition once the released sugars are converted into ethanol. Moreover, the liquid fuel and overall energy efficiency of the SSF process is higher than that of the SHF process (PETERSEN et al., 2015).

Cellulosic ethanol is obtained by fermentation of D-glucose from cellulose fraction, while hemicellulosic ethanol is obtained by fermentation of pentoses, mainly D-xylose, from hemicellulose fraction. Cellulosic and hemicellulosic ethanol production are very similar and comprises four stages as shown in Figure 2-9. For the cellulosic ethanol production through biochemical conversion, the use of enzymes is mandatory, so that the pretreatment is a critical step as it promotes enzyme access to the cellulose matrix during the hydrolysis step. Unfortunately, the high cost of the enzymes impedes the expansion of an industry converting cellulosic biomass into liquid fuels on a large scale (LYND et al., 2008). In contrast, pentoses sugars for hemicellulosic ethanol production may be obtained through acid pretreatment as discussed in item 2.3.2.1. Hemicellulosic ethanol production would be less costly when compared to cellulosic ethanol due to not having the requirement for enzymes. However, the necessity of extra steps to remove the toxic compounds (discussed in item 2.3.2.1.2) and specific pentose fermenting microorganisms hamper the process. On the other hand, although the conversion of D-xylose into ethanol is slower than the D-glucose, the possibility of the overall ethanol yields increase by the end of the process is a great goal to keep the researches in this area (JONKER et al., 2015; MATHEW et al., 2016).

Despite the possibility to integrate the metabolism of pentoses and hexoses, the ability of wild strain of fermenting both these sugars concomitantly and produce ethanol is still uncertain. On the other hand, metabolic engineering has been utilized to improve, redirect or generate new metabolic reactions or whole pathways in microorganisms. Instead of only metabolizing D-glucose, *Saccharomyces cerevisiae* has been genetically modified towards conversion of other substrates, in addition to D-glucose, into ethanol such as L-

arabinose (BECKER; BOLES, 2003), D-xylose (MATSUSHIKA et al., 2009) and even though toxic compounds, such as acetic acid (WEI et al., 2015). Although it shows to be a very promising method, many issues are related with the use of genetically modified strains, such as unknown risks for the human health and random transference of the genetic material among the strains (RENAULT, 2002). Therefore the potential of D-xylose fermenting strains must be improved to maintain the ethanol productivity measures of the current starch- and cane sugar based ethanol systems, being tolerant to toxic compounds from the hemicellulosic hydrolyzate, ethanol, low pH, high osmolarity and high temperatures (JORDAN et al., 2012).

Saccharomyces cerevisiae and *Zymomonas mobilis* are very well-known strains related to D-glucose metabolism and its conversion into ethanol, whereas *Pichia* (*Scheffersomyces*) *stipitis* and *Candida shehatae* are capable of synthesizing the enzyme apparatus necessary to D-xylose fermentation (DELGENES; MOLETTA; NAVARRO, 1996). *S. cerevisiae* has traditionally been used in large-scale ethanolic fermentation of D-glucose obtained from both sucrose and starch and it is therefore well adapted to the industrial context. It produces ethanol under anaerobic fermentation through Embden-Meyerhof glycolytic pathway with stoichiometric yields and tolerates a wide spectrum of inhibitors and elevated osmotic pressure (HAHN-HÄGERDAL et al., 2007; KASPAR VON MEYENBURG, 1969; VAN MARIS et al., 2006). D-glucose is transported via facilitated diffusion from extracellular into intracellular medium, thus D-glucose uptake only requires a concentration gradient across the plasma membrane. Once in the cytoplasm, D-glucose undergoes a ten-step reaction and at the end of the process, two pyruvate molecules are formed with a balance of two ATP per D-glucose. In anaerobic fermentative cultures the NADH formed by glyceraldehyde-3-phosphate dehydrogenase is reoxidized via alcoholic fermentation. This essential redox balancing involves the combined activity of pyruvate decarboxylase and alcohol dehydrogenase (VAN MARIS et al., 2006).

D-xylose and L-arabinose metabolism are performed either by fungi or bacteria, and is dependent on a specific enzyme apparatus as illustrated in Figure 2-15. Both of these pathways converge for D-xylulose 5P and end up into pentose phosphate pathway. However, more attention has been paid for D-xylose metabolism due to its higher concentration in hemicellulosic hydrolysate than L-arabinose. In most fungi and D-xylose-fermenting yeasts such as *Scheffersomyces stipitis*, *Candida shehatae*, D-xylose is converted to D-xylulose by two oxidoreductases involving cofactors NAD(P)H and NAD(P)⁺ through the type I pathway. NAD(P)H-dependent xylose reductase enzymes reduces D-xylose to xylitol, which

is oxidized to D-xylulose by the enzyme NAD⁺-dependent xylitol dehydrogenase. The conversion ends by the phosphorylation of D-xylulose into D-xylulose 5-phosphate as result of the xylulokinase enzyme action. D-xylulose is further metabolized through the pentose phosphate pathway (PPP). Generally, bacteria do not pursue D-xylose reductase and xylitol dehydrogenase enzymes. In this case the most bacteria such as *Escherichia coli* and *Streptomyces* sp., D-xylose is directly isomerized to D-xylulose by D-xylose isomerase through the type II pathway without xylitol formation (HAHN-HÄGERDAL et al., 2007; MATSUSHIKA et al., 2009).

Figure 2-15: D-xylose and L-arabinose metabolic pathway in fungi and bacteria

Source: Hahn-Hägerdal et al. (2007)

2.4. Integration of first and second generation bioethanol production

According to Leong (2004) there are many critical issues in agriculture, such as the exponential growth of the in human population, destruction of natural landscapes, destruction of environmental quality due to the exposure to agrochemicals, erosion of soils, salinization of soils, exhaustion and contamination of fresh water resources, among others. As long as first generation ethanol production is based on crops and requires the land

cultivation, the resources must be better availed to contribute to the preservation of the environment. Even though Brazil is a large-extension country with productive and arable land besides the favorable weather with potential for sugarcane crops expansion, countries in different conditions must think on a process which requires smaller cultivation area to obtain the feedstock. Moreover, although the sugarcane bagasse is burnt to produce steam and electricity, sugars that could be recovered and converted into ethanol to increase the overall yield of the process are burnt along with the fibers. In addition, the burn releases CO₂ into the atmosphere, contributing to the greenhouse effect.

According to Dias et al. (2012), second generation ethanol would contribute profitably with the conventional autonomous distillery with the whole sugarcane utilization. Carbohydrates should be extracted prior the burning of sugarcane trash and the bagasse. Jonker et al. (2015) statements that significant gains can be obtained when pentose fermentation to ethanol is accessible. Although the overall ethanol yield would be mainly determined by the first generation part of the installation, the total ethanol yield of sugarcane ethanol would be superior to the energy generated from the sugarcane. In addition, the exact amount of lignocellulosic material available for hydrolysis could be determined according to the thermal requirements of the entire process, since a portion of the material available must be burnt in the boilers to supply the energy requirements of the integrated plant. Thus, lower energy demand of the production process leads to higher amounts of bagasse and trash available for hydrolysis (DIAS et al., 2011). A proposal of first and second generation ethanol production integration is shown in Figure 2-16.

Figure 2-16: Integration of first and second generation ethanol production.

Source: modified from Dias et al. (2012)

According to Figure 2-16, the first generation site would remain unchanged and sugarcane bagasse and trash are joined into a pretreatment step for pentose sugars recovery. The resultant cellulignin is hydrolyzed for obtaining D-glucose. Unreacted solids are utilized for steam and electric energy cogeneration. Recovered sugars from the lignocellulosic biomass are added to the juice during the concentration step. The concentrated sugars are fermented by specific yeasts, which are recycled. Ethanol anhydrous is obtained after distillation and dehydration steps (DIAS et al., 2012)

2.5. Future perspectives

It is clear that changes must be made towards the preservation of the environment and alternatives processes must be developed in order to reduce pollution. However, the efforts made over the time to improve the technological processes should be valued. The integration of well-known technologies and new-developed technologies are worthy in the sense that current processes may be improved and optimized furnishing a total utilization of feedstocks along with costs reduction. Therefore, the integration of first and second generation ethanol represents a step over the future in regards of sustainability and green technology.

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CHAPTER 3

3. The Impact of Short Duration Fungal Pretreatment of Sugarcane Bagasse on Sugar Accessibility

Abstract

A short-duration biological pretreatment using four environmentally isolated fungal strains was evaluated for increased accessibility of fermentable sugars from sugarcane bagasse (SCB) and subsequent conversion into ethanol. A fractional factorial design 2^{4-1} was used to assess the impact of the solid-state fermentation parameters of agitation, SCB size, moisture level and pH on ligninolytic and hydrolytic enzyme activities and lignin transformation over 21 days. Acid-precipitable polymeric lignin (APPL) was released and metabolized by all strains tested. None of the strains produced cellulase under the conditions employed in the study. Using optimized conditions identified through the fractional factorial experiment, xylanase activity was successfully reduced from a maximum of 30 U/mg to 5 U/mg. Thus, optimal culture conditions were identified in which the fungal strains were able to depolymerize lignin in a short period of time with no significant consumption of fermentable sugars.

2.6. Introduction

Vegetal biomass, mainly agricultural waste, requires pretreatment through physical, chemical, physicochemical or biological processes in order to improve the yield of second-generation bioethanol. The advantages and drawbacks of various pretreatments have been extensively studied with respect to treatment time, final ethanol yield and end of process waste generation (SATHITSUKSANOH; GEORGE; ZHANG, 2013).

Research and development attention has recently been directed toward green and sustainable approaches aimed at avoiding contamination of water or expensive wastewater treatments. Biological pretreatment shows promise as an alternative for lignin removal and sugar recovery from vegetal biomass, since this is a green method which does not generate toxic residues (reviewed by CHANDEL et al. 2013). However, for biological pretreatment to be industrially practical, protracted incubation times and the microbial consumption of sugars must be minimized. Microorganisms usually require lengthy incubation times to

degrade lignin through the secretion of ligninolytic enzymes such as laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) which open pores into the lignocellulosic matrix releasing lignin intermediates and allowing hydrolytic enzymes to reach the polysaccharide (DIAS et al., 2010).

Laccase belongs to a large and diverse family of blue multi-copper enzymes and catalyzes the oxidation of a wide range of aromatic compounds using molecular oxygen as a terminal electron acceptor. It is capable of oxidizing both phenolic and non-phenolic moieties of lignin by one-electron abstraction, resulting in radicals, which can bind each other. The non-phenolic oxidation is dependent on the co-presence of primary laccase substrates. Lignin peroxidases (LiPs) are H_2O_2 -dependent heme-containing enzymes, which have the ability of degrade lignin by oxidizing its phenolic and non-phenolic substructures (RENGANATHAN; GOLD, 1986). This enzyme appears to be a more effective delignifying agent than laccase (KEILIN; MANN, 1939). Even though the overall structure of MnP is similar to LiP, with two structural calcium ions, this enzyme is unique with respect to reducing the substrate Mn^{+2} to Mn^{+3} (SUNDARAMOORTHY et al., 1994). It is not necessary for these three ligninolytic enzymes to be synthesized simultaneously by a lignin-degrading microorganism. Enzyme production depends on the feedstock, available nutrients and experimental conditions (ARORA; CHANDER; GILL, 2002; FUJIAN; HONGZHANG; ZUOHU, 2001).

To ensure high ethanol yields, the ideal biological pretreatment strain should neither use hexose nor pentose sugars from vegetal biomass as energy source. Furthermore, it is crucial that the strain not produce hydrolytic enzymes such as cellulase and xylanase. In pursuit of these ideal strain characteristics, four environmentally isolated fungal strains were evaluated for their capacity to biodelignify SCB. The extracellular enzymatic activities produced by the fungal strains and lignin depolymerization, as followed by the appearance of APPL, were analyzed. The best culture conditions for biodelignification were statistically optimized.

3.2. Material

Raw SCB samples were obtained from Fazenda Invernada, Bioserv, Unica (Vale do Rosário, São Paulo, Brazil) dried under sunlight until 10% humidity as measured in an infrared weighter (Top Ray – Bel Engineering) at 100°C. The dried biomass was milled to 20, 40 and 60 mesh size and stored in a sealed container until further use. The strains tested for the biological SCB pretreatment (JLS6-A6, JLS6-A7, JLS6-A12 and JLS6-F6) are part of the

Molecular Microbial Biochemistry Laboratory culture collection (University of Ontario Institute of Technology (UOIT)). Statistica (version 12) software was used for regression and statistical analyses of the data. The statistical significance of the regression coefficients was 95%.

3.3. Methods

3.3.1. Microbial strains and inoculation

For the biodelignification process, four environmental isolates JLS6-A6, JLS6-A7, JLS6-A12 and JLS6-F6 were selected after a preliminary screen for biodelignification potential and were verified as lignin degraders by *in vitro* plate assays. The strains were maintained on Potato Dextrose Agar (PDA) plates at 4°C. To prepare the inoculum for the solid-state fermentation of SCB, 1 cm² agar plugs of mycelium were cut along the edge of the actively growing colonies, which had been cultivated on PDA plates for 4 days at 30°C.

3.3.2. Screening of significant factors using fractionary factorial design

A fractionary factorial design 2^{4-1} with a central point performed in triplicate was employed to evaluate the physical and nutritional factors of agitation, SCB size, moisture level and pH for effect on APPL, LiP, laccase, xylanase and cellulase production for the strains JLS6-A6, JLS6-A7, JLS6-A12 and JLS6-F6. Each variable was studied at two extreme levels, i.e. high (+1) and low (-1) during the screening process (Table1). The response variable was the xylanase activity (U/mg of protein) (y).

3.3.3. Solid state fermentation and biodelignification

As described in Table 1, 125-mL conical flasks containing 0.5 g of dried SCB with different mesh sizes (20, 40 and 60 mesh) were autoclaved at 121°C for 20 min each four times, with two days break in between each autoclaving to ensure sterility. Autoclaved SCB was moistened with different volumes (4, 8 and 12 mL) of basal mineral salt medium (Shirling and Gottlieb, 1966) with different pH adjustments (5.5, 7.0 and 8.5) and inoculated with two mycelium containing agar plugs of each strain to be tested. The agar plugs were aseptically crushed and homogenized prior to inoculation. The cultures were incubated at 30°C in the dark under either agitated or stationary conditions for 21 days. A control experiment was performed where SCB samples were treated identically but without fungal inoculation. The fermentation flasks were sampled every 7 days by adding 5 mL of autoclaved Type I water to each flask, swirled vigorously to mix the contents and

withdrawing 5 mL of enzyme containing extract. The sample was centrifuged at 1942 x g for 5 min to remove the SCB. The liquid fraction was assayed for APPL and enzyme activity, while the solid SCB residues were oven-dried at 85°C to constant weight for the final determination of weight loss and change in chemical composition.

A confirmation experiment was performed using the optimum parameters identified from the fractionary factorial design experiment for biodelignification with the lowest xylanase activity in triplicate for each of the four strains. For this, 125-mL conical flasks containing 0.5 g of SCB were used. In addition, a scaled-up experiment was performed using the same cultivation conditions in 500-mL conical flasks containing 30.0 g of SCB.

3.3.4. Acid-precipitable polymeric lignin (APPL)

The released lignin intermediates were assayed as APPL. The APPL content in each sample was determined by acidifying a 0.19-mL portion of the aqueous fraction to pH 1 to 2 with 0.01 mL of 12 M HCl, and recording the optical density of the acidified sample at 600 nm (CRAWFORD; POMETTO; CRAWFORD, 1983). The results are expressed as APPL/mL of extract.

3.3.5. Ligninase enzyme assays

3.3.5.1. Lignin peroxidase

LiP activity (EC 1.11.1.14) was assayed by determining the oxidation of azure B dye (ARCHIBALD, 1992). One unit of enzyme activity has been expressed as an O.D. decrease of 0.1 units per minute per mL of the culture filtrate.

3.3.5.2. Laccase

Laccase (EC 1.10.3.2) activity was measured based on the oxidation of the substrate 5 mM 2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) at 420 nm with 0.1 M sodium acetate buffer (pH 5) at 30°C according Atalla et al., 2010 with modifications. The mixture was incubated for 5 min at 30°C and the absorbance was measured immediately in one-minute intervals. One unit of laccase activity (U) was defined as the activity of enzyme that catalyzes the conversion of 1 mole of ABTS ($\epsilon_{420} = 36 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) per min.

3.3.6. Hydrolytic enzyme assays

Xylanase (EC 3.2.1.8), endoglucanase (E.C. 3.2.1.4) and exoglucanase (E.C. 3.2.1.91) were assayed by measuring the formation of reducing sugar by the dinitrosalicylic acid (DNS) method (MILLER, 1959). The activity was quantified according to Dhillon et al. (2000) with modifications, as follow.

3.3.6.1. Xylanase

Xylanase assay was carried out with 50 mM, sodium phosphate buffer (pH 7.0) and 2% xylan. The xylanase was assayed at 30°C and after 30 min DNS reagent was added to stop the reaction. The solution was heated in a boiling water bath for 15 min and then cooled to ambient temperature. Absorbance was measured at 550 nm. One unit of xylanase activity (U) is defined as the amount of enzyme that released 1 μ mole reducing sugar equivalent to xylose per min under the above assay conditions.

3.3.6.2. Cellulase

Endoglucanase and exoglucanase assay were with 50 mM, sodium phosphate buffer (pH 7.0) and 2% CMC and 2% avicel respectively. The solution was heated in a boiling water bath for 15 min and then cooled to ambient temperature. For assay of avicelase activity, a centrifugation step was included to remove the microcrystalline cellulose (Avicel) before reading the absorbance at 550 nm. One unit of endoglucanase activity and avicelase activity (U) is defined as the amount of enzyme that released 1 μ mole reducing sugar equivalent to glucose per min under the above assay conditions.

3.3.7. Determination of Protein Content

Protein content of the crude and purified enzyme extracts were estimated following the method of Bradford (1976) using bovine serum albumin as standard. The results for enzyme activity were expressed per mg of protein (UA/mg of protein).

3.4. Results

The 2G ethanol-producing techniques usually employ a type of chemical or physico-chemical pre-treatment. Commercially purified enzymes can also be applied to lignocellulosic material in order to depolymerize and solubilize the carbohydrates for fermentation. Unlike the regular pretreatments, biological pre-treatment is an environmental friendly way to recover the sugars from the lignocellulosic biomass, besides it is cheaper

than the use of purified enzymes to degrade lignin. In addition, the long time required for the microorganisms to produce the enzymatic apparatus to degrade lignin is being overcome.

3.4.1. Acid-Precipitable Polymeric Lignin

APPLs are lignin fragments which have an increased number of free phenolic hydroxyl, α -carbonyl, and carboxylic acid groups as compared with native lignin. These intermediates are water-soluble and precipitate from aqueous solution upon acidification (CRAWFORD; POMETTO; CRAWFORD, 1983). The variation of APPL concentration during the 21-day time course experiment followed production of the ligninolytic enzymes which work synergistically; lignin consumption by the strains was characterized by the decrease in APPL concentration. The best experimental condition for APPL production for all the strains was after 14 days of incubation in dampened, static-grown cultures with a basic pH and the largest mesh size tested (20 mesh; run 3), ranging from 1.33 to 4.88 APPL/mL of filtrate. JLS6-A7 showed the best APPL (Figure 3-1b) production of all the strains (4.88 APPL/mL) followed by JLS-A12 (Figure 3-1c) and JLS-F6 (Figure 3-1d) (3.00 and 2.91 APPL/mL respectively) and JLS-A6 (Figure 3-1a) (1.33 APPL/mL).

Other researchers have been conducting their researches towards the short-term biodelignification. Mohanram et al. (2015) reported that the white rot fungi *Trametes hirsuta* MTCC136 and *Myrothecium roridum* LG7 showed satisfactory performance during rice straw delignification within a short incubation time of 7 days. Moreover, the depolymerized lignin was rich in APPL (MOHANRAM et al., 2015). The strains used in this study are not classified yet, but they showed the ability of producing APPL in a short time, as well. APPL is known as acid-precipitable polymeric lignin characterized by its a water-solubility and consists of a heterogeneous mixture of molecular weight components of >20,000 (CRAWFORD; POMETTO; CRAWFORD, 1983). APPLs were precipitated from culture filtrates after they had been acidified to pH 1 - 2. All tested strains in this work were able to depolymerize lignin, showing the potential of producing lignolytic enzymes when growing in SCB. In addition, the quantity of APPL released was dependent on the experimental condition (Figure 3 1). In this case, the lignolytic enzymes production was affected by the variables tested (agitation, SCB particle size, pH and solid:liquid ratio). There are different types of ligninolytic enzymes, so they may be influenced in different ways for different variables. In this study two lignolytic enzymes were tested: laccase and LiP.

Figure 3-1: APPL concentration after 7, 14 and 21 days of biological pretreatment of sugarcane bagasse by the strains (a) JLS6-A6, (b) JLS6-A7, (c) JLS6-A12 and (d) JLS6-F6.

Source: Personal file

3.4.2. Enzyme assays

Since the goal of the biodelignification process is lignin removal while preserving the fermentable sugars inherent in the lignocellulosic material for subsequent fermentation to ethanol, the lowest xylose consumption by the strains was evaluated using statistical tools. Considering that short incubation time would be preferred by industrial scale to save energy, space for storage and time, and since the strains used in this study were able to degrade xylan and metabolize xylose, a time-frame of 14 days was chosen to promote lignin degradation while conserving as much pentose sugar as possible in the SCB structure. Our results showed that xylanase activity decreased from day 7 to day 14 when the culture flasks were incubated under high agitation (100 rpm) conditions (Figure 3-2). While laccase and LiP were barely influenced by the tested variables, xylanase activity was clearly affected by the high level of agitation and solid:liquid ratio, where very low activity of this enzyme was observed for JSL6-A6, JSL6-A7, JSL6-A12 strains. Even though it was not tested in this study, nutritional parameters such as xylose and lactose may repress xylanase production (GESSESSE; MAMO, 1999).

The highest laccase activity observed was 0.52, 0.54, 0.43 and 0.49 U/mg of protein for JLS6-A6, JLS6-A7, JLS6-A12, JLS6-F6 respectively after 7-day incubation. Its production occurred under a mild agitation condition in a neutral to acid aqueous environment with the preference for the lower SCB mesh size. After this time, no laccase activity was detected. Laccases are phenol oxidases whose function is not dependent on the addition of H_2O_2 (EGGERT et al., 1996). Its production may be triggered by nitrogen depletion in the medium (JANUSZ et al., 2015) or induced by nitrogen and copper, while the oxygen supply has no effect on laccase activity levels (COLLINS; DOBSON, 1997). In this study, SCB supplemented with trace minerals and yeast extract were used to establish a suitable growth environment since, for example, copper is not an element of sugarcane composition. Although basal mineral salt medium contains a small amount of ammonium sulphate as a source of nitrogen, it does not seem to support laccase induction, due to the four strains showing low laccase activity. The maximum laccase activity was detected after 7 days incubation, which decreased during the following two weeks. Furthermore, the oxygen supplied through the agitation conditions used in this study did not trigger a significant induction of laccase activity either.

As was observed for laccase, LiP activity was very low during the time-course experiment. Maximum LiP activity was observed after 7 days of incubation. JLS6-A6 (0.33

U/mg of protein) and JLS6-A12 (0.31 U/mg of protein) showed maximum LiP activity with the same conditions as observed for maximum laccase production. JLS6-A12 and JLS6-F6 showed a maximum activity that was 55% lower than JLS6-A6 (0.18 U/mg of protein) at run 8 and run 5, respectively. The analyses of the data showed that laccase and LiP were barely influenced by the tested variables, with very low activity during the 21-days course of experiment. LiP isozymes are differentially regulated in response to environmental stress when fungi grow under nitrogen limited conditions (HOLZBAUR; TIEN, 1988; LI; ALIC; GOLD, 1994) or nitrogen sufficiency (MESTER; DE JONG; FIELD, 1995). Moreover, LiP production and regulation, in manganese ions (Mn^{2+}) deficient cultures is correlated to low levels of manganese containing superoxide dismutase. In the case of the white rot fungus *Phanerochaete chrysosporium*, despite the low activity level of this enzyme in Mn^{2+} -deficient cultures, the presence of H_2O_2 is mandatory for the expression of the *lip-H2* gene, which encodes for the major LIP isoenzyme produced (MATITYAHU et al., 2015). As it was observed for laccase, LiP activity was very low during the time-course experiment. The absence of LiP in the experiments suggests that sugarcane could negatively affect this activity. According to Mester, De Jong, and Field (1995), low LiP is detected in the presence of a high manganese concentration, and MnP is remarkably stimulated under this condition. On the other hand, MnP activity is very low in the absence of manganese, whereas LiP production is enhanced. In this context, the results suggest that MnP is likely to be the enzyme responsible for the high APPL levels and lignin degradation, since low laccase and LiP activities were observed.

Besides ligninolytic enzymes, hydrolytic enzymes may also be produced by microorganisms when growing in sugar-containing substrates. As long as sugars are the main energy source for the microorganism, xylanase and cellulase are usually synthesized in presence of hemicellulose and cellulose, respectively. In this study, no cellulolytic activity was detected during 21 days of incubation. In the same way, Singhal, Jaiswal, and Thakur (2015) reported that the production of cellulose degrading enzymes was negligible during the biopulping of bagasse by *Cryptococcus albidus*. Therefore, cellulose is not metabolized and glucose remains in the SCB after the biodelignification process; no reducing sugars were found in the extract. As long as cellulose is conserved, the strains might metabolize pentose sugars from hemicellulose as an energy source.

In this study, hemicellulase was produced simultaneously with ligninolytic enzymes by the four strains during the biodegradation of SCB. The strains were found to produce considerable amounts of xylanase during the 21-day time course experiment. In addition,

xylanase activity was higher than laccase and LiP activities. Xylanase production was affected by the experimental condition, reaching activities from 0 to 31.83 U/mg of protein for JLS6-A6 (Figure 3-2a), 0 to 42.94 U/mg of protein for JLS6-A7 (Figure 3-2b), 0 to 31.96 U/mg of protein for JLS6-A12 (Figure 3-2c) and 0 to 48.26 U/mg of protein JLS6-F6 (Figure 3-2d) in the first week, depending on the incubation condition. Dong et al. (2013) also reported hemicellulase production during the biodelignification of SCB by white rot fungi. The strains were found to produce considerable amounts of xylanase during the 21-day time course experiment. In addition, xylanase activity was higher than laccase and LiP activities, indicating that the strains were able to remove lignin and compromise the hemicellulose matrix. This is a very interesting result since production of this enzyme could be optimized and applied to the saccharification of the pre-treated SCB, decreasing the hydrolysis cost (GAO et al., 2011). Moreover, xylanase may open pores in the SCB structure, allowing cellulase permeation during the saccharification step.

Figure 3-2: Xylanase activity after 7, 14 and 21 days of biological pretreatment in of sugarcane bagasse by the strains (a) JLS6-A6, (b) JLS6-A7, (c) JLS6-A12 and (d) JLS6-F6.

Source: Personal file

Xylanase activity fluctuated during the experiment and might be gene regulated according to carbon source availability. Moreover, xylanase activity was clearly affected by the high level of agitation and solid:liquid ratio. This enzyme is generally synthesized by certain fungal species when using lignocellulosic biomass as substrate due to the presence of hemicellulose, which is rich in xylose. Archana and Satyanarayana (1997) reported the production of extracellular thermostable xylanase by *Bacillus licheniformis* A99 under solid-state fermentation using wheat bran moistened with tap water as substrate at 50°C. The peak of xylanase production was found in 72 h. In the same way, high xylanase production was observed by Gessesse and Mamo (1999) using wheat bran moistened (1:0.5 to 1:1.5) and supplemented with 10% (w/w) Na₂CO₃. It is an interesting approach for biopulping due to agroindustrial waste is low cost feedstock (Ho, 2015), but not for biodelignification. In this case, the sugars must be conducted to an ethanol production step, so that the ligninolytic enzymes must be the unique pool of enzymes extensively produced by the microorganisms. Besides the actual interest of this study in explore the potential of biodelignification of the strains, the production of an enzymatic extract are likely to be explored in further experiments.

3.4.3. Statistical analysis

Fractionary Factorial Design was applied to evaluate the influence of agitation (X₁), SCB mesh size (X₂), solid:liquid ratio (X₃) and medium pH (X₄) (Table 3-1). The ANOVA analysis of biodelignification by JLS6-A6 (Table 3-2), JLS6-A7 (Table 3-3), JLS6-A12 (Table 3-4), and JLS6-F6 (Table 3-5) shows a high correlation coefficient for all strains (R²=0.91, 0.91, 0.87 and 0.93, respectively). Therefore, equation (1), equation (2) and equation (3) are predictive of xylanase production in the investigated range of factors for JLS6-A6, JLS6-A12 and JLS6-F6 respectively, since the lack of fit was not significant (p<0.05). The correlation coefficient for JLS6-A7 was 0.91, however the statistical analysis of this strain showed the lack of fit significant (p>0.05) indicating that the model is not predictive for xylanase production.

The effects of xylanase activity and significative variables after 14 days of biodelignification by fungal isolates are shown on in Figure 3-3, Figure 3-4, Figure 3-5 and Figure 3-6. For JLS6-A6, only agitation and SCB size were significant (p<0.05) with a negative effect (Figure 3-3). Agitation was the most significant input variable while solid:liquid ratio, pH and the interaction between the variables was not significant. The best culture conditions to achieve the lowest xylanase activity for this strain therefore requires an

agitation of 100 rpm and SCB of 20 mesh size. Agitation was the only significant variable for JLS6-A12, with a negative effect; high agitation rates further lower xylanase activity (Figure 3-5). While the other variables were tested, the interaction between them was not significant. Thus, the important growth parameter for supporting the lowest xylanase activity of JLS6-A7 is agitation at high level (100 rpm) Figure 3-4. Finally, the only significant variable for JLS6-F6 was the SCB size with a negative effect, therefore, the higher the SCB mesh size, the lower the xylanase activity (Figure 3-6). The other variables and the interaction between the variables were not significant. Therefore, the best growth condition to achieve the lowest xylanase activity for JLS6-F6 includes agitation at 100 rpm.

Table 3-1 Fractionary design 2^{4-1} investigating effects of agitation (X_1), SCB mesh size (X_2), solid:liquid ratio (X_3) and medium pH (X_4) on xylanase activity after 14 days of biodelignification by fungal isolates JLS6-A6, JLS6-A7, JLS6-A12, JLS6-F6.

Run	X_1	X_2	X_3	X_4	Xylanase Activity (U/mg of protein)			
					JLS6-A6	JLS6-A7	JLS6-A12	JLS6-F6
1	-1	-1	-1	-1	21.79	19.65	18.59	17.61
2	+1	-1	-1	+1	6.99	0.76	3.90	12.32
3	-1	+1	-1	+1	12.64	2.50	11.37	0.00
4	+1	+1	-1	-1	0.07	8.11	10.32	1.92
5	-1	-1	+1	+1	22.97	26.51	33.00	18.31
6	+1	-1	+1	-1	0.02	0.84	0.42	1.48
7	-1	+1	+1	-1	6.29	25.30	23.14	9.67
8	+1	+1	+1	+1	0.25	2.34	0.21	0.24
9 (CP)	0	0	0	0	1.85	4.98	3.05	5.53
10 (CP)	0	0	0	0	5.04	5.88	3.17	8.29
11 (CP)	0	0	0	0	4.62	3.19	8.85	2.18

Variables		Levels		
		-1	0	+1
X_1	Agitation (rpm)	0	50	100
X_2	Sugarcane bagasse size (mesh)	60	40	20
X_3	Solid:liquid ratio	1:8 Dampened	1:16	1:24 Submerged
X_4	pH	5.5	7.0	8.5

Table 3-2: ANOVA for xylanase activity results after 14 days of biodelignification by JLS6-A6

	SS	df	MS	F	p
<i>Agitation</i>	397.06	1	397.06	132.17	0.01
<i>SCB size</i>	132.19	1	132.20	44.00	0.02
Solid:Liquid	17.88	1	17.88	5.95	0.14
pH	26.94	1	26.94	8.97	0.10
Agitation x SCB size	45.79	1	45.80	15.24	0.06
Agitation x Solid:Liquid	0.33	1	0.33	0.11	0.77
Agitation x pH	0.02	1	0.09	0.01	0.95
Lack of Fit	55.44	1	55.44	18.45	0.05
Pure Error	6.01	2	3.00		
Total SS	681.66	10			

SS: sum of squares; **df:** degrees of freedom; **MS:** medium square

$$y = 7.50 - 7.05X_1 - 4.07X_2 - 1.50X_3 + 1.83X_4 + 2.40X_1X_2 - 0.21X_1X_3 - 0.05X_1X_4 \quad (1)$$

Table 3-3: ANOVA for xylanase activity after 14 days of biodelignification by JLS6-A7.

	SS	df	MS	F	p
<i>Agitation</i>	479.16	1	479.16	255.56	0.01
SCB size	11.33	1	11.33	6.04	0.13
<i>Solid:Liquid</i>	71.81	1	71.81	38.30	0.03
<i>pH</i>	59.33	1	59.33	31.65	0.03
<i>Agitation x SCB size</i>	92.52	1	92.52	49.35	0.02
<i>Agitation x Solid:Liquid</i>	156.12	1	156.12	83.27	0.01
Agitation x pH	12.71	1	12.71	6.78	0.12
<i>Lack of Fit</i>	80.33	1	80.33	42.84	0.02
Pure Error	3.75	2	1.88		
Total SS	967.06	10			

SS: sum of squares; **df:** degrees of freedom; **MS:** medium square

Table 3-4: ANOVA for xylanase activity after 14 days of biodelignification by JLS6-A12

	SS	df	MS	F	p
<i>Agitation</i>	634.65	1	634.66	57.81	0.02
SCB size	14.75	1	14.75	1.34	0.37
Solid:Liquid	19.74	1	19.74	1.79	0.31
pH	1.99	1	1.99	0.18	0.71
Agitation x SCB size	67.78	1	67.78	6.17	0.13
<i>Agitation x Solid:Liquid</i>	197.80	1	197.80	18.08	0.05
Agitation x pH	10.75	1	10.75	0.98	0.43
Lack of Fit	125.99	1	125.98	11.48	0.08
Pure Error	21.96	2	10.98		
Total SS	1095.41	10			

SS: sum of squares; **df:** degrees of freedom; **MS:** medium square

$$y = 10.55 - 8.91X_1 + 1.36X_2 + 1.57X_3 - 0.50X_4 + 2.91X_1X_2 - 4.97X_1X_3 - 1.16X_1X_4 \quad (2)$$

Table 3-5: ANOVA for xylanase activity after 14 days of biodelignification by JLS6-F6.

	SS	df	MS	F	p
Agitation	109.76	1	109.76	11.73	0.08
SCB size	179.45	1	179.46	19.18	0.05
Solid:Liquid	0.58	1	0.58	0.06	0.83
pH	0.01	1	0.01	0.01	0.99
Agitation x SCB size	26.75	1	26.76	2.86	0.23
Agitation x Solid:Liquid	65.42	1	65.42	6.99	0.12
Agitation x pH	41.10	1	41.10	4.39	0.17
Lack of Fit	12.15	1	12.16	1.30	0.37
Pure Error	18.71	2	9.36		
Total SS	453.95	10			

SS: sum of squares; **df:** degrees of freedom; **MS:** medium square

$$y = 7.04 - 7.41X_1 - 9.47X_2 - 0.54X_3 + 0.05X_4 + 3.66X_1X_2 - 5.72X_1X_2 + 4.53X_1X_4 \quad (3)$$

Figure 3-3: Pareto chart showing the effect of the variables (a) and response surface showing the impact of the significant variables (b) on xylanase activity after 14 days of biodelignification by JLS6-A6.

Source: Personal file

Figure 3-4: Pareto chart showing the effect of the variables (a) and response surface showing the impact of the significant variables (b) on xylanase activity after 14 days of biodelignification by JLS6-A7.

Source: Personal file

Figure 3-5: Pareto chart showing the effect of the variables (a) and response surface showing the impact of the significant variables (b) on xylanase activity after 14 days of biodelignification by JLS6-A12.

Source: Personal file

Figure 3-6: Pareto chart showing the effect of the variables (a) and response surface showing the impact of the significant variables (b) on xylanase activity after 14 days of biodelignification by JLS6-F6.

Source: Personal file

3.4.4. Confirmation experiment

In order to save the maximum quantity of sugars in the SCB the confirmation experiment was conducted towards xylanase activity reduction. Dry weight measurements of SCB after biological pretreatment showed a slight increase ranging from 1.57% to 1.85% for the three strains subjected to confirmation experiment. On the other hand, the control showed a decrease of 0.76%. The weight increase of treated samples can be explained by the fungal growth on the SCB. The mycelia proliferation added mass to the overall weigh, and in this case, the mass balance was unfeasible. Regarding the weight decrease observed for the controls, it can be explained by the leaching of water soluble compounds including sugars from the SCB and by the reduction of the initial 10% humidity after drying the SCB at 85°C until constant weight. The low xylanase activity found after 14 days of confirmation experiment confirms the quality of the method, indicating that the predicted condition is reliable. Once no reducing sugars were found in the culture supernatant, the strains may be metabolizing the low quantity released by the low xylanase activity as energy source while consuming APPL. APPL results were higher than the control, indicating a release of 60% to 84% more APPL when the SCB was subjected to the pretreatment. This data suggests that the biodelignification process is indeed taking place but is due to enzyme activities that were not assayed as part of this study.

A unique cultivation condition was determined by the fractional factorial experiment to support the lowest xylanase activity for all strains except JLS6-A7 for which results showed none of the tested parameters were statistically significant. Therefore, the confirmation experiments were performed in triplicate with moistened SCB (solid:liquid 1:10) at pH 8.5 milled to a 20 mesh size and incubated at 30°C with agitation at 100 rpm for 14 days.

Dry weight measurements of SCB after biological pretreatment showed a slight increase of 1.85%, 1.79% and 1.57% for JLS6-A6, JLS6-A12 and JLS6-F6, respectively, while the control showed a decrease of 0.76% (Figure 3-7a). The weight increase of treated samples is due to fungal growth on the SCB, while the weight decrease observed for the controls is due to the leaching of water soluble compounds including sugars from the SCB and by the reduction of the initial 10% humidity after drying the SCB at 85°C until constant weight.

As expected, low xylanase activity was observed in the confirmation experiment using the physical and nutritional parameters identified by the fractionary factorial

experiment. Additionally, no significant amounts of reducing sugars were found in the culture supernatants of any of the strains tested. JLS6-A12 showed the highest xylanase activity (5.28 ± 0.32 U/mg of protein) followed by JLS6-F6 (5.24 ± 0.29 U/mg of protein) and JLS6-A6 (4.20 ± 0.21 U/mg of protein).

No significant LiP activity was observed during the 14-day experiment, only 0.01 U/mg of protein was observed for JLS6-A6, JLS6-A12 and JLS6-F6. The APPL concentration was similar among the three strains (Figure 3-7b). It was found 0.72 ± 0.01 APPL/mL for JLS6-A6, 0.79 ± 0.02 APPL/mL for JLS6-F6 and 0.83 ± 0.02 APPL/mL for JLS6-A12. In addition, the APPL results obtained from the supernatant of the cultures were higher when compared to the control (0.45 ± 0.01 APPL/mL).

Figure 3-7: Natural and biological pre-treated SCB weight (a) and APPL concentration (b) after 14 days of biological pretreatment in confirmation experiment.

Source: Personal file

3.4.5. Scale-up

The scale-up experiment was conducted under the same experimental conditions as the confirmation experiment. However, in contrast to the confirmation experiment, neither xylanase activity nor LiP activity was detected. Laccase activity (0.01 U/mg of protein) was detected for JLS6-A6 and JLS6-A12 during the scale-up experiment, while it was observed only for JLS6-A6 in the confirmation experiment, with similar results. Considerable amounts of reducing sugars were detected in the scale-up experiment (Figure 3-8a). The control showed higher levels of reducing sugars and APPL than the inoculated SCB, indicating that the strains are metabolizing the leached sugars and the APPL (Figure 3-8b). In addition, these results suggest the possibility of the genetic regulation of xylanase, laccase and LiP production.

Figure 3-8: Reducing sugars concentration (a) and APPL concentration (b) after 14 days of biological pretreatment in scale-up experiment.

Source: Personal file

As long as there is a readily available energy source for the microorganism, hydrolytic enzyme production is not necessary to obtain substrate carbon from the feedstock. Notably, the SCB dry weight analysis yielded the same profile that was observed between the confirmation experiment and the scale-up experiment, where all the biologically pretreated SCB showed a slight weight increase while the untreated control SCB showed a weight decrease (Figure 3-9).

Figure 3-9: Untreated and biological pre-treated SCB weight after 14 days of biological pretreatment in scale-up experiment.

Source: Personal file

3.5. Conclusion

This study showed an exploratory strategy regarding nutritional and physical parameters towards the synthesis of ligninolytic and hydrolytic enzymes by four novel fungi strains growing under solid-state fermentation. The results highlight the efficiency of the statistical tools regarding the obtaining of low hydrolytic activity, saving the sugar fraction for ethanol production and the potential of those strains for xylanase production.

4.6. Acknowledgements

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CHAPTER 4

4. Taguchi method-optimized fungal pretreatment increases the release of reducing sugars from sugarcane bagasse during enzymatic hydrolysis

Abstract

Environmental concerns have driven the development of fungal pretreatments as a green technique for second-generation ethanol production to minimize generation of waste, to save energy and to conserve water. A novel fungal strain, JLS6-A6, showed high manganese peroxidase (MnP) yield and high efficiency during the biodelignification of sugarcane bagasse (SCB) after a short incubation period. Different experimental conditions were investigated utilizing Taguchi L₈ orthogonal array. High levels of reducing sugars were released from SCB pretreated by JLS6-A6 after cellulase hydrolysis. The optimum saccharification condition was achieved at pH 8.5 with dampened biomass whereas SCB size and agitation were not significant variables. Compared to the control, simultaneous saccharification and fermentation (SSF) of unmilled pretreated SCB increased glucose release by 37.9% and yielded three times the ethanol production. These results confirm the potential of JLS6-A6 to be employed as a biological pretreatment for SCB.

2.7. Introduction

Nowadays, a major concern is the search for a substitute for fossil fuel. One such substitute is cellulosic ethanol, an environmentally friendly renewable energy source, which contributes to reducing CO₂ emissions and minimizes the harmful effects of global warming due to the release of greenhouse gases into the atmosphere. Ethanol, obtained from the fermentation of sugars from sugarcane juice, molasses or starchy materials such as corn, is a clean burning oxygenated biofuel referred to as first generation ethanol (1G). Agro-industrial waste may also be used as feedstock for bioethanol production due to its sugar composition, giving rise to second generation ethanol (2G). The integrated process of producing 1G and 2G from the same plant would be more profitable than from a 1G ethanol autonomous distillery. However, this is a prospect for the future since the cost of 2G production is affected by process operation, process conditions, operating costs and market prices. Besides, it requires pentose and hexose fermentation (MACRELLI; GALBE;

WALLBERG, 2014). Hexose fermentation is a well-known process in which the yeast *Saccharomyces cerevisiae* is employed to quickly convert glucose into ethanol during 1G production, especially in Brazil (BASSO et al., 2008). On the other hand, pentose fermentation is a very delicate process. Pentose-converting strains are usually sensitive to inhibitors and may be product-inhibited due to the presence of a high ethanol concentration during the fermentation (CHAUDHARY; GHOSH, 2014).

Brazil is the top sugarcane-producing country, with 653 million tons of sugarcane recovered in 2014 (<http://www.unicadata.com.br>). SCB has considerable potential as a feedstock for biofuels and other chemicals as it is composed of the lignocellulosic carbohydrates cellulose and hemicellulose. However, lignocellulosic biomass is difficult to manage due to the presence of lignin, a polyphenolic barrier that provides protection to the plant against microbial attack. Due to the recalcitrance of the plant cell wall to degradation, efficient enzymatic hydrolysis is restricted by substrate accessibility (ZENG et al., 2014).

Enzymatic hydrolysis of lignocellulosic biomass can be improved through chemical, physical, physico-chemical and biological pretreatment techniques that alter both its physical and chemical structure (AGBOR et al., 2011). Biological pretreatment consists of a solid-state fermentation process in which microorganisms grow on the lignocellulosic biomass by selectively degrading lignin. Under ideal biological pretreatment conditions, the cellulose and hemicellulose are expected to remain untouched by microbial action. Implementation of an efficient solid-state fermentation requires the optimization of nutrients, moisture content, aeration, pH, temperature, inoculum size and the strain of microorganism (ISROI et al., 2011).

The best-studied microbial delignification agents are white rot fungi as they selectively degrade lignin with marginal effect on carbohydrates. White rot fungi are amenable to simultaneous saccharification and fermentation (SSF) since their vegetative growth does not require aeration, is supported by minimal amounts of water, and low nutrient concentrations. These fungi degrade biomass by a complex mixture of cellulases, hemicellulases and ligninases, reflecting the complexity of the lignocellulosic substrate. Enzyme production by white rot fungi is dependent on substrate, incubation time, temperature and moisture. Lignin degradation is achieved through the action of oxidases and peroxidases that generate non-specific and highly reactive free radicals. The main enzymes involved in lignin degradation by fungi and bacteria are lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and laccase; the roles of which have been

reviewed by Chandel et al. (2013). MnP and LiP structures are very similar in that they are heme-containing glycoproteins which require hydrogen peroxide as an oxidant (POULOS et al., 1993; SUNDARAMOORTHY et al., 1994). Laccase, a copper-containing oxidase, utilizes molecular oxygen as oxidant to oxidize phenolic rings to phenoxyl radicals (BERTRAND et al., 2002).

Biological saccharification of sugarcane bagasse may be performed by separate hydrolysis and fermentation (SHF) or SSF. SSF uses either suspended or immobilized microorganisms (WIRAWAN et al., 2012) or free enzymes in the saccharification medium (SANTOS et al., 2012b). Operation costs can be reduced by recycling the enzymes or by recycling the solid fraction after enzymatic hydrolysis (VISSER et al., 2015). SSF is more advantageous than SHF in that it reduces processing time by combining hydrolysis and fermentation. Moreover, the liquid fuel and overall energy efficiency is higher when compared to SHF in a life cycle analysis (PETERSEN et al., 2015).

There are two main approaches for optimization of the fermentation process. The first involves multiple interaction experiments wherein one factor at a time is altered. The second involves varying several factors simultaneously and then determining potential interactions using statistical analysis, also known as design of experiments (DoE). Because DoE enables visualization of the interactions among experimental variables, it yields more information per experiment than the more traditional and time-consuming method of single variable manipulation. By identifying interactions among variables, one can predict the response data in areas not directly covered by experimentation. Exploratory studies and optimization of biological parameters towards the production of various bioproducts using DoE have taken place in the past few years, reducing costs and the number of experiments. Taguchi design utilizes two, three and mixed level fractionary factorial designs which ensures good performance in the design stage of products or processes. It proceeds with a selection of parameters and levels to be explored, investigation and analysis of the process response in terms of the selected outputs and tests the configurations under which the process is predicted to give optimal responses upon conducting new experiments (SANTANGELO et al., 2014).

The goals of this work were the biodelignification pretreatment using novel strains to overcome the recalcitrance of SCB for efficient recovery of fermentable sugars followed by the production of 2G by *Saccharomyces cerevisiae* in a SSF process. A scale-up of the best condition, as determined by application of the Taguchi method, was conducted to

evaluate the performance of the selected strain regarding enzyme production in the presence of higher amounts of feedstock.

4.2. Material

4.2.1. Sugarcane bagasse

Raw sugarcane bagasse samples were obtained from Fazenda Invernada, Bioserv, Unica (Vale do Rosario, São Paulo, Brazil), dried under sunlight until 10% humidity measured with an infra-red weighter (Top Ray – Bel Engineering) at 100°C. The dried biomass was milled and sieved through a 20 and 60 mesh and stored in a sealed container.

4.2.2. Microbial strains and inoculation preparation

Seven environmental isolates, six fungal strains JLS6-A6, JLS6-A7, JLS6-A12, JLS6-F6, JLS6-G4, JLS6-H9 and one bacterial strain JLS3-C7 (*Streptomyces*) were selected after a preliminary screen for biodelignification potential and were verified as lignin degraders. The strains were maintained on Potato Dextrose Agar (PDA) plates stored at 4°C. To prepare the inoculum for the SSF of SCB, mycelium agar plugs of 1 cm² were cut along the edge of the actively growing colonies, which had been cultivated on PDA plates for 4 days at 30°C.

Inoculum for the fermentation step of the SSF was prepared by transferring *S. cerevisiae* cells to a 150-mL conical flask containing 50 mL of cultivation medium consisting of (g/L): glucose, 30.0; peptone, 20.0 and yeast extract, 10.0. Cultures were grown at 30°C with agitation at 150 rpm for 24 hours. After that, cultures were centrifuged (2000 x g) for 15 minutes and the cell pellet was resuspended in sterile Type I water.

4.3. Methods

4.3.1. Screening of significant factors for SSF applying Taguchi Design

Taguchi L₈ design was employed to evaluate the important factors affecting SSF after SCB pretreatment by the strains JLS6-A6, JLS6-A7, JLS6-A12, JLS6-F6, JLS6-G4, JLS6-H9 and JLS3-C7. The experimental design is given in Table 1. Four physical and nutritional variables (agitation, SCB size, moisture level and pH) were selected to study the effect of all JLS6 strains.

4.3.2. Statistical analysis

Statistica (version 12) software was used for regression and graphical analysis of the data. The level of significance was 95%. The optimum concentrations or condition of each variable was obtained from the graphical analysis.

4.3.3. Biodelignification and solid state fermentation

As described in Table 1, 0.5 g of dried SCB of different mesh sizes (20 and 60) was added to 125-mL conical flasks, and autoclaved at 121°C for 20 min each four times, with two days break in between each autoclaving to ensure sterility. Autoclaved SCB was moistened with different volumes (4 and 12 mL) of Basal Mineral Salts medium (g/L): $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 0.0064 g; $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.0011 g; $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$, 0.0079 g; $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.0015 g; $(\text{NH}_4)_2\text{SO}_4$, 2.64 g; KH_2PO_4 , 2.38 g; K_2HPO_4 , 4.31 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 1.00 g; yeast extract, 3.00 g; with different pH adjustments (5.5 and 8.5) and inoculated with two mycelium-containing agar (PDA) plugs each prepared from the six fungal strains. The agar plugs were aseptically crushed and homogenized prior to inoculation. All the strains were incubated at 30°C in the dark under either agitated or static conditions for 21 days. A control experiment was performed where SCB samples were treated identically but without inoculation.

The fermentation flasks were sampled every 7 days for 21 days by adding 5 mL of autoclaved Type I water to each flask, swirling vigorously to mix the contents and withdrawing 5 mL of enzyme containing extract. The sample was centrifuged at 1942 x g for 5 min to remove the SCB. The liquid fraction was assayed for acid-precipitable polymeric lignin (APPL) and enzyme activity. The solid SCB residues obtained after 21 days were oven-dried at 85°C and subjected to the saccharification process to quantify reducing sugars released from the substrate.

A confirmation experiment using the best of the seven strains was performed in triplicate, using the optimum parameters identified from the Taguchi design for high glucose release during saccharification. For this, 125-mL conical flasks containing 0.5 g of milled SCB (20 mesh size) were used. In addition, a scale-up experiment was performed using the same cultivation conditions in 500-mL conical flasks containing 30.0 g of SCB and basal salts mineral medium. Unmilled SCB was subjected to the same experimental conditions for comparison.

4.3.4. Mycelia removal

After incubation, the mycelia were removed from samples according to Dong et al., (2013) with some modifications. The method consists of the immersion of 0.25 g of SCB residues in 5 mL of acetic acid: nitric acid (8:1, v/v) for 1 h and then washing with deionized water until the wash water was neutral. The mycelium-free SCB residues were dried at ambient temperature to avoid any additional structural changes, and then subjected to the saccharification process according to item 4.3.5 below.

4.3.5. Saccharification of pretreated sugarcane bagasse

For the saccharification, 0.06 g of dried pretreated SCB was placed into 50-mL screw capped tubes and 10 ml of 50 mM citrate buffer pH 4.8 was added. After “swelling” for 2 h, 30 μ L of diluted (1:10) cellulase from *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich) with 2.5 U mL^{-1} CMCase activity was added. The tubes were incubated at 30°C, 100 rpm for 24 h. The reducing sugar equivalents were determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Controls were submitted to the same procedures.

4.3.6. Simultaneous Saccharification and Fermentation (SSF)

SSF experiments were performed in 125-mL Erlenmeyer flasks with 55 ml of 50 mM citrate buffer pH 4.8. Biodelignified substrate was previously autoclaved for 15 min at 110°C. Sterile 0.05 M citrate buffer pH 4.8 and cellulase from *Trichoderma reesei* ATCC 26921 (Sigma-Aldrich) were added and SSF reaction mixtures were incubated at 30°C, 100 rpm. After 24 h the medium was supplemented with (g/L): yeast extract, 1.00; peptone, 1.00; NH_4Cl , 2.00; KH_2PO_4 , 1.00; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.30 and *Saccharomyces cerevisiae* cells (0.5 g/L) previously prepared (item 4.2.2) were added. The fermentation was carried out for 72 h using the same experimental conditions.

4.3.7. Assays

4.3.7.1. Acid-precipitable polymeric lignin (APPL)

The released lignin intermediates were assayed as APPL. The APPL content in each solid state fermentation sample was determined by acidifying a 190 μ L portion of the aqueous fraction to pH 1.0 to 2.0 with 10 μ L of 12 M HCl, and recording the optical density of the acidified sample at 600 nm (CRAWFORD; POMETTO; CRAWFORD, 1983). The results are expressed as APPL/mL of extract.

4.3.7.2. Reducing sugars

Reducing sugars liberated during solid-state fermentation cultivation, saccharification and SSF experiments were determined according to the dinitrosalicylic acid (DNS) method (MILLER, 1959). The results for reducing sugars were expressed as g/L of supernatant.

4.3.7.3. Enzyme assays

In each of the enzyme assays, enzyme-containing extract obtained from solid-state fermentation was used as the enzyme sample.

4.3.7.3.1. Ligninolytic enzymes

LiP activity (EC 1.11.1.14) was assayed by determining the oxidation of azure B dye (ARCHIBALD, 1992). The reaction mixture contained 32 μM Azure B and 100 μM H_2O_2 in 50 mM Na tartrate buffer (pH 4.5, 25°C). The dye and H_2O_2 were made up as 100x stock solutions. The reaction was initiated by adding hydrogen peroxide. One unit of enzyme activity has been expressed as an O.D. decrease of 0.1 units per minute per mL of the culture filtrate.

MnP activity was determined by monitoring the oxidation of 0.01% phenol red (oxidation product ($\epsilon_{610} = 2.2 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) at 610 nm in 20 mM H_2O_2 (Kuwahara et al., 1984). One unit of MnP activity (UA) has been expressed as an O.D. decrease of 0.1 units per minute per mL of the culture filtrate.

Mn-Ind oxidative activity was determined through the oxidization of Reactive Black 5 ($\epsilon_{598} = 3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) an azo dye that is not oxidized by Mn^{3+} or LiP. The assay was carried out with 0.10 mM Reactive Black 5 in 100 mM tartrate at pH 3.5 in presence of 0.10 mM H_2O_2 (PÉREZ-BOADA et al., 2005)). One unit of Mn-Ind oxidative activity (UA) has been expressed as an O.D. decrease of 0.1 units per minute per mL of the culture filtrate.

VP activity was monitored at 238 nm in regard to the formation of the Mn^{3+} -tartrate complex ($\epsilon_{238} = 6.5 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) from 0.10 mM MnSO_4 in 100 mM tartrate at pH 5.0 in the presence of 0.10 mM H_2O_2 (PÉREZ-BOADA et al., 2005)). One unit of VP activity (UA) has been expressed as an O.D. decrease of 0.1 units per minute per mL of the culture filtrate.

Laccase (EC 1.10.3.2) activity was measured based on the oxidation of the substrate 5 mM 2,2'-azino-bis (3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) at 420 nm with 0.1 M sodium acetate buffer (pH 5) at 30°C according (ATALLA et al., 2010 modified). The

mixture was incubated for 5 min at 30°C and the absorbance was measured immediately in one-minute intervals. One unit of laccase activity (UA) was defined as the activity of enzyme that catalyzes the conversion of 1 mole of ABTS ($\epsilon_{420} = 36 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) per minute.

4.3.7.3.2. Hydrolytic enzymes

Xylanase (EC 3.2.1.8) was assayed by measuring the formation of reducing sugars (xylose) by the DNS acid method (MILLER, 1959). The assay was carried out with 50 mM sodium phosphate buffer (pH 7.0) and 2% xylan. The xylanase was assayed at 30°C and after 30 min DNS reagent was added to stop the reaction. The solution was boiled in a water bath for 15 min and then cooled to ambient temperature. Absorbance was measured at 550 nm. One unit of xylanase activity (UA) is defined as the amount of enzyme that released 1 μmole reducing sugars equivalent to xylose per min under the above assay conditions (DHILLON et al., 2000 modified).

Endo-glucanase (E.C. 3.2.1.4) and exo-glucanase (E.C. 3.2.1.91) were assayed by measuring the formation of reducing sugars (glucose) by the DNS method (Miller, 1959) with 50 mM sodium phosphate buffer (pH 7.0) and 2% carboxymethylcellulose (CMC) and 2% avicel respectively. The solution was boiled in a water bath for 15 min and then cooled to ambient temperature. For the assay of avicelase activity, a centrifugation step was included to remove the microcrystalline cellulose (Avicel) before recording the absorbance at 550 nm. One unit of endo-glucanase activity and avicelase activity (UA) is defined as the amount of enzyme that released 1 μmole reducing sugars equivalent to glucose per min under the above assay conditions (DHILLON et al., 2000 modified).

4.3.7.4. Quantification of sugars, ethanol and organic acids

The concentration of sugars (D-xylose, D-glucose, L-arabinose) and ethanol was determined by High-Performance Liquid Chromatography (HPLC) equipped with refraction index detection (Waters 410; Milford, MA, USA), column BIO-RAD AMINEX HPX-87H (7.8 x 300 mm) (Bio-Rad, Hercules, CA, USA) at 45°C. As eluent, 0.005 M sulfuric acid was used with 0.6 mL/min flow rate. Samples were previously diluted with Type I water (1:10) and filtered by *Sep-Pak* C18 filters prior to injection into HPLC (20 μL).

4.3.8. Calculations

Sugars (xylose, glucose and arabinoses) and acetic acid consumption (%) during fermentation were determined according to equation 1, where X is the consumption (%), X_i ,

initial sugar/acetic acid concentration (g/L) and X_f , final sugar/acetic acid concentration (g/L).

$$X = \left(\frac{X_f - X_i}{X_i} \right) \cdot 100 \quad (1)$$

Conversion of xylose, as a substrate, into ethanol, as a product ($Y_{P/S}$), was determined according to equation 2, where P_f is the final concentration of ethanol (g/L), P_i , initial concentration of ethanol (g/L), S_f , final concentration of substrate (g/L), S_i , initial concentration of substrate (g/L).

$$Y_{P/S} = \frac{\Delta P}{\Delta S} = \frac{P_f - P_i}{S_f - S_i} \quad (2)$$

Volumetric productivity (QP) (g/L.h) of ethanol was calculated according to equation 3, where P_f is the final concentration of ethanol (g/L), P_i , initial concentration of ethanol (g/L) and t , time of fermentation (h).

$$Q_P = \frac{P_f - P_i}{t} \quad (3)$$

4.1 Results and Discussion

Microbial lignin removal of various feedstocks has been the subject of study by several research groups. The microbial enzyme production profile depends on the feedstock composition, the strain, the incubation time and the growth medium (ZHAO; DE KOKER; JANSE, 1996). The strains used for biodelignification were selected according their ability to degrade lignin in a preliminary screen (data not shown). As expected, laccase, LiP and MnP activities were detected during the time course experiment. MnP activity was assayed for the confirmation experiment only and showed high activity whereas laccase and LiP activities were very low during both optimization and confirmation experiments, ranging from 0.03 to 0.11 UA/mL and 0 to 0.09 UA/mL, respectively (for all experiments performed with all strains).

Similar results are reported in the literature, as those obtained by Dias et al. (2010), who evaluated the performance of the white rot fungi Euc-1 and *Irpex lacteus*, on corn stover lignin degradation. The strain Euc-1 had a maximum production of laccase of 0.10 U/mL after 46 days of incubation time and LiP activity peak of 0.08 U/mL after 35 days. No laccase activity was detected in the case of *I. lacteus*, but the maximum LiP activity (0.06 U/mL) was detected after 23 days. Dong et al. (2013) evaluated the biodelignification performance of *Pleurotus ostreatus*, *Phanerochaete chrysosporium* PC2 and *Lentinula edodes* in biodelignification of SCB. *P. ostreatus* is a white rot basidiomycete and well-known laccase isoenzyme producer (PALMIERI et al., 2000). As expected, laccase activity for *P. ostreatus*

PO45 was very high when compared to the other strains, and a peak of 35 U/mL and 40 U/mL for laccase production was found on week 6 and week 9, respectively. However, no significant amount of laccase was found for *P. chrysosporium* PC2 and *L. edodes* after 12 weeks. Arora et al. (2002) did not find laccase activity for *P. chrysosporium* after 32 days of incubation using corn stover as feedstock. In addition, low laccase activity was detected for *Daedalea flavida* (0.45 U/mL after 16 days), *Dichomitus squalens* (0.15 U/mL after 8 days) after 32 days of incubation with corn stover as feedstock. On the other hand, Adhi et al. (1989) found LiP production during corn lignocellulose degradation in agitated cultures by streptomycetes, where *Streptomyces viridosporus* T7A reached the maximum peroxidase activity (0.11 U/mL) in the stationary-growth phase, 5 days earlier than *Streptomyces badius* 252 (0.35 U/mL).

No significant cellulolytic activity was detected during the time-course experiment for any of the strains tested, meaning the glucose polymerized as cellulose was conserved. This is an essential feature for a biodelignification agent to be useful for ethanol production. This result is in agreement with Ma and Ruan (2015), who detected low activity levels for hydrolytic enzymes such as CMCase and xylanase during mycelial growth of the white-rot fungus *Coprinus comatus* on corn stover. However, xylanase activity was found simultaneously with ligninolytic enzymes by the seven strains during the biodegradation of SCB, showing higher activity than laccase and LiP. Dong et al. (2013) and Adhi et al. (1989) also reported production of hydrolytic enzymes during the biodelignification of SCB by white rot fungi and corn by *Streptomyces*, respectively. According to Adhi et al. (1989) the peak activities for CMCase and xylanase were higher for *S. viridosporus* T7A (0.09 U/mL and 0.30 U/mL) when compared to *S. badius* 252 (0.04 U/mL and 0.06 U/mL) during 14 days of experiment with high agitation (200 rpm) and aeration (0.5 liter/min).

Xylanase production was hardly affected by the experimental condition or the time. Activities ranged from 0 to 11 U/mL for JLS6-A6, JLS6-A7, JLS6-A12, JLS6-F6 and 0 to 13 U/mL for JLS6-G4 and JLS6-H9 strains during the time-course experiment, depending on the incubation condition. The fluctuation of the xylanase activity suggests that this enzyme may be gene regulated according to the need for carbon and energy. JLS3-C7 strain did not show significant xylanase activity. Analysis of uninoculated, sterile controls showed low reducing sugars levels indicating that sugars are being leached from the biomass under the experimental conditions, as these controls remained sterile during the time-course

experiment. Reducing sugars levels in the solid state fermentation were equal to or lower than the sterile controls even in the situations where high xylanase activity was observed.

4.4.1. Biological pretreatment effect on reducing sugars released during the saccharification process

Samples of each of the seven biologically treated SCB and untreated SCB was subjected to saccharification in order to assess the yield of reducing sugars released after 24 h. Reducing sugars were detected in concentrations similar to or lower than the control for all strains, except JLS6-A6 which showed higher concentration of reducing sugars when compared to the sterile control. This results shows that among the seven strains tested, JLS6-A6 produced a greater amount of enzymes in a shorter time enhancing the saccharification by opening the pores in the SCB structure under the experimental conditions. In this case, high moisture level (S), high SCB size (20 mesh size), agitation (100 rpm) and pH 8.5 (run 1) (Table 4-1) was the best condition for pretreatment which is reflected by 51% (0.24 g/L) more reducing sugars release when compared to the control. On the other hand, reducing sugars released in run 5 (dampened, 20 mesh size, 100 rpm and pH 5.5) and 8 (dampened, 60 mesh size, static and pH 5.5) was lower in comparison to the control and the other experimental conditions (Table 4 1). Since JLS6-A6 strain was shown to be the best strain for releasing sugars from the SCB, the SSF was optimized for this strain, as described below.

Table 4-1: Orthogonal array Taguchi L₈ experimental design. Factors used for biological pretreatment of SCB by JLS6-A6.

Run	A	B	AxB	C	BxC	AxC	D	RS* (g/L)
1	2	2	1	2	1	2	2	0.23
2	2	2	1	1	2	1	1	0.19
3	2	1	2	2	1	1	1	0.19
4	2	1	2	1	2	2	2	0.20
5	1	2	2	2	2	2	1	0.16
6	1	2	2	1	1	1	2	0.19
7	1	1	1	2	2	1	2	0.21
8	1	1	1	1	1	2	1	0.17
Factors				Levels				
				1	2			
A	Moisture level			Dampened		Submerged		
B	SCB mesh size			60		20		
C	Agitation (rpm)			0		100		
D	pH			5.5		8.5		

* RS released after 24 h of saccharification

4.4.2. Optimization of saccharification parameters involving the biologically pretreated SCB by Taguchi method

4.4.2.1. Statistical optimization using Taguchi Orthogonal Array Design

The optimization of the fermentation process is possible when the microbe shows a positive performance by producing high amounts of the product of interest. All the variables involved in the fermentation process may be evaluated in order to increase the performance of the strain (THIRY; CINGOLANI, 2002). In this study, the Taguchi experimental design matrix, a standard orthogonal array L₈, was used to examine the impact of four factors, namely, moisture level, SCB size, agitation and pH in two levels (Table 4-1) on the saccharification of the biologically pretreated sugarcane bagasse by JLS6-A6. The lower and upper levels of optimized factors were selected based on preliminary experiments (data not shown), which showed the suitable conditions for fungal growth on SCB. Each of the eight-saccharification experiments denoted by “runs” was carried out as described in Table 4-1 where the concentration (g/L) of the released reducing sugars after 24 h of saccharification is also shown.

The analysis of variance (ANOVA) was applied to determine which factors and interaction between factors were statistically significant (Table 4-2). The controlling factors were identified, with the magnitude of the effects qualified and the statistically significant effects determined. Accordingly, the optimal conditions were determined by combining the levels of factors that had the greatest effect value. The factors in the experimental design considered to be statistically significant at a 95% confidence limit were used to determine the ratio (*F*) and the *p*-value ($p < 0.05$). The pH was the most significant variable with 89% of confidence, followed by the moisture level with 85% and the interaction between the moisture level and SCB size. The variables SCB size, agitation and

the other interactions were not significant. These results are illustrated in the Figure 4-1. Pretreated SCB with a high moisture level and at pH 8.5 was the best condition for biodelignification prior to the saccharification process as a higher quantity of reducing sugars was released under these conditions compared to delignification under dampened conditions at pH 5.5. The optimized delignification condition established in this work is in contrast with the moisture level and pH conditions described in the literature. According to Moreno et al. (2014), solid state fermentations are usually performed at a moisture content between 45 and 85% with an inoculum level of 1-10 mg/g substrate (dry weight), at pH 4-5 and temperatures between 15 and 40°C for 1-12 weeks, depending on the white rot fungi

strain used. This underscores the importance of optimizing solid state fermentations specifically for the biodelignification agent and the feedstock used.

Table 4-2: ANOVA for effects of factors after saccharification of SCB biodelignification by JLS6-A6.

Effect	Sum of Squares	Degree of Freedom	Mean of Squares	F	p value
Moisture level (A)	0.01	1	0.01	17.50	0.15
SCB mesh size (B)	0	1	0	0.01	0.94
Interaction (A) x (B)	0.01	1	0.01	9.81	0.20
Agitation (C)	0.01	1	0.01	2.77	0.34
Interaction (B) x (C)	0.01	1	0.01	2.16	0.38
pH (D)	0.01	1	0.01	31.38	0.11
Residual	0.01	1	0.01		

Figure 4-1: The effect of variables on the saccharification of the biologically pretreated SCB bagasse by JLS6-A6.

Source: Personal file

4.4.2.2. Confirmation Experiment and Scale-up

The confirmation experiment was carried out in triplicate according to the optimized condition and then scaled-up. Samples for the confirmation experiment were taken every 7 days and for the scale-up experiment, after 21 days. Since it had been determined that neither

the mesh size of the SCB nor the agitation were significant, the conditions of the experiment were set with a SCB at 20 mesh size and cultivation under static conditions. A parallel experiment was carried out using unmilled SCB under the same optimized experimental conditions, to evaluate the performance of the strains regarding the enzyme production on the whole SCB fiber. The choice for the static solid-state fermentation is due to the energy saving which would reduce the overall cost of the process.

Very low and similar activity for xylanase was detected after 7 days (0.55 ± 0.03 UA/mL and 0.38 ± 0.05 UA/mL) of solid state fermentation when 20 mesh size SCB and unmilled SCB was used as substrate, respectively (Figure 4-2a). Higher xylanase activity was observed after 14 days (1.95 ± 0.03 UA/mL and 2.34 ± 0.01 UA/mL) and 21 days (2.14 ± 0.18 UA/mL and 2.18 ± 0.17 UA/mL) for 20 mesh size and unmilled SCB, respectively. The scale-up experiment showed very low xylanase activity for 20 mesh size (0.44 UA/mL) after 21 days of incubation, as compared to the confirmation experiment. However, no xylanase activity was observed when unmilled SCB was the substrate for scale-up.

Figure 4-2: Xylanase activity and reducing sugars concentration during the confirmation experiment of biological pretreatment by JLS6-A6 for milled and unmilled SCB.

Source: Personal file

Due to the ability of JLS6-A6 to produce xylanase, the reducing sugars content of the solid-state fermentation supernatant was tested to evaluate the available levels of xylose and others monomeric reducing sugars (Figure 4-2b). A low concentration of reducing sugars was observed during the experimentation period for both the 20 mesh size and unmilled SCB (less than 0.17 g/L). Reducing sugars levels in the inoculated flasks were similar to the uninoculated SCB control and for the 20 mesh size SCB during the 21 days of experimentation. The reducing sugars concentration increased 41% and 29% for 20 mesh size and unmilled SCB respectively, whereas APPL increased 10 times for 20 mesh size and no increase was observed for unmilled SCB during the time course experiment in the uninoculated controls. These results indicate that reducing sugars and APPL were being leached from the SCB, except for unmilled SCB, which showed only reducing sugars leaching. The uninoculated SCB showed evidence of leaching during the first two weeks with the leached reducing sugars concentration remaining constant. The lack of relationship between the xylanase activity and the reducing sugars released from the SCB can be explained by the consumption of these sugars by the fungal strain as an energy source during the growing phase.

Neither Mn-Ind or VP activities were detected over the course of the confirmation experiment. The levels of laccase and LiP activities that were observed during the time-course confirmation experiment and scale-up experiment were not significant. However, in the confirmation experiment MnP activity was observed after 7 days (0.05 ± 0.01 UA/mL and 0.38 ± 0.01 UA/mL) and 21 days (0.16 ± 0.03 UA/mL and 0.30 ± 0.04 UA/mL) for 20 mesh size and unmilled SCB respectively, where higher activity was found for unmilled SCB (Figure 4-6). For the scale-up experiment MnP was the most active enzyme at 3.96 UA/mL and 0.78 UA/mL for 20 mesh size and unmilled SCB respectively. According to Adhi et al. (1989), the drop in the activities of peroxidase, cellulase and xylanase is followed by production of APPL. APPL from the inoculated flasks was observed to be at higher levels than the sterile control, suggesting that MnP is oxidizing the phenolic compounds and releasing the detected APPL. The fluctuation in APPL levels suggests that the strain is metabolizing the small lignin compounds in parallel with reducing sugars. This situation contrasts to the APPL levels observed in the scale-up experiment, which were similar to the control, meaning that all the APPL was being metabolized by the strain during the scale-up experiment, as was the reducing sugars.

Figure 4-3: MnP activity during the confirmation experiment of biological pretreatment by JLS6-A6 for milled and unmilled SCB.

4.4.3. Simultaneous Saccharification and Fermentation

Milled and unmilled SCB was subjected to SSF after the biological pretreatment by JLS6-A6 for both the confirmation experiment for 7, 14 and 21 days and the scale-up experiment for 21 days. Reducing sugars release and consumption and ethanol production were investigated.

4.4.3.1. Confirmation experiment

Three different controls were used to evaluate the reducing sugars yield from pretreated SCB during the confirmation experiment. In order to evaluate the efficiency of the JLS6-A6 pretreatment, the first control corresponds to un-pretreated SCB subjected to SSF (C1). The second represents un-pretreated SCB (C2) in order to analyze the amount of leached sugars. Finally, the third control was the un-pretreated SCB not subjected to saccharification to evaluate *S. cerevisiae* performance during the fermentation of the leached sugars (C3).

After 24 h of saccharification, 47%, 81% and 48% of reducing sugars was released from 20 mesh size SCB pretreated for 7, 14 and 21 days, respectively (Figure 4-4). Lower amounts of reducing sugars were found for unmilled SCB for the same period (7%, 36% and 30%) (Figure 4-5), suggesting that the cellulose was less accessible and that the pretreatment time was not sufficient in this case to enhance access to this polymer. No significant amounts of xylose, arabinose or cellobiose were detected for 20 mesh size nor unmilled SCB. Glucose was completely consumed and low amounts of acetic acid present in SSF medium was metabolized after 24 h of fermentation by *S. cerevisiae*.

Figure 4-4: Reducing sugars concentration during simultaneous saccharification and fermentation of biological pretreated milled SCB by JLS6-A6 after 7 (a), 14 (b) and 21 (c) days and controls.

SCB: sugarcane bagasse; **C1:** un-pretreated SCB subjected to SSF; **C2:** un-pretreated SCB; **C3:** un-pretreated SCB not subjected to saccharification.

No changes in reducing sugars concentration was observed in C2, confirming that cellulase is the key to the process at this point. This result was confirmed by C3 and, as expected, no reducing sugars was metabolized by the yeast. The reducing sugars yield after saccharification of pretreated SCB was up to two times higher than the reducing sugars found

for C1, emphasizing the efficiency of the biological pretreatment. As long as the *S. cerevisiae* glucose conversion into ethanol is very quick, this yeast metabolizes ethanol as an energy source for biomass growth; no ethanol was detected.

Figure 4-5: Reducing sugars concentration during simultaneous saccharification and fermentation of biological pretreated unmilled SCB by JLS6-A6 after 7 (a), 14 (b) and 21 (c) days and controls.

SCB: sugarcane bagasse; **C1:** un-pretreated SCB subjected to SSF; **C2:** un-pretreated SCB; **C3:** un-pretreated SCB not subjected to saccharification.

4.4.3.2. Scale-up experiment

The ethanol production observed in the scale-up experiment was considerable, where the $Y_{P/S}$ was 2.7 fold higher for the pretreated unmilled SCB ($Y_{P/S} = 0.19\text{g/g}$) compared to the un-pretreated unmilled SCB ($Y_{P/S} = 0.07\text{g/g}$). On the other hand, the $Y_{P/S}$ for 20 mesh size pretreated SCB ($Y_{P/S} = 0.45\text{g/g}$) was 2.14 fold higher than unmilled pretreated SCB and 1.29 times higher than 20 mesh size un-pretreated SCB. These results shows that even though lower amount of ligninolytic enzymes were produced during the biodelignification process, the milled SCB is more feasible to the SSF. This fact can be explained by the increasing of the surface area after the milling process.

Higher concentration of acetic acid ($0.29\text{g/L} \pm 0.06$) was found in 20 mesh size SCB saccharification medium when compared to unmilled SCB ($0.04\text{g/L} \pm 0.01$). No significant amounts of xylose and cellobiose were released during the saccharification for both 20 mesh size and unmilled pretreated and un-treated SCB. All released glucose was converted into ethanol after 24 h and the ethanol concentration was constant during the 72 h of fermentation and not metabolized by *S. cerevisiae*, most probably due to the constant glucose release by the cellulase enzyme or the consumption of the acetic acid during the fermentation. As expected, cellobiose and xylose were not metabolized by the strain.

4.4.4. Acid wash of sugarcane bagasse for mycelia removal

Samples of JLS6-A6 strain biologically pretreated SCB that was obtained according to Taguchi design (Table 4-1) were washed with a mix of acetic acid and nitric acid as a way of removing the fungal mycelia as described by Dong et al. (2013); the results are shown in Table 4-3 and Figure 4-6. There was a considerable difference in the reducing sugars release between the pretreated acid washed SCB and the pretreated but not acid washed SCB. While the not acid washed SCB released 0.19 g/L to 0.23 g/L reducing sugars after the saccharification, the acid washed SCB reducing sugars release ranged from 0.36 g/L to 0.67 g/L, suggesting that the acid used during the washing step is changing the SCB structure.

According the studies reviewed by Alvira et al. (2010), an acid pretreatment can solubilize the hemicellulosic fraction of the biomass and make the cellulose more accessible to enzymes; the most widely used and tested approaches are based on diluted sulfuric acid. However, nitric acid and acetic acid are known to effect vegetable biomass, causing structural changes under severe conditions. Hydrolysis of lignocellulosic material using diluted nitric acid was studied by Brink (1996) in a two-step reaction. The biomass was

subjected to a first stage hydrolysis under 140°C to 220°C at a pH of 2.0 to 3.0 to cause hydrolysis of the hemicelluloses and a second stage hydrolysis at 160°C to 240°C. It was found that lignin did not solubilize in the acid during this process. Tan et al. (2010) showed that a mixture of acetic acid and peroxide under high temperature (60-80°C) might be used as a hydrolytic agent to break down the lignin from the vegetable biomass with low amounts of hemicellulose removal. In the context of the present study, nitric acid and acetic acid contribute to the structural changes of the vegetal biomass even under the mild conditions used and are therefore not indicated to remove the mycelia from the biologically pretreated SCB as was suggested by Dong et al (2013).

Table 4-3: Reducing sugars release after the saccharification of the un-washed and acid washed biological pretreated SCB by JLS6-A6.

Run	Reducing sugars release (g/L)					
	Unwashed SCB			Acid washed SCB		
	Cellulase ^a	Control ^b	% Release ^c	Cellulase ^a	Control ^b	% Release ^c
1	0.23	0.15	53.3	0.45	0.17	165.0
2	0.19	0.16	18.8	0.47	0.17	176.5
3	0.19	0.15	26.7	0.42	0.17	147.1
4	0.20	0.15	33.3	0.63	0.19	231.6
5	0.17	0.18	0	0.36	0.18	100.0
6	0.19	0.15	26.7	0.59	0.20	195.03
7	0.21	0.15	40.0	0.67	0.20	135.0
8	0.17	0.18	0	0.66	0.19	247.4

^a Biological pretreated SCB + 2.5 U ml⁻¹ CMCase activity; ^b Biological pretreated SCB with no cellulase addition; ^c Percentage of reducing sugars released in relation to the control; ^d Relationship between reducing sugars released during saccharification of un-washed and washed SCB.

Milled (60 and 20 mesh size) and unmilled natural SCB were subjected to the saccharification process before the acid wash and after 1 h of acid wash. The results were compared against those of the biological pretreatment. Similar reducing sugars concentrations were observed after the saccharification of the unmilled natural SCB bagasse and the 60 and 20 mesh size, suggesting that the physical treatment by itself does not affect the enzymatic hydrolysis. Moreover, the reducing sugars concentration obtained from the enzyme-treated samples is also similar to the control, meaning that the cellulase enzyme was not able to reach the cellulose polymer in the natural SCB thus the reducing sugars detected was leached from the fibers. However, the reducing sugars release during the saccharification process of the treated SCB was higher than the natural SCB, indicating that JLS6-A6 had an important role towards the cellulose exposure in the short-time course pretreatment.

Figure 4-6: Reducing sugars released after 24 h of saccharification (blue) of: pretreated SCB by JLS6-A6 according to Taguchi design (a), pretreated SCB by JLS6-A6 according to Taguchi design after acid wash to remove fungal mycelia (b) natural milled and unmilled SCB (c) and natural milled and unmilled SCB after acid wash (d). The control (red) is related to the SCB without enzyme addition.

Source: Personal file

4.5. Conclusion

The new fungal strain JLS6-A6 showed high potential of synthesizing the enzyme MnP and efficiency in short-time lignin removal from sugarcane bagasse. The saccharification of acid-washing biodelignified SCB removed efficiently the fungal mycelia, increasing the ethanol yield after saccharification. However, the acid-washing process must be explored further. Therefore, this study is a promising alternative of green second generation bioethanol production, overcoming the lagging period of pretreatment by microbial strains.

4.6. Acknowledgements

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CHAPTER 5

5. Acetic acid removal from hemicellulosic hydrolyzate by the yeast *Issatchenkia occidentalis* Y1'a is increased in absence of glucose

Abstract

Second-generation (2G) ethanol is an alternative to increase the world ethanol production utilizing agro-industrial residue crops and minimizing the use of fossil fuels. Fossil fuels burn releases gases i.e. carbon dioxide (CO₂), which contributes with the greenhouse effect and consequently global warming increases. A pretreatment step to expose the sugars is mandatory to produce 2G ethanol. This process must be low cost and high efficient. In spite of diluted-acid pretreatment corresponds to this prerequisites, metabolism inhibitors are generated during the process due to the severity necessary to disrupt the plant cell walls. In this study, we report that the yeast *I. occidentalis* Y1'a was able to grow in sugarcane bagasse (SCB) hemicellulosic hydrolyzate with no supplementation, using acetic acid as energy source. In the presence of glucose, the acetic acid removal was delayed. As long as acetic acid is considered an inhibitor of the metabolism of microorganisms during the ethanol production, this find characterizes an efficient and low cost hemicellulosic hydrolyzate biodegradation. The fermentation step was successful with an ethanol yield ($Y_{P/S}$) of 0.26 g/g and volumetric ethanol productivity (Q_p) of 0.14 g/L.h indicating that the diluted-acid pretreatment followed by biodegradation with *I. occidentalis* Y1'a and pentoses fermentation by *Candida shehatae* UFMG 52.2 is a promising way of producing 2G bioethanol.

5.1. Introduction

New transportation fuels are strictly necessary to reduce the fossil fuels usage and consequently the necessity of petrol. The burn of based-on petrol fuels releases high amounts of gases, mainly CO₂, that contribute with greenhouse effect and climate changes. Fuels obtained from plants are known as biofuels. They are green fuels and their carbon cycle is considered closed, once the CO₂ released during their burn is reabsorbed by the plants during the photosynthesis process. The 2G biofuels are obtained from sugars extracted from lignocellulosic biomass, such as agricultural, urban and industrial residues, which

composition is based on cellulose, hemicellulose and lignin (PRASAD; SINGH; JOSHI, 2007). As long as cellulose is a polymer of glucose and hemicellulose is composed by different types of sugars, those residues can be used as source of fermentable sugars. However, the extraction of sugars present in lignocellulosic biomass requires previous stages of pretreatment to break the lignocellulosic structure down. Several physical, physico-chemical and chemical pretreatments methods have been reviewed in the literature (AGBOR et al., 2011; ALVIRA et al., 2010; HENDRIKS; ZEEMAN, 2009; KUMAR et al., 2009a, 2009b; MOSIER et al., 2005). Among them, sulfuric diluted-acid pretreatment, a chemical pretreatment, has been shown to be one of the promising technologies to disrupt the lignocellulosic biomasses, once it yields high recovery of hemicellulosic sugars (80-90%). Moreover, a little portion of lignin is dissolved during the pretreatment, increasing cellulose susceptibility to enzymes during the saccharification step (MARTIN et al., 2007; YANG; WYMAN, 2004)

Two fractions are generated after the diluted-acid pretreatment, a solid fraction and a liquid fraction. The solid fraction is mainly composed by cellulose and lignin (cellulignin). Small amount of hemicellulose can be found whether the acid hydrolyses was not completely effective (SUN; CHENG, 2005). The liquid fraction corresponds to the hemicellulosic hydrolyzate, which is rich in pentoses sugars, such as xylose, arabinose, mannose, etc that were released from hemicellulose fraction. In addition to pentose sugars, the hemicellulosic hydrolyzate also contains small amount of other glucose, weak acids, furanic compounds and phenolic compounds (MARTIN et al., 2007; SUN; CHENG, 2005). Depending on the concentration, weak acids, furanic compounds and phenolic compounds are toxic to fermenting microorganisms, inhibiting their metabolism (CANILHA et al., 2011; LARSSON et al., 1999), so that they must be removed from the hemicellulosic hydrolyzate prior the fermentation step. In this case, a detoxification step is essential to overcome the metabolism of sugars by the fermenting microorganism (FONSECA et al., 2011, 2013; YU et al., 2011).

The furan aldehydes 5-hydroxymethylfurfural (HMF) and furfural originate from the degradation of pentose sugars and hexoses, respectively. As those molecules are highly reactive they are capable of contributing to the formation of reactive oxygen species, causing damage to proteins, nucleic acids and cell organelles (SANCHEZ; BAUTISTA, 1988) consequently there is a reduction in enzymatic and biological activities of yeasts, DNA breaks and inhibition of protein synthesis and RNA (MODIG; LIDÉN; TAHERZADEH, 2002).

Weak acids such as acetic acid, levulinic acid and formic acid. Acetic acid is released upon the hydrolysis of acetyl groups from hemicellulose, while levulinic and formic acids are degradation products of the solubilized sugars from polysaccharides subjected to thermochemical acid catalysis. Formic acid is a degradation product of both furfural and HMF, whilst levulinic acid is formed by the degradation of HMF (ULBRICHT; NORTHUP; THOMAS, 1984). The toxic effects of weak acids are related to the pK value. At pH values below the weak acid's pKa value, these acids are found mainly in its undissociated form. In this case, acids are soluble and can diffuse through the plasma membrane into the interior of the microbial cell (PAMPULHA; LOUREIRO-DIAS, 1989). Due to the neutral intracellular pH (around 7.4) the dissociation of these acids is favored, promoting a reduction in the intracellular pH to values lower than the physiological limits, causing cell death and consequently reduction in the ethanol production rate (LOHMEIER-VOGEL; SOPHER; LEE, 1998).

Various phenolic and aromatic compounds, e.g. syringaldehyde, 4-hydrozibenzaldehyd and vanillin are formed from the degradation of the lignin molecular structure during the lignocellulosic biomass pretreatment (FONSECA et al., 2011).. Although different microorganism species exhibit different sensitivities towards phenolic compounds (GALLUCCI et al., 2014; PIZZOLITTO et al., 2015; PUUPPONEN-PIMIA et al., 2001) and the mechanism of toxicity has not been completely elucidated. It is believed that these inhibitors are able to interact with the cell membrane causing efflux of cellular metabolites and ions (HEIPIEPER; KEWELOH; REHM, 1991), therefore, influencing its function and changing the protein-lipid ratio present in its composition (FONSECA et al., 2011; HERIBERT; GERD; HANS-JÜRGEN, 1990).

The partial or total removal of these inhibitors from the hemicellulosic hydrolyzate is a key step to increase the ethanol productivity by the microorganisms. Several physical and physico-chemical methods have been developed and applied to remove these degradation products from the lignocellulosic as reviewed by (MUSSATTO; ROBERTO, 2004). The choice of the method depends on both the type of hemicellulose hydrolyzate and the specie of microorganism used for fermenting the sugars, since each microorganism has a different degree of tolerance to inhibitors (LARSSON et al., 1999). The major drawback of many of detoxification methods is that they generate additional waste. The biological detoxification method is an alternative to physical and physico-chemical regarding the removal of toxic compounds from hemicellulosic hydrolyzate. It is a green method where

microorganisms (CAO et al., 2013; FONSECA et al., 2011, 2013; ZHANG et al., 2013) or their enzymes (VITHANAGE et al., 2015) are utilized with little waste generation and no need for effluent treatment, but it requires long period of time and there is a possibility of sugar consumption by the microorganism in parallel to the conversion of the inhibitors. Even though genetically modified microorganisms tolerant to inhibitor compounds have been studied (HASUNUMA et al., 2014), this is an unknown direction due to the uncertain of these strains behavior when in the environment. Thus, alternative microorganisms and experimental conditions must be explored.

The goal of this study was to evaluate the consumption of inhibitors from SCB-derived hemicellulosic hydrolyzate during biological detoxification by the yeast *I. occidentalis* Y1'a. The supplementation of the hemicellulosic hydrolyzate was explored using the Box and Behken 2³ design; so that the influence of a carbon sources (glucose) and two different nitrogen sources (yeast extract and peptone) were evaluated. In addition, the efficiency of hemicellulosic ethanol production by the yeast *C. shehatae* UFMG HM 52.2 was assessed.

5.2. Material

Raw SCB samples were obtained from Fazenda Invernada, Bioserv, Unica (Vale do Rosario, Brazil). The yeasts *I. occidentalis* Y1'a and *C. shehatae* UFMG 52.2 were kindly provided by the Laboratory of Microbiology from the Centre for Study of Social Insects from São Paulo State University.

Statistica (version 12) software was used for regression and statistical analyses of the data. The statistical significance of the regression coefficients was 95%.

5.3. Methods

5.3.1. Inoculum preparation

The strains were grown in Agar Sabouraud 2% (w/w) medium by agar plate method and incubated at 30°C for 48h. Inoculum of *I. occidentalis* Y1'a was grown in YPD medium containing (g/L): yeast extract, 10; peptone, 20; glucose, 20 (LÓPEZ et al., 2004), while inoculum of *C. shehatae* UFMG 52.2 was grown in medium containing (g/L): yeast extract, 10; peptone, 20; xylose, 30. Distilled water and solutions of peptone, yeast extract and sugar were autoclaved separately at 110°C for 15 min and mixed up aseptically in a 1000 mL conical flask with 400 mL of final medium volume for each strain. Regarding *I. occidentalis*

Y1'a, cultivations were carried out at 30°C and 200 rpm for 48 h in rotatory shaker while *C. shehatae* UFMG 52.2 was incubated under the same conditions for 24h. The cultivations were centrifuged at 2600 x g for 20 min, and the supernatant was discarded and the cells were washed and resuspended with NaCl 0.9% (w/v). Cell concentration was determined by OD ($\lambda = 600\text{nm}$) and inoculum was standardized in 0.5 g/L of cells for both strains.

5.3.2. Hemicellulosic hydrolyzate

SCB was sundried until moisture content of 10% as measured in an infra-red weightier at 100°C. Acid pre-treatment was carried out in a 50 L vertical rotatory reactor at 121°C for 15 min with H₂SO₄ 10%. After the reaction, the reactor content was filtered to separate the solid fraction (cellulignin) from the hemicellulosic hydrolyzate, which corresponds to the liquid fraction. Hemicellulosic hydrolyzate was concentrated 5 fold using a vacuum evaporator apparatus at 70°C due to the high total sugar content and stored at 10°C. Natural and pretreated SCB were chemically characterized according to NREL/TP-510-42618 protocol (SLUITER et al., 2004).

5.3.3. Experimental design

A Box Behnken 2³ design with triplicate at the central point was applied in order to evaluate the *I. occidentalis* Y1'a capacity to grow in SCB hemicellulosic hydrolyzate. The impact of the independent variables: concentration of yeast extract (g/L), concentration of peptone (g/L) and concentration of glucose (g/L) was evaluated in the removal of acetic acid. Total phenol concentration was also evaluated during the time-course experiment. The pH of the hemicellulosic hydrolyzate was adjusted with NaOH to 4.0 according to previous studies (SOARES, 2012), autoclaved at 110°C for 15 min and centrifuged (2600 x g) for 20 min to remove suspended solids. A volume of 55 mL of sterile hemicellulosic hydrolyzate was poured into each 125 mL conical flask, which was supplemented according to the Box and Behnken matrix condition with glucose, yeast extract and peptone (Table 5-3). The experiment was conducted at 30°C, 300 rpm for 96h. Samples were taken every 24h within a period of 96h.

The best nutritional condition for biological detoxification was validated by an experimental run, which was performed in triplicate, and tested for ethanol production by *C. shehatae* UFMG 52.2.

5.3.4. Supplementation of biot detoxified hemicellulosic hydrolyzate

Biot detoxified hemicellulosic hydrolyzate was supplemented with (g/L): yeast extract, 3; malt extract, 3 and ammonium sulfate, 5; and incubated in shaker at 30°C, 200 rpm for 48h. Samples were taken with 24h and 48h to evaluate sugar consumption, ethanol production, $Y_{P/S}$ and Q_p .

5.3.5. Analytical methods

5.3.5.1. Phenolic compounds

Total phenolic compounds were quantified according to Folin-Ciocalteu method (SINGLETON; ORTHOFER; LAMUELA-RAVENTOS, 1999).

5.3.5.2. Sugars, ethanol and acetic acid

The concentration of xylose, glucose, arabinose, acetic acid and ethanol were determined by High-Performance Liquid Chromatography (HPLC) equipped with refraction index detection (Waters 410; Milford, MA, USA), column BIO-RAD AMINEX HPX-87H (7.8 x 300 mm) (Bio-Rad, Hercules, CA, USA) at 45°C. The eluent was 0.005 M sulfuric acid at 0.6 mL/min flow rate. Samples were previously diluted (1:10) and filtered with Sep Pak C18 filter.

5.3.5.3. Furan compounds

Furfural and HMF concentration were determined by HPLC equipped with UV detector (Waters 2487/USA) and column Eclipse XDB-C18 5 μ m (4.6 x 150 mm) at 25°C. The eluent was acetonitrile: water (1:8) with 1% of acetic acid with pH adjusted to 3.0 with phosphoric acid. The flow rate was adjusted to 0.9 mL/min. Samples were previously filtered using Schleicher & Schuell membrane 0.45 μ m.

5.3.6. Calculations

Sugars (xylose, glucose and arabinoses) and acetic acid consumption (%) during fermentation were determined according to equation (1), where X is the consumption (%), X_i , initial sugar/acetic acid concentration (g/L) and X_f , final sugar/acetic acid concentration (g/L).

$$X = \left(\frac{X_f - X_i}{X_i} \right) \cdot 100 \quad (1)$$

The conversion of xylose into ethanol was determined by the substrate –product conversion factor ($Y_{P/S}$), according to equation (2), where P_f is the final concentration of ethanol (g/L), P_i , the initial concentration of ethanol (g/L), S_f , the final concentration of substrate (g/L), S_i , the initial concentration of substrate (g/L).

$$Y_{P/S} = \frac{\Delta P}{\Delta S} = \frac{P_F - P_I}{S_F - S_I} \quad (2)$$

Volumetric productivity (Q_P) (g/L.h) of ethanol was calculated according to equation (3), where P_f the is the final concentration of ethanol (g/L), P_i , the initial concentration of ethanol (g/L) and t , the time of fermentation (h).

$$Q_P = \frac{P_F - P_I}{t} \quad (3)$$

5.4. Results

5.4.1. Chemical composition of sugarcane bagasse

The composition of SCB and its compact structure before and after the acid hydrolysis pretreatment are shown in Table 5-1 and Figure 5-1a and Figure 5-1b.

An increase in cellulose and lignin proportion in acid-pretreated SCB samples was observed (Table 5-1). On the other hand, the proportion of hemicellulose decreased after the acid pretreatment, due to its depolymerization to sugars in the aqueous fraction. These results can be explained by the physical structure of each polymer. Cellulose is an unbranched and linear homopolymer composed of D-glucose units joined by glycosidic β (1→4) bonds. This polymer shows a particular arrangement which results in crystalline and amorphous regions. Crystalline regions are highly organized, making cellulose insoluble in water and in most solvents, and also confer cellulose resistance to enzymatic attack and action of chemicals (SILVA et al., 2009). Compared to cellulose, hemicellulose is more susceptible to acid hydrolysis because it offers greater accessibility to mineral acids commonly used as catalysts. This reactivity is usually attributed to the hemicellulose amorphous nature (SANTOS et al., 2012a).

The total content of cellulose (37.44%), hemicellulose (28.31%) and lignin (22.22%) in SCB was approximately 88% (Table 5-1). These results are in agreement with those obtained by (RODRIGUES et al., 2010). Acid hydrolysis pretreatment promoted the disorganization of SCB fiber after partial removal of the hemicellulose fraction (Figure 5-1b). The physical structure of the solid residue, composed by cellulose (47.25%) and lignin

(26.47%) and hemicellulose (23.35%), is shown in Table 5-1. The maintenance of the acetyl radical proportion in both untreated and pretreated SCB indicates that acetyl radicals were released as acetic acid during hemicellulose depolymerization. There was no change in the ash content, indicating that the acid pretreatment did not alter the mineral composition of SCB. However, complete removal of extractives during pretreatment was observed (Table 5-1).

Table 5-1: Chemical composition of sugarcane bagasse.

(% w/w)	Natural	Pre-treated
Cellulose	37.44	47.25
Hemicellulose	28.31	23.35
Xylose	21.69	18.45
Arabinose	2.68	1.48
Acetyl radicals	2.49	2.33
% Sugars	65.75	70.60
Total Lignin	22.22	26.47
Ashes	1.48	1.49
Extractives	3.19	-
Total	92.64	98.56

Figure 5-1: Scanning electron microscopy (SEM) with 500 x magnification of (a) natural SCB and (a) sulfuric acid pretreated.

Source: Personal file

5.4.2. Physico-chemical characterization of hemicellulosic hydrolyzate

The composition of crude and concentrated hemicellulosic hydrolyzate is shown in Table 5-2. The hydrolysis conditions utilized generated a hydrolyzate with 19.7 g/L of sugars, mainly xylose (77%), followed by arabinose (12%) and glucose (11%). The concentration of xylose (15.10 g/L) in the no concentrated hemicellulosic hydrolyzate was

about 7 fold higher than glucose concentration (2.23 g/L) confirming, as expected, that the hemicellulose was more susceptible to acid hydrolysis than the cellulose.

Acid hydrolysis of lignocellulosic biomass generates fermentation inhibitors, such as furfural, acetic acid and HMF, which adversely affect the microorganism growth, sugars consumption, and cell metabolism (KIM et al., 2013). After SCB acid hydrolysis, a small concentration of acetic acid (3.28 g/L) was observed in the hemicellulosic hydrolyzate (Table 5-2). Acetic acid is formed after the release of acetyl groups present in the hemicellulose during the acid pretreatment (ULBRICHT; NORTHUP; THOMAS, 1984). Low concentration of sugar degradation products, such as furfural (0.20 g/L) and HMF (0.02 g/L) was observed (Table 5-2). Studies from Kim et al. (2013) indicate that an inhibitory effect of HMF and furfural on microbial metabolism, characterized by reduction of the glucose consumption, was found when HMF concentration was 1 g/L in the culture medium. Concentrations higher than 4 g/L completely inhibited microbial metabolism. Furthermore, the toxic effect of furfural was more pronounced than HMF toxic effect. Furfural concentration around 1 g/L inhibited about 50% the glucose metabolism, whereas concentrations above 3 g/L completely inhibited glucose consumption, affecting cell growth and ethanol production. Therefore, the concentration of furfural and HMF found in crude hemicellulosic hydrolyzate (0.20 g/L and 0.02 g/L, respectively) and concentrated hemicellulosic hydrolyzate (0.25 g/L and 0.02 g/L, respectively) are not expected to be high enough to inhibit microbial metabolism.

Table 5-2: Partial composition of the hemicellulosic hydrolyzate before and after the concentration step.

Component (g/L)	Hydrolyzate	
	Raw	Concentrated (CF = 5)
D-xylose	15.10	86.38
D-glucose	2.23	10.54
L-arabinose	2.41	11.11
Acetic Acid	3.28	1.23
Furfural	0.20	0.25
HMF	0.02	0.02
pH	0.67	0.11

CF: concentration factor

Crude hydrolyzate was concentrated using a concentration factor (CF) equals five, in order to facilitate the SCB hemicellulosic hydrolyzate storage. Although sugars concentration in hemicellulosic hydrolyzate increased proportionally to the CF employed, the concentration of acetic acid was reduced due to its volatilization during the process as

high temperature (70°C) was applied under vacuum. A slightly increase (1.25 times) in the furfural concentration was observed, whereas HMF concentration did not suffer any variation (Table 5-2). As long as acetic acid, furfural and HMF are volatile compounds, this fact can be explained by the easiness with which they are dragged by vapors, and by the boiling point drops due to the vacuum, regardless the temperature applied to the process (RODRIGUES et al., 2001). Those results are in accordance to Parajo; Domínguez; Dominguez (1996) who observed partially removal of volatile compounds and increase of nonvolatile products of hemicellulosic hydrolyzate after vacuum evaporation process.

5.4.3. Evaluation of hemicellulosic hydrolyzate supplementation using the factorial design Box and Behnken 2³ during the biodegradation process

The earliest total consumption of acetic acid was observed in runs 5 and 6 after 48h, followed by the runs 9 and 10 (Table 5-3). Taking into account industrial processes and considering the reduction of costs and time, 48h was the time of choice for statistical analysis. The coefficient of determination (R^2) was 0.89. The Table 5-4 shows the ANOVA, where only the linear term of the variable glucose concentration was significant at 5% level ($p = 0.01$), with a negative effect (-34.71). The impact of the effects of the variables and of the interaction between the variables are shown in Figure 5-2a. In this way, glucose concentration showed a strong influence in acetic acid removal and high concentrations of glucose in the hemicellulosic hydrolyzate might inhibit or retard the consumption of acetic acid by the yeast, independent of the yeast extract (Figure 5-2b) or peptone (Figure 5-2c). Zhang et al. (2013) evaluated the influence of nitrogen sources during the biodegradation of hydrolyzate of oil palm empty fruit bunch by *Enterobacter* sp. FDS8. In line with our results, the authors also found that yeast extract and peptone as supplement did not increase the degradation rates of the toxic compounds, in this case furfural and HMF. In addition, $(\text{NH}_4)_2\text{SO}_4$ and NaNO_3 considerably reduced the degradation rates, especially for furfural.

Table 5-3: Supplementation of the hemicellulosic hydrolyzate and acetic acid removal (%) after 48h of biodetoxification with the yeast *I. occidentalis* Y1'a.

Run	Independent Variables			Dependent Variable
	X ₁	X ₂	X ₃	Acetic Acid Removal (%)
1	-1	-1	0	84.38
2	+1	-1	0	80.05
3	-1	+1	0	86.80
4	+1	+1	0	81.97
5	-1	0	-1	100.00
6	+1	0	-1	100.00
7	-1	0	+1	52.60
8	+1	0	+1	54.76
9	0	-1	-1	91.74
10	0	+1	-1	92.63
11	0	-1	+1	69.75
12	0	+1	+1	68.43
13 (PC)	0	0	0	80.69
14 (PC)	0	0	0	84.08
15 (PC)	0	0	0	87.23

Variables (g/L)		Levels		
		-1	0	+1
X ₁	Yeast Extract	10	20	30
X ₂	Peptone	10	20	30
X ₃	Glucose	0	10	20

Table 5-4: Analysis of variance (ANOVA) for the effect of the variables yeast extract, peptone and glucose concentration on the removal of acetic acid from the hemicellulosic hydrolyzate by the yeast *I. occidentalis* Y1'a after a 48 h-detoxification.

Variables	SS	DF	MS	F	p
YE (L)	6.13	1	6.13	0.58	0.53
YE (Q)	18.67	1	18.67	1.75	0.32
P (L)	1.91	1	1.91	0.18	0.71
P (Q)	8.86	1	8.86	0.828	0.46
G (L)	2409.22	1	2409.22	225.21	0.01
G (Q)	89.06	1	89.06	8.33	0.10
YE (L) x P (L)	0.06	1	0.06	0.01	0.95
YE (L) x G (L)	1.17	1	1.17	0.11	0.77
P (L) x G (L)	1.22	1	1.22	0.11	0.77
Lack of fit	288.56	3	96.19	8.99	
Error	21.40	2	10.70		
TSS	2847.57	14			

SS: sum of squares; DF: degree of freedom; MS: mean square; TSS: total sum of squares; YE: yeast extract; P: peptone; G: glucose; (L): linear term; (Q): quadratic term.

Figure 5-2: Pareto chart indicating the effect of the variables and interactions between the variables after 48 h of hemicellulosic hydrolyzate detoxification (a). Response of surface related to acetic acid consumption and (b) glucose concentration versus yeast extract concentration (c) glucose concentration and peptone concentration, after 48h of hemicellulosic hydrolyzate detoxification by the yeast *I. occidentalis* Y1'a.

Source: Personal file

5.4.4. Evaluation of sugars consumption and toxic compounds removal from hemicellulosic hydrolyzate during biodegradation process

The initial concentration of sugars in the biodegradation medium are shown in Table 5-5. The theoretical glucose concentration regards the concentration of glucose in the 5 fold concentrated hemicellulosic hydrolyzate, as determined in Table 5-2, whereas the real glucose concentration regards the final concentration of glucose in the 5 fold concentrated hemicellulosic hydrolyzate after the supplementation. The difference between theoretical and real values is due to the glucose released during the acid pretreatment. Glucose assimilation by the yeast was followed by ethanol production after 24 h of cultivation in the hemicellulosic hydrolyzate supplemented with 10g/L (Figure 5-3 and Figure 5-5) and 20 g/L (Figure 5-6) of glucose. In addition, the amount of ethanol was proportional of the amount of glucose in the culture medium. In the absence of glucose, ethanol was slightly consumed during the biodegradation process as carbon source (Figure 5-5 and Figure 5-6). Xylitol production was also observed, accompanied by xylose metabolism (Figure 5-3, Figure 5-4, Figure 5-5 and Figure 5-6). No xylitol consumption was observed until the end of the experiment most probably due to xylose was still in abundance in the cultivation medium as an alternative carbon source.

Xylose consumption by *I. occidentalis* Y1'a ranged from 14.79 to 36.02% and was influenced by the supplementation of the hemicellulosic hydrolyzate. The highest consumption of such sugar were combined to higher yields of xylitol (9.10 g/L), which production was initiated after 48 h of cultivation, after all glucose was depleted and consumption of ethanol started. Therefore, xylose was not the preferred carbon source of this yeast, which is capable of synthesize the enzyme apparatus necessary for xylose-into-xylitol conversion. Soares, (2012) observed the consumption of xylose ranging from 1.75% to 7.17% and from 1.02% to 4.99% by the yeasts *I. occidentalis* M1 and *I. occidentalis* Y1'a, respectively after 96 h detoxification of hemicellulosic hydrolyzate from SCB at different pH values (4.0; 4.5 and 5.0) and agitations (100, 200 and 300 rpm). Fonseca et al. (2011) observed a consumption of 2.5% and 49.6% of xylose after 72 h of concentrated and unconcentrated hemicellulosic hydrolyzate from SCB detoxification by yeast *I. occidentalis* CCTCC M 206097. Sugar consumption by the yeast *Coniochaeta ligniaria*. Cao et al., (2013) observed a consumption of 20% of xylose after 48 h detoxification of hemicellulosic hydrolyzate from corn stover. Therefore, different physical and nutritional parameters

applied to the detoxification process, and the type of hemicellulosic hydrolyzate impact on the metabolism of the yeast.

Table 5-5: Initial concentration of sugars in the cultivation medium.

Theoretical glucose concentration	Glucose (g/L)			Xylose (g/L)	Arabinose (g/L)
	0*	10 g/L	20 g/L		
Real glucose concentration	4.87	13.49	22.55	41.16	5.59

* Culture medium no supplemented with glucose.

No arabinose consumption was observed during the time-course biodetoxification process, indicating that this sugar is not metabolized by *I. occidentalis* Y1'a (Figure 5-3, Figure 5-4, Figure 5-5 and Figure 5-6). Similarly, the strains *C. ligniaria* (CAO et al., 2013), *S. cerevisiae* (FONSECA et al., 2013) and *Enterobacter* sp. FDS8 (ZHANG et al., 2013) did not consume arabinose during the biodetoxification of corn straw, rice straw and oil palm empty fruit bunch acid hydrolyzate, respectively.

Figure 5-3: Central point average of sugars consumption from the hemicellulosic hydrolyzate supplemented with 10 g/L of glucose and their conversion into ethanol and xylitol during 96 h of biodetoxification by the yeast *I. occidentalis* Y1'a.

Source: Personal file

Figure 5-4: Sugars consumption from the no-glucose supplemented hemicellulosic hydrolyzate and their conversion into ethanol and xylitol during 96 h of biodegradation by the yeast *I. occidentalis* Y1'a in the run 5 (a), 6 (b), 9 (c) and 10 (d).

Source: Personal file

Figure 5-5: Sugars consumption from the hemicellulosic hydrolyzate supplemented with 10 g/L of glucose and their conversion into ethanol and xylitol during 96 h of biodegradation by the yeast *I. occidentalis* Y1'a in the run 1 (a), 2 (b), 3 (c) and 4 (d).

Source: Personal file

Figure 5-6: Sugars consumption from the hemicellulosic hydrolyzate supplemented with 20 g/L of glucose and their conversion into ethanol and xylitol during 96 h of biodegradation by the yeast *I. occidentalis* Y1'a in the run 7 (a), 8 (b), 11 (c) and 12 (d).

Source: Personal file

No glucose supplemented medium showed higher acetic acid consumption, ranging from 91.7% to 100% (run 5, 6, 9 and 10 in Table 5-3) as shown in Figure 5-7b. Moreover, acetic acid was almost totally consumed during the first 24 h of the detoxification process (up to 89.2%), which was accompanied by the reduction in acidity of the culture media with a slight increase in pH. However, glucose was metabolized prior to acetic acid in glucose-added medium (Figure 5-3, Figure 5-5 and Figure 5-6). In the culture medium supplemented with 10 g/L of glucose (Figure 5-3 and Figure 5-6), a complete consumption of acetic acid was observed after 72h of detoxification (Figure 5-7a), whereas the consumption of acetic acid was observed only after 96h in the media supplemented with 20 g/L of glucose (Figure 5-7c). These results show that, besides ethanol and xylose, acetic acid was a second-plan nutritional source of choice when compared to glucose, most probably due to the easiness for the microorganism to synthesize the enzymatic apparatus to use glucose as an energy source. Those results evince the influence of glucose in the acetic acid metabolism by the yeast *I. occidentalis* Y1'a. Therefore, for a rapid uptake of this acid, it is ideal a low initial glucose concentration in hemicellulosic hydrolyzate.

I. occidentalis Y1'a showed better performance regarding acetic acid removal when compared to the fungal strain used by Yu et al. (2011). While *Aspergillus nidulans* (FLZ10) removed 41.6% of acetic acid from the hydrolyzate with cellulose and 53.6% of acetic acid after 72h of pretreatment, *I. occidentalis* Y1'a removed up to 87% of acetic acid after 48h of biodetoxification. Cao; Nagarajan; Loh (2009) reported the removal of 37.1% and 92.8% of acetic acid after 24 h and 48 h, respectively, during the detoxification of a hydrolyzate from the acid pretreatment of corn stover by the yeast *C. ligniaria*. Fonseca et al. (2011) reported a consumption of 100% and 6.1% of acetic acid from unconcentrated and concentrated SCB hemicellulosic hydrolyzate, respectively, after 72 h of detoxification by the yeast *I. occidentalis* CCTCC M 206097. Acetic acid has a role as carbon source in Krebs cycle and contributes directly to intermediate compounds formation, which are indispensable to cell metabolism, energy (ATP) and coenzymes (NADH and FADH₂) production. NADH and FADH₂ are carried to the electron transport chain where they are oxidized to form ATP, that can be used for the cellular biomass formation (SHERIDAN; COLIN; CHALK, 1990). Thus, in the absence of glucose, acetic acid was used by *I. occidentalis* Y1'a likely as a carbon source for the maintenance of vital functions of the cell and biomass proliferation.

Figure 5-7: Acetic acid removal from the hemicellulosic hydrolyzate during 96 h of biodegradation by the yeast *I. occidentalis* Y1'a from the hemicellulosic hydrolyzate supplemented with 10 g/L of glucose (a) no supplemented with glucose (b) and supplemented with 20 g/L of glucose (c).

Source: Personal file

In contrast to acetic acid removal, phenolic compounds removal showed to be independent of glucose concentration in the culture medium. The concentration of these compounds remained constant during the first 48h of incubation in the no-glucose added medium (Figure 5-8b). On the other hand, the phenolic compounds concentration fluctuated when the medium was supplemented with 10 g/L of glucose (Figure 5-8b). A considerable increase in phenolic compounds concentration was observed after 48h on biodegradation in the hemicellulosic hydrolyzate supplemented with 20 g/L of glucose. Neither yeast extract nor peptone concentration showed to actuate in the metabolism of those compounds. After 72h, the concentration of phenolic compounds increased for all of the experiments, regardless the supplementation.

In the present study, phenolic compounds concentration was ascertained using Folin-Ciocalteu method. As reported by Everette et al. (2010), besides phenolic compounds Folin-Ciocalteu reagent reacts to thiol derivatives, nucleotide bases, proteins, fatty acids, and vitamin derivatives at different ranges. In addition, Ikawa et al. (2003) identified a strong reaction between certain nitrogen compounds and this reagent, mainly those containing the free -OH group. It is well known that hemicellulosic hydrolyzate is a very complex medium and diverse sub-products can be found during the metabolism of inhibitor compounds, thus this method might show high accuracy regarding hydrolyzate analysis (FONSECA et al., 2013). Moreover, many types of phenolic compounds may be released from lignin structure during diluted-acid pretreatment, such as benzoic acid, ferulic acid, *p*-hydroxybenzaldehyde, dimethoxybenzoic acid, syringaldehyde and vanillin. These compounds may be metabolized by the microorganism and consequently converted into other compounds during the biological treatment, as reported by Handerson and Farmer (1955). These authors detected the conversion of vanillin and ferulic acid into vanillic acid before the breaking of benzene ring, and syringaldehyde into syringic acid by soil fungi. On the other hand, no intermediate was traced from *p*-hydroxybenzaldehyde. In a next study, Henderson (1961) reported that *p*-hydroxybenzoic acid is the principal intermediate from *p*-hydroxybenzaldehyde, hydroxybenzoic acids from the corresponding mono-methoxybenzoic acids, protocatechuic acid from *p*-hydroxybenzoic acid, *p*-hydroxybenzoic and protocatechuic acids from benzoic acid and vanillic acid from 3:4-dimethoxybenzoic acid. Therefore, the interconverting and the breaking of those compounds may release new compounds which may be detected by the Folin-Ciocalteu reagent, characterizing the increase or decrease of the phenolic compounds concentration observed in Figure 5-8.

Figure 5-8: Phenolic compounds metabolism from the hemicellulosic hydrolyzate supplemented with 10 g/L of glucose (a) no-glucose supplemented hemicellulosic hydrolyzate (b) and supplemented with 20 g/L of glucose (c) during 96 h of biodegradation by the yeast *I. occidentalis* Y1'a estimated using Folin-Ciocalteu Reagent.

Source: Personal file

The results found in this work contrast with those obtained by Wang et al. (2010), who observed that the phenol compounds biodegradation by the fungus *Paecilomyces variottu* JH6 was related to the presence of glucose in the culture medium. On the other hand, the profile of phenolic compounds consumption was directly influenced by the glucose

concentration in the medium or by the concentration of peptone in some cases. In addition, the results suggested that initially the glucose and acetic acid were the carbon sources of choice for the cellular metabolic activity maintenance, possibly due to the cell apparatus is adapting to phenolic compounds content, which may improve the biodegradation process, mainly in the case of recycling of cells (ZHANG et al., 2013). After consumption of these substrates, the phenolic compounds were used as a substrate for the yeast, with a consumption ranging from 39.2% to 53.7%. Moreover, Henderson (1961) reported that the fungi *Pullularia pullulans* could oxidize vanillic acid and grow on it as sole carbon source, indicating that not only the phenolic compounds may be a carbon source for the microorganisms, but also the intermediates formed during its metabolism.

5.4.5. Confirmation experiment

The results for confirmation experiment indicated that the yeast was capable of consuming acetic acid from the unsupplemented hemicellulosic hydrolyzate. After 24h, 55% of the acetic acid was removed and the glucose resulting from the pretreatment process was totally consumed by the yeast. After 48h, around 81% of the acetic acid was metabolized by the yeast. There was no consumption of arabinose and xylose after 96h of detoxification. In addition, low concentration of ethanol (1 g/L) was observed after 24h of incubation, which was completely consumed during the time of incubation.

5.4.6. Ethanol production from the hemicellulosic hydrolyzate biologically detoxified

After the evaluation of the nutritional parameters, the unsupplemented hemicellulosic hydrolyzate was chosen for the biodegradation process by the yeast *I. occidentalis* Y1'a. Unsupplemented hemicellulosic hydrolyzate was biodegraded for 48h and centrifuged in order to remove the yeast cells and the solid particles. The supernatant was supplemented according to the nutrients required for the metabolism of the yeast *C. shehatae* UFMG 52.2 and inoculated with 0.5 g/L cells.

I. occidentalis Y1'a effectively removed around 79% of the acetic acid from the hemicellulosic hydrolyzate after 48h of detoxification Figure 5-9. Lower acetic acid removal was also observed during the fermentation period, which suggests that *I. occidentalis* Y1'a cells may have not been completely removed during the centrifugation process and remained active in the hemicellulosic hydrolyzate. Furthermore, this information indicates the possibility of future studies aimed at co-cultivation using *I. occidentalis* Y1'a and *C.*

shehatae UFMG 52.2 yeasts. However, one major drawback in co-cultivation is the competition of the microorganisms by the substrate, as observed by Yu et al. (2011) during a simultaneous biodetoxification and fermentation process utilizing a fungal strain and *Saccharomyces cerevisiae*. Co-cultivation between *I. occidentalis* Y1'a and *C. shehatae* UFMG 52.2 yeasts may be successful, since *I. occidentalis* Y1'a did not metabolize xylose in presence of *C. shehatae* UFMG 52.2, as shown in Figure 5-9, while the yeast was able to convert this sugar into ethanol.

C. shehatae UFMG 52.2 was strongly inhibited by acetic acid and could not metabolize the sugars from sugarcane hemicellulosic hydrolyzate without a previous detoxification step. In line with this result, Fonseca et al. (2013) also observed strongly inhibition of the yeast *Pichia stipitis* in untreated rice straw and olive tree pruning hydrolyzate. However, *C. shehatae* UFMG 52.2 consumed about 49% of xylose present in the biodetoxified hemicellulosic hydrolyzate after 48 h of fermentation and produced around 8.5 g/L of ethanol. The ethanol yield ($Y_{P/S}$) was 0.26 g/g and the volumetric ethanol productivity (Q_p) 0.14 g/L.h. These results show higher performance when compared to Fonseca et al. (2013), who found $Y_{P/S} = 0.17$ g/g and $Q_p = 0.09$ g/L.h, for ethanol produced by *Pichia stipitis* after 96 h of fermentation of rice straw hemicellulosic hydrolyzate biodetoxified by *S. cerevisiae* for 6h.

Figure 5-9: Ethanol production by the yeast *I. occidentalis* Y1'a during the biodetoxification process of the no supplemented hemicellulosic hydrolyzate and by the yeast *C. shehatae* UFMG 52.2 during the fermentation process from the detoxified hemicellulosic hydrolyzate. The initial glucose content regards the glucose released from hemicellulose and cellulose during the diluted-acid pretreatment.

Source: Personal file

5.5. Conclusions

The evaluation of the results indicates that the yeast *I. occidentalis* Y1'a is a promising microorganism for the biological detoxification of SCB hemicellulosic hydrolyzate, since it was able to remove around 80% of acetic acid in only 48 h, providing economic advantages to the process. Moreover, the yeast *I. occidentalis* Y1'a did not show a considerable consumption of xylose, which was converted into ethanol during the fermentation step by the yeast *C. shehatae* UFMG 52.2. Yeasts have worked synergically, suggesting the possibility of future studies involving detoxification and simultaneous fermentation in one pot.

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6. General conclusion

This study showed an exploratory strategy regarding nutritional and physical parameters towards the synthesis of ligninolytic and hydrolytic enzymes by seven novel fungi strains growing under solid-state fermentation. The results highlight the efficiency of the statistical tools regarding the obtaining of low hydrolytic activity, saving the sugar fraction for ethanol production and the potential of those strains for xylanase production. In addition, the new fungal strain JLS6-A6 showed high potential of synthesizing the enzyme manganese peroxidase and efficiency in short-time lignin removal from sugarcane bagasse. Moreover, the yeast *I. occidentalis* Y1'a is a promising microorganism for the biological detoxification of sugarcane bagasse hemicellulosic hydrolyzate, since it was able to remove around 80% of acetic acid in only 48 h, providing economic advantages to the process. The yeast did not show considerable consumption of xylose, which was converted into ethanol during the fermentation step by the yeast *C. shehatae* UFMG 52.2. Both of the yeasts have worked synergically, suggesting the possibility of future studies involving detoxification and simultaneous fermentation in one pot. Therefore, this study is a promising alternative of green second generation bioethanol production, overcoming the adaptation phase of microbial strains during pretreatment of lignocellulosic feedstock and detoxification of hemicellulosic hydrolyzate.