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Pretreatment of Sugarcane Bagasse Hemicellulose Hydrolysate for Xylitol Production by *Candida guilliermondii*

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ABSTRACT

In order to remove or reduce the concentrations of toxic substances present in the sugarcane bagasse hemicellulose hydrolysate for xylose-to-xylitol bioconversion, the hydrolysate was pretreated by changing the initial pH level through the combination of different bases and acids with or without the subsequent addition of activated charcoal. Attention was given to the influence of the fermentation time as well.

The experiments were based on multivariate statistical concepts, with the application of fractional factorial design techniques to identify the important variables in the process. Subsequently, the levels of these variables were quantified by the response surface methodology, which permitted the establishment of the best pretreatment procedure with a xylose-to-xylitol bioconversion efficiency of 86.2%. This procedure consisted in increasing the pH of the hydrolysate from 0.5 to 7.0 with CaO and reducing it to 5.5 with H_3PO_4. Next, the hydrolysate was treated with activated charcoal (2.4%). The highest xylitol yield (0.79 g/g) corresponded to a productivity of 0.47 g/L/h.

Index Entries: Sugarcane bagasse; hydrolysate; pretreatment; xylitol; *Candida guilliermondii*.

INTRODUCTION

Sugarcane bagasse consists of 25 to 35% hemicellulose, of which D-xylose is the major component. This pentose can be directly fermented to xylitol by *Candida guilliermondii* as an alternative to the production of xylitol

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(1,2), which today is obtained by chemical process *(3)*. However, various toxic compounds such as furfural, hydroxymethylfurfural, and acetic acid are formed during hydrolysis of hemicellulose. Once present in the hydrolysate, these compounds are potential inhibitors of the microbial metabolism *(1,4)*.

Xylose in high concentrations favors the xylitol production by the yeast *(5,6)*. This could be achieved by concentrating the hydrolysate before using it as a culture medium. However, the more concentrated the hydrolysate, the lower the xylose consumption and xylitol production, because of the simultaneous increase in the concentrations of toxic compounds *(7)*. According to these authors, the inhibitory effect could be overcome by using an adequate hydrolysate pretreatment *(8)*, a hydrolysate-adapted yeast strain *(1)*, and/or bioreactors inoculated with a high cell concentration *(2)*.

Different procedures for pretreating the bagasse hemicellulose hydrolysate were tested with a view to improving the xylose-to-xylitol fermentation efficiency.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

Cells of *Candida guilliermondii* FTI 20037 *(9)* maintained at 4°C on malt-extract agar slant were inoculated in the culture medium containing 30 g of xylose supplemented with the following nutrients (g/L): $(NH_4)_2SO_4$ (2.0), $CaCl_2.2H_4O$ (0.1), and rice bran (20.0). Fifty milliliters of this medium was placed into 125-mL Erlenmeyer flasks and agitated at 200 rpm, at 30°C for 24 h. The cells were then centrifuged at 2200*g* for 15 min and washed in sterile distilled water. A suspension was prepared with the cell mass in sterile distilled water and utilized as the inoculum. For the experiments, the initial cell concentration was 0.45 g/L.

Preparation and Treatment of Hemicellulose Hydrolysate

The sugarcane bagasse was introduced into a 250-L reactor and mixed with concentrated H_2SO_4 (100 mg of acid per gram of dry matter) with a solid:liquid ratio of 1:10. After hydrolysis (121°C, 10 min), the hydrolysate containing (g/L) glucose (2.1), xylose (15.7), arabinose (2.3), acetic acid (3.9), furfural (0.06), and hydroximethylfurfural (0.05) was filtered and concentrated under vacuum at 70°C to increase the xylose concentration threefold. The hydrolysate thus obtained had the following composition (g/L): glucose (5.9), xylose (50.2), L-arabinose (6.5), acetic acid (6.9), furfural (0.03), hydroxymethylfurfural (0.15). The hydrolysate was treated as follows: the initial pH (0.5) was raised to 7.0 or 10.0 with CaO or $Ca(OH)_2$ (commercial powder); the hydrolysate was then acidified with concentrated H_2SO_4 or H_3PO_4 to pH 5.5, with or without the subsequent addition of 3% w/v

activated charcoal (refined powder). The charcoal was mixed with the hydrolysate under agitation (200 rpm) at 30°C for 1 h. In all the treatments the precipitate resulting from pH adjustment and from addition of activated charcoal was removed by vacuum filtration. These treatments resulted in several hydrolysates that were autoclaved at 111°C, 0.5 atm, to be used as culture media.

Medium and Fermentation Conditions

The hydrolysates containing 43–48 g/L xylose were supplemented with the same nutrients described in the inoculum preparation. Fermentations were carried out in 125-mL Erlenmeyer flasks containing 50 mL of the culture medium (pH 5.5), on a rotary shaker at 200 rpm at 30°C. The influence of the fermentation time was also evaluated (45 or 63 h of incubation).

Analytical Methods

The concentrations of glucose, L-arabinose, xylose, xylitol, acetic acid, furfural, and hydroxymethylfurfural were determined by high-pressure liquid chromatography (*2*). Cell concentration was determined by optical density at 600 nm.

Experimental Design

The effects of the treatments and fermentation time were appraised with the application of 2^{5-1} fractional factorial design with two replicates (*11,12*), to identify the variables important to the process (Table 1). The factors and their respective levels represented by the signs (−) and (+), were as follows: [A] acid (H_2SO_4, H_3PO_4), [B] base (CaO, $Ca(OH)_2$), [pH] pH (7.0, 10.0), [CA] activated charcoal (0.3%), and [FT] fermentation time (45 or 63 h). After statistical analysis, the factors with significant effects were used for additional experiments (2^2 full factorial design with a centered face and three replicates at the center point). The intermediary level for the selected factors was 1.5% of activated charcoal and 54 h of fermentation time.

The statistical analysis was performed using the STATGRAPHICS statistical software version 6.0 and STATISTICA program version 5.0.

RESULTS AND DISCUSSION

Table 1 presents the experimental matrix as well as the results achieved for the fermentation parameters of the xylose-to-xylitol bioconversion by *C. guilliermondii* as a function of different pretreatments of the sugarcane bagasse hemicellulose hydrolysate. The analysis of the estimated effect (Table 2) shows that for xylitol yield (Y p/s), the significant main effects ($p < 0.05$) are: fermentation time, interaction between acid

Table 1
Experimental Design and Fermentative Parameters in the Bioconversion Xylose-to-Xylitol by *C. guilliermondii* as a Function of Different Treatments of the Sugarcane Bagasse Hemicellulose Hydrolysate for the 2^{5-1} Fractional Fatorial Design[a]

Treatment	[A]	[B]	[pH]	[CA]	[FT]	$Y_{p/s}$ [b]	Q_p [c]
1	-	-	-	-	+	0.58	0.35
2	+	-	-	-	-	0.55	0.42
3	-	+	-	-	-	0.56	0.44
4	+	+	-	-	+	0.51	0.34
5	-	-	+	-	-	0.55	0.46
6	+	-	+	-	+	0.53	0.36
7	-	+	+	-	+	0.56	0.35
8	+	+	+	-	-	0.57	0.37
9	-	-	-	+	-	0.55	0.50
10	+	-	-	+	+	0.51	0.37
11	-	+	-	+	+	0.47	0.34
12	+	+	-	+	-	0.70	0.54
13	-	-	+	+	+	0.58	0.39
14	+	-	+	+	-	0.62	0.51
15	-	+	+	+	-	0.61	0.49
16	+	+	+	+	+	0.60	0.35

[a] Average of two replicate experiments.
[b] $Y_{p/s}$, xylitol produced (g)/xylose consumed (g).
[c] Q_p, volumetric productivity (g/L/h).

and activated charcoal, and the interaction between fermentation time and activated charcoal. With respect to productivity, charcoal and fermentation time have significant main effects ($p < 0.05$). Also significant is the effect of the interaction between these two factors (Table 2).

Despite the results of other researchers *(1,2,8,13,14,15)*, our experiments did not show a significant improvement in fermentation with overlining to pH 10.0. The base and pH did not present significant effects (at the 5% level) for any of the parameters evaluated. The increase in the pH of the hydrolysate to 7.0 or 10.0 during the treatment with base did not affect the fermentative parameters. Raising the pH to 7.0 implied a reduction in the amounts of base and acid to be added as well as in the time required for the treatment, which consequently affects the cost of the process. As for the base factor, according to Roberto et al. *(8)*, treating the sugarcane bagasse hydrolysate with CaO and $Ca(OH)_2$ increased the xylose consumption to 95 and 98%, respectively, whereas with KOH the consumption was only 56%. Increasing the pH of the hydrolysate with Ca^{2+} ions may cause precipitation of the toxic compounds present in the

Table 2
Effect Estimates, Standard Errors, *T*-Test for the Yield and Productivity in the Xylose-to-Xylitol Bioconversion by *C. guilliermondii*, in the 2^{5-1} Fractional Factorial Design

Factors and	Yield			Productivity		
Interactions	estimate	Standard errors	t	Estimate	Standard errors	t
Average	0.562	± 0.008	-	0.408	± 0.006	-
[A]	0.017	± 0.016	1.064	-0.006	± 0.012	0.468
[B]	0.016	± 0.016	0.985	-0.017	± 0.012	1.403
[pH]	0.027	± 0.016	1.695	-0.003	± 0.012	0.260
[CA]	0.028	± 0.016	1.774	0.049	± 0.012	4.104*
[FT]	-0.046	± 0.016	2.877*	-0.109	± 0.012	9.090*
[A] x [B]	0.028	± 0.016	1.774	0.003	± 0.012	0.260
[A] x [pH]	-0.011	± 0.016	0.670	-0.018	± 0.012	1.506
[A] x [CA]	0.038	± 0.016	2.404*	0.019	± 0.012	1.610
[A] x [FT]	-0.026	± 0.016	1.616	0.006	± 0.012	0.468
[B] x [pH]	0.003	± 0.016	0.197	-0.022	± 0.012	1.818
[B] x [CA]	0.017	± 0.016	1.064	0.006	± 0.012	0.468
[B] x [FT]	-0.029	± 0.016	1.852	-0.008	± 0.012	0.675
[pH] x [CA]	0.021	± 0.016	1.301	-0.001	± 0.012	0.052
[pH] x [FT]	0.027	± 0.016	1.695	0.013	± 0.012	1.091
[CA] x [FT]	-0.034	± 0.016	2.168*	-0.039	± 0.012	3.273*

[a] Significant at 5% probability level.

hydrolysate, which can then be removed by filtration *(16)*. Since the base did not have a significant effect, the treatment with CaO was preferred, for its low cost.

As is evident from Table 2, the activated charcoal has a positive main effect and the effect of the interaction between the charcoal and the acid is also positive. Thus, H_3PO_4 was selected for the next experiments. A 2^2 factorial design with a centered face and three replicates at the center point was used to obtain the mathematical model representing this fermentative process by the response-surface methodology. The matrix referring to this design and to the fermentative parameters Y p/s and Qp) is shown in Table 3. As can be seen, treatment 4 provided the highest xylitol yield (0.79 g/g), whereas treatment 1 provided the lowest yield (0.62 g/g). This difference is equivalent to 22%. With respect to productivity, the variation between maximum and minimum values (0.52 g/L/h for treatment 2 and 0.40 g/L/h for treatment 8), corresponded to an increase of 23%, which confirms the

Table 3
Experimental Design and Fermentative Parameters of the Xylose-to-Xylitol Bioconversion by *C. guilliermondii* as a Function of Different Treatments of Sugarcane Bagasse Hemicellulose Hydrolysate for the 2^2 Factorial Design with a Centered Face and Three Replicates at the Center Point

Treatment	[CA]	[FT]	$Y_{p/s}$	Q_p
1	-1	-1	0.62	0.44
2	+1	-1	0.75	0.52
3	-1	1	0.64	0.41
4	+1	1	0.79	0.47
5	-1	0	0.63	0.44
6	+1	0	0.74	0.50
7	0	-1	0.73	0.49
8	0	1	0.76	0.40
9	0	0	0.76	0.47
10	0	0	0.75	0.44
11	0	0	0.76	0.51

Table 4
Regression Coefficients, Standard Errors, *T*-Test, and Significance Level for the Model Representing the Yield in the Xylose-to-Xylitol Bioconversion by *C. guilliermondii* in the 2^2 Factorial Design with a Centered Face and Three Replicates at the Center Point

Factors	coefficients	Standard errors	t	P
Constant	0.7511	±0.00655	114.6194	-
X_1	0.065	±0.00523	12.4648	0.0001*
X_2	0.015	±0.00523	2.8765	0.0347*
X_1X_2	0.005	0.00639	0.7829	0.4691
X_1^2	-0.0576	±0.00803	-7.1813	0.0008*
X_2^2	0.0024	0.00803	0.2951	0.7798

[a] Significant at 5% probability level.

influence of the treatments on the xylose-to-xylitol bioconversion. The maximum value of xylitol yield (0.79 g/g) was obtained when 3% w/v activated charcoal was utilized. Parajo et al. *(17)* found that the treatment of eucalyptus hydrolysate with activated charcoal (0.5%) increased the xylitol yield from 0.4 to 0.6 g/g, as compared with the untreated hydrolysate.

Table 4 presents the regression coefficients, standard errors, *t* values, and significance levels for the model representing xylitol yield. At 5% probability level, the activated charcoal, the fermentation time, and the charcoal quadratic term presented significant effects. The conversion efficiency increased by 17% when charcoal was used and by 4% when the fermentation time was changed from 45 to 63 h. The linear and quadratic

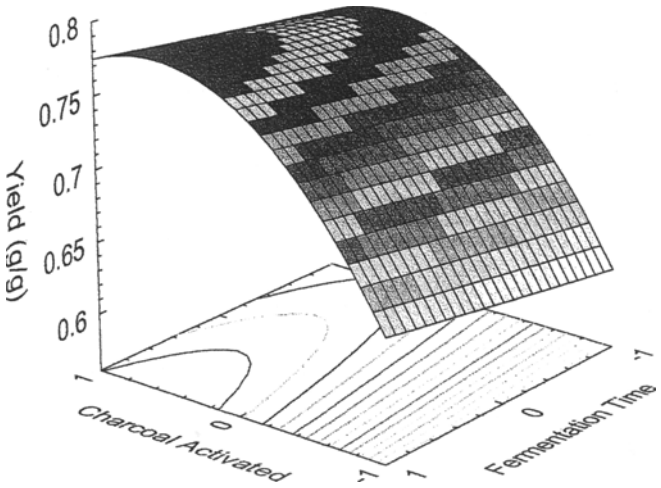

Fig. 1. Response surface and contour lines described by the Y_1 model representing the yield in the xylose-to-xylitol bioconversion by *C. guilliermondii* in sugarcane bagasse hemicelullosic hydrolysate.

terms presented an optimum significance level. The regression variance analysis shows that the mathematical model is significant. This is confirmed by the determination coefficient ($R^2 = 0.98$), which indicates that the selected model is suitable for the process and allows an estimation of 98% variance as a function of the activated charcoal concentration and fermentation time. The mathematical model for the xylitol yield is represented by the equation *(1)*:

$$Y_1 = 0.754 + 0.070\ X_1 + 0.015\ X_2 - 0.057\ X_1^2 \qquad (1)$$

where Y_1 represents the yield, X_1 the activated charcoal ratio, and X_2 the fermentation time.

Solving this mathematical model for the optiminal conditions predicts a yield of 0.79 g/g using 2.4% charcoal and a fermentation time of 63 h. This optiminal region can be observed in Fig. 1, which depicts the response surface and contour lines described by the Y_1 model.

Table 5 presents the regression coefficient, standard errors, t values, and significance level for the model representing the xylose-to-xylitol bioconversion productivity at 5% significance level. In this case, charcoal and fermentation time have significant effects. The results lead to the conclusion that using charcoal in the hydrolysate treatment increases the xylitol productivity by 14%, whereas changing the fermentation time from 45 to 63 h reduces it by 12%. The regression variance analysis and the coefficient determination of the model ($R^2 = 0.78$) reveal that the mathematical model is significant and can be expressed by the equation *(2)*:

Table 5
Regression Coefficients, Standard Errors, *T*-Test, and Significance Level for the Model Representing the Productivity of the Xylose-to-Xylitol Bioconversion by *C. guilliermondii* in the 2^2 Fatorial Design with a Centered Face and Three Replicates at the Center Point

Factors	Coefficients	standard errors	t	P
Constant	0.4695	± 0.0137	34.3671	-
X_1	0.0333	± 0.0109	3.0662	0.0279*
X_2	-0.0283	± 0.0109	-2.6062	0.0479*
X_1X_2	-0.005	± 0.0133	-0.3755	0.7227
X_1^2	0.0063	± 0.0167	0.3775	0.7213
X_2^2	-0.0187	± 0.0167	-1.1168	0.3149

[a] Significant at 5% probability level.

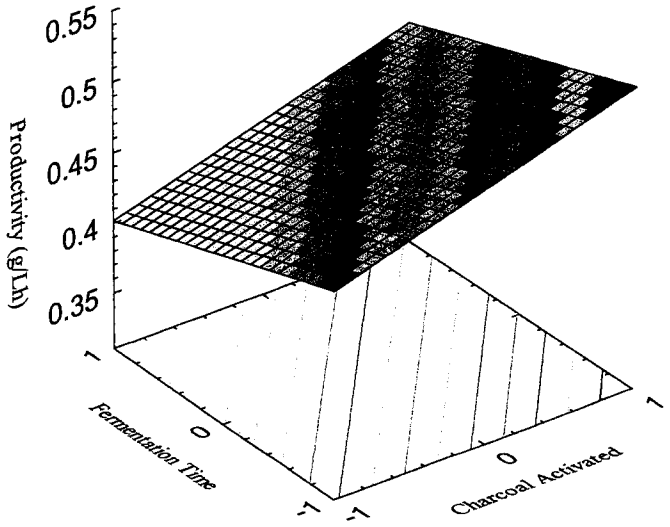

Fig. 2. Response surface and contour lines described by the Y_2 model representing the productivity in the xylose-to-xylitol bioconversion by *C. guilliermondii* in sugarcane bagasse hemicelullosic hydrolysate.

$$Y_2 = 0.463 + 0.033\ X_1 - 0.031\ X_2 \quad (2)$$

where Y_2 represents the productivity, X_1 the activated charcoal ratio, and X_2 the fermentation time.

Figure 2 shows the response surface and the contour lines described by the Y_2 model. The best results were predicted from coded values 1 and -1, corresponding to 3% charcoal and 45 h of fermentation time, respectively. The mathematical model makes it possible to obtain the maximum point for these factors, resulting in a productivity of 0.52 g/L/h.

CONCLUSION

Developing a new methodology for the pretreatment of sugarcane bagasse hemicellulose hydrolysate is indispensable for increasing xylose-to-xylitol conversion efficiency. An extensive factorial design showed that the best conditions for this bioconversion were: increasing the pH level to 7.0 with CaO, reducing it to 5.5 with H_3PO_4, and adding 2.4% of activated charcoal. Under these conditions the highest xylitol yield (0.79 g/g) and productivity (0.52 g/L/h) were obtained after 63 and 45 h of fermentation time, respectively. The charcoal reduced the concentration of the toxic compounds furfural and hydroxymethylfurfural in the hydrolysate, whereas the concentration of acetic acid was not significantly affected. From previous studies conducted in our laboratory *(1,2,10)*, which are in agreement with other reports *(4,16,18)*, it is evident that the acetic acid inhibits the xylose metabolism of the yeast. The effect of this acid mainly depends on its concentration level *(10)* and the pH of the fermentation. Phenolic compounds, derived from the breakdown of lignin, have also been considered as inhibitors of various bioconversion processes employing hydrolysates obtained from lignocellulosic materials *(8,17,19)*. Although the concentration levels of these compounds were not measured in our experiments, activated charcoal has been reported by several authors as able to remove phenolics from hydrolysate *(8,17,20)*.

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