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Xylose Reductase Activity of *Candida guilliermondii* During Xylitol Production by Fed-Batch Fermentation

Selection of Process Variables

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Abstract

Xylose reductase activity of *Candida guilliermondii* FTI 20037 was evaluated during xylitol production by fed-batch fermentation of sugarcane bagasse hydrolysate. A 2^{4-1} fractional factorial design was used to select process variables. The xylose concentrations in the feeding solution (S_F) and in the fermentor (S_0), the pH, and the aeration rate were selected for optimization of this process, which will be undertaken in the near future. The best experimental result was achieved at S_F = 45 g/L, S_0 = 40 g/L, pH controlled at 6.0, and aeration rate of 1.2 vvm. Under these conditions, the xylose reductase activity was 0.81 U/mg of protein and xylitol production was 26.3 g/L, corresponding to a volumetric productivity of 0.55 g/(L·h) and a xylose xylitol yield factor of 0.68 g/g.

Index Entries: Xylitol; sugar cane bagasse; *Candida guilliermondii*; xylose reductase; fed batch.

Introduction

Xylitol is a five-carbon sugar alcohol of wide application in food, pharmaceutical, and odontologic industries owing to its anticariogenic and cariostatic properties *(1–3)*. Although xylitol is currently obtained by hydrogenation of xylose produced from xylan-containing plant materials, its microbial production is gaining interest as an alternative method. The

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Table 1
Basic Composition of Hydrolysate Before Overlime Treatment

	Concentration factor		
Components	1	3	4
Glucose	0.97	2.76	3.88
Xylose	16.00	48.67	65.54
Arabinose	1.50	4.54	5.78
Acetic acid	2.42	5.38	5.78
Furfural	0.19	0.21	0.23
Hydroxymethylfurfural	0.30	0.90	1.12

efficiency and productivity of this fermentation chiefly depends on the microorganism and the process conditions employed. Xylose reductase (XR) and xylitol dehydrogenase (XD) also play an important role in xylose metabolism by yeasts. The continued understanding of the mechanisms regulating the activities of these enzymes will allow the establishment of optimum conditions for the production of xylitol.

In the present study, we evalulated XR activity during xylitol production by *Candida guilliermondii* FTI 20037 from sugarcane bagasse hemicellulosic hydrolysate by fed-batch process using an exponential feeding rate. Different levels of initial aeration and pH control, as well as of xylose concentration both in the fermentor (S_0) and in the feeding solution (S_F), were used. A fractional factorial design was employed to verify the effects of these variables on XR activity.

Materials and Methods

Preparation of Hemicellulosic Hydrolysate

Sugarcane bagasse was hydrolyzed in a 250-L reactor at 121°C for 10 min with H_2SO_4 (solid:liquid ratio of 1:10). The hydrolysate was concentrated under vacuum at 70°C to increase xylose concentration, as shown in Table 1. The hydrolysates were treated in order to reduce the concentrations of toxic substances generated by acid hydrolysis. The initial pH was raised to 7.0 with CaO and acidified to pH 5.5 with H_3PO_4. Subsequently, 2.4% (w/v) activated charcoal was added to the hydrolysates, which were then left under agitation (200 rpm) at 30°C for 1 h *(4)*. The precipitates resulting from all stages of the treatment were removed by vacuum filtration.

Microorganism

The experiments were conducted with *C. guilliermondii* FTI 20037 as described by Barbosa et al. *(5)*. The yeast stock culture was maintained on malt-extract agar slants at 4°C. The inoculum was cultivated in a medium composed of 30 g/L of xylose and supplemented with the following nutrients: 20 g/L of rice bran extract, 0.1 g/L of $CaCl_2$(2H_2O, 5 g/L of and

Table 2
Parameters Obtained from Kinetic Study of Batch Process

	S_0 (g/L)	
Parameter	20	40
t_0 (h)	29	13
μ_0 (h^{-1})	0.035	0.014
X_0 (g/L)	3.4	2.7
$(Y_{X/S})_0$ (g/g)	0.059	0.084

$(NH_4)_2SO_4$. The cultivation was carried out in 125-mL Erlenmeyer flasks containing 50 mL of medium, on a rotatory shaker set at 200 rpm, at 30°C for 24 h.

Fermentation Conditions

The fermentation medium used for obtaining the initial culture was composed of hydrolysate (concentration factor of 4, Table 1) and the nutrients just described. The experiments were carried out in a 1.5-L fermentor (Bioflo III; New Brunswick) at 30°C, 300 rpm, an aeration rate of 0.4 vvm, and a medium work volume of 650 mL. The initial cell concentration was 1 g/L (dry wt).

The process employed was fed-batch mode, and the feeding was initiated when the xylose concentration in the fermentor reached 20 and 40 g/L. Feeding media (prepared with hydrolysate) with different xylose concentration levels were employed. To keep these levels at about 20 and 40 g/L, an exponential feeding rate (ϕ) was used, according to the following mathematical model *(6)*:

$$\phi = A \cdot \exp(\mu_0 \cdot t) \tag{1}$$

in which

$$A = \frac{\mu_0 \cdot X_0 \cdot V_0}{(Y_{X/S}) \cdot (S_F - S_0)} \tag{2}$$

The parameters μ_0, X_0, S_0, and $(Y_{X/S})_0$ were obtained from a previous kinetic study of batch process (Table 2).

Experimental Design

A 2^{4-1} fractional factorial design as described by Box et al. *(7)* was used (Table 3).

Preparation of Cell-Free Extracts

Cells were harvested by centrifugation at 800*g* and washed in phosphate buffer (50 n*M*, pH 7.2), and the cell pellets were stored in a freezer. For enzymatic analysis, cell extracts were thawed and disrupted by a sonic

Table 3
2^{4-1} Fractional Factorial Design Matrix

Experiment	x_1	x_2	x_3	x_4	S_0 (g/L)	S_F (g/L)	pH[a]	Aeration rate (vvm)
1	–1	–1	–1	–1	20	45	NC	1.2
2	+1	–1	–1	+1	40	45	NC	2.0
3	–1	+1	–1	+1	20	68	NC	2.0
4	+1	+1	–1	–1	40	68	NC	1.2
5	–1	–1	+1	+1	20	45	C	2.0
6	+1	–1	+1	–1	40	45	C	1.2
7	–1	+1	+1	–1	20	68	C	1.2
8	+1	+1	+1	+1	40	68	C	2.0

[a]NC, pH not controlled; C, pH controlled at 6.0 with 1.0 *N* H_2SO_4.

disrupter. Cell homogenates were then centrifuged at 6700*g* (MR 1812; Jouan, Winchester, VA) at 4°C for 10 min, and the supernatant solution was used for enzymatic assays.

Enzyme Assays

XR and XD activities were determined spectrophotometrically at 340 nm at 25°C *(8)*. Enzyme units were defined as micromoles of NAD(P)H or NAD^+ oxidized/reduced using an extinction coefficient of 6.22×10^{-3}. Specific activities were expressed as units per milligram of protein based on protein determinations according to the method of Bradford *(9)* using bovine serum albumin as the standard.

Analytical Methods

Glucose, xylose, and xylitol concentrations were determined by liquid chromatography *(10)*. Cell concentration was measured by turbidimetry at 600 nm.

Results and Discussion

Figure 1 presents the results of xylitol production and the consumption of xylose and acetic acid for the sugarcane bagasse fermentation. The results indicate that *C. guilliermondii* was able to accumulate xylitol in all the conditions tested. However, the amount of xylitol formed was strongly dependent on the experimental conditions.

Table 4 shows the results for the fed-batch fermentation of sugarcane bagasse hydrolysate. The best experimental result for XR activity (0.81 U/mg of protein) and xylose xylitol yield factor (0.68 g/g) was achieved in experiment 6. Under these conditions, the volumetric productivity was 0.55 g/(L·h). In this experiment, the xylose xylitol yield factor was 49% higher than that attained with batch fermentation (data not shown), for the same amount of xylitol. This demonstrates that the

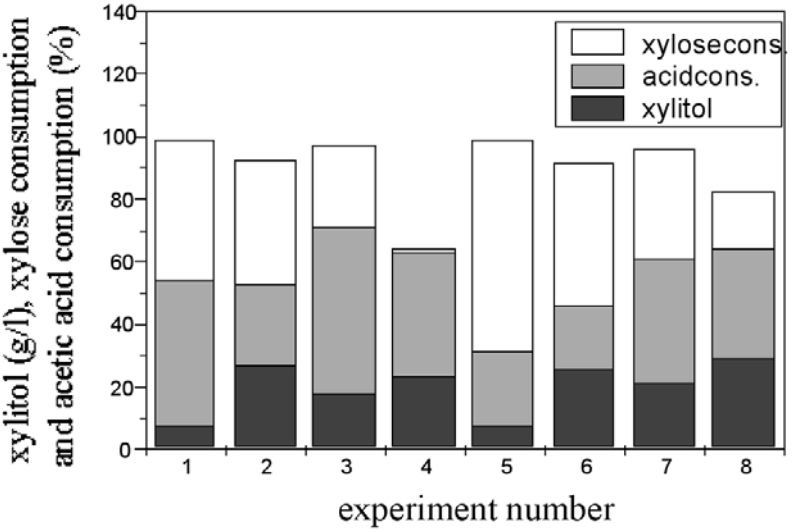

Fig. 1. Xylitol production and xylose and acetic acid consumption after 48 h of fermentation of sugarcane bagasse hydrolysate under different conditions.

Table 4
Fermentative Parameters for Fed-Batch Fermentation of Sugarcane Bagasse Hydrolyzate

Experiment	XR (U/mg protein)	XR/XD	$Y_{P/S}$ (g/g)	Q_{PV} (g/[L·h])
1	0.55	1.13	0.64	0.17
2	0.81	1.80	0.66	0.57
3	0.63	1.50	0.63	0.37
4	0.62	2.50	0.61	0.48
5	0.61	1.39	0.64	0.16
6	0.81	1.40	0.68	0.55
7	0.79	1.94	0.65	0.45
8	0.78	1.49	0.67	0.62

fed-batch process is effective for xylitol production from sugarcane bagasse hydrolysate by *C. guilliermondii*.

The experimental results presented in Table 4 were used to estimate the main effects of the variables and their interaction effects over the fermentation parameters. A normal probability plot was used to verify the significance of these effects. For XR activity, the normal probability plot (Fig. 2) shows that the main effects [S_0] and [pH] and the interaction [pH] × [AR] were significant.

The interaction between pH and aeration rate affects XR activity probably owing to the acetic acid inhibition of the metabolism. The effect of this acid mainly depends on its concentration level and the pH of the fermentation *(10–12)*.

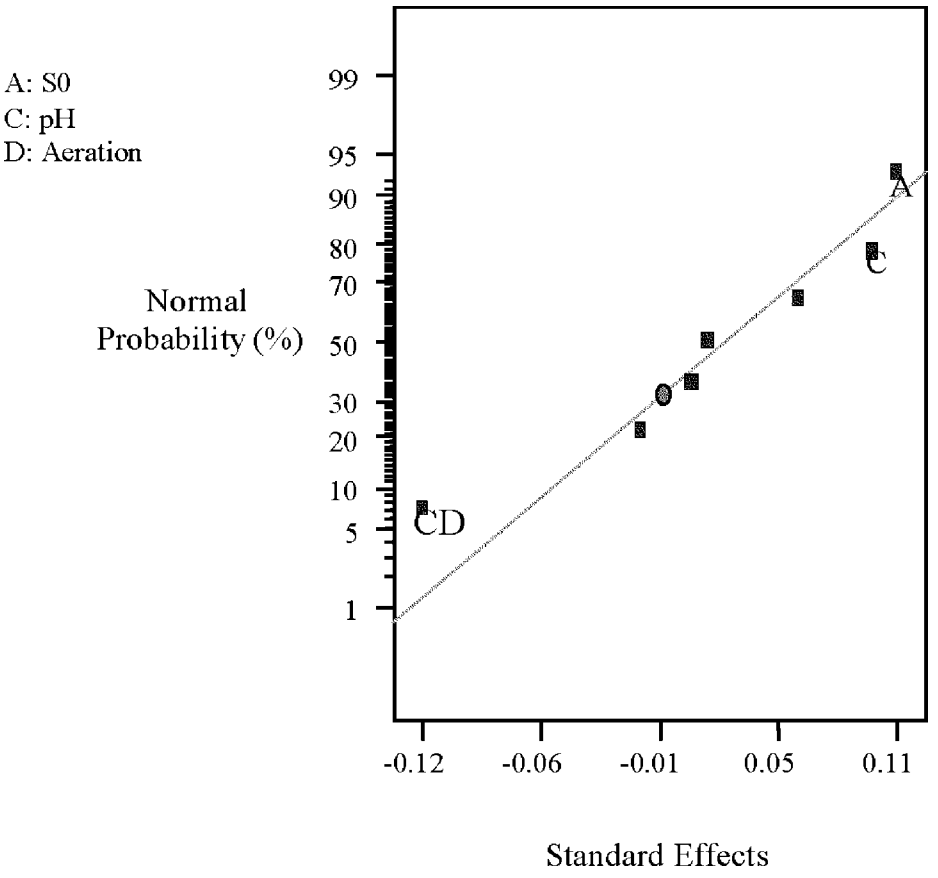

Fig. 2. Normal probability plot for XR activity.

Figures 3 and 4 present the normal probability plot for XR/XD activity ratio and volumetric productivity, respectively. As can be seen in Fig. 3 for the XR/XD ratio, the main effects [S_0] and [S_F] and the interaction [S_F]×[AR] were significant. For the volumetric productivity, the effects [S_0] and [S_F] and their interaction were significant (Fig. 4). The analysis of variance (ANOVA) for these responses is shown in Table 5. The ANOVA demonstrates that the variables studied have significant effects over the fermentative and enzymatic parameters.

The variables S_0, S_F, pH, and aeration were selected for the optimization of this process, which will be undertaken in the near future.

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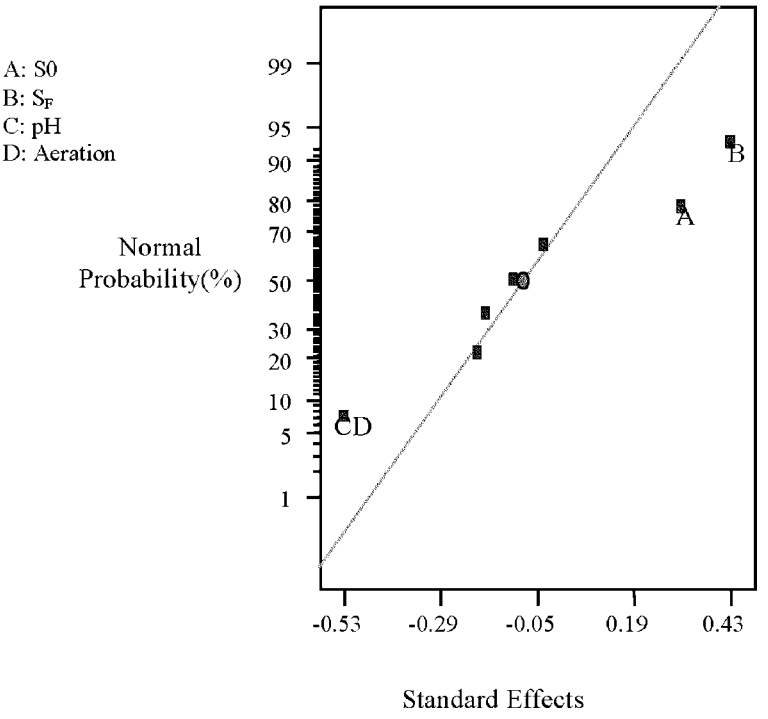

Fig. 3. Normal probability plot for XR/XD activity ratio.

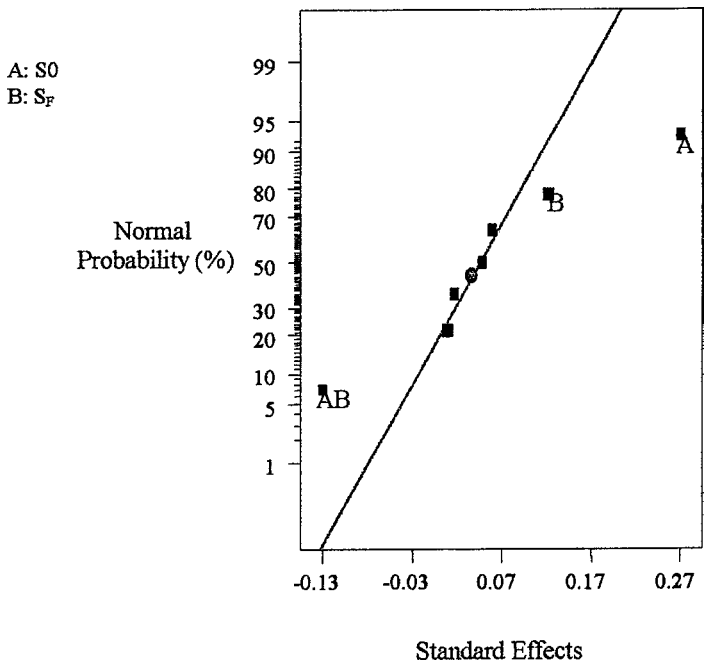

Fig. 4. Normal probability plot for xylitol volumetric productivity.

Table 5
Estimated Effects, t Value, and p Value for XR Activity, XR/XD Ratio, and Volumetric Productivity for Fed-Batch Fermentation of Sugarcane Bagasse Hydrolysate

	XR			XR/XD			Q_{pv}		
Variable	Effects	t Value	p Value	Effects	t Value	p Value	Effects	t Value	p Value
$[S_0]$	0.1080	2.9268[a]	0.0613[a]	0.3075	2.5350[a]	0.0850[a]	0.2668	6.7716[b]	0.0025[b]
$[S_i]$	—	—	—	0.4275	0.5243[b]	0.0388[b]	0.1212	3.0761[b]	0.0371[b]
[pH]	0.0965	2.6152[a]	0.0795[a]	—	—	—	—	—	—
[AR]	0.0175	0.4742	0.6726	–0.1975	1.6282	0.2019	—	—	—
[pH] [AR]	0.1200	3.2520[b]	0.0475[b]	—	—	—	—	—	—
$[S_i]$ [AR]	—	—	—	–0.5275	4.3487[b]	0.0224[b]	—	—	—
$[S_0]$ $[S_i]$	—	—	—	—	—	—	–0.1272	3.2284[b]	0.0320[b]
R^2	0.8971			0.9309			0.9426		

[a]significant at 10% level.
[b]significant at 5% level.

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