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Bioconversion of Rice Straw Hemicellulose Hydrolysate for the Production of Xylitol

Effect of pH and Nitrogen Source

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ABSTRACT

Xylitol production by the yeast *Candida guilliermondii* was evaluated in a rice straw hemicellulose hydrolysate under different conditions of initial pH and nitrogen source. Xylitol production was significantly affected ($p < 0.05$) by the nitrogen source, pH, and the interaction between these factors. The best yield and productivity were observed at initial pH of 5.3 in medium containing ammonium sulfate as nitrogen source. Under these conditions, the xylitol yield factor ($Y_{p/s}$) was 0.68 g/g and volumetric productivity (Q_p) was 0.51 g/L.h.

Index Entries: Rice straw; hemicellulose hydrolysate; xylitol; xylose; *Candida guilliermondii*.

INTRODUCTION

Lignocellulosic materials in the form of agricultural and forest residues accumulate in large amounts all over the world every year. Cereal straw is a major subproduct of agriculture in developing countries, with an annual production of more than 560 million tons in Asian countries *(1)*. In Brazil, these residues are also quite abundant, especially in the southern region where approx 3.85 t of straw are generated per each ha of rice planted. The availability and the possibility of converting these materials into different products by biotechnological processes have called the attention of several researchers to the utilization of the total biomass components. Over the last few years, several studies have been performed aimed at the utilization of the xylose-rich hemicellulosic fraction as a substrate for the production of ethanol *(2,3)*, microbial protein *(4)*, acetic acid *(5)*, and xylitol *(6–8)*.

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Xylitol is a xylose pentitol of wide application in the food, pharmaceutical, and dental industry owing to its sweetening power equivalent to sucrose and anticariogenic properties *(9,10)*. Furthermore, xylitol can be used by diabetic patients, since its metabolism is not insulin dependent *(10)*. Despite these many advantages, the use of xylitol has been limited by its high price. This cost is a result of the extensive purification steps needed for the preparation of a pure xylose solution, which is essential for the chemical process *(11)*. The fermentative process of xylitol production has been considered an alternative to the chemical process, since it does not require initial xylose purification *(12)*. Although many yeast species are able to convert xylose to xylitol with high yields in semisynthetic media *(13–16)*, the fermentation conditions may differ from those employed with media containing hemicellulose hydrolysate. This is because of the substrate complexity, which normally includes different compounds that are toxic to the microbial metabolism. By using appropriate environmental conditions, such as initial pH value *(17)* and inoculum concentration *(18)*, negative effects can be minimized.

Research on the utilization of lignocellulosic residues by fermentative processes has been carried out in our laboratory since 1987 *(2,7,8)*. The possibility of converting xylose to xylitol using rice straw hemicellulose hydrolysate obtained by acid hydrolysis has been demonstrated recently *(19)*. In the present article, we report the fermentation results using rice straw hemicellulose hydrolysate under different conditions of initial pH and nitrogen source by the yeast *Candida guilliermondii*.

MATERIALS AND METHODS

Microorganisms

The yeast *C. guilliermondii* FTI 20037, previously selected by Barbosa et al. *(15)*, was used for the study. Cultures were maintained on malt extract agar at 4°C.

Inoculum Preparation

The inoculum was prepared by growing cells in 125-mL Erlenmeyer flasks containing 50 mL medium of the following composition: 20.0 g/L xylose, 3.0 g/L $(NH_4)_2SO_4$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, and 20.0 g/L rice bran. The culture was incubated at 30°C for 24 h under shaking (200 rpm). The cells were separated by centrifugation at 1600*g* for 10 min and used to prepare a suspension in sterile distilled water at the concentration of 3.7 g/L dry wt. This suspension was used to inoculate the fermentation medium to obtain an initial cell concentration of 0.5 g/L.

Preparation and Treatment of Hemicellulose Hydrolysate

The raw material used was rice straw from the region of Lorena, São Paulo, Brazil. Before extraction of the hemicellulose fraction, the straw was dried in the sun and reduced to 100-mm long and 1-mm thick fragments. The hemicellulose hydrolysate was obtained by acid hydrolysis in a 25-L steel reactor (AISI 316) under the following conditions: temperature of 145°C, reaction time of 20 min, and H_2SO_4 (0.7% [w/v]) using a liquid-to-solid ratio of 10:1. After the hydrolysis, the liquid fraction (hemicellulosic) was concentrated at 70°C under vacuum until a xylose concentration of approx 80 g/L was reached. The concentrated hydrolysate (pH 1.37) was treated with NaOH (pellets) to a pH of 10.0 and adjusted to 5.0 by addition

of H_2SO_4 (72% [w/w]). After each change in pH, the precipitate was removed by centrifugation at 1000*g* for 15 min.

Medium and Fermentation Conditions

The hydrolysate treated as described above was submitted to pH adjustment to 4.0, 5.0, and 6.0 with NaOH or H_2SO_4 and then supplemented with the same nutrients as used for inoculum culture, except xylose. The effect of the nitrogen source was verifed by keeping fixed the C/N ratio, using ammonium sulfate (3.0 g/L) or urea (1.3 g/L) to provide a concentration of 0.6 g nitrogen/L. The nutrient solutions were sterilized separately by autoclaving at 121°C for 20 min, except the urea solution that was sterilized by filtration through membranes with 0.22-μm pores. All experiments were carried out in 125-mL Erlenmeyer flasks containing 50 mL of the fermentation medium with shaking (200 rpm) at 30°C. The fermentations were monitored by periodic analyses of the samples.

Analytical Methods

The concentrations of xylose, glucose, arabinose, xylitol, and acetic acid were determined by high-performance liquid chromatography (HPLC) using a model HP 1082B Hewlett-Packard (Miami, FL) apparatus, under the following conditions: Aminex (Bio-Rad, Hercules, CA) HPX87H column at 45°C, 0.01*N* sulfuric acid as eluent, flow rate 0.6 mL/min, detector with a 16× refraction index, and 20-μL sample volume.

Furfural and hydroxymethylfurfural were analyzed by HPLC using the apparatus described under the following conditions: RP18 (HP-79916B) column at 25°C, acetonitrile:water (1:8) and 1% acetic acid as eluent, flow rate of 0.8 mL/min, visible UV-light detector at 276 nm, and 20-μL volume sample.

Cell growth was monitored by measuring absorbance at 600 nm using a Micronal model B34211 spectrophotometer. Cell concentration was calculated by a calibration curve (dry wt × absorbance) prepared with cells grown in hydrolysate supplemented with the same nutrients and using the same conditions as those employed for inoculum preparation. Incubation time was 48 h.

Statistical Analysis

The effect of pH, nitrogen source, and the interaction of these two factors was evaluated by a two-way factorial design *(20)*. The analysis of variance was done using the Statgraphics statistical software version 2.7.

RESULTS AND DISCUSSION

Chemical Composition of the Hydrolysate

The chemical composition of the rice straw hemicellulose hydrolysate obtained by acid hydrolysis is given in Table 1. The hydrolysis conditions used resulted in a hydrolysate containing a mixture of xylose, glucose, and arabinose at the proportions of 70, 20, and 10%, respectively. The presence of furfural and hydroxymethylfurfural, products of pentoses and hexoses degradation, respectively, was also detected. These compounds represent a sugar loss, and their pres-

Table 1
Composition of Rice Straw Hemicellulose Hydrolysate Obtained by Acid Hydrolysis

Components	Concentration, g/L	
	Original hydrolysate	Concentrated hydrolysate
Xylose	16.44	79.3
Glucose	4.40	22.6
Arabinose	2.35	13.4
Furfural	0.41	0.13
Hydroxymethylfurfural	0.096	0.51
Acetic acid	1.40	1.82

ence in the hydrolysates is undesirable owing to their inhibitory effects on microbial metabolism *(21,22)*. In addition to these two compounds, acetic acid resulting from acetylated sugars was also observed. This has a negative effect on different bioconversion processes *(23–26)*.

The hydrolysate evaporation (under vacuum) gave variations on the original component levels (Table 1). Furfural concentration was reduced in about 30%, whereas hydroxymethylfurfural concentration increased proportionally to water removal during evaporation, by about five times, owing to the low volatility of this compound. The levels of these compounds present in rice straw hydrolysate, however, were lower than those reported as inhibitors of the growth of the *Saccharomyces cerevisiae* yeast *(18)*.

Regarding acetic acid levels, only a small variation (1.40–1.82 g/L) was attained during the evaporation process. Higher acetic acid concentrations on the order of 10 g/L have been detected in wood and sugar cane bagasse hemicellulose hydrolysates *(27,28)*. According to these authors, acetic acid was found to be the most important inhibitor to fermentation. The low concentration attained in rice straw values is expected to improve the xylitol yields achieved by *C. guilliermondii*.

Influence of pH and of Nitrogen Source

The results of the fermentation runs using rice straw hemicellulose hydrolysate supplemented with $(NH_4)_2SO_4$ or urea as nitrogen source at different initial pH values are illustrated in Figs. 1–3 (A,B). Independently of the nitrogen source employed at pH 4.5, no sugar consumption and a slight cell growth were observed during the first 15 h of fermentation (Fig. 1A,B). These results suggest the existence of a phase of yeast adaptation as a function of the low initial pH value utilized. The presence of acetic acid in the hydrolysate (Table 1) may have contributed to this behavior, since the toxic effect of acetic acid is a function of the concentration of nondissociated acid and therefore pH dependent *(29)*. These observations were confirmed by the behavior of cell growth in the fermentations carried out at initial pH of 5.3 and 6.0 (Figs. 2 and 3, A,B). Under these conditions, high growth rates and simultaneous utilization of xylose and glucose were observed since the beginning of fermentation. Regarding xylitol production, the higher and similar values were observed in media at pH 5.3 and 6.0, independently of the nitrogen source utilized (Figs. 2 and 3, A,B). However, at pH 4.5 (Fig. 1A,B) the use of urea promoted a 25%

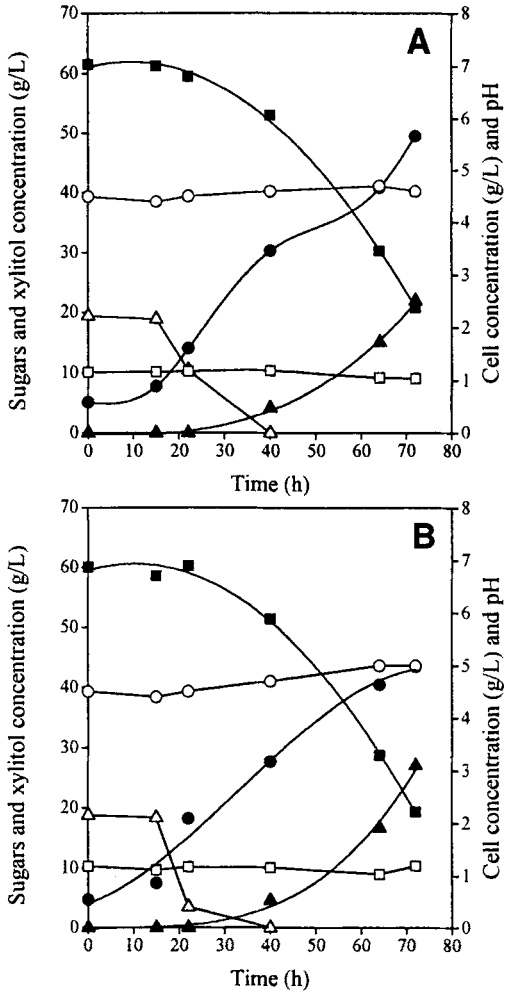

Fig. 1. Fermentation of rice straw hemicellulose hydrolysate by *C. guilliermondii* using $(NH_4)_2SO_4$ **(A)** or urea **(B)** as nitrogen source at pH 4.5. △, glucose; ■, xylose; □, arabinose; ▲, xylitol; ●, cell mass; and ○, pH.

increase in xylitol formation when compared to ammonium sulfate. This behavior can be related to the increase in the pH medium owing to the release of ammonia and CO_2 by urea hydrolysis *(30)*. Furthermore, the consumption of acetic acid by the yeast may also have contributed to this raise in the pH value *(19)*. A similar behavior in terms of pH variation in the presence of urea was observed in *Candida shehatae* cultures on medium containing xylose as a carbon source *(31)*.

With respect to the fermentative parameters (Table 2), it can be seen that the highest xylitol yields were obtained under different conditons of pH and nitrogen source. These yields were 0.68 g/g for the medium containing ammonium sulfate at pH 5.3 and 0.66 g/g in the presence of urea at 4.5. Despite similar yields, higher volumetric productivity was attained when ammonium sulfate was used as nitrogen source. These results reveal the important role of the nitrogen source as a function of pH in the bioconversion of xylose to xylitol using hemicellulose hydrolysates. According to Silva et al. *(16)*, in a semisynthetic medium, the amount of xylitol

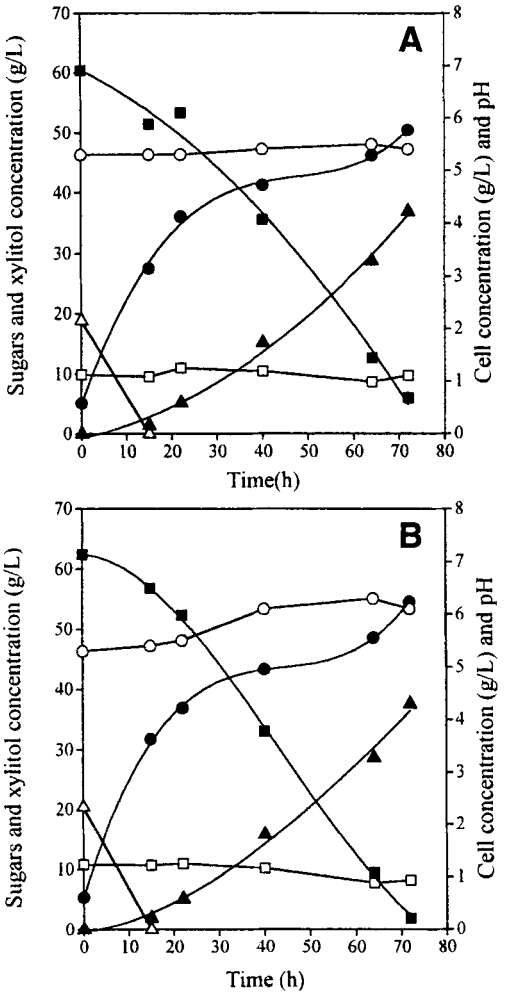

Fig. 2. Fermentation of the rice straw hemicellulose hydrolysate by *C. guilliermondii* using $(NH_4)_2SO_4$ **(A)** or urea **(B)** as nitrogen source at pH 5.3. △, glucose; ■, xylose; □, arabinose; ▲, xylitol; ●, cell mass; and ○, pH.

produced by the yeast *C. guilliermondii* was not affected by the type of nitrogen source utilized. The composition of the fermentation medium may possibly account for these differences.

The results concerning the influence of pH and nitrogen source on xylitol production were also analyzed statistically by analysis of variance of the major effects and their interactions (Table 3). The data revealed a significant effect ($p < 0.05$) of these factors and their interaction in the production of xylitol by *C. guilliermondii* in a rice straw hydrolysate. The effect of the interaction was probably caused by the increase in the pH level when urea was used as nitrogen source at pH 4.5 (Fig. 1 B).

These results demonstrate that the effect of pH cannot be analyzed individually because of the interaction with nitrogen source and possibly with other medium components, such as acetic acid, which is commonly present in hemicellulose hydrolysates.

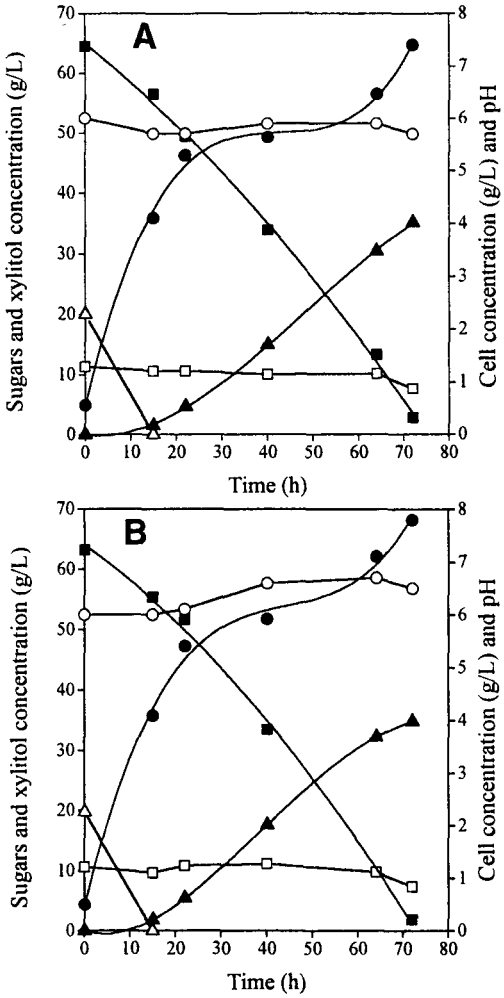

Fig. 3. Fermentation of the rice straw hemicellulose hydrolysate by *C. guilliermondii* using $(NH_4)_2SO_4$ **(A)** or urea **(B)** as nitrogen source at pH 6.0. △, glucose; ■, xylose; □, arabinose; ▲, xylitol; ●, cell mass; and ○, pH.

Table 2
Effect of pH and Nitrogen Source on Xylitol Fermentation of Rice Straw Hemicellulose Hydrolysate by *Candida guilliermondii* after 72 h

Nitrogen source[a]	Ammonium sulfate			Urea		
Initial pH	4.5	5.3	6.0	4.5	5.3	6.0
Xylitol, g/L	21.86	36.92	35.16	27.11	37.57	34.84
Q_p, g/L·h	0.32	0.51	0.49	0.38	0.49	0.48
$Y_{p/s}$, g/g	0.54	0.68	0.57	0.66	0.62	0.57
$Y_{x/s}$, g/g	0.08	0.07	0.08	0.07	0.07	0.09
Efficiency, %	60	74	62	72	64	62

[a]Qp = xylitol volumetric productivity; Yp/s, g xylitol produced/g xylose consumed; $Y_{x/s}$, g cells produced/g (xylose + glucose) consumed; efficiency was calculated assuming a theoretical maximum of 0.917 g/g *(1)*.

Table 3
Analysis of Variance for Xylitol Production
by *C. guilliermondii* as a Function of pH and Nitrogen Source (NS)

Source of variation	Degrees of freedom	Sum of squares	Means square	Calculated F	Level of significance
Main effects	3	572.25	190.75	72.83	0.0000
NS	1	15.60	15.60	5.96	0.0311[a]
pH	2	556.54	278.32	106.27	0.0000[a]
Interactions	2	26.52	13.26	5.06	0.0254
NS × pH	2	26.52	13.26	5.06	0.0254[a]
Residue	12	31.43	2.62		
Total (corr.)	17	630.19			

[a]Significant at the 5% level of probability.

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REFERENCES

1. Srnivasan, M. C., Ramarao, R., Deshmukh, S. S., and Sahasrabudhe, N. A. (1983), *Enzyme Microb. Technol.* **5(4),** 269–272.
2. Roberto, I. C., Lacis, L., Barbosa, M. F. S., and Mancilha, I. M. (1991), *Process Biochem.* **26,** 15–21.
3. Lawford, H. G. and Rousseau, J. D. (1993), *Biotechnol. Lett.* **15(5),** 505–510.
4. Meyer, P. S., du Preez, J. C., and Kilian, S. G. (1992), Syst. Appl. Microbiol. **15(1),** 161–165.
5. Brownell, J. E. and Nakas, J. P. (1991), *J. Ind. Microbiol.* **7,** 1–6.
6. Strehaino, P. and Dupuy, M. L. (1990), French Patent 2,641,545.
7. Roberto, I. C., Felipe, M. G. A., Lacis, L., Silva, S. S., and Mancilha, I. M. (1991), *Bioresource Technol.* **36(3),** 271–275.
8. Felipe, M. G. A., Mancilha, I. M., Vitolo, M., Roberto, I. C., and Silva, S. S. (1993), *Arquivos de Biologia e Tecnologia* **36(1),** 103–114.
9. Bar, A. (1986), in *Alternative Sweeteners,* O'Brien-Nabors, L. and Gelardi, R., eds., Marcel Dekker, New York, pp. 185–216.
10. Pepper, T. and Olinger, P. M. (1988), *Food Technol.* **42(10),** 98–106.
11. Melaja, J. and Hämäläinen, L. (1977), US Patent 4,008,285.
12. Heikkilä, H., Nurmi, J., Rahkila, L., and Töyrylä, M. (1992), US Patent 5,081,026.
13. Nolleau, V., Preziosi-Belloy, L., Delgenes, J. P., and Navarro, J. M. (1993), *Curr. Microbiol.* **27,** 191–197.
14. Meyrial, V., Delgenes, J. P., Moletta, R., and Navarro, J. M. (1991), *Biotechnol. Lett.* **13(4),** 281–286.
15. Barbosa, M. F. S., Medeiros, M. B., Mancilha, I. M., Schneider, H., and Lee, H. (1988), *J. Ind. Microbiol.* **3,** 241–251.
16. Silva, S. S., Mancilha, I. M., Queiroz, M. A., Felipe, M. G. A., Roberto, I. C., and Vitolo, M. (1994), *J. Basic Microbiol.* **34(3),** 205–208.
17. van Zyl, C., Prior, B. A., and du Preez, J. C. (1991), *Enzyme Microb. Technol.* **13,** 82–86.
18. Chung, I. S. and Lee, Y. Y. (1985), *Biotechnol. Bioeng.* **27,** 308–315.
19. Roberto, I. C., Mancilha, I. M., Souza, C. A., Felipe, M. G. A., Sato, S., and Castro, H. F. (1994), *Biotechnol. Lett.* **16(11),** 1211–1216.
20. Box, G. E. P., Hunter W. G., and Hunter, J. S. (1978), in *Statistics for Experimenters,* Wiley, New York, pp. 228–244.

21. Tran, A. V. and Chambers, R. P. (1985), *Biotechnol. Lett.* **7(11),** 841–846.
22. Weigert, B., Klein, C., Rizzi, M., Lauterbach, C., and Dellweg, H. (1988), *Biotechnol. Lett.* **10(12),** 895–900.
23. Ferrari, M. D., Neirotti, E., Albornoz, C., and Saucedo, E. (1992), *Biotechnol. Bioeng.* **40,** 753–759.
24. Bjorling, T. and Lindman, B. (1989), *Enzyme Microb. Technol.* **11(4),** 240–246.
25. Meyer, P. S., du Preez, J. C., and Kilian, S. G. (1992), *J. Ind. Microbiol.* **9,** 109–113.
26. Felipe, M. G. A., Vieira, D. C., Vitolo, M., Silva, S. S., Roberto, I. C., and Mancilha, I. M. (1995), *J. Basic Microbiol.* **35,** 171–177.
27. Perego, P., Converti, A., Palazzi, E., Del Borghi, M. D., and Ferraiolo, G. (1990), *J. Ind. Microbiol.* **6,** 157–164.
28. van Zyl, C., Prior, B. A., and du Preez, J. C. (1988), *Appl. Biochem. Biotechnol.* **17,** 357–369.
29. Lawford, H. G. and Rousseau, J. D. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 301–322.
30. Phaff, H. J., Miller, M. W., and Mark, E. W. (1978), in *The Life of Yeasts,* 2nd ed., Harvard University Press, Cambridge, MA, pp. 168,169.
31. Jeffries, T. W. (1985), *Biotechnol. Bioeng. Symp.* **15,** 149–166.