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Ethanol Production from Sugarcane Bagasse Hydrolysate Using *Pichia stipitis*

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Abstract The objective of this study was to evaluate the ethanol production from the sugars contained in the sugarcane bagasse hemicellulosic hydrolysate with the yeast *Pichia stipitis* DSM 3651. The fermentations were carried out in 250-mL Erlenmeyers with 100 mL of medium incubated at 200 rpm and 30 °C for 120 h. The medium was composed by raw (non-detoxified) hydrolysate or by hydrolysates detoxified by pH alteration followed by active charcoal adsorption or by adsorption into ion-exchange resins, all of them supplemented with yeast extract (3 g/L), malt extract (3 g/L), and peptone (5 g/L). The initial concentration of cells was 3 g/L. According to the results, the detoxification procedures removed inhibitory compounds from the hemicellulosic hydrolysate and, thus, improved the bioconversion of the sugars into ethanol. The fermentation using the non-detoxified hydrolysate led to 4.9 g/L ethanol in 120 h, with a yield of 0.20 g/g and a productivity of 0.04 g L^{-1} h^{-1}. The detoxification by pH alteration and active charcoal adsorption led to 6.1 g/L ethanol in 48 h, with a yield of 0.30 g/g and a productivity of 0.13 g L^{-1} h^{-1}. The detoxification by adsorption into ion-exchange resins, in turn, provided 7.5 g/L ethanol in 48 h, with a yield of 0.30 g/g and a productivity of 0.16 g L^{-1} h^{-1}.

Keywords Sugarcane bagasse hemicellulosic hydrolysate · Fermentation inhibitors · Ethanol · *Pichia stipitis*

Introduction

Brazil is the largest producer of sugarcane in the world. In 2008, more than 570 million tons of sugarcane was processed by the Brazilian sugar-alcohol mills, leading to the production

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of roughly 32 million tons of sugar and 27 billion liters of ethanol [1]. Each ton of sugarcane processed by the mills generates approximately 140 kg of dry bagasse [2]; thus, it can be inferred that Brazilian mills produced around 80 million tons of sugarcane bagasse only in the 2008 crop.

Between 60% and 90% of the bagasse generated during the production of sucrose and ethanol is used by the mills as a combustible for electricity and heat generation [3]. The excess still causes environmental and storage concern [4]. Therefore, many researchers suggest that this excess could be used to prepare animal feed, pulp and paper, and many other goods [5, 6]. In such a scenario, more ethanol would be produced from the same amount of sugarcane processed [7].

Sugarcane bagasse contains 38–50% cellulose, 25–30% hemicellulose, 22–27% lignin, and 1–3% ash [8, 9]. Its hemicellulose can be successfully hydrolyzed in the presence of dilute acids, generating hydrolysates rich in fermentable sugars like xylose, glucose, and arabinose [10]. Inhibitory compounds like acetic acid, furfural, hydroxymethylfurfural, and lignin derivatives are also liberated in the hydrolysate during the dilute acid hydrolysis [11]. Such compounds act as inhibitors of the microbial metabolism, hindering the bioconversion of sugars into desired products [12]. The degree of inhibition depends on the type and concentration of the inhibiting compounds [13]. Synergistic effects among different types of inhibitors were already demonstrated in a hydrolysate-based medium [14].

In order to reduce the concentrations of inhibitory compounds found in hemicellulosic hydrolysates, and thus improve the bioconversion of sugars into desired products, different methods like pH adjustment [15, 16], active charcoal adsorption [17, 18], and ion-exchange resins adsorption [19, 20], either alone or in combination [21, 22], have been proposed as detoxification strategies.

In the present study, we performed the bioconversion of sugars derived from the sugarcane bagasse into ethanol. For this, we used a strain of the naturally pentose-fermenting yeast *Pichia stipitis*. The fermentation medium was composed of raw (non-detoxified) hydrolysate or by hydrolysates detoxified by pH alteration followed by active charcoal adsorption or by adsorption into ion-exchange resins.

Material and Methods

All reagents used in this study were of analytical grade. Ingredients used to supplement the fermentation medium were from Oxoid. Sugarcane bagasse was acquired from Usina Vale do Rosário (Morro Agudo, S.P.), air-dried, and milled to pass through a 20-mesh sieve.

Sugarcane Bagasse Hydrolysis

The hydrolysis of the sugarcane bagasse with dilute sulfuric acid was performed as follows: Initially, the bagasse and the aqueous acid solution (2.0%, *w*/*v*) were loaded into a 200-mL stainless steel container (19×7 cm) using a ratio of 1.75:10 (*w*/*v*) among the sugarcane bagasse (dry weight) and the acid solution. The container was tightly sealed and immersed in a silicone bath provided with electrical heating. The heater was turned on, and when the temperature reached the programmed value (150 °C), the residence time started to be counted. At the due time (30 min), the hydrolysis was stopped by immersing the container into an ice bath, which quenched the reaction. Both the heating and the cooling times were negligible. After removing the screw cap from the container, the hemicellulosic hydrolysate was separated from the pretreated solids by filtration.

The hydrolysate was characterized to quantify the concentrations of cellobiose, glucuronic acid, glucose, xylose, arabinose, acetic acid, furfural, hydroxymethylfurfural (HMF), lignin derivatives, total solids, and ash.

Hydrolysate Detoxification

In order to reduce the amounts of the main fermentation inhibitors, the hydrolysate was detoxified by two different methods: pH alteration followed by active charcoal adsorption or adsorption into ion-exchange resins.

The detoxified hydrolysates were also characterized to quantify the concentrations of cellobiose, glucuronic acid, glucose, xylose, arabinose, acetic acid, furfural, HMF, lignin derivatives, total solids, and ash.

Alteration of pH Followed by Adsorption into Active Charcoal

The operation cycle of pH alteration followed by active charcoal adsorption (pHAC) treatment was as follows: (1) calcium oxide was added to the hydrolysate until pH 7.0 and the precipitate removed by filtration; (2) phosphoric acid was added to the hydrolysate until pH 5.5 and the precipitate removed in the same fashion; and (3) 2.5% active charcoal powder (Synth) was added to the hydrolysate which was then agitated at 200 rpm and 30 °C for 1 h. The solids were removed by vacuum filtration.

Adsorption into IER

The ion-exchange resins A-860S (macroporous strong base anion exchanger), A-500PS (macroporous type I strong base anion exchanger) and C-150 (macroporous strong acid cation exchanger) were used in the present study in sequence. Physicochemical properties are reported by the manufacturer (Purolite).

The operation cycle of the ion-exchange resins (IER) treatment was as follows: (1) resins were regenerated with solutions of 10% NaOH and 5% HCl for anion-exchangers and cation-exchanger, respectively; (2) resins were washed with deionized water; (3) resins were maintained in contact with the sugarcane bagasse hydrolysate (1:2, *v*/*v*, respectively); and (4) resins were washed with deionized water. These operations were carried out in Erlenmeyer flasks in a rotary shaker at 200 rpm and 30 °C for 1 h. After each one of the operations, the resins were recovered by filtration.

Microorganism and Inoculum Preparation

P. stipitis DSM 3651, acquired from Coleção de Culturas Tropical–Fundação André Tosello (Campinas, S.P.), was used in the experiments. The inoculum was grown by transferring a loopful of cells into 500-mL Erlenmeyer flasks containing 200 mL of synthetic medium consisting of (g/L) xylose (30.0), yeast extract (3.0), malt extract (3.0), and peptone (5.0). The cells were then incubated in a rotatory shaker at 30 °C and 200 rpm for 24 h, collected by a 30-min centrifugation at 2,000×*g*, and resuspended in sterile distilled water.

Hydrolysate Fermentation

The fermentations were carried out in 250-mL Erlenmeyer flasks containing 100 mL of medium inoculated with 3.0 g cells (dry weight) per liter. The medium was composed of the

hydrolysate (untreated, treated by pHAC or treated by IER) supplemented with (g/L) yeast extract (3.0), malt extract (3.0), and peptone (5.0). The flasks were maintained in a rotatory shaker at 30 °C and 200 rpm for 120 h. Samples were periodically collected to determine the concentrations of sugars, acetic acid, ethanol, cells, and pH.

All the hydrolysates were sterilized by autoclaving at 111 °C for 15 min before the use.

Analytical Methods

Cellobiose, glucose, xylose, arabinose, formic acid, acetic acid, and glucuronic acid concentrations were determined by HPLC (Waters) using a refraction index detector (2414) and a Biorad Aminex HPX-87H column at 45 °C. Sulfuric acid 0.01 N at a flow rate of 0.6 mL/min was used as eluent, and the injection volume was of 20 μL.

Furfural and HMF concentrations were also determined in the same HPLC system, but using a UV–VIS detector (2489) at 276 nm and a Hewlett-Packard RP18 column at 25 °C. Acetonitrile/water (1:8) supplemented with 1% acetic acid (volume basis) was used as the eluent at a flow rate of 0.8 mL/min. The injection volume was of 20 μL.

Lignin derivatives were determined by spectrophotometry (Beckman DU640B) according to Carvalho et al. [14].

Ash and total solids were determined by gravimetry according to Browning [23].

Cell concentrations were determined by turbidimetry using the aforementioned spectrophotometer. The absorbance measurements at 600 nm were correlated with the cell concentrations (g/L) through the following calibration curve:

$$y = 1.3479x + 0.021 \quad (1)$$

Sugars-to-ethanol conversion yield ($Y_{P/S}$) was calculated as the ratio between ethanol production and sugars consumption, sugars-to-cell conversion yield ($Y_{X/S}$) was calculated as the ratio between cell growth and sugars consumption, and volumetric productivity (Q_P) was calculated as the ratio between ethanol production and fermentation time.

Results and Discussion

Hydrolysate Detoxification

To be employed in the ethanol production, the sugarcane bagasse was submitted to acid hydrolysis under conditions that promoted significant release of its hemicellulosic sugars. Several compounds inhibitory to the microbial metabolism were also liberated and/or formed during the pretreatment (Table 1).

To minimize the content of inhibitory compounds, known to lead to slow kinetics and limited yields during the fermentations [24, 25], the hydrolysate was submitted to detoxification procedures before being used as the fermentation medium by pHAC or adsorption into IER.

As can be seen in Table 1, the concentration of xylose in the hydrolysate detoxified by IER was very similar to that determined in the non-detoxified hydrolysate. The detoxification of the hydrolysate by pHAC, however, led to a loss of approximately 11% in the content of xylose, similar to what was previously reported by other authors [26–28]. Glucose and arabinose concentrations decreased approximately 37% and 39% after IER treatment and 11% and 7% after pHAC treatment, respectively. Cellobiose was not detected in the hydrolysate.

Table 1 Composition of the non-detoxified (raw) hydrolysate, of the hydrolysate detoxified by pHAC, and of the hydrolysate detoxified by adsorption into IER.

Hydrolysate	pH	CEL (g/L)	GA (g/L)	XYL (g/L)	GLU (g/L)	ARA (g/L)	AC (g/L)	HMF (g/L)	FURF (g/L)	LD (g/L)	TS (g/L)	Ash (g/L)
Non-detoxified	0.6	ND	0.8	23.5	3.0	2.1	2.7	0.04	0.3	2.1	38.8	1.7
pHAC	5.8	ND	ND	20.8	2.7	1.9	2.9	ND	ND	0.1	30.0	4.3
IER	3.1	ND	ND	23.6	1.9	1.3	0.3	ND	0.04	0.1	35.9	0.2

CEL cellobiose, *GA* glucuronic acid, *XYL* xylose, *GLU* glucose, *ARA* arabinose, *AC* acetic acid, *HMF* hydroxymethylfurfural, *FURF* furfural, *LD* lignin derivatives, *TS* total solids, *ND* not detected

Regarding inhibitory compounds, HMF was completely removed from the hydrolysate after both detoxification treatments. A similar behavior was also observed for furfural, which was detected in a very small quantity (0.04 g/L) only in the hydrolysate treated by IER. The acetic acid concentration in the hydrolysate treated by pHAC was very similar to that determined in the non-detoxified hydrolysate, which shows that this treatment does not remove this compound. The IER treatment, on the other hand, removed 88% of the acetic acid present in the hydrolysate. Both detoxification treatments led to a significant removal of lignin derivatives of about 95%.

Carvalheiro et al. [28] also observed that acetic acid could not be removed from a spent grain hemicellulosic hydrolysate by active charcoal adsorption, while HMF and furfural concentrations were considerably decreased (68% and 92%, respectively) after this treatment. Villarreal et al. [20] observed that only small quantities of acetic acid could be removed from eucalyptus hemicellulosic hydrolysate by active charcoal adsorption, while this compound could be entirely removed from the hydrolysate by ion-exchange resins adsorption. Chandel et al. [29] observed that the detoxification of a sugarcane bagasse hemicellulosic hydrolysate with ion-exchange resins led to a high removal of acetic acid (83%), while the detoxification with pH alteration or active charcoal adsorption caused high reductions in the concentration of lignin derivatives (39% or 46%, respectively).

As can be seen in Fig. 1, both detoxification treatments led to an efficient removal of coloring compounds from the sugarcane bagasse hemicellulosic hydrolysate.

Hydrolysate Fermentation

Figure 2 shows the profiles of sugars and acetic acid consumptions, ethanol production, and cell growth during the bioconversions in the three hydrolysates, namely, non-detoxified, detoxified by pHAC, or detoxified by IER. As can be seen, glucose was consumed by the yeast in the first 48 h of fermentation when using the non-detoxified and the IER hydrolysates and in the first 24 h of fermentation when using the pHAC hydrolysate. Both the arabinose and the acetic acid were consumed together with the xylose in all the three fermentations, which confirms the versatility of this yeast in the utilization of all the main carbon sources encountered in hemicellulosic hydrolysates [15, 24]. Most importantly, the time necessary for the complete consumption of xylose, the main monosaccharide found in the hydrolysate, decreased considerably when using the detoxified hydrolysates instead of the non-detoxified hydrolysate.

The concentrations of ethanol and cells and the fermentative parameters determined for the three fermentations are presented in Table 2. As can be seen, the rate of ethanol

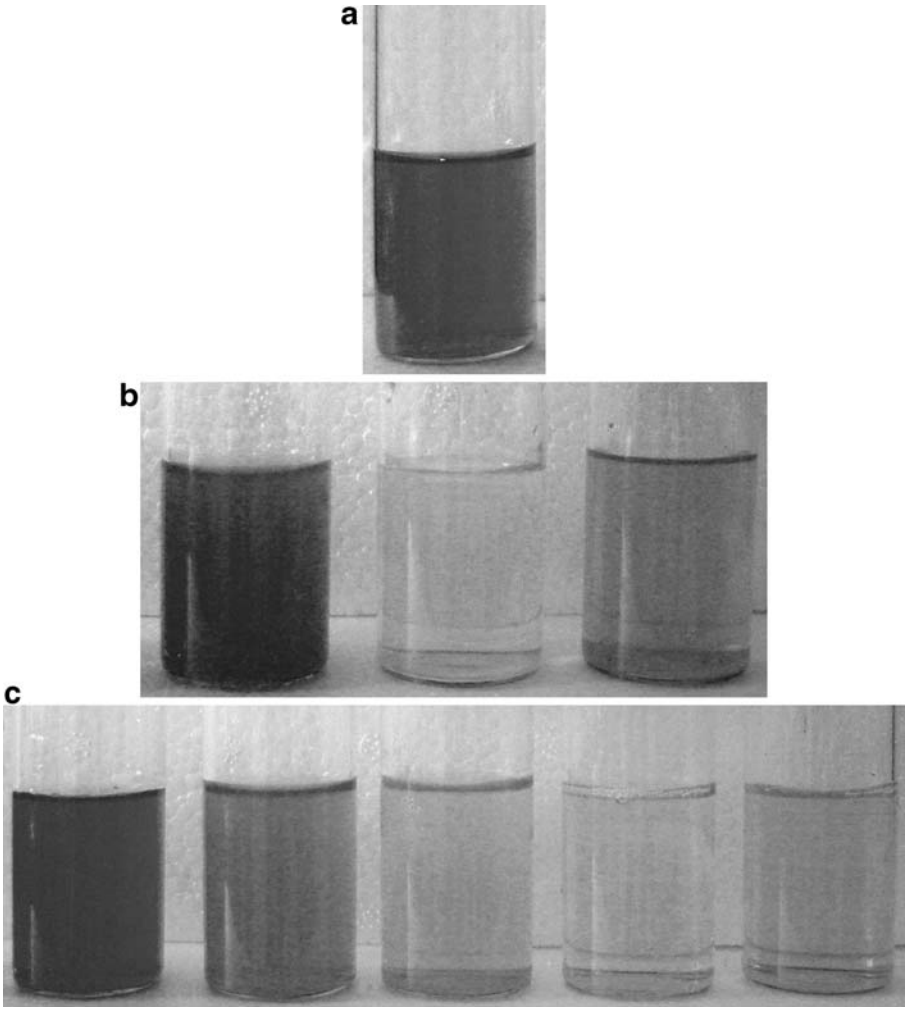

Fig. 1 Sugarcane bagasse hemicellulosic hydrolysate. **a** Untreated hydrolysate. **b** *Left to right*, hydrolysate after pH alteration, after active charcoal adsorption, and after autoclaving. **c** *Left to right*, hydrolysate after adsorption into resin A-860S, after adsorption into resin A-500PS, after adsorption into resin C-150, after centrifugation, and after autoclaving

production was considerably increased when using the treated hydrolysates. In fact, the time required for complete xylose consumption could be reduced in 60% by detoxifying the hydrolysates before the fermentations. The ethanol concentration achieved in the IER hydrolysate (7.5 g/L) was the highest, while that obtained using the non-detoxified hydrolysate (4.9 g/L) was the lowest. The sugars-to-ethanol conversion yields ($Y_{P/S}$) and volumetric productivities (Q_P) were 0.20 g/g and 0.04 g L^{-1} h^{-1} for the non-detoxified hydrolysate, 0.30 g/g and 0.13 g L^{-1} h^{-1} for the pHAC hydrolysate, and 0.30 g/g and 0.16 g L^{-1} h^{-1} for the IER hydrolysate, respectively. Such results are compatible with the understanding that the detoxification procedures reduced the concentrations of toxic compounds in the hydrolysate and improved its fermentability. Other

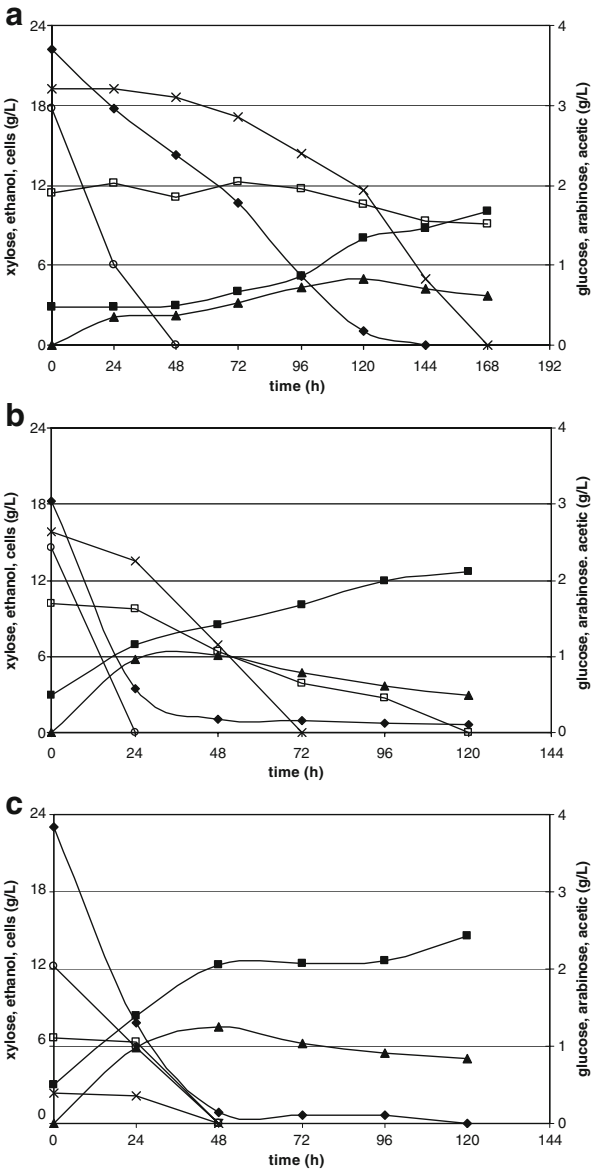

Fig. 2 Concentration of xylose (*diamond*), glucose (*circle*), arabinose (*empty square*), acetic acid (*ex*), ethanol (*triangle*), and cells (*filled square*) during the bioconversions in the three hydrolysates: **a** non-detoxified, **b** detoxified by pH alteration followed by active charcoal adsorption, and **c** detoxified by adsorption into ion-exchange resins

researchers have also documented that improvements in sugars-to-ethanol conversion were achieved when using detoxified hydrolysates [29–31] and that the hydrolysates treated by adsorption into IER performed better than those detoxified by other methods [29, 32].

Table 2 Fermentative parameters determined for the non-detoxified hydrolysate and for the hydrolysates detoxified by pHAC or by adsorption into IER.

Hydrolysate	Time (h)	Ethanol (g/L)	Cells (g/L)	$Y_{P/S}$ (g/g)	$Y_{X/S}$ (g/g)	Q_P (g L^{-1} h^{-1})
Non-detoxified	120	4.9	8.0	0.20	0.21	0.04
pHAC	48	6.1	8.5	0.30	0.27	0.13
IER	48	7.5	12.3	0.30	0.37	0.16

Initial cell concentration, 3 g cells (dry weight) per liter

$Y_{P/S}$ sugars-to-ethanol conversion yield, $Y_{X/S}$ sugars-to-cells conversion yield, Q_P volumetric productivity

Table 3 presents a selected compilation of literature data on ethanol bioproduction with different strains of the yeast *P. stipitis* in media prepared with hemicellulosic hydrolysates from different raw materials. As can be seen, the best sugars-to-ethanol conversion yield (0.30 g/g) and productivity (0.16 g L^{-1} h^{-1}) achieved in the present study are still low. In this context, it should be mentioned that we did not optimize the cell cultivation conditions before performing the present study; many of the authors referenced in Table 3 did. It is also likely that some undesirable compounds were still present in the detoxified hydrolysates and negatively affected the growth and ethanol production by the yeast. Such a behavior has already been reported [24].

Conclusion

The results achieved in the present study show that both detoxification strategies, pH alteration followed by active charcoal adsorption and adsorption into ion-exchange resins, reduce the content of inhibitory compounds of the hydrolysate. In consequence, the detoxified hydrolysates exhibit improved bioconversion rates and yields when compared to the non-detoxified hydrolysate. Adsorption into ion-exchange resins seemed to be a better detoxification strategy, promoting higher productivity and lower loss of fermentable sugars.

Table 3 Selected past studies on sugars-to-ethanol conversion by *P. stipitis* using hydrolysates prepared from different raw materials.

Microorganism	Raw material	$Y_{P/S}$ (g/g)	Q_P (g L^{-1} h^{-1})	Reference
P. stipitis NRRL Y-7124	Corncob	0.34	0.11	Eken-Saraçoglu and Arslan [15]
P. stipitis NCIM 3498	Wood	0.39	0.30	Gupta et al. [33]
P. stipitis FPL-Y-606	Mixed wood	0.49	0.26	Sreenath and Jeffries [34]
P. stipitis CBS 5773	Paper sludge	0.25	0.33	Marques et al. [35]
P. stipitis NRRL Y-7124	Rice straw	0.32	0.24	Silva [36]
P. stipitis CBS 5773	Sugarcane bagasse	0.38	0.46	Roberto et al. [37]
P. stipitis NRRL Y-7124	Sunflower seed hull	0.32	0.07	Telli-Okur and Eken-Saraçoglu [38]
P. stipitis NRRL Y-7124	Water hyacinth	0.19	0.02	Nigam [24]
P. stipitis NRRL Y-7124	Wheat straw	0.36	0.30	Nigam [26]

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References

1. Conab (Companhia Nacional de Abastecimento). Retrieved September 2009 from www.conab.gov.br.
2. Cenbio (Centro Nacional de Referência em Biomassa). Retrieved August 2003 from www.cenbio.org.br.
3. Fapesp (Fundação de Amparo a Pesquisa do Estado de São Paulo). Propriedades do bagaço da cana-de-açúcar. Revista Pesquisa FAPESP, 30ª ed., Abril 1998.
4. Sun, J. X., Sun, X. F., Zhao, H., & Sun, R. C. (2004). *Polymer Degradation and Stability, 84*, 331–339.
5. Selman-Housein, G., López, M. A., Ramos, O., Carmona, E. R., Arencibia, A. D., Menéndez, E., et al. (2000). *Developments in Plant Genetics and Breeding, 5*, 189–193.
6. Baudel, H. M., Zaror, C., & Abreu, C. A. M. (2005). *Industrial Crops and Products, 21*, 309–315.
7. Cerqueira Leite, R. C., Leal, M. R. L. V., Cortez, L. A. B., Griffin, W. M., & Scandiffio, M. I. G. (2009). *Energy, 34*, 655–661.
8. Pandey, A., Soccol, C. R., Nigam, P., & Soccol, V. T. (2000). *Bioresource Technology, 74*, 69–80.
9. Aguilar, R., Ramírez, J. A., Garrote, G., & Vásquez, M. (2002). *Journal of Food Engineering, 55*, 304–318.
10. Carvalho, W., Batista, M. A., Canilha, L., Santos, J. C., Converti, A., & Silva, S. S. (2004). *Journal of Chemical Technology and Biotechnology, 79*, 1308–1312.
11. Silva, S. S., Matos, Z. R., & Carvalho, W. (2005). *Biotechnology Progress, 21*, 1449–1452.
12. Felipe, M. G. A. (2004). In B. C. Saha & K. Hayashi (Eds.), *Lignocellulose biodegradation, ADC Symposium series 889*. Washington: American Chemical Society, pp. 300–315.
13. Palmqvist, E., & Hahn-Hagerdal, B. (2000). *Bioresource Technology, 74*, 17–24.
14. Carvalho, W., Santos, J. C., Canilha, L., Almeida e Silva, J. B., Felipe, M. G. A., Mancilha, I. M., et al. (2004). *Process Biochemistry, 39*, 2135–2141.
15. Eken-Saraçoglu, N., & Arslan, Y. (2000). *Biotechnological Letters, 22*, 855–858.
16. Martinez, A., Rodriguez, M. E., Wells, M. L., York, S. W., Preston, J. F., & Ingram, L. O. (2001). *Biotechnology Progress, 17*, 287–293.
17. Lee, W. G., Lee, J. S., Shin, C. S., Park, S. C., Chang, H. N., & Chang, Y. K. (1999). *Applied Biochemistry and Biotechnology, 77–79*, 547–559.
18. Mussatto, S. I., & Roberto, I. C. (2001). *Biotechnological Letters, 23*, 1681–1684.
19. Carvalho, W., Canilha, L., Mussatto, S. I., Dragone, G., Morales, M. L. V., & Solenzal, A. I. N. (2004). *Journal of Chemical Technology and Biotechnology, 79*, 863–868.
20. Villarreal, M. L. M., Prata, A. M. R., Felipe, M. G. A., & Almeida e Silva, J. B. (2006). *Enzyme Microbial Technology, 40*, 17–24.
21. Alves, L. A., Felipe, M. G. A., Almeida e Silva, J. B., Silva, S. S., & Prata, A. M. R. (1998). *Applied Biochemistry and Biotechnology, 70-72*, 89–98.
22. Canilha, L., Almeida e Silva, J. B., & Solenzal, A. I. N. (2004). *Process Biochemistry, 39*, 1909–1912.
23. Browning, B. L. (1967). *Method of wood chemistry*. New York: Wiley.
24. Nigam, J. N. (2002). *Journal of Biotechnology, 97*, 107–116.
25. Hahn-Hägerdal, B., Jeppsson, H., Olsson, L., & Mohagheghi, A. (1994). *Applied Microbiology and Biotechnology, 41*, 62–72.
26. Nigam, J. N. (2001). *Journal of Biotechnology, 87*, 17–27.
27. Nilvebrant, N. O., Reimann, A., Larsson, S., & Jonsson, L. J. (2001). *Applied Biochemistry and Biotechnology, 91–93*, 35–49.
28. Carvalheiro, F., Duarte, L. C., Lopes, S., Parajó, J. C., Pereira, H., & Gírio, F. M. (2005). *Process Biochemistry, 40*, 1215–1223.
29. Chandel, A. K., Kapoor, R. K., Singh, A., & Kuhad, R. C. (2007). *Bioresource Technology, 98*, 1947–1950.
30. Karimi, K., Emtiazi, G., & Taherzadeh, M. J. (2006). *Process Biochemistry, 41*, 653–658.
31. Cheng, K. K., Cai, B. Y., Zhang, J. A., Ling, H. Z., Zhou, Y. J., Ge, J. P., et al. (2008). *Biochemical Engineering Journal, 38*, 105–109.
32. Larsson, S., Reimann, A., Nilvebrant, N., & Jönsson, L. J. (1999). *Applied Biochemistry and Biotechnology, 77–79*, 91–103.
33. Gupta, R., Sharma, K. K., & Kuhad, R. C. (2009). *Bioresource Technology, 100*, 1214–1220.
34. Sreenath, H. K., & Jeffries, T. W. (2000). *Bioresource Technology, 72*, 253–260.
35. Marques, S., Alves, L., Roseiro, J. C., & Girio, F. M. (2008). *Biomass Bioenergy, 32*, 400–406.
36. Silva, J. P. A. (2007). Master dissertation, Universidade de São Paulo, Escola de Engenharia de Lorena, Lorena, São Paulo, Brazil.
37. Roberto, I. C., Lacis, L. S., Barbosa, M. F. S., & Mancilha, I. M. (1991). *Process Biochemistry, 26*, 15–21.
38. Telli-Okur, M., & Eken-Saraçoglu, N. (2008). *Bioresource Technology, 99*, 2162–2169.