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Application of Factorial Design to the Study of Xylitol Production from Eucalyptus Hemicellulosic Hydrolysate

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Abstract

This study deals with the bioconversion of xylose into xylitol by *Candida guilliermondii* FTI 20037 using eucalyptus hemicellulosic hydrolysate obtained by acid hydrolysis. The influence of various parameters (ammonium sulfate, rice bran, pH, and xylose concentration) on the production of xylitol was evaluated. The experiments were based on multivariate statistical concepts, with the application of factorial design techniques to identify the most important variables in the process. The levels of these variables were quantified by the response surface methodology, which permitted the establishment of a significant mathematical model with a coefficient determination of $R^2 = 0.92$. The best results (xylitol = 10.0 g/L, yield factor = 0.2 g/g, and productivity = 0.1 g/[L·h]) were attained with hydrolysate containing ammonium sulfate (1.1 g/L), rice bran (5.0 g/L), and xylose (initial concentration of 60.0 g/L), after 72 h of fermentation. The pH of fermentation was adjusted to 8.0 and the inoculum level utilized was 3 g/L.

Index Entries: Factorial design; eucalyptus hemicellulosic hydrolysate; *Candida guilliermondii*; xylitol.

Introduction

Wood from eucalyptus is a most suitable raw material for the production of pulp and paper. This type of tree is cultivated by reforestation, and because it grows rapidly, an entire eucalyptus forest can be replaced in a few years after the trees have been cut. The production of paper and other

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materials derived from wood can thus be ensured without destroying native forests. For these reasons the industrial use of this type of wood has been largely stimulated in Brazil in recent years *(1)*. However, only 51.7% of the total dry weight of eucalyptus trees is utilized by the Brazilian paper mills, the branches and foliage remaining in the fields as lignocellulosic residues. By means of acid hydrolysis, such residues originate a hemicellulosic hydrolysate rich in sugars that can be fermented by several microorganisms *(2)* and converted into products of commercial interest, such as ethanol *(3)*, microbial protein *(4,5)* and xylitol *(6–9)*.

Xylitol ($C_5H_{12}O_5$), a sugar alcohol obtained from xylose, is also generated during the metabolism of carbohydrates in animals and humans. Its concentration in the human blood varies from 0.03 to 0.06 mg/100 mL *(10)*. Xylitol is also present in fruits and vegetables *(11)*, but at low concentrations, which makes its production from these sources economically unfeasible *(12)*. As a sweetener, xylitol can very well substitute for conventional sugars *(13)*. Its sweetening power is comparable to that of sucrose and is higher than that of sorbitol and mannitol *(14)*. Furthermore, xylitol has anticariogenic properties. Because it is not consumed by *Streptococcus mutans*, one of the microorganisms of the buccal flora, xylitol prevents the formation of acids that attack the tooth enamel *(15)*. In addition to reducing dental caries, xylitol also promotes tooth enamel remineralization by reversing small lesions. This happens because when in contact with xylitol, the saliva seems to be favorably influenced; xylitol's chemical composition induces a significant increase in calcium ions and phosphate *(16)*. Owing to groups in its molecules, xylitol does not cause food to have darkening reactions of the Maillard type *(10)*. Therefore, xylitol is appropriate for food processing at high temperatures. Because it cannot be fermented by yeasts, its utilization for the preparation of syrups and soft drinks is highly advantageous, dispensing with the need for product pasteurization and the addition of preservatives to allow for 5-mo storage in sealed flasks and bottles. For all these characteristics, xylitol is a feedstock of great interest to food, odontological, and pharmaceutical industries *(10)*.

The present study deals with the bioconversion of xylose into xylitol by the yeast *Candida guilliermondii* FTI 20037 in concentrated *Eucalyptus* wood hydrolysates. A fractional factorial design followed by response surface methodology permitted the observation of the influences of pH, xylose concentration, and nutrients, such as $(NH_4)_2SO_4$ and rice bran, on xylitol production.

Materials and Methods

Preparation of Hemicellulose Hydrolysate

The hemicellulose hydrolysate was obtained by cooking *Eucalyptus grandis* chips (acid hydrolysis described by Almeida e Silva *[17]*) averaging $20 \times 10 \times 5$ mm and containing 29% moisture. The ratio of chips (w/v) to 0.35% sulfuric acid solution was 1:4.5, the temperature was 156°C, and the

Table 1
Factor Levels Used for 2^4 Centered Face Factorial Design with Two Center Points

Factor	Abbreviation	Level (–1)	Level (0)	Level (+1)
Ammonium sulfate (g/L)	[AS]	1.0	1.5	3.0
Rice bran (g/L)	[RB]	5.0	12.5	20.0
pH	[pH]	4.0	6.0	8.0
Xylose concentration (g/L)	[XC]	15.0	30.0	45.0

time of hydrolysis was 27 min. The hydrolysate obtained was maintained in 50-L containers stored in a cold chamber at 4°C. To increase xylose content, the hydrolysate was concentrated three times in a 4-L evaporator operating at 75°C. Analyses made before and after concentrating the hydrolysate revealed the following composition: 2.54 and 6.50 g/L of glucose, 19.17 and 59.21 g/L of xylose, 0.41 and 0.98 g/L of arabinose, 5.03 and 6.33 g/L of acetic acid, 1.25 g/L of phenols (the content of phenols was measured only before the three concentrations, not after). The hydrolysate was treated as reported by Alves et al. *(18)*. The treatment consisted of raising the initial pH from 1.9 to 7.0 with CaO and decreasing it to 5.5 with H_3PO_4. Next, 10% activated charcoal was added to the hydrolysate under 200 rpm agitation for 1 h at 30°C. At each pH alteration and after treatment with charcoal, the precipitate formed was removed by vacuum filtration in a porcelain funnel and Klabin qualitative filter paper. The pH was then adjusted with H_3PO_4 or 5 N NaOH, according to the values determined by the statistical design (Table 1), and the hydrolysate was autoclaved at 111°C for 15 min. After treating the hydrolysate, the content of xylose was 15 g/L in the hydrolysate before the first concentration, and 45 g/L after the third concentration. Last, the nutrients were added to the hydrolysate, as given in Table 1.

Preparation of Microorganism and Inoculum

The yeast *C. guilliermondii* FTI 20037, described by Barbosa et al. *(2)*, was maintained at 4°C on malt-extract agar slants. The cells, aged for 3–5 d, were inoculated in the culture medium containing 30.0 g/L of D-xylose, 7.0 g/L of D-glucose, 20.0 g/L of rice bran extract, 2.0 g/L of $(NH_4)_2SO_4$, and 0.1 g/L of $CaCl_2 \cdot 2H_2O$. Fifty milliliters of this medium was placed into 125-mL Erlenmeyer flasks and incubated at 200 rpm at 30°C for 24 h. Afterward, the cells were collected by centrifugation (2000*g* for 15 min) and resuspended in sterile distilled water. From this suspension an adequate volume (0.5–1.0 mL) was taken to attain the desired inoculum concentration (3.0 g/L or 10^8 cells/mL).

Fermentation Conditions

Erlenmeyer flasks (125 mL) containing 50 mL of treated hydrolysate were supplemented with different combinations of nutrients used for the

fermentation runs (according to the experimental matrix) on a rotary shaker at 200 rpm at 30°C for 72 h.

Analytical Methods

Glucose, arabinose, xylose, xylitol, and acetic acid concentrations were determined by high-performance liquid chromatography as previously described by Canettieri *(19)*. To determine the content of the phenolic compounds in the original hydrolysate, the colorimetric method described by Kim and Yoo *(20)* and Guerra *(21)* was employed.

Statistical Analysis

A 2^4 centered face factorial design with center point *(22,23)* was used to study the effects of the combined components of the medium for xylitol production. Ammonium sulfate, rice bran, pH, and xylose concentrations were selected as experimental factors. Table 1 gives their levels. The experimental data were analyzed by the response surface regression procedure (Statgraphics Program version 6.0). This method is based on the polynomial model, which can be represented by Eq. 1:

$$Y_i = b_0 + \sum b_i x_j + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 + e_i \quad (1)$$

in which Y_i is the response variable (dependent); b is the regression coefficients; X is the independent variables or experimental factor levels; and e_i is the experimental error.

Results and Discussion

Based on the results obtained by Canettieri *(19)* in the process of xylose conversion into xylitol from eucalyptus hemicellulosic hydrolysate, the present study was conducted to determine optimum factor levels and to improve the process.

The experimental matrix as well as the results achieved for the production of xylitol are displayed in Table 2. These results are low in comparison with the results obtained with bagasse hemicellulosic hydrolysate. High xylitol production (34.0 g/L) was attained by growing *C. guilliermondii* FTI 20037 in bagasse hydrolysate containing 54.5 g/L of initial xylose using a 24-h-old inoculum (3 g/L) at pH 5.5 *(24)*. From the results presented in Table 2, it is possible to conclude that the amount of xylitol formed was strongly dependent on the pH. When the pH of the hydrolysate was low (4.0) the formation of xylitol was lower (runs 1–4, 9–12, 21, and 23). The highest xylitol concentration (7.61 g/L) was found in hydrolysate with the pH adjusted to 8.0 (run 13). In studying the effect of the pH level on the cultivation of the same yeast in bagasse hydrolysate, Felipe et al. *(25)* also detected a strong inhibition in the production of xylitol at pH ≤ 4.5. It is known that during hydrolysis, acetic acid is formed as a consequence of the deacetylation of the acetylated pentosan and that the acetic acid toxicity is pH dependent. By virtue of its ability to traverse the cell membrane freely,

Table 2
Experimental Matrix Used in 2^4 Centered Face Factorial Design with Two Center Points and Amounts of Xylitol Produced

Run	[AS]	[RB]	[pH]	[XC]	Xylitol (g/L)
1	–1	–1	–1	–1	0.65
2	+1	–1	–1	–1	0.56
3	–1	+1	–1	–1	0.71
4	+1	+1	–1	–1	1.66
5	–1	–1	+1	–1	5.06
6	+1	–1	+1	–1	2.50
7	–1	+1	+1	–1	2.93
8	+1	+1	+1	–1	2.63
9	–1	–1	–1	+1	0.09
10	+1	–1	–1	+1	0
11	–1	+1	–1	+1	0.53
12	+1	+1	–1	+1	0
13	–1	–1	+1	+1	7.61
14	+1	–1	+1	+1	5.32
15	–1	+1	+1	+1	6.82
16	+1	+1	+1	+1	5.56
17	–1	0	0	0	6.45
18	+1	0	0	0	2.64
19	0	–1	0	0	4.54
20	0	+1	0	0	3.02
21	0	0	–1	0	0.37
22	0	0	+1	0	3.99
23	0	0	0	–1	0.86
24	0	0	0	+1	6.43
25	0	0	0	0	1.80
26	0	0	0	0	2.04

the undissociated form of acetic acid acts as a membrane protonophore and causes its inhibitory effect by bringing about the acidification of the cytoplasm *(26)*. The literature also reports that with *C. guilliermondii*, the pH range for optimal growth with glucose and xylose is 5.5–6.0 *(25)*. The acetic acid content of hemicellulose hydrolysates varies in the range of 2.0–10.0 g/L, depending on the type of biomass used *(26)*. Acetic acid concentrations higher than 3.0 g/L inhibit the ability of *C. guilliermondii* to convert xylose into xylitol *(25)*. Probably, the acetic acid present in these hydrolysates (6.0 g/L) acts as an inhibitor of the yeast metabolism. According to Herrero et al. *(27)*, the acetic acid toxicity has been attributed to its undissociated form. The acid then diffuses into the cell, causing a decrease in the cytoplasmic pH. Once inside the cell, the acid dissociates owing to the relatively high intracellular pH. Therefore, the proton gradient through the membrane cannot be maintained. The loss of energy and of the transport of various nutrients occurs as a consequence. The results presented in Table 2 were used to estimate the main effects of the variables and their interaction effects.

Table 3
Estimated Effects, Standard Errors, and Student's *t*-Test for Xylitol Production Using 2^4 Centered Face Factorial Design with Two Center Points

Factor and interaction	Estimate	Standard error	Student's *t* value
Average	2.611	±0.684	—
[AS]	–0.952	±0.382	2.492[a]
[RB]	–0.600	±0.467	1.285
[pH]	4.285	±0.438	9.783[a]
[XC]	1.616	±0.385	4.197[a]
[AS][RB]	0.668	±0.403	1.657
[AS][pH]	–0.931	±0.363	2.565[a]
[AS][XC]	–0.188	±0.382	0.492
[RB][pH]	–0.406	±0.377	1.077
[RB][XC]	0.247	±0.403	0.613
[pH][XC]	1.219	±0.461	2.644[a]
$[AS]^2$	2.191	±0.918	2.387[a]
$[RB]^2$	0.756	±0.926	0.816
$[pH]^2$	–2.640	±1.585	1.666
$[XC]^2$	0.006	±0.491	0.012

[a]Significant at 5% probability level (t = 2.20156).

The regression coefficients for a polynomial model in order to predict and quantify the level of the factors for the xylitol production were also estimated. The effects of pH and xylose concentration were positive and significant (Table 3). The model suggests the existence of a positive interaction between these factors. This indicates that simultaneous increases in the levels of these factors cause the xylitol production to increase. Also, the ammonium sulfate factor was significant, but had a negative effect. Rice bran did not have a significant effect, and hence a very low amount of this nutrient was utilized in the fermentation (5 g/L). Only the significant effects at a probability level below 5% were used to determine the quadratic model.

Table 4 presents the regression coefficients, standard errors, student's *t* values, and significance levels for the model representing xylitol production. The regression variance analysis shows that the mathematical model is significant ($p < 0.05$). This is confirmed by the determination coefficient ($R^2 = 0.92$), which indicates that the selected model is suitable for the process and allows an estimation of 92% variance as a function of ammonium sulfate, pH, and xylose concentration.

The mathematical model for xylitol production represented only by the significant terms is described in Eq. 2:

$$Y = 3.53 - 0.98X_1 + 1.62X_3 + 1.01X_1^2 \quad (2)$$

in which Y is the concentration of xylitol (grams/liter) for the model; and X_1 and X_3 are the ammonium sulfate and xylose concentration, respectively.

Table 4
Regression Coefficients, Standard Errors, Student's *t*-Test, and Significance Level for Model Representing Xylitol Production by *C. guilliermondii* in Eucalyptus Hydrolysate Using 2^4 Centered Face Factorial Design with Two Center Points

Factor[a]	Coefficient	Standard error	Student's *t* value	*p*
Constant	1.609	0.305	5.266	0.0000
X_1	–0.492	0.187	–2.628	0.0166[b]
X_2	1.923	0.165	11.669	0.0000[b]
X_3	0.756	0.163	4.644	0.0002[b]
X_1X_2	–0.486	0.175	–2.775	0.0121[b]
X_2X_3	0.865	0.153	5.636	0.0000[b]
X_1^2	1.010	0.347	2.913	0.0089[b]

[a]X_1 represented the [AS]; X_2 the [pH], and X_3 the [XC].
[b]Significant at 5% probability level.

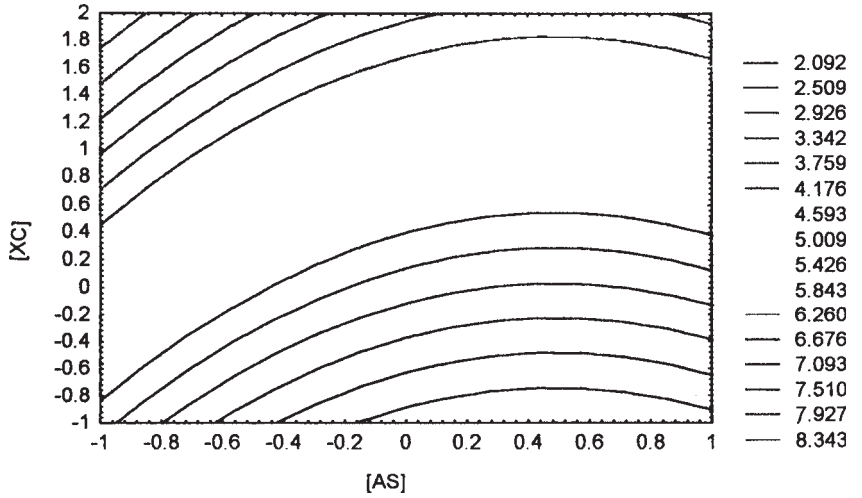

Fig. 1. Contour lines described by the quadratic model for xylitol production in eucalyptus hemicellulosic hydrolysate.

Solving this mathematical model for the optimal conditions predicts a production of 8.34 g/L of xylitol for the coded values of –0.90 and 1.97, corresponding to 1.1 g/L of ammonium sulfate and 60.0 g/L of xylose concentration, respectively, with the pH of hydrolysate initially adjusted to 8.0. This optimal region can be observed in Fig. 1, which depicts the contour plots showing the expected xylitol values as a function of the pH described by the *Y* model. The model indicated that the best results could be achieved by increasing the xylose concentration in the hydrolysate. However, the five- and sixfold increases in the original concentration led to sugar and volume losses after treating the hydrolysate, which makes this procedure unfeasible. Each of the observed xylitol production values, $Y_{j(o)}$, is com-

Table 5
Observed Responses and Predicted Values for Xylitol Production

Obs. Nr.	Actual value ($Y_{j(o)}$)	Predicted value ($Y_{j(p)}$)	Residual ($Y_{j(o)} - Y_{j(p)}$)
1	0.65	0.80	–0.15
2	0.56	0.84	–0.28
3	0.71	1.10	–0.39
4	1.66	0.94	0.71
5	5.06	4.65	0.41
6	2.50	2.05	0.45
7	2.93	3.90	–0.97
8	2.63	1.83	0.80
9	0.09	–0.05	0.14
10	0.00	0.23	–0.23
11	0.53	0.50	0.03
12	0.00	0.31	–0.31
13	7.61	7.40	0.20
14	5.32	6.12	–0.80
15	6.82	7.62	–0.80
16	5.56	5.30	0.26
17	6.45	4.93	1.52
18	2.64	3.23	–0.59
19	4.54	3.20	1.34
20	3.02	2.88	0.14
21	0.37	–0.01	0.38
22	3.99	3.65	0.33
23	0.86	2.00	–1.14
24	6.43	5.99	0.44
25	1.80	2.63	–0.83
26	2.04	2.70	–0.66

pared with the values predicted from the model, $Y_{j(p)}$, in Table 5. The comparison of the residuals with the error variance S_e^2(=3.41) indicates that none of the individual residual exceeds twice the square root of the residual variance. All of these considerations indicate an excellent adequacy of the regression model *(28)*.

A separate experiment using the predicted optimal conditions was conducted, and the experimental xylitol production found was 10.0 g/L, whereas the value predicted by the model was 8.3 g/L. This experimental finding is in close agreement with the model prediction, considering the standard deviation. Xylitol production can be improved with the study of wood hydrolysate treatment, because in this study we used a treatment proposed by Alves et al. *(18)* for sugarcane bagasse. Parajó et al. *(29)* found that the ratio of hydrolysate to charcoal was an influential variable, and obtained a xylitol production of 20.0 g/L using an initial xylose concentration of 45.0 g/L.

Conclusion

The result obtained with the model was 10.0 g/L whereas the predicted value was 8.3 g/L. However, this result can still be improved by studying the composition of the eucalyptus hydrolysate in more detail, in order to identify and quantify the toxic compounds that inhibit the growth of the *C. guilliermondii* FTI 20037, and consequently affect the production of xylitol. The model proposed can be considered suitable for the process of xylitol production by *C. guilliermondii* in eucalyptus hemicellulosic hydrolysate.

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