Metabolic Behavior of Immobilized *Candida guilliermondii* Cells During Batch Xylitol Production from Sugarcane Bagasse Acid Hydrolyzate

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Abstract: *Candida guilliermondii* cells, immobilized in Ca-alginate beads, were used for batch xylitol production from concentrated sugarcane bagasse hydrolyzate. Maximum xylitol concentration (20.6 g/L), volumetric productivity (0.43 g/L · h), and yield (0.47 g/g) obtained after 48 h of fermentation were higher than similar immobilized-cell systems but lower than free-cell cultivation systems. Substrates, products, and biomass concentrations were used in material balances to study the ways in which the different carbon sources were utilized by the yeast cells under microaerobic conditions. The fraction of xylose consumed to produce xylitol reached a maximum value (0.70) after glucose and oxygen depletion while alternative metabolic routes were favored by sub-optimal conditions. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* **79**: 165–169, 2002.
Keywords: xylitol; sugarcane bagasse hydrolyzate; immobilized cells; carbon material balance; metabolic behavior; *Candida guilliermondii*

INTRODUCTION

Biotechnological production of xylitol, a special sweetener with outstanding organoleptic and anticariogenic properties, could be a cheaper alternative to the present chemical reduction (Winkelhausen and Kuzmanova, 1998). In fact, several researchers are pursuing the development of an economical technique for xylitol bioproduction from hemicellulose hydrolyzates made from sugarcane bagasse (Chen and Gong, 1985), hardwood (Preziosi-Belloy et al., 1997), rice straw (Roberto et al., 1996), and other lignocellulosic materials (Parajó et al. 1998).

Xylitol can be formed as an intermediary of D-xylose fermentation by several yeasts. Xylose is firstly reduced by xylose reductase (XR) to xylitol, which is then oxidized to xylulose by xylitol dehydrogenase (XDH). Xylulose is subsequently phosphorylated by xylulose kinase to xylulose 5-phosphate, which is finally converted into intermediates of the Embden–Meyerhof pathway (Hahn-Hägerdal et al., 1994). The oxygen transfer rate influences xylitol accumulation more than any other parameter (Vandeska et al., 1995). When yeasts with high NADPH-dependent XR activity are used, such as *Candida guilliermondii* (Nolleau et al., 1995), low oxygen availability prevents complete NADH regeneration by the respiratory chain, leading to xylitol accumulation (Winkelhausen and Kuzmanova, 1998). For this reason, microaerobic conditions were used in this study.

Toxic compounds released during the acid hydrolysis hinder the bioconversion of xylose contained in hemicellulose hydrolyzates. One of the proposed methods for minimizing the toxicity of such hydrolyzates is the use of high cell concentrations (Parajó et al., 1996), which can be ensured by cell immobilization within Ca-alginate beads (Carvalho et al., 2000). An unsolved problem is the presence of other sugars, such as glucose, which can inactivate the xylose transport system (Nobre et al., 1999) or repress the induction of XR activity (Sugai and Delgenes, 1995). Therefore, additional information on pentose-fermenting yeasts metabolism is necessary. A carbon material balance, a powerful and general tool for this purpose (Converti et al., 1999), was used to estimate the carbon source splitting up among the different xylose-consuming ways simultaneously active in *Candida guilliermondii*, as well as the relative contribution of each hydrolyzate component to the formation of the main products.

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MATERIALS AND METHODS

Preparation and Detoxification of the Sugarcane Bagasse Hydrolyzate

The hemicellulose hydrolyzate was obtained by acid hydrolysis as described by Carvalho et al. (2000). After hydrolysis, the liquid fraction was concentrated threefold at 70°C under vacuum. To remove the inhibitors more effectively, the concentrated hydrolyzate was neutralized with CaO, acidified to pH 5.5 with H_3PO_4 and finally treated for 1 h with 2.5% active charcoal at 200 rpm and 30°C. All the precipitates were removed by vacuum filtration.

Microorganism and Immobilization Technique

Candida guilliermondii FTI 20037 cells were maintained on agar malt extract slant at 4°C. A loopful of cells was transferred to 125-mL Erlenmeyer flasks containing 50 mL of medium constituted of xylose (30 g/L), $(NH_4)_2SO_4$ (3 g/L), $CaCl_2$ (0.1 g/L), and rice bran extract (20% v/v). The cells for the inoculum were cultivated at 30°C for 24 h in a rotary shaker at 200 rpm, collected by centrifugation (2,000*g*, 15 min), washed and resuspended in sterile distilled water, and then immobilized by entrapment in Ca-alginate beads. An adequate volume of the cell suspension was added to a solution of sodium alginate previously heated to 121°C for 15 min. The final concentration of sodium alginate was 20 g/L and the final concentration of cells was 3 g/L (dry weight). Cell-gel beads (2.70 ± 0.10 mm in diameter) were produced by dripping the cell suspension in a 11 g/L $CaCl_2$ solution, using a 19 G (1-½ inch) needle and a peristaltic pump. The cell-gel beads were maintained in the $CaCl_2$ solution at 4°C for 24 h, washed with sterile distilled water, and introduced into the fermentation flasks.

Medium and Fermentation Conditions

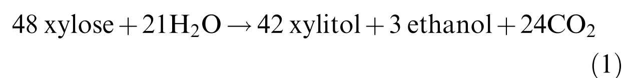
The detoxified hydrolyzate was heated to 110°C for 15 min and supplemented with the same nutrients as described in the inoculum preparation. Erlenmeyer flasks (125 mL) containing 10 mL of immobilized biocatalysts (void volume neglected) and 40 mL of fermentation medium were maintained in a rotary shaker at 200 rpm and 30°C for 72 h.

Analytical Methods

The concentrations of glucose, xylose, arabinose, xylitol, ethanol, and acetic acid were determined by HPLC as described by Carvalho et al. (2000). The cell concentration used in the immobilization step was determined by absorbance at 600 nm and correlated with the cell dry weight through a corresponding calibration curve. The suspended cell concentration in the fermentation medium was determined by direct count in a Neubauer chamber and correlated with the cell dry weight. The immobilized cell concentration (concerning the volume of the beads) was analyzed in the same way, after the Ca-alginate beads were dissolved in a 2% potassium citrate solution. Both immobilized and suspended cell concentrations were finally related to the reactor working volume (50 mL).

Carbon Material Balance

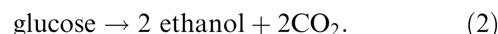
Carbon material balances were based on the productions of xylitol, arabitol, ethanol, CO_2, and biomass, as well as on the consumptions of xylose, arabinose, glucose, and acetic acid. The following equation, proposed by Barbosa et al. (1988) for the redox-balanced production of xylitol under microaerobiosis, was used,



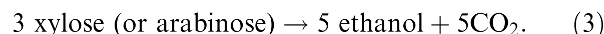
$$48\ \text{xylose} + 21H_2O \rightarrow 42\ \text{xylitol} + 3\ \text{ethanol} + 24CO_2 \quad (1)$$

taking into account the parallel metabolism necessary to obtain energy. A similar equation was assumed for arabitol production from arabinose.

The excess production of ethanol observed with respect to this stoichiometry (lacking in synthetic medium) suggested that the several carbon sources present in the hydrolyzate were responsible for the activation of alternative routes, thus complicating the interpretation of the metabolic results. Therefore, the excess ethanol was ascribed to alcoholic fermentation, which was assumed to consume the carbon sources, namely glucose, xylose, and arabinose (listed here in order of preference), according to the following equations:



$$\text{glucose} \rightarrow 2\ \text{ethanol} + 2CO_2. \quad (2)$$



$$3\ \text{xylose (or arabinose)} \rightarrow 5\ \text{ethanol} + 5CO_2. \quad (3)$$

The same order of preference was applied to biomass growth. The minimal formula $CH_{1.79}O_{0.50}N_{0.20}$ reported by Roels (1983) for yeast dry biomass was used for the calculation of carbon consumption due to cell growth. All the carbon source fractions not consumed by these routes, including those of acetic acid, were considered as having been utilized for respiration.

The amount of CO_2 produced was estimated as the difference between total starting carbon (from xylose, glucose, arabinose, and acetic acid) and carbon consumed for ethanol, xylitol, arabitol, and biomass productions. Blank tests were periodically performed in the way described by Converti and Domínguez (2001), with a 5-L reactor equipped with a continuous CO_2 analyzer in order to determine the difference between the estimated CO_2 production values and the amounts actually produced.

Table I. Experimental results of microaerobic fermentation of sugarcane bagasse acid hydrolyzate by *C. guilliermondii* cells immobilized in Ca-alginate beads (averages of two repetitions).

Time (h)	0	12	24	36	48	60	72
Xylose (g/L)	48.00	37.50	26.80	14.20	4.32	0.51	0
Glucose (g/L)	4.75	0.09	0	0	0	0	0
Arabinose (g/L)	4.23	3.57	3.12	3.05	2.99	2.62	2.09
Acetic acid (g/L)	3.69	2.84	2.01	1.31	0.76	0.35	0.04
Xylitol (g/L)	0	2.68	7.21	14.50	20.60	21.30	19.10
Ethanol (g/L)	0	2.25	3.89	4.50	4.70	4.75	4.76
Arabitol (g/L)	0	0.21	0.56	0.61	0.64	0.74	0.83
X (g/L)	0	0.80	1.66	1.98	2.25	3.01	3.50
X_{im} (g/L)	1.29	2.68	3.46	3.50	3.53	3.56	3.58
CO_2 (g/L)	0	11.40	16.70	20.50	27.50	31.70	33.10

X: Suspended cell concentration; X_{im}: Immobilized cell concentration.

RESULTS AND DISCUSSION

The experimental data shown in Table I describe the fermentation profile of the cells in the sugarcane bagasse hemicellulose hydrolyzate. The xylitol concentration (20.6 g xylitol/L), volumetric productivity (0.43 g xylitol/L · h), and product yield (0.47 g xylitol/g xylose consumed) obtained after 48 h of fermentation were higher than those reported by Domínguez et al. (1999). Unfortunately, these values are still lower than those obtained by free-cell cultivation systems (Parajó et al., 1998; Rodrigues et al., 1998).

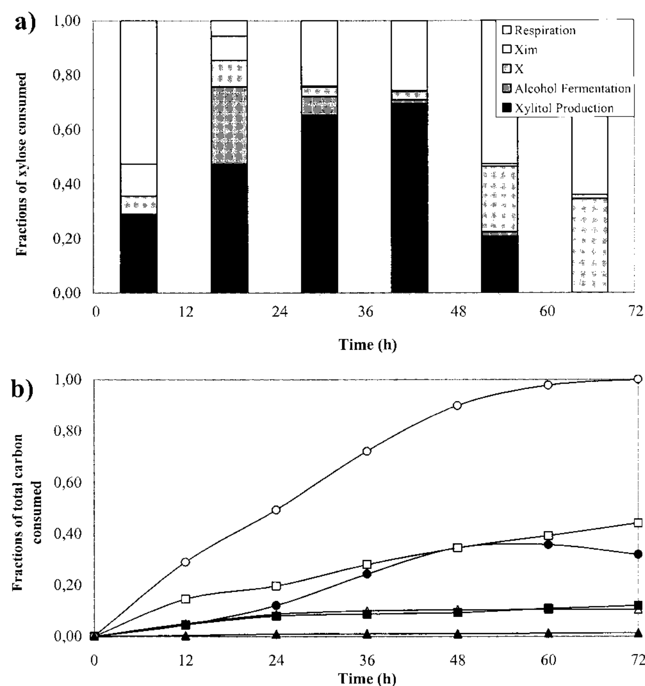


Figure 1. Microaerobic fermentation of sugarcane bagasse acid hydrolyzate by *C. guilliermondii* cells immobilized in Ca-alginate beads. (a) Fractions of xylose consumed by different metabolic pathways during the different fermentation phases. (b) Fractions of total carbon consumed for the formation of different fermentation products: (●) xylitol; (Δ) ethanol; (■) total biomass; (□) carbon dioxide; (▲) arabitol; (○) total products.

Similar concentrations of suspended and immobilized cells observed at the end of the run (about 3.5 g/L) show that the immobilized-cell system was subject to cell leakage from the alginate beads. Yahashi et al. (1996) also observed the presence of leaked cells in the fermentation medium while using Ca-alginate immobilized cells for xylitol production in synthetic xylose medium. In fact, according to Chen and Huang (1989) and to Quirós et al. (1995), cell growth in calcium alginate beads leads to the appearance of crater-like pores on the gel surface, which become the main passage for the cell leakage from the gels. As can be seen in Table I, the high final concentration of suspended cells, absent at the start of the run, was due to their faster growth with respect to the immobilized cells, mainly after the first 24 h of cultivation. Hence, the following conclusions about the metabolic behavior apply to a mixed immobilized/free-cell system instead of to an immobilized-cell system.

As can be seen in Figure 1a, the fraction of xylose consumed to produce xylitol reached a maximum value (0.70) between 36 and 48 h of fermentation, after glucose and oxygen depletion, while alternative metabolic routes were favored by sub-optimal conditions. Xylose fermentation to ethanol was practically non-existent at the

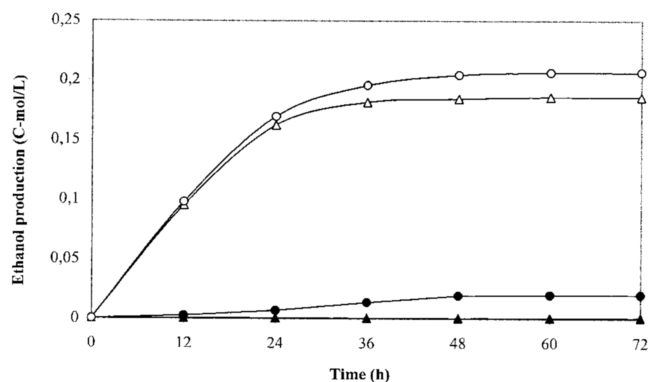


Figure 2. Ethanol produced from total carbon by different metabolic activities (C-mol/L): (●) xylitol production; (Δ) alcoholic fermentation; (▲) arabitol production; (○) total ethanol.

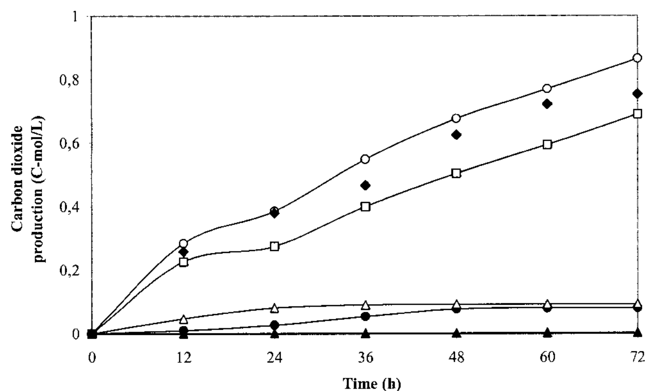


Figure 3. Carbon dioxide produced from total carbon by different metabolic activities (C-mol/L): (●) xylitol production; (△) alcoholic fermentation; (□) respiration; (▲) arabitol production; (○) total carbon dioxide (estimated); (◆) total carbon dioxide (experimental).

start of the run, due to at least four different causes: (a) presence of a more easily fermentable carbon source (glucose); (b) catabolite repression of the induction of XR activity (Sugai and Delgenes, 1995); (c) inactivation of the xylose transport system (Nobre et al., 1999); and (d) presence of oxygen favoring growth and respiration. The ethanol production was suppressed also at the end of the run, when the main fermentable substrates were nearly exhausted. During these phases, respiration appeared to gain relative importance, consuming more than 52% and 64% of xylose.

Since glucose is assimilated better than xylose by pentose-fermenting yeasts, cell growth during the start of the run was assumed to take place preferentially at the expense of glucose and subsequently of xylose. According to this hypothesis, it was verified that cell growth accounted for 18–19% xylose consumption at the start of fermentation (up to 24 h), whereas it was nearly negligible during the subsequent phases. This demonstrates the validity of adopting microaerobic conditions to initially stimulate cell growth and, subsequently, xylitol production.

As shown in Figure 1b, total carbon was consumed very rapidly during the first 12 h of fermentation, mainly due to respiration and, to some extent, to cell growth as well as to the production of ethanol and xylitol. After glucose and oxygen depletion, the carbon consumption rate progressively decelerated. Although the splitting up of xylose among the different metabolic routes was qualitatively similar to that of total carbon, xylitol was the main xylose product, whereas carbon dioxide was the main total carbon product, due to respiration of minor carbon sources (like acetic acid) and fermentation of glucose.

Figure 2 shows the fraction of ethanol progressively formed from total carbon by alcoholic fermentation and the fractions associated with xylitol and arabitol productions. As expected (Hsiao et al., 1982), more than 90% of ethanol came from alcoholic fermentation within the first 24 h, whereas only negligible fractions were associated with the reduction of both xylose and arabinose to their respective pentitols. With respect to Eq. (1), the excess ethanol observed mainly during the first 12 h of fermentation was ascribed to glucose fermentation through Eq. (2). Xylose was the major carbon source for ethanol production after complete glucose depletion.

Also the CO_2 release was initially very rapid because of the oxygen availability for respiration. As shown in Figure 3, respiration was responsible for most of the CO_2 produced throughout the run, while the alcoholic fermentation promoted a significant CO_2 production only at the start of the run. The difference between experimental and estimated values of the progressive CO_2 production never exceeded 15%, which demonstrates a satisfactory reliability of the proposed model.

These results show that the secondary carbon sources play an important role in the fermentation of hemicellulose hydrolyzates and confirm the validity of carbon material balances in the study of the metabolic behavior of fermentation systems.

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