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Environmental parameters affecting xylitol production from sugar cane bagasse hemicellulosic hydrolyzate by *Candida guilliermondii*

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The bioconversion of xylose to xylitol by *Candida guilliermondii* FTI 20037 cultivated in sugar cane bagasse hemicellulosic hydrolyzate was influenced by cell inoculum level, age of inoculum and hydrolyzate concentration. The maximum xylitol productivity (0.75 g L^{-1} h^{-1}) occurred in tests carried out with hydrolyzate containing 54.5 g L^{-1} of xylose, using 3.0 g L^{-1} of a 24-h-old inoculum. Xylitol productivity and cell concentration decreased with hydrolyzate containing 74.2 g L^{-1} of xylose.

Keywords: xylitol; sugar cane bagasse; *Candida guilliermondii*

Introduction

Sugar cane bagasse is a plentiful and inexpensive source of xylose, a pentose that represents 82% of the whole hemicellulose fraction. The xylose present in the bagasse hydrolyzate can be converted directly into xylitol by *Candida guilliermondii* FTI 20037 [15]. This bioconversion becomes an alternative for the catalytic hydrogenation of xylose in wood hydrolyzates, the current xylitol production method. Xylitol has high commercial value due to its sweetening, anticariogenic properties and clinical applications [1,2,22,23].

Although xylitol production from xylose by microorganisms grown in synthetic media has already been reported [3,4,6,13,17,21], there are few data about this bioconversion carried out in hydrolyzates of lignocellulosic materials. Moreover, the main problem in using such hydrolyzates is the presence of substances toxic to yeast such as furfural, acetic acid and hydroxymethylfurfural (5-HMF), which are produced during acid hydrolysis. The damage these compounds cause to yeast metabolism depends on the concentration, the intracellular physiological conditions, dissolved oxygen concentration and pH of the medium. Fermentation tests using *C. guilliermondii* with increasing concentrations of furfural and 5-HMF showed that these compounds inhibit cell growth in concentrations above 1.0 and 1.5 g L^{-1}, respectively [16]. According to these authors, growth inhibition was caused by the action of furfural and 5-HMF on several key glucolytic enzymes. Acetic acid has been also reported as a strong inhibitor of the xylose to xylitol fermentation by *C. guilliermondii* [6]; in the fermentation of a semisynthetic medium, the presence of this acid in concentrations higher than 3.0 g L^{-1} reduced the xylitol yield and productivity. The inhibitory action of the acetic acid has been attributed to the undissociated acid concentration, which also makes it pH-dependent [8].

The inhibitory effect could be overcome by using an adequate hydrolyzate pretreatment, a hydrolyzate-adapted yeast strain and/or bioreactors inoculated with high concentration of cells. Among the yeast strains that ferment xylose to xylitol in the hydrolyzates of agroindustrial residues, *Candida guilliermondii* FTI 20037 has been identified as a yeast that can efficiently perform this bioconversion.

This study deals with the effects of cell inoculum level, age of inoculum and hydrolyzate concentration upon xylitol production from sugar cane bagasse hemicellulosic hydrolyzate using *C. guilliermondii* FTI 20037.

Materials and methods

Microorganism and inoculum preparation

Candida guilliermondii FTI 20037 described by Barbosa *et al* [3] was maintained as a slant on malt extract agar at 4°C. The cells, aged for 7–10 days, were transferred to 125-ml Erlenmeyer flasks containing 50 ml of the medium: 30.0 g L^{-1} D-xylose, 7.0 g L^{-1} D-glucose, 2.0 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$ and 20.0 g L^{-1} rice bran. The flasks were incubated in a gyratory shaker (200 rpm) at 30°C for 16, 24 or 48 h. Afterwards, the cells were collected by centrifugation (2000 × *g*, 15 min), rinsed thoroughly with sterile distilled water, centrifuged 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 (0.1, 0.5, 1.3, 3.0 or 6.0 g L^{-1}).

Preparation of the hemicellulosic hydrolyzate

A stainless steel reactor was filled with ground sugar cane bagasse and sulfuric acid (0.1 g H_2SO_4 g^{-1} dry matter) and hydrolysis was carried out at 140°C for 20 min, as described by Pessoa Jr [14]. The hydrolyzate was filtered

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twice under vacuum and concentrated to a xylose concentration of 37.6 g L^{-1} (10.0°Brix), 54.5 g L^{-1} (14.5°Brix), and 74.2 g L^{-1} (18.3°Brix) in a 4-L vacuum concentrator at 58°C. The concentrated hydrolyzate was treated as follows: first, the pH was increased to 10.0 with CaO (commercial grade), then reduced to 5.5 with H_2SO_4. After each pH alteration, the hydrolyzate was centrifuged (2000 × *g*, 15 min) and the precipitate was discarded.

The treated hydrolyzate was autoclaved at 110°C for 15 min, supplemented with 2.0 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$ and 20.0 g L^{-1} rice bran, and then employed as the fermentation medium.

Fermentation conditions

The fermentation tests were carried out in triplicate in 125-ml Erlenmeyer flasks, containing 50 ml of the fermentation medium described above, which were incubated on a gyratory shaker (200 rpm) at 30°C for 45 h. The parameters studied included cell inoculum level and age, and sugar concentration in the hydrolyzate.

Analytical methods

The fermentations were followed by measuring the consumption of glucose, xylose and arabinose, production of cell mass and xylitol, acetic acid concentration, and pH. The concentrations of glucose, xylose, arabinose, xylitol and acetic acid were determined by high performance liquid chromatography (Hewlet-Packard model 1082B) using a refractive index (RI) detector and a Bio-Rad HPX87H (300 × 7.8 mm) column, under the following conditions: 0.01 N H_2SO_4 as eluant; 0.6 ml min^{-1} flow rate; column temperature 45°C; detector attenuation 16×; sample volume 20 μl. Cell concentration of the inoculum was determined by comparing the optical density of a cell suspension against a standard curve (absorbance at 600 nm × dry cell weight). Cell concentration of the hydrolyzate was measured directly by counting in a Neubauer chamber [15].

Results and discussion

Effect of cell inoculum level

The effect of cell inoculum level (0.1, 0.5, 1.3, 3.0 and 6.0 g L^{-1}) was studied in hydrolyzate containing 37.6 g L^{-1} of xylose and a 24-h-old inoculum. From Figure 1 it is clear that in all fermentation runs glucose was completely consumed after 22 h, whereas the arabinose concentration (about 7 g L^{-1}) remained approximately constant during the fermentation run. Moreover, xylose consumption was complete after 45 h of fermentation using 3.0 g L^{-1} of a 16- or 24-h inoculum in tests carried out with hydrolyzate containing 37.6 g L^{-1} of xylose. A decrease of around 40% in acetic acid concentration and an increase in pH were also observed in the presence of inoculum concentrations up to 3.0 g L^{-1}. These phenomena were similar to those observed in fermentations with *C. guilliermondii* cultivated in a semi-synthetic medium [6] and with *Pichia stipitis*, *Candida blankii* and *Candida utilis* grown in hemicellulosic hydrolyzates [7,11,19,20]. The ability of *C. guilliermondii* FTI 20037 to metabolize acetic acid is useful in xylose to xylitol conversion using lignocellulosic hydrolyzate as the main medium constituent. The acetic acid is always present

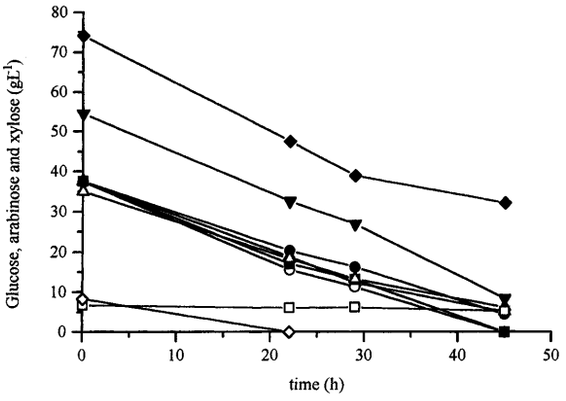

Figure 1 Xylose consumption by *Candida guilliermondii*: 24-h inoculum age, 37.6 g L^{-1} xylose and cell inoculum level (g L^{-1}): 1.3 (—●—), 3.0 (—■—) and 6.0 (—▲—); 37.6 g L^{-1} xylose, cell inoculum level 3.0 g L^{-1} and inoculum age (h): 16 (—○—), 24 (—■—), and 48 (—△—); 24-h inoculum age, cell inoculum level 3.0 g L^{-1} and xylose concentration (g L^{-1}): 54.5 (—▼—) and 74.2 (—◆—); arabinose (—□—), and glucose (—◇—) consumption are also shown.

in this medium, resulting from hydrolysis of lignocellulose. At an initially high yeast concentration (6.0 g L^{-1}), acetic acid was accumulated in the medium causing a decrease in the pH of the medium. These results could be due to the occurrence of some modifications in the metabolic pathways of *C. guilliermondii* FTI 20037. A possible explanation for the increase in the acetic acid concentration in the medium is that *C. guilliermondii* might have converted xylose into acetic acid in agreement with the results found by Mahmourides *et al* [9] and Neirinck *et al* [12] for *Pachysolen tannophilus*. According to Neirinck *et al* [12], xylose metabolism by *P. tannophilus* is associated with an interaction between cell density and oxygen limitation. It can also be noted (Table 1) that the xylitol yield and productivity decreased to 58% and 50% respectively and final cell concentration increased by increasing the inoculum concentration from 3.0 to 6.0 g L^{-1}. According to Nolleau *et al* [13], a perturbation in yeast metabolism could be related to the dissolved oxygen in the medium, which becomes limiting as the cell concentration increases. Thus, a limited oxygen supply to cells causes incomplete xylose metabolization leading to acetic acid accumulation, among other possible effects. The acetic acid, in turn, would interfere with several points of the yeast metabolism, such as xylose uptake through the cytoplasmic membrane, inhibition of xylose reductase activity and/or biosynthesis. According to Chung and Lee [5] and Yu *et al* [24], there is an optimum inoculum concentration under which the fermentation rate promoted by the yeast grown in lignocellulosic hydrolyzate would be enhanced.

It must be pointed out that acetic acid, glucose and xylose were metabolized simultaneously (data not shown), as already observed for *P. stipitis* [20] and *C. blankii* [10,11].

Effect of inoculum age

The effect of inoculum age (16, 24 and 48 h) was evaluated for hydrolyzate containing 37.6 g L^{-1} of xylose and 3.0 g L^{-1} of inoculum. Modifications in xylose consumption (Figure 1) and xylitol production (Figure 2) were observed

Table 1 Effect of cell inoculum level, age of inoculum and hydrolyzate concentration on final cell concentration (X), xylitol productivity (P) and xylitol yield ($Y_{p/s}$). The initial and final acetic acid concentration (HAc) and pH are also shown. All tests were carried out using 45-h cultures

Inoculum level ($g\ L^{-1}$)[a]	Inoculum age (h)[b]	Xylose ($g\ L^{-1}$)	X (cells ml^{-1}) $\times 10^{-8}$	$Y_{p/s}$ ($g\ g^{-1}$)	P ($g\ L^{-1}\ h^{-1}$)	HAc ($g\ L^{-1}$)	pH
0.1	24	37.6	0.60	0.75	0.52	3.78[c]/2.28[d]	5.3[c]/6.1[d]
0.5	24	37.6	1.37	0.75	0.52	3.78/2.03	5.3/6.1
1.3	24	37.6	1.63	0.71	0.52	3.78/2.35	5.3/5.9
3.0	16	37.6	1.07	0.58	0.48	3.77/2.88	5.3/6.5
3.0	24	37.6	1.03	0.62	0.52	3.78/2.55	5.3/5.9
3.0	24	54.5	1.46	0.74	0.75	4.70/3.72	5.3/5.9
3.0	24	74.2	0.66	0.51	0.57	4.89/6.27	5.3/5.0
3.0	48	37.6	1.12	0.51	0.33	4.00/2.69	5.3/5.5
6.0	24	37.6	1.29	0.36	0.26	3.78/4.91	5.2/4.7

[a]Cell inoculum level.
[b]Age of the inoculum.
[c]Initial acetic acid concentration and pH.
[d]Final acetic acid concentration and pH.
$Y_{p/s}$ theoretical = 0.917 $g\ g^{-1}$ [3].

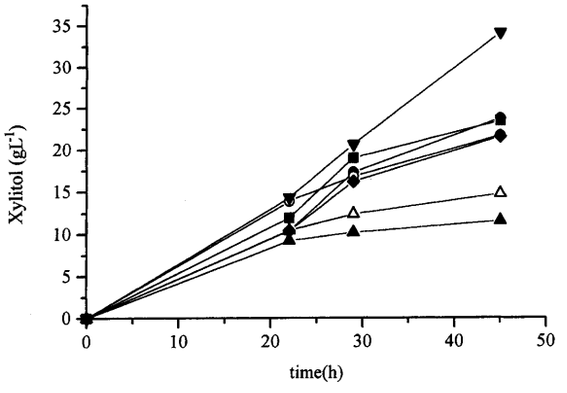

Figure 2 Xylitol production by *Candida guilliermondii*: 24-h inoculum age, 37.6 g L^{-1} xylose and cell inoculum level (g L^{-1}): 1.3 (—●—), 3.0 (—■—) and 6.0 (—▲—); 37.6 g L^{-1} xylose, cell inoculum level 3.0 g L^{-1} and inoculum age (h): 16 (—○—), 24 (—■—) and 48 (—△—); 24-h inoculum age, cell inoculum level 3.0 g L^{-1} and xylose concentration (g L^{-1}): 54.5 (—▼—) and 74.2 (—◆—).

in tests carried out with 16-, 24- or 48-h inoculum. The highest xylitol productivity (0.52 g L^{-1} h^{-1}) occurred when a 24-h inoculum was employed, while a decrease of about 36% was observed with a 48-h inoculum (Table 1). As proposed by Sreenath *et al* [18], who observed similar behavior for a 24-h inoculum of *C. shehatae*, older cells would have their growth capabilities reduced owing to the influence of the culture conditions employed. However, the age of the inoculum influenced only the xylitol productivity (Table 1). Meanwhile, the final cell concentration remained practically unchanged and 24–33% of the acetic acid in the medium was metabolized. It might also be possible that old cells of *C. guilliermondii* FTI 20037 have their xylose-to-xylitol bioconversion capability depressed due to the inhibition of intracytoplasmic xylose reductase activity (XR). Tests to confirm this observation, by measuring the XR activity during fermentation, are needed.

Effect of sugar concentration

The effect of sugar concentration (37.6, 54.5 and 74.2 g L^{-1}) was evaluated utilizing 3.0 g L^{-1} inoculum containing 24-h cells. In tests carried out with hydrolyzate containing 54.5 g L^{-1} xylose, the highest xylitol production (34.4 g L^{-1}) was observed (Figure 2), which corresponds to an increase of 44% in the xylitol productivity as compared to hydrolyzate containing 37.6 g L^{-1} of xylose (Table 1). However, the utilization of a more concentrated hydrolyzate (74.2 g L^{-1} xylose) resulted in a pronounced diminution of xylose consumption and xylitol production (Figures 1,2) with a decrease of 24% in xylitol productivity (Table 1). In this case, the final cell concentration diminished (around 50%) and the acetic acid accumulated in the medium during fermentation (around 28%). Thereby, controlling the acetic acid content in the medium (preferably absent or lower than 3.78 g L^{-1}) is a fundamental approach to improving the xylose to xylitol bioconversion. More accurate studies are needed on this subject, mainly on eventual interaction between dissolved oxygen and acetic acid concentration.

Finally, a high xylitol production, 34.0 g L^{-1} (±1.54), corresponding to a 4.53% coefficient of variation, was attained by growing *C. guilliermondii* FTI 20037 in sugar cane bagasse hydrolyzate containing 54.5 g L^{-1} of xylose using a 24-h-old inoculum (3.0 g L^{-1}). It was also observed that this yeast can assimilate acetic acid, suggesting that these cells may act as a medium-detoxifying agent, which is a positive aspect for the microbiological process of xylitol production using xylose-rich lignocellulose hydrolyzate.

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