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Effects of Environmental Conditions on Xylose Reductase and Xylitol Dehydrogenase Production by *Candida guilliermondii*

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Abstract

The effects of environmental conditions, namely initial pH (2.5–7.0) and temperature (25 and 35°C), on xylose reductase and xylitol dehydrogenase levels, as well as on xylitol production, were evaluated. Although the fermentative parameter values increased with an increase in pH and temperature (the maximum $Y_{p/s}$ and Q_p were 0.75 g/g and 0.95 g/[L·h], respectively, both attained at pH 6.0, 35°C), the highest xylose reductase activities (nearly 900 IU/mg of protein) were observed at an initial pH varying from 4.0 to 6.0. Xylitol dehydrogenase was favored by an increase in both initial pH and temperature of the medium. The highest xylitol dehydrogenase specific activity was attained at pH 6.5 and 35°C (577 IU/mg of protein).

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

Introduction

Xylitol has sweetening and anticariogenic properties *(1–5)*, and for this reason has caught the attention of food, odontological, and pharmaceutical industries. The conversion of sugars from lignocellulosic biomass into xylitol by xylose-fermenting yeasts represents a cheaper alternative than the chemical process to produce this polyol.

The xylose-xylitol bioconversion by *Candida guilliermondii* occurs through the induction of xylose reductase (aldose reductase EC 1.1.1.21) dependent of nicotinamide adenine dinucleotide phosphate (reduced form). The oxidation of xylitol to xylulose by xylitol dehydrogenase (EC

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1.1.1.9) dependent of nicotinamide adenine dinucleotide (oxidized form) is followed by phosphorylation to xylulose-5-phosphate catalyzed by xylulose kinase (EC 2.7.1.17), which then enters into the pentose phosphate pathway *(6–8)*. The xylose metabolism in yeasts by the phosphopentose pathway and subsequent glucose-6-phosphate oxidation generates 2 mol of NAD(P)H/mol of CO_2 liberated, providing the NAD(P)H regeneration necessary to reduce xylose to xylitol. The production of xylitol by yeasts is essentially related to the pool of NAD(P)H *(9)*. Nevertheless, the complete absence of NADH-linked xylose reductase activity provides xylitol accumulation at high levels, since it allows an imbalance of the NAD^+/NADH under anaerobic conditions *(10,11).* The excretion of xylitol in the culture medium by NAD(P)H-xylose reductase in yeasts is also related to the dissolved oxygen concentration, since at low aeration rates NADH cannot be oxidized to NAD, leading to an increase in the NADH:NAD^+ ratio. Consequently, xylitol oxidization to xylulose is avoided.

The fermentation of sugarcane bagasse hydrolysate by yeasts is critical, because the hydrolysate contains substances inhibitory to microbial metabolism, mainly acetic acid. The conversion of xylose to xylitol by *C. guilliermondii* FTI 20037 is inhibited by acetic acid at concentrations higher than 3.0 g/L *(12)*. However, the inhibition depends on the pH of the culture medium *(13–15).* Nevertheless, the utilization of xylose derived from lignocellulosic residues has proved entirely viable. The present study was undertaken to verify the effects of initial pH (2.5–7.0) and temperature (25 and 35°C) on the production of xylose reductase and xylitol dehydrogenase, as well as to find a correlation between the enzyme levels and the bioconversion of xylose to xylitol.

Materials and Methods

Microorganism

The experiments were conducted with *C. guilliermondii* FTI 20037 described by Barbosa et al. *(16)*. The yeast was maintained on malt-extract agar slants at 4°C.

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). A portion of the hydrolysate was further concentrated under vacuum at 70°C to increase xylose concentration threefold. The hydrolysates were then treated in order to reduce the concentrations of toxic substances. The initial pH was raised to 7.0 with CaO (commercial powder), and acidified to pH 5.5 with H_3PO_4. Subsequently, 2.4% (w/v) of activated charcoal (refined powder) was added to the hydrolysates which were then left under agitation (200 rpm) at 30°C, for 1 h *(17)*. The precipitates resulting from all the stages of the treatment were removed by vacuum filtration.

Inoculum Preparation, Media, and Fermentation Conditions

Inoculum was grown in nonconcentrated bagasse hemicellulosic hydrolysate containing 18.8 g/L of xylose, 1.7 g/L of glucose, 1.6 g/L of arabinose, and 3.2 g/L of acetic acid. The hydrolysate was supplemented with the following nutrients: 2.0 g/L of $(NH_4)_2SO_4$, 0.1 g/L of $CaCl_2 \cdot 2H_2O$, and 20.0 g/L of rice bran extract. The inoculum was prepared in 125-mL Erlenmeyer flasks containing 50 mL of medium at initial pH 5.5 and incubated on a rotary shaker (200 rpm) at 30°C for 24 h.

For the fermentation tests, concentrated bagasse hemicellulosic hydrolysate containing 51.7 g/L of xylose, 4.1 g/L of glucose, 4.4 g/L of arabinose, and 5.0 g/L of acetic acid was employed. The hydrolysate was supplemented with the same nutrients used for the inoculum preparation. The medium (50 mL) was placed in 125-mL Erlenmeyer flasks and fermented at 200 rpm for 48 h, at 25 and 35°C, with initial pH ranging from 2.5 to 7.0. The initial cell concentration in all the experiments was 1.0 g/L.

Preparation of Cell-Free Extracts

Cells were harvested by centrifugation at 800*g*, and washed in phosphate buffer (50 m*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 disruption technique using a Sonics & Materials (Newtown, CT) (VC-1OO) 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

Xylose reductase and xylitol dehydrogenase activities were determined spectrophotometrically at 340 nm at room temperature *(18)*. Enzyme units were defined as nmol of NAD(P)H or NAD^+ oxidized/reduced using an extinction coefficient of 6.22×10^{-3}. Specific activities were expressed as IU/mg of protein based on protein determinations according to the method of Bradford *(19)* using bovine serum albumin as the standard.

Analytical Methods

Glucose, xylose, xylitol, and acetic acid concentrations were determined by liquid chromatography *(20)*. Cell concentration was measured by turbidimetry at 600 nm.

Results and Discussion

Figure 1 shows the consumption of glucose and xylose during the fermentations of sugarcane bagasse hydrolysates at initial pH varying from 2.5 to 7.0 and temperatures of 25 and 35°C. According to the results, the assimilation of sugars was totally inhibited when the initial pH values were <3.5. Increasing the temperature of the medium favored the glucose assimi-

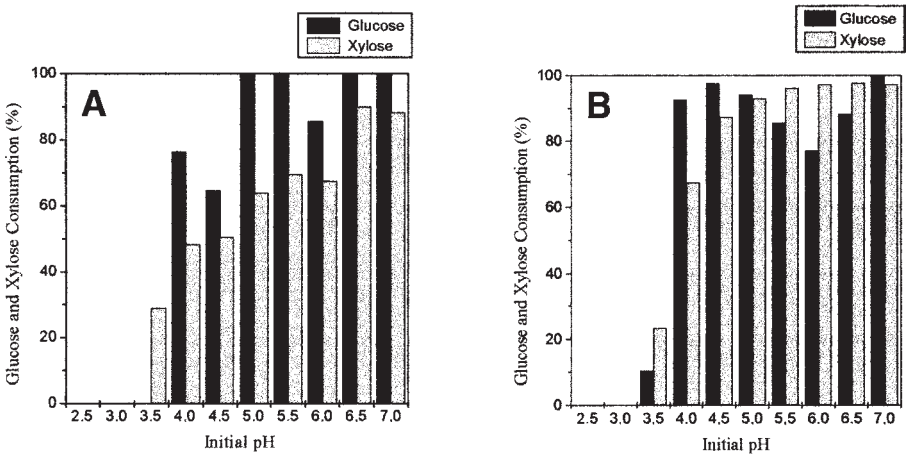

Fig. 1. Glucose and xylose consumption (%) after 48 h of fermentation of sugarcane bagasse hydrolysate by *C. guilliermondii* under different initial pH values and temperatures **(A)** 25°C; **(B)** 35°C.

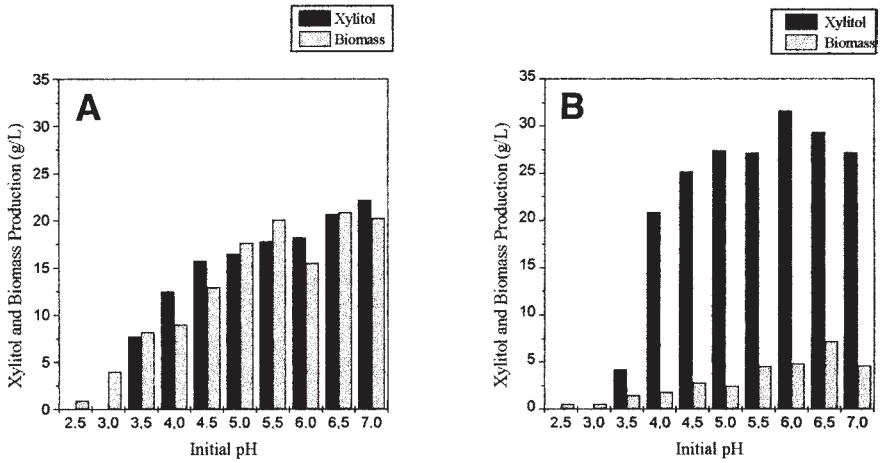

Fig. 2. Xylitol and biomass production (g/L) after 48 h of fermentation of sugarcane bagasse hydrolysate by *C. guilliermondii* under different initial pH values and temperatures **(A)** 25°C; **(B)** 35°C.

lation at pH values <4.5. Above this pH value, all the glucose was rapidly consumed independently of the temperature employed. It should be emphasized that the glucose uptake preceded the xylose consumption, as previously observed for the same yeast grown in sugar cane bagasse *(21,22)* or in eucalyptus hydrolysate *(20)*, and for *Pichia stiptis (23)* and *Pachysolen tannophilus (24)* grown in synthetic medium. In previous work employing *C. guilliermondii* grown in bagasse hemicellulosic hydrolysate, sugar consumption and xylitol production were observed when the initial pH was ≥4.5, and the highest xylitol production rates were attained when the pH values employed were 5.5, 6.5, and 7.5 *(15)*.

Table 1
Acetic Acid Consumption (%) after 48 h by *C. guilliermondii* FTI 20037 Grown in Sugarcane Bagasse Hydrolysate Under Different Initial pH Values and Temperatures

	Acetic acid (%)	
Initial pH	25°C	35°C
3.5	60	46
4.0	66	71
4.5	73	71
5.0	58	42
5.5	38	48
6.0	36	40
6.5	22	37
7.0	44	42

Figure 1 also shows that the assimilation of xylose was optimized when the initial pH and temperature were increased. As can be seen, >90% of the xylose was consumed at 35°C in fermentabions at pH values from 5.5 to 7.0.

Figure 2 presents the effects of pH and temperature on xylitol production by *C. guilliermondii*. As expected, no xylitol was detected in the medium when initial pH values of 2.5 and 3.0 were employed. At 25°C, increasing the pH from 3.5 to 7.0 caused the xylitol production to triple. However, the effect of the pH on xylitol production was more evident at 35°C. At this temperature, the maximum xylitol concentration, 31.64 g/L, which corresponded to an increase of 13%, was attained at pH 6.0 after 48 h. A similar result had already been found for *Candida boidinii.* In this case, the xylitol production also increased when the initial pH was raised from 4.0 to 7.0, but decreased when the pH value was >7.0 *(25)*.

In the present work, pH changes were verified in fermentations in which sugar consumption occurred, independently of the temperature used (data not shown). The pH increase during the fermentations was owing to the assimilation of the acetic acid present in the bagasse hydrolysate (Table 1) as well as to the decrease in the concentration of the substrate and to the increase in xylitol in the medium. Higher pH values coincided with higher acetic acid assimilation rates (initial pH 4.5 and 5.0).

The acetic acid consumption was previously observed in experiments with *C. guilliermondii* grown in bagasse hydrolysate *(22)* and in synthetic medium *(12)*, as well as with *P. stipitis (26)*, *Candida blanki,* and *Candida utilis (27,28)*, and *Paecilomyces varioui (29).* Although acetic acid is assimilated by microorganisms, it can also inhibit xylose metabolism in yeasts. The acetic acid toxicity depends on pH *(30,31)*, temperature *(32)*, aeration *(33)*, xylose:acetic acid ratio *(34)*, and mainly on the acetic acid concentration in the culture medium *(12)*.

Table 2
Xylitol Yield ($Y_{p/s}$) During Xylose Bioconversion by *C. guilliermondii* FTI 20037 Grown in Sugarcane Bagasse Hydrolysate Under Different Initial pH Values and Temperatures

	$Y_{p/s}$ (g/g) at 25°C				$Y_{p/s}$ (g/g) at 35°C			
	Time (h)				Time (h)			
Initial pH	T_{12}	T_{24}	T_{36}	T_{48}	T_{12}	T_{24}	T_{36}	T_{48}
3.5	0	0.62	0.58	0.57	0.1	0.2	0.18	0.39
4.0	0	0.37	0.47	0.53	0.35	0.48	0.58	0.66
4.5	0	0.57	0.51	0.69	0.32	0.54	0.61	0.64
5.0	0.18	0.42	0.51	0.53	0.51	0.63	0.69	0.65
5.5	0.19	0.38	0.25	0.53	0.47	0.63	0.63	0.60
6.0	0.16	0.44	0.49	0.56	0.64	0.70	0.73	0.75
6.5	0	0.65	0.63	0.62	0.59	0.62	0.71	0.64
7.0	0.43	0.38	0.48	0.52	0.47	0.65	0.55	0.60

Table 3
Xylitol Productivity (Q_p) During Xylose Bioconversion by *C. guilliermondii* FTI 20037 Grown in Sugarcane Bagasse Hydrolysate Under Different Initial pH Values and Temperatures

	Q_p (g/[L·h]) at 25°C				Q_p (g/[L·h]) at 35°C			
	Time (h)				Time (h)			
Initial pH	T_{12}	T_{24}	T_{36}	T_{48}	T_{12}	T_{24}	T_{36}	T_{48}
3.5	0	0.04	0.10	0.16	0.03	0.02	0.03	0.09
4.0	0	0.13	0.22	0.26	0.10	0.20	0.31	0.43
4.5	0.08	0.18	0.23	0.33	0.15	0.32	0.35	0.52
5.0	0.09	0.20	0.31	0.31	0.37	0.53	0.73	0.57
5.5	0.07	0.22	0.20	0.37	0.44	0.69	0.70	0.57
6.0	0.07	0.24	0.39	0.38	0.68	0.95	0.82	0.66
6.5	0	0.28	0.27	0.43	0.61	0.78	0.84	0.61
7.0	0.08	0.30	0.36	0.46	0.55	0.80	0.67	0.57

According to Table 2, the initial pH strongly influenced the xylitol yield, which was null when the pH was <3.5. Increasing the temperature increased the xylose-xylitol conversion rate. The maximum $Y_{p/s}$ value (0.75 g/g) was achieved at pH 6.0 and 35°C, after 48 h. Table 3 shows that the initial pH had a main positive effect on productivity (Q_p), especially at 35°C. The maximum Q_p value (0.95 g/[L·h]) was obtained at pH 6.0 and 35°C, after 24 h. High values of Q_p were also attained at pH 6.0 and 6.5, after 36 h. Probably owing to the inoculum adaptation to the hydrolysate, the Q_p values in the present work were much higher than those observed for the

Table 4
Specific Activities of NAD(P)H-XR in Cell Extracts of *C. guilliermondii* FTI Grown in Sugarcane Bagasse Hydrolysate Under Different Initial pH Values and Temperatures

	NAD(P)H-NR (IU/mg of protein) at 25°C				NAD(P)H-XR (IU/mg of protein) at 35°C			
	Time (h)				Time (h)			
Initial pH	T_{12}	T_{24}	T_{36}	T_{48}	T_{12}	T_{24}	T_{36}	T_{48}
3.5	232	192	233	310	—	—	162	260
4.0	462	434	844	848	310	861	525	638
4.5	215	320	533	523	267	613	592	472
5.0	428	1142	490	611	396	613	839	704
5.5	199	290	476	542	458	567	525	475
6.0	291	476	396	455	411	692	874	852
6.5	283	455	369	398	391	567	557	536
7.0	171	327	305	373	626	554	441	633

[a] T_0 (25°C) = 359 IU/mg of protein. T_0 (35°C) = 331 IU/mg of protein.

fermentation of bagasse hydrolysate by *C. guilliermondii* at initial pH 5.5 at 30°C (0.57 g/[L·h]) *(24)*.

The results of cell growth at different pH values and temperatures are shown in Fig. 2. The temperature strongly influenced the cell growth at all pH values studied. Biomass production increased at 25°C. Probably, the temperature increase impaired one or more metabolic pathways related to cell growth, but not those related to xylitol production, which, on the contrary, was enhanced when the temperature was increased. Cell growth was, however, favored by the initial pH increase, independently of the temperature used.

Tables 4 and 5 show the specific activities of xylase reductase and xylitol dehydrogenase in cell extracts of *C. guilliermondii* grown in bagasse hydrolysate under different pH values and temperatures, except for pH 2.5 and 3.0. Because cell growth was negligible at low initial pH values (2.5 and 3.0), xylase reductase and xylitol dehydrogenase activities could not be detected in the enzymatic assays.

As shown in Table 4, values of xylase reductase activity attained after 12 h of fermentation decreased in relation to those obtained at zero time of fermentation (enzymes from inoculum) for the cells grown at 25°C (initial pH 3.5, 4.5, 5.5, 6.0, 6.5, and 7.0). This fact was also observed for xylitol dehydrogenase activity (Table 5). The decrease observed for xylase reductase and xylitol dehydrogenase activities in comparison with the values observed at zero time was probably due to catabolic repression of both enzymes by glucose present in the hydrolysate (4.1 g/L), since the glucose uptake was rapid and preceded xylose consumption. This behavior was also described for the yeast *P. tannophilus* grown in a medium containing

Table 5
Specific Activities of NADH-XD in Cell Extracts of *C. guilliermondii* FTI Grown in Sugarcane Bagasse Hydrolysate Under Different Initial pH Values and Temperatures

	NAD^+-XD (IU/mg of protein) at 25°C				NAD^+-XD (IU/mg of protein) at 35°C			
	Time (h)				Time (h)			
Initial pH	T_{12}	T_{24}	T_{36}	T_{48}	T_{12}	T_{24}	T_{36}	T_{48}
3.5	—	9	54	68	—	—	20	102
4.0	79	24	270	244	22	199	211	372
4.5	37	156	139	77	36	278	229	252
5.0	42	201	80	143	167	181	—	229
5.5	44	100	109	108	161	316	319	318
6.0	35	72	110	79	92	211	340	370
6.5	49	152	84	216	104	307	577	297
7.0	39	143	155	178	296	419	368	391

[a] T_0 (25°C) = 106 IU/mg of protein. T_0 (35°C) = 127 IU/mg of protein.

glucose as the carbon source, in which a low value of xylase reductase activity was found (0.03 UI/mg of protein). However, when this yeast was grown in xylose, the xylase reductase activity was high (0.280 UI/mg of protein), which confirms that xylase reductase is induced by xylose *(35)*. Induction of xylase reductase and xylitol dehydrogenase enzymes was also observed for different species of yeasts *(23,24,36–40)*. In the present study, enzyme activity was enhanced after glucose depletion owing to the induction by xylose and possibly by L-arabinose present in the hydrolysates. The induction of xylase reductase and xylitol dehydrogenase by L-arabinose was verified in the cultivation of *C. guilliermondii (39)* and *Candida tennis (40)*. In experiments with *C. tenuis* grown at pH 5.5, xylase reductase and xylitol dehydrogenase activities increased during the exponential growth phase, but decreased due to the depletion of xylose, the inducing substrate *(40).*

In the present study, increasing the temperature from 25 to 35°C decreased the cell growth, although the xylose uptake and production was enhanced under all the pH values tested. At 25°C, xylose was probably utilized for biomass production, although the xylitol dehydrogenase and xylase reductase levels were systematically high. Alexander *(18)* observed a decrease in xylase reductase and xylitol dehydrogenase activities as the temperature was raised from 50 to 37°C during the growth of *P. tannophilus* using xylose as the substrate. In addition, even with a reduction in enzyme activity, cell growth and cell division occurred, which suggests that the low xylase reductase activity is not a limiting factor for xylose utilization or that there is an alternative pathway for xylose utilization by *P. tannophilus (18).*

According to Table 4, the highest xylase reductase activity was attained at pH 5.0 and 25°C (1142 IU/mg of protein) after 24 h. High values of xylase

reductase activity were also achieved at pH 4.0 and 25°C after 48 and 36 h of incubation (848 and 844 IU/mg of protein, respectively) as well as at pH 4.0 after 36 h (861 IU/mg of protein) and at pH 6.0 after 48 h (874 IU/mg of protein) at 35°C.

When the yeast *C. guilliermondii* was grown in synthetic medium containing xylose, the maximum xylase reductase and xylitol dehydrogenase activity values were, respectively, 1096 IU/mg of protein (initial pH 3.0) and 255 IU/mg of protein (initial pH 6.5) *(41).*

In the present study, although high pH values and temperatures favored xylitol production, high concentrations of this product had no effect on the xylitol dehydrogenase activity. Probably xylitol is not an inductor of XD in *C. guilliermondii.* In fact, induction of xylase reductase and xylitol dehydrogenase enzymes by xylitol was not observed in *C. tenuis (40).* Table 5 also shows that the highest xylitol dehydrogenase activity (577 IU/mg of protein) occurred at pH 6.5 and 35°C.

Conclusion

From the results presented herein, it is possible to conclude that the environmental conditions necessary to produce xylitol with high yields are different from those necessary to obtain high xylase reductase activities. This should be considered for the xylitol production using either the conventional fermentation method or immobilized enzyme.

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