0273-2289/98/70–72—0127$10.25

Xylose Reductase Production by *Candida guilliermondii*

S. M. A. Rosa,[1] M. G. A. Felipe,[2] S. S. Silva,[2] and M. Vitolo*[1]

[1]*Depto. Tecnologia Bioquímico-Farmacêutica, FCF, University of São Paulo. P.B. 66083, 05389-970, São Paulo, SP, Brasil. FAX: 0055-11-8156386;* [2]*Faculdade de Engenharia Química de Lorena, Depto. Biotecnologia, 12600–000, Lorena, SP, Brasil*

ABSTRACT

The effect of pH, time of fermentation, and xylose and glucose concentration on xylitol production, cell growth, xylose reductase (XR), and xylitol dehydrogenase (XD) activities of *Candida guilliermondii* FTI 20037 were determined. For attaining XR and XD activities of 129–2190 U/mg of protein and 24–917 U/mg of protein, respectively, the cited parameters could vary as follows: initial pH: 3.0–5.0; xylose: 15–60 g/L; glucose: 0–5 g/L; and fermentation time: 12–24 h. Moreover, the high XR and XD activities occurred when the xylitol production by the yeast was less than 19.0 g/L.

Index Entries: Xylose reductase; *Candida guilliermondii*; xylitol dehydrogenase.

INTRODUCTION

The xylose reductase (EC.1.1.1.21) (XR), an enzyme found mainly in yeasts, catalyzes the conversion of xylose into xylitol, a product with sweetening and anticarious properties *(1)*.

The xylose–xylitol bioconversion is an alternative of economic interest to the catalytic hydrogenation of pure xylose, which is currently obtained from wood hydrolysates. Two reasons can explain this. First, some yeasts convert xylose into xylitol at high yield, either in sugarcane bagasse or in wood hydrolysates, without previous purification of xylose *(2)*. Second, the bagasse is a plentiful residue available from ethanol distilleries, so it is by far cheaper than wood. Nevertheless, even the fermentative process could lead to an unsuitable xylitol production, because of perturbations on the microbial metabolism caused by factors such as oxygen limitation

* Author to whom all correspondence and reprint requests should be addressed.

(3), pH, and temperature (4); presence of inhibitors (furfural and acetic acid, among others); and/or sugars other than xylose (5). Thus, carrying out the xylose–xylitol conversion directly with the XR, those problems would be overcome. In this case, however, the enzyme must be readily available and inexpensive.

Of course, the main sources of XR would probably be the xylose-fermenting yeasts belonging to the genera *Pachysolen, Pichia, Candida, Hansenula,* and *Debaryomyces (6).*

Although XR production and characterization are well-documented for the species *Pichia stipitis (7), Pachysolen tannophilus (8),* and *Candida shehatae (9),* little information is available for the XR of *Candida guilliermondii,* despite the fact that this yeast is a good xylitol producer *(10).* Indeed, the strain *C. guilliermondii* FTI 20037 was already fully described and adapted to the sugar cane and wood hemicellulosic hydrolysates, which are useful raw materials for bioconversions *(2,10,11,12).*

This paper deals with the effect of initial pH, fermentation time, and xylose and glucose concentrations on the xylose reductase production by *C. guilliermondii* FTI 20037. Conditions under which low xylitol dehydrogenase activity occurred were also considered.

METHODS

Microorganism and Inoculum Preparation

C. guilliermondii FTI 20037, described by Barbosa et al. *(10),* was used in all experiments. The stock culture was maintained at 4°C on malt-extract agar slants. A loopful of the stock culture was transferred into a 125-mL Erlenmeyer flask containing 50 mL of the following medium: xylose, 30.0 g/L; rice bran, 20.0 g/L; $(NH_4)_2SO_4$, 2.0 g/L; $CaCl_2{\cdot}2H_2O$, 0.1 g/L. The initial pH was adjusted to 5.0. The flask was incubated in a rotary shaker at 200 rpm and 30°C for 24 h. After that, the cells were separated by centrifugation (2000 *g;* 10 min), rinsed twice with distilled water, and the cell cake was resuspended in an adequate volume of distilled water to attain a final concentration of 5 g dry cell/L.

Growth Conditions and Cell Disruption

Five mL of the cell suspension was introduced into a 125-mL Erlenmeyer flask containing 45 mL of the medium: rice bran, 20 g/L; $(NH_4)_2SO_4$, 2.0 g/L; $CaCl_2{\cdot}2H_2O$, 0.1 g/L; xylose (15, 30, 60, 90, 120, or 240 g/L) and glucose (0, 5, 10, 20 or 30 g/L). The initial pH was adjusted to 3.0, 5.0, or 6.5. The flasks were left at 30°C for 12 h, 24 h and 36 h in a rotary shaker (200 rpm). At each time three flasks were drawn, and the suspensions centrifuged (5000*g;* 10 min). Using high-pressure liquid chromatography (HPLC) the xylose, xylitol, and glucose concentrations were measured in the supernatant. The cell cake was washed twice with distilled water,

resuspended in 0.1 *M* phosphate buffer (pH 7.2), and submitted to disruption in a vortex, in the presence of 0.5-mm glass beads for 6 min. Then the suspension was left resting in an ice bath for 3 min. The vortexing/resting cycle was repeated six times, leading to a cell disruption of about 70%. This determination was accomplished through microscope observation, and by counting into a Neubauer chamber (area = 1/400 mm^2; height = 0.100 mm) the number of intact cells before and after vortexing/resting. The cell-free extract was employed for xylose reductase and xylitol dehydrogenase activities measurement.

Analytical Methods

Xylose, xylitol, and glucose were measured by HPLC (Shimadzu [Kyoto, Japan] LC-10AD), using a refractive index (RI) detector and a Bio-Rad (Hercules, CA) HPX87H (300 × 7.8 mm) column under the following conditions: 0.01 *N* H_2SO_4 as the eluent; 0.6 mL/min flow rate; column temperature 45°C; detector attenuation: 16X; sample volume 20 μL. Cell concentration, expressed as g dry matter/L, was measured by filtering 5 mL of cell suspension through a Millipore (Bedford, MA) membrane (0.22-μm pore diameter), followed by drying in an oven at 105°C for 2 h.

The measurement of xylose reductase activity (XR) was carried out in the following reaction medium: 5m*M* xylose, 0.1 *M* phosphate buffer (pH 7.2), 3.0 μ*M* NADPH, and 0.1 *M* β-mercaptoethanol. For xylitol dehydrogenase (XD), the reaction medium employed was: 5m*M* xylitol, 0.5 *M* Tris buffer (pH 8.6), 2.5 μ*M* NADP, and 0.1 *M* β-mercaptoethanol. The reactions were initiated by the addition of xylose or xylitol, and the variation of absorbance was spectrometrically monitored (340 nm) against the reaction time at 25°C.

Protein in the cell-free extract was determined by the dye-binding technique, using Coomassie blue reagent *(13)* and bovine serum albumin (Sigma, St. Louis, MO >99% purity) as the standard protein. One XR or XD unit (U) was defined as the amount of enzyme catalyzing, respectively, the formation of 1 μmol NADP/min or 1 μmol NADPH/min. The specific activity was expressed as U/mg of protein.

RESULTS AND DISCUSSION

Table 1 shows that 66.4 g/L of xylose was completely consumed after 48 h of fermentation, the xylitol concentration was equal to 43 g/L, which led to a volumetric productivity (VP) of 0.90 g/Lh. When this VP is compared to that attained by Meyrial et al. *(14)* for *C. guilliermondii* NRC 5578, which was equal to 0.30 g/Lh, it is concluded that *C. guilliermondii* FTI 20037 has a superior xylitol producing capability. It is also observed that the pH of the medium decreased 3 pH units (from 5.0 to 2.0) after 48 h of fermentation. This behavior is quite common among yeasts, because of the high permeability to cations and hydrogen ions presented by the cell

Table 1
Xylose Consumption, Xylitol and Biomass Production, and pH Variation of Medium for *C. guilliermondii* FTI 20037, Cultivated Batchwise in a Synthetic Medium

Time (h)	Xylose (g/L)	Biomass (g/L)	Xylitol (g/L)	pH
0	66.4	0.50	0	5.0
16	48.5	2.80	17.6	2.8
24	31.0	3.76	27.4	2.5
48	0	5.29	43.4	2.2
51	0	5.50	46.8	2.1
62	0	6.20	47.6	2.0

60 g/L of xylose; 20 g/L of rice bran; 2 g/L of ammonium sulfate; and 0.1 g/L of calcium chloride.

membrane *(15)*. Moreover, the cell and xylitol concentrations increased, respectively, 14.6 and 8.8% between 48 h and 62 h (Table 1). Probably, some reserve substance synthesized during the high cell-growth rate phase was lately converted into biomass. Meanwhile the extra xylitol into the medium would result from a late excretion of this substance still accumulated inside the cells. However, the fermentative parameters for xylitol production are affected by factors such as pH, temperature, dissolved oxygen, and xylose and glucose concentration *(3–5)*.

Tables 2 and 3 show that the xylitol yield factor ($Y_{p/s}$) and VP diminished markedly for initial xylose and glucose concentrations higher than 90.0 g/L and 5.0 g/L, respectively. Regarding xylose, the result is in accordance with Silva et al. *(16)*, who set 100 g/L as the upper limit for the initial xylose concentration without affecting xylitol production by *C. guilliermondii*. Furthermore, according to Du Preez et al. *(17)* and Silva et al. *(16)*, under high initial xylose concentration (higher than 100 g/L), the yeast would be submitted either to the dissolved oxygen limitation or to the increased osmotic pressure of the culture medium. In addition, the cell yield factor ($Y_{x/s}$) diminished 71% as the initial xylose concentration varied from 15.0 g/L to 240 g/L (Table 2), but increased 75% in a medium containing 60.0 g/L of xylose, and the initial glucose concentration was varied from 0 to 30.0 g/L (Table 3). In this case, it is clear that glucose is a more suitable carbon source for cell growth than xylose.

As the reaction sequence xylose/xylitol/xylulose (the initial steps of the pentose pathway in the yeast) are catalyzed by XR and XD, the effect of pH, xylose, and glucose on their production by *C. guilliermondii* FTI 20037 were evaluated.

From Table 4, it is clear that the initial pH of the medium affects the XR and XD activities. At pH 3.0 and 6.5, the XR activity decreased during the fermentation, but the opposite occurred at pH 5.0. The same occurred

Table 2
Xylose Conversion into Xylitol ($Y_{p/s}$), Xylitol Volumetric Productivity (VP), and Xylose Conversion into Biomass ($Y_{x/s}$) for *C. guilliermondii* FTI 20037 Cultivated in a Synthetic Medium for 36 h at Different Xylose Concentrations

Xylose (g/L)	$Y_{p/s}$ (g/g)	$Y_{x/s}$ (g/g)	VP (g/Lh)
15	0.23	0.63	0.096
30	0.42	0.22	0.34
60	0.57	0.15	0.71
90	0.58	0.19	0.80
120	0.33	0.13	0.44
240	0.00	0.096	0.00

Table 3
Effect of Initial Glucose Concentration on Xylitol Production by *C. guilliermondii* FTI 20037 Cultivated in a Synthetic Medium for 36 h and in Presence of Xylose at Initial Concentration of 60 g/L

Glucose (g/L)	$Y_{p/s}$ (g/g)	$Y_{x/s}$ (g/g)	VP (g/Lh)
0	0.50	0.14	0.69
5	0.56	0.16	0.64
10	0.49	0.22	0.41
20	0.20	0.32	0.10
30	0.30	0.55	0.11

Table 4
Effect of Initial pH of Culture Medium on Xylose Reductase (XR) and Xylitol Dehydrogenase (XD) Activities of *C. guilliermondii* FTI 20037.

pH (Initial)	Time (h)	Biomass (g/L)	Xylose (residual) (g/L)	Xylitol (g/L)	U/mg protein	
					XR	XD
	12	3.4	53.5	0.00	1097	184.5
3.0	24	4.7	38.5	9.52	665.3	167.0
	36	5.1	23.4	20.2	572.2	142.3
	12	5.0	52.4	0.00	327.9	47.30
5.0	24	6.7	34.2	11.6	673.8	191
	36	7.0	12.6	25.2	801.7	171.2
	12	2.1	58.1	0.00	889.2	255.1
6.5	24	8.7	32.3	10.6	690.5	211.7
	36	9.6	10.5	22.3	505.0	183.5

In all tests the initial biomass and xylose concentrations were 0.5 g dry matter/L and 60.0 g/L, respectively.

Table 5
Effect of Initial Xylose Concentration on Xylose Reductase (XR) and Xylitol Dehydrogenase (XD) Activities of *C. guilliermondii* FTI 20037

Xylose (initial) (g/L)	Time (h)	Biomass (g/L)	Xylose (residual) (g/L)	Xylitol (g/L)	U/mg protein	
					XR	XD
	12	7.2	6.8	0.0	319.6	135.2
	(0.34 g/Lh)[a]					
15	24	8.6	0.15	0.0	2189.5	917.2
	36	9.9	0.0	3.4	213.0	145.2
	12	6.4	24.8	2.3	452.9	152.8
	(0.25 g/Lh)[a]					
30	24	6.5	11.8	19.0	2033.5	777.0
	36	7.1	0.45	12.4	288.3	136.0
	12	5.9	50.7	1.7	824.9	117.4
	(0.26 g/Lh)[a]					
60	24	6.7	36.9	20.4	694.2	242.0
	36	7.1	15.3	25.5	365.3	275.6
	12	5.6	75.4	0.0	1201.7	393.0
	(0.23 g/Lh)[a]					
90	24	6.1	54.6	21.3	528.1	224.7
	36	6.9	40.9	28.7	320.9	303.2
	12	5.1	100	0.0	1172.3	493.5
	(0.22 g/Lh)[a]					
120	24	5.8	75.3	11.7	554.1	217.3
	36	5.9	71.5	15.8	292.3	327.0
	12	3.9	226	0.0	213.9	140.4
	(0.20 g/Lh)[a]					
240	24	5.2	214	0.0	258.8	185.1
	36	5.8	210	0.0	264.7	296.4

In all tests X_o = 0.5 g dry matter/L and initial pH = 5.0.

[a] Growth rate calculated as follows: $(X_{24} - X_o)/24$, where X_{24} and X_o were the biomass concentration (g dry matter/L) at t = 24 h and t = 0 h, respectively.

for XD, except that at pH 5.0 its activity increased until t = 24 h, and decreased after that. Furthermore, at pH 3.0, 5.0, and 6.5, the xylose consumption between t = 12 h and t = 36 h were 56, 76, and 82%, respectively. It must be pointed out that until t = 24 h the xylitol production was low, but XR (665.3 U/mg of protein) and XD (167.0 U/mg of protein) activities were high. This is evidence that xylose was directed to biomass formation. When t > 24 h, the growing rate slows down and the xylitol accumulates in the medium, reaching a final concentration of 25.2 g/L at pH 5.0 (Table 4). The XR:XD ratio always diminishes as the fermentation time or the pH increases (Table 7). Hence, to attain a good yield for XR, the initial pH should be between 3.0 and 5.0, and the fermentation carried out up to 12 h.

Table 6
Effect of Initial Glucose Concentration on Xylose Reductase (XR) and Xylitol Dehydrogenase (XD) Activities of *C. guilliermondii* FTI 20037

Glucose (initial) (g/L)	Time (h)	Xylose (residual) (g/L)	Biomass (g/L)	Xylitol (g/L)	U/mg protein	
					XR	XD
	12	50.7	5.9	1.7	824.9	117.4
0	24	36.9	6.7	20.4	694.2	242.0
	36	15.3	7.1	25.5	365.3	275.6
	(1.4 g/Lh)[a]					
	12	53.3	3.9	0.0	158.4	27.6
5	24	32.9	7.2	10.1	782.5	70.5
	36	18.4	7.9	23.2	300.6	62.5
	(1.2 g/Lh)[a]					
	12	49.3	4.2	0.0	122.4	46.8
10	24	40.9	7.0	5.9	285.2	64.0
	36	29.8	7.1	14.9	396.9	68.7
	(0.84 g/Lh)[a]					
	12	56.5	5.0	0.0	411.8	45.4
20	24	52.7	6.5	0.0	135.7	46.3
	36	41.3	7.3	3.7	246.2	45.4
	(0.52 g/Lh)[a]					
	12	55.2	6.5	0.0	178.9	29.6
30	24	46.6	7.8	0.0	138.0	35.5
	36	46.6	8.4	4.0	61.8	38.6
	(0.37 g/Lh)[a]					

In all tests the initial xylose concentration (XY_o) was equal to 60 g/L. The initial pH and cell concentration were 5.0 and 0.5 g dry matter/L, respectively.

[a] Xylose consumption rate calculated as follows: $(XY_{36} - XY_o)/36$, where XY_{36} and XY_o were the xylose concentrations at $t = 36$ h and $t = 0$ h, respectively.

The highest activities of XR (2189.5 U/mg of protein) and XD (917.2 U/mg of protein) occurred after 24 h of fermentation, at an initial xylose concentration of 15.0 g/L (Table 5). Taking into account that, under these conditions, no xylitol was formed, and that the highest growth rate (0.34 g/Lh) occurred, it can be assumed that the biosynthesis of XR and XD is linked to the cell growth. This is confirmed by the fact that, at 90.0 g/L of xylose, when xylitol is formed (21.32 g/L) and the growth rate diminished to 0.24 g/Lh, the XR and XD activities decreased 76%. Table 5 shows also that, in the 30.0 g/L of the initial xylose-test, the xylitol concentration decreases by 35%, probably because of its consumption by the cell as an alternative carbon source, since after $t = 24$ h the xylose concentration in the medium was low.

It is quite important to establish the effect of glucose on the XR and XD activities of *C. guilliermondii*, because this hexose is always present in lignocellulosic hydrolysates, which presently is the main constituent of the

Table 7
Effect of pH, Xylose, and Glucose Concentration on XR:XD Ratio at Different Fermentation Times

Parameter		Fermentation time (h)		
		12	24	36
pH	3.0	5.95	3.98	4.02
	5.0	6.93	3.52	4.68
	6.5	3.49	3.26	2.75
Xylose (g/L)	15	2.36	2.38	1.47
	30	2.96	2.62	2.12
	60	7.03	2.87	1.33
	90	3.06	2.35	1.06
	120	2.38	2.55	0.89
	240	1.52	1.40	0.89
Glucose (g/L)	0	5.40	8.20	3.76
	5	5.74	11.1	4.81
	10	2.62	4.46	5.78
	20	9.07	2.93	5.42
	30	6.04	3.89	1.60

fermentative medium for xylitol production by yeasts *(16)*. Therefore, it is clear from Table 6 that XR and XD activities of *C. guilliermondii* were sensitive to the glucose present in the culture medium. The highest XR (782.5 U/mg of protein) was attained at 5.0 g/L of glucose, but the high XD activity (81.2 U/mg of protein) occurred in the absence of glucose. Regarding the xylitol formation, a negative effect occurs when the initial glucose concentration is higher than 20.0 g/L. The authors can see that, between t = 12 h and t = 36 h, the xylose consumption rate, which was 1.4 g/Lh (absence of glucose) or 1.2 g/Lh (in the presence of 5.0 g/L of glucose), decreased at least 45% for glucose concentration above 10.0 g/L. Taking into account that XR and XD are inducible enzymes *(18,19)*, and that glucose can interfere in the xylose-transport mechanism across the cell membrane *(16)*, the authors hypothesize that the diminution of xylose uptake by *C. guilliermondii* FTI 20037 led to the observed diminution of XR and XD activities.

According to the data presented, the production of XR should take into account the conditions under which XR:XD ratio is between 2.36 and 11.1 (Table 7). Thereby, a suitable XR production could be attained by setting the parameters studied as follows: initial pH: 3.0–5.0; xylose: 15–60 g/L; glucose: 0–5 g/L; and fermentation time: 12–24 h.

ACKNOWLEDGMENTS

To Conselho Nacional De Desenvolvimento Científico E Tecnológico for sponsoring this work.

REFERENCES

1. Emodi, A. (1978), *Food Technol.* **32,** 28–32.
2. Felipe, M. G. A., Vitolo, M., and Mancilha, I. M. (1996), *Acta Biotechnol.* **16,** 73–79.
3. Delgenes, J. P., Moletta, R., and Navarro, J. M. (1989), *Biotechnol. Bioeng.* **34,** 398–402.
4. Forage, R. G., Harrison, D. E. F., Pitt, D. E. (1985), in *Comprehensive Biotechnology*, vol. 1, Moo-Young, M., ed., Pergamon, New York, pp. 251–280.
5. Nolleau, V., Preziosi, B. L., Delgenes, J. P., and Delgenes, J. M. (1993), *Curr. Microbiol.* **27,** 191–197.
6. Onishi, H. and Suzuki, T. (1966), *Agricultural Biol. Chem.* **30,** 1139–1144.
7. Steven, R. and Lee, H. (1992), *J. Gen. Microbiol.* **138,** 1857–1863.
8. Smiley, K. L. and Bolen, P. L. (1982), *Biotechnol. Lett.* **4,** 607–610.
9. Du Preez, J. C., Driessel, B., and Prior, B. A. (1989), *Arch. Microbiol.* **152,** 143–147.
10. Barbosa, M. F. S., Medeiros, M. B., Mancilha, I. M., Schneider, H., and Lee, H. (1988), *J. Ind. Microbiol.* **3,** 241–251.
11. Roberto, I. C., Felipe, M. G. A., Mancilha, I. M., Vitolo, M., and Silva, S. S. (1995), *Bioresource Technol.* **51,** 255–257.
12. Silva, S. S., Dimas, J., Felipe, M. G. A., and Vitolo, M. (1997), *J. Ind. Microbiol.* **63/65,** 557–563.
13. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
14. Meyrial, V., Delgenes, J. P., Moletta, R., and Navarro, J. M. (1991), *Biotechnol. Lett.* **13,** 281–286.
15. Weitzel, G., Pilatus, U., and Rensing, I. (1987), *Exp. Cell Res.* **170,** 64–79.
16. Silva, S. S., Vitolo, M., Pessoa, A., Jr, and Felipe, M. G. A. (1996), *J. Basic Microbiol.* **36,** 187–191.
17. Du Preez, J. C., Bosch, M., and Prior, B. A. (1986), *Enzyme Microb. Technol.* **8,** 360- 364.
18. Bicho, P. A., Runnals, P. L., Cunninghan, J. D., and Lee, H. (1988), *Appl. Environ. Microbiol.* **54,** 50–54.
19. Lee, H. (1992), *FEMS Microbiol. Lett.* **92,** 1–4.