

# Use of Immobilized *Candida* Cells on Xylitol Production from Sugarcane Bagasse

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In this study we used the yeast *Candida guilliermondii* FTI 20037 immobilized by entrapment in Ca-alginate beads (2.5–3 mm diameter) for xylitol production from concentrated sugarcane bagasse hemicellulosic hydrolysate in a repeated batch system. The fermentation runs were carried out in 125- and 250-ml Erlenmeyer flasks placed in an orbital shaker at 30 °C and 200 rpm during 72 h, keeping constant the proportion between work volume and flask total volume. According to the results, cell viability was substantially high (98%) in all fermentative cycles. The values of parameters xylitol yield and volumetric productivity increased significantly with the reutilization of the immobilized biocatalysts. The highest values of xylitol final concentration (11.05 g/l), yield factor (0.47 g/g) and volumetric productivity (0.22 g/lh) were obtained in 250-ml Erlenmeyer flasks containing 80 ml of medium plus 20 ml of immobilized biocatalysts. The support used in this study (Ca-alginate) presented stability in the experimental conditions used. The results show that the use of immobilized cells is a promising approach for increasing the xylitol production rates.

## Introduction

Xylitol is a polyalcohol with a sweetening power comparable to that of sucrose (Bär, 1986). This polyol, used in provision industries in the Scandinavian countries (Hahn-Hägerdal *et al.*, 1994), has stood out in the last years for its anticariogenic properties (Pepper and Olinger, 1988) and for its clinical applications as a sugar substitute in cases of diabetes, obesity, deficiency in glucose 6P dehydrogenase enzyme and problems in the metabolism of lipids (Ylikahri, 1979).

From the discovery of the ability of certain yeasts to ferment xylose as a source of carbon and energy (Schneider *et al.*, 1981; Slininger *et al.*, 1982), the interest of scientists in xylitol production by biotechnological means has increased worldwide, since this process has several advantages over the conventional chemical process (Ojamo *et al.*, 1988). In fact, several researchers have pursuing the development of an economical and feasible technique for xylitol bioproduction from lignocellulosic materials.

The bioconversion of xylose into xylitol can be made by the use of mold, bacteria, yeasts or purified enzymes (Winkelhausen and Kuzmanova,

1998). The yeast *Candida guilliermondii* FTI 20037, selected in our laboratories (Barbosa *et al.*, 1988), has proven suitable for this bioprocess, mainly when lignocellulosic materials like eucalyptus chips (Felipe *et al.*, 1996a), rice straw (Roberto *et al.*, 1996) and sugarcane bagasse (Silva *et al.*, 1997) are used as sources of xylose.

Lignocellulosic biomass is a potential renewable source of carbohydrates that can be used as substrates in biotechnological processes. Before biological utilization, the biomass needs to be hydrolysed in order to remove the sugars from the polymeric fraction. Acid hydrolysis of the hemicellulosic fraction produces mainly pentose sugars, xylose being the major component (Parajó *et al.*, 1996). Sugarcane bagasse is an appropriate raw material for xylitol production, since its hemicellulosic fraction contains 80% of xylose (Kuhad and Singh, 1993). Besides, from the ecological point of view, the adoption of technologies employing this material would prevent serious storage problems and environmental pollution caused by the surplus of bagasse (Burgi, 1988). On the other hand, hemicellulosic hydrolysates obtained through acid hydrolysis contains several inhibitory substances,

and they constitute the main obstacle to the use of such hydrolysates as a fermentation medium (Felipe *et al.*, 1997a).

The adaptation of the microorganism to the hydrolysate (Chen and Gong, 1985; Felipe *et al.*, 1996b; Sene *et al.*, 1998), the recycle of cells (Sene *et al.*, 1998) and the use of high cell concentrations (Chung and Lee, 1985; Felipe *et al.*, 1996a; Felipe *et al.*, 1997a) are procedures for reducing the toxic effects of the hydrolysate on the activity of the microorganisms and for improving the fermentative parameters of xylitol production. Fermentations in repeated batch mode are appropriate for this bioprocess because they promote a good adaptation of the cells to the hydrolysate. In this sense, the immobilized cell system has been recommended not only for maximizing the fermented broth fraction discharged at the end of each batch but also for facilitating the reutilization of the biocatalysts, for minimizing times and separation costs, and for making the cells more stable (Gamarra *et al.*, 1986; Dervakos and Webb, 1991; Lothar and Oetterer, 1995; Domínguez, 1998).

Among the methods of cell immobilization, is the entrapment in Ca-alginate hydrogel a simple and low-cost technique, mainly used for the immobilization of viable cells, preserving the cell viability (Vitolo and Carreira, 1992; Nolan *et al.*, 1994; Lothar and Oetterer, 1995; Jen *et al.*, 1996; Yahashi *et al.*, 1996; Roca *et al.*, 1996).

There are few publications on the use of immobilized cells applied to xylitol production (Silva and Afschar, 1994; Yahashi *et al.*, 1996; Roca *et al.*, 1996; Domínguez, 1998). Although obtained with the use of synthetic medium, these studies prove the technical viability of this procedure as an alternative to increase productivity and process efficiency.

This study evaluates the xylitol production from concentrated sugarcane bagasse hydrolysate in repeated batch fermentations with the use of immobilized cells of the yeast *Candida guilliermondii* FTI 20037.

## Materials and Methods

### *Preparation of the sugar cane bagasse hydrolysate*

The hemicellulosic hydrolysate was obtained by acid hydrolysis of sugar cane bagasse in a 250 liters steel reactor at 121 °C for 10 min, using a catalyst/

bagasse ratio of 100 mg of sulfuric acid per gram of bagasse (dry-weight). After the hydrolysis was completed, the liquid fraction was separated by centrifugation and concentrated at 70 °C under vacuum, in order to obtain a final xylose concentration of 38 g/l. The concentrated hydrolysate was treated by raising the pH to 7.0 with calcium oxide, by reducing it to 5.5 with phosphoric acid and by adding 2.5% active charcoal under agitation (200 rpm) at 30 °C for 1 h (Alves *et al.*, 1998). In all the treatments the precipitate resulting from pH adjustment and from addition of activated charcoal was removed by vacuum filtration.

### *Medium and fermentation conditions*

The hydrolysate was heated at 110 °C for 15 min, and supplemented with ammonium sulfate (3 g/l), calcium chloride (0.1 g/l) and rice bran (20 g/l) before being utilized as a fermentation medium.

The fermentations were carried out in 125- and 250-ml Erlenmeyer flasks containing 50 and 100 ml of work volume, respectively (ratio of 1/4 between immobilized biocatalysts and fermentation medium) in repeated batch mode. The flasks were placed in an orbital shaker at 200 rpm and 30 °C for 72 h. At the end of each fermentation run, the fermented medium was discarded and the immobilized biocatalysts were re-fed with fresh medium.

### *Microorganism and inoculum cultivation*

*Candida guilliermondii* FTI 20037 described by Barbosa *et al.* (1988) was maintained as a slant on malt extract agar at 4 °C. A loopful of cells were transferred to 125-mL Erlenmeyer flasks containing 50 ml of medium constituted of xylose (30 g/l), ammonium sulfate (3 g/l), calcium chloride (0.1 g/l) and rice bran (20 g/l). The inoculum was cultivated in an orbital shaker at 200 rpm and 30 °C for 24 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.

### *Cell immobilization*

The yeast cells were immobilized by entrapment in calcium alginate (SG 800 – Sanofi do Brasil Indústria e Comércio Ltd., Brazil). An adequate

volume of the cellular suspension obtained was added to a solution of sodium alginate previously heated at 121 °C for 15 min, the final concentration of sodium alginate being 1% w/v and the final cellular concentration being 1 g/l (dry-weight). Cell-gel beads about 2.5–3 mm in diameter were produced by dripping this mixture into a gently stirred solution of calcium chloride (0.1 M in Ca<sup>2+</sup>). A 19-G needle (1½inches) and a peristaltic pump were used to make the beads. The cell-gel beads were maintained in calcium chloride (0.1 M in Ca<sup>2+</sup>) at 4 °C for 24 h. Afterwards, the beads were washed with sterile distilled water and added to the fermentation flasks.

*Analytic methods*

The glucose, xylose, xylitol and acetic acid concentrations were determined by HPLC using a refraction index detector and a Bio-Rad HPX 87:H column under the following conditions: H<sub>2</sub>SO<sub>4</sub> 0.01 N as eluent, flow of 0.6 ml/min, temperature of the column 45 °C and sample volume to 20 µl.

The cell concentration of the inoculum was determined by absorbance at 600 nm and correlated with the dry-weight of cells (g/l) through a corresponding calibration curve. The concentration of free cells in the fermentation medium was estimated by direct count in a Neubauer chamber.

**Results and Discussion**

As can be verified from Table I, the glucose was consumed quickly in all the fermentations, since

the presence of this sugar was not detected in the medium after 24 h. A fast consumption was also verified by Felipe *et al.* (1997a), using different inoculum concentrations.

As can be observed in Figures 1 and 2, the xylose consumption and xylitol formation rates were increased significantly in the second batch fermentation under both conditions. In 125-ml flasks, after 48 h of fermentation, the xylose concentrations decreased by 76 and 100 %, while the xylitol concentrations increased by 3.15 and 10.08 g/l in the first and second batch fermentations, respectively. Similar results were observed in fermentations carried out in 250-ml flasks. In the same period, the xylose concentrations decreased by 86 and 98%, while the xylitol concentrations increased by 6.51 and 10.74 g/l in the first and second batch fermentations, respectively.

The significant improvement in the xylitol yield and productivity in the second batch (Table II) can

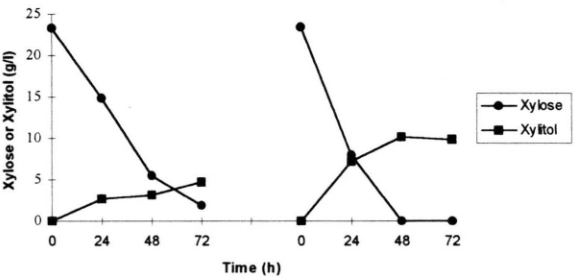


Fig. 1. Xylose consumption and xylitol production during the first and second batch fermentations in 125-ml Erlenmeyer flasks.

Table I. Variation of the pH and of the glucose, xylose, xylitol, acetic acid and free cells concentrations in the fermentation medium as a function of time of fermentation in 125-ml (data in brackets) and 250-ml Erlenmeyer flasks (averages of two repetitions).

| Time (h)                     | 1 <sup>st</sup> Batch |                  |                |                | 2 <sup>nd</sup> Batch |                |                  |                 |
|------------------------------|-----------------------|------------------|----------------|----------------|-----------------------|----------------|------------------|-----------------|
|                              | 0                     | 24               | 48             | 72             | 0                     | 24             | 48               | 72              |
| Glucose (g/l)                | 1.39<br>(1.39)        | 0<br>(0)         | 0<br>(0)       | 0<br>(0)       | 1.28<br>(1.28)        | 0<br>(0)       | 0<br>(0)         | 0<br>(0)        |
| Xylose (g/l)                 | 23.23<br>(23.23)      | 13.74<br>(14.77) | 3.36<br>(5.51) | 0.34<br>(1.87) | 23.36<br>(23.36)      | 9.82<br>(7.90) | 0.43<br>(0)      | 0<br>(0)        |
| Xylitol (g/l)                | 0<br>(0)              | 3.03<br>(2.62)   | 6.51<br>(3.15) | 7.26<br>(4.76) | 0<br>(0)              | 6.96<br>(7.12) | 10.74<br>(10.08) | 11.05<br>(9.90) |
| Acetic acid (g/l)            | 2.10<br>(2.10)        | 1.32<br>(1.36)   | 1.11<br>(0)    | 0<br>(0)       | 2.70<br>(2.70)        | 1.78<br>(0.94) | 0.35<br>(0)      | 0<br>(0)        |
| (Cells/ml) × 10 <sup>8</sup> | 0<br>(0)              | 0.57<br>(0.83)   | 1.36<br>(1.93) | 2.05<br>(2.23) | 0<br>(0)              | 0.76<br>(0.90) | 1.47<br>(2.10)   | 1.95<br>(2.73)  |
| pH                           | 5.00<br>(5.00)        | 5.06<br>(5.13)   | 5.15<br>(5.20) | 5.36<br>(5.33) | 5.06<br>(5.06)        | 5.17<br>(5.37) | 5.74<br>(5.56)   | 6.09<br>(5.72)  |

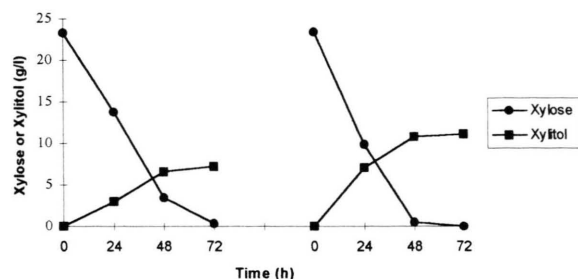


Fig. 2. Xylose consumption and xylitol production during the first and second batch fermentations in 250-ml Erlenmeyer flasks.

be explained by the limitation of oxygen and nutrients in the pellets during the first batch fermentation. In this phase the cells contained inside the pellets usually grow more slowly. After a initial time, it is possible to observe a preferential colonization in the periphery of the pellets where the limitations of mass and oxygen transfer are smaller. In this area, after the colonization, the cells begin to exhibit a stronger metabolic activity, with a consequent increase in the fermentative parameters (Omar, 1993; Dominguez, 1998).

On the other hand, recycling and adaptation of the cells to the hydrolysate result in better fermentative parameters, possibly due to the adaptation of the cells to the toxic compounds generated during the acid hydrolysis of lignocellulosics which interfere with cell growth and xylitol production rates (Sene *et al.*, 1998).

The increase of 95 and 47% in the yield factor and of 200 and 120% in productivity obtained by reutilizing immobilized cells in 125- and 250-ml Erlenmeyers flasks, respectively, enhances the possibility of using immobilized biocatalysts for the xylitol production in medium composed of sugar-cane bagasse hemicellulosic hydrolysates.

Table II. Yield factor ( $Y_p/s$ ), volumetric productivity ( $Q_p$ ) and fermentation efficiency ( $\eta$ ) of the xylose-xylitol bioconversion in the first and second batches in 125-ml (data in brackets) and 250-ml Erlenmeyer flasks.

|                       | $Y_p/s$ [g/g]  | $Q_p$ [g/l $\times$ h] | $\eta$ [%]       |
|-----------------------|----------------|------------------------|------------------|
| 1 <sup>st</sup> Batch | 0.32<br>(0.22) | 0.10<br>(0.07)         | 34.90<br>(24.00) |
| 2 <sup>nd</sup> Batch | 0.47<br>(0.43) | 0.22<br>(0.21)         | 51.25<br>(46.90) |

After 72 h of fermentation, the presence of acetic acid was not evidenced in the fermentation medium (Table I). On the contrary, this acid was found in the cultivation of free cells (Felipe *et al.*, 1997b). The use of this compound by the yeast is a favorable characteristic in fermentations that have lignocellulosic hydrolysates as main components, since the presence of acetic groups is common in the hemicellulosic fraction of such materials.

Table I shows that acetic acid consumption rate was higher in 125-ml flasks, probably due to the concentration of dissolved oxygen in the medium. Parajó *et al.* (1997) observed that the fermentations are faster and less affected by inhibitors when the hydrolysate medium is more aerated. According to Nollet *et al.* (1993), the limited supply of oxygen to the cells leads to the accumulation of acetic acid in the medium. It can thus be assumed that the dissolved oxygen concentration was higher in 125-ml flasks because they contained a smaller volume of medium and were agitated at the same rate as the 250-ml flasks.

This hypothesis is reinforced by determining the cell concentrations suspended in the culture medium (Table I), as well as the xylitol yield factor (Table II). The lower xylitol yields and higher cell free concentrations attained in 125-ml flasks suggest that the dissolved oxygen concentration in the medium was higher than that ideal for xylitol production, xylose being used for biomass production. In agreement with Gírio *et al.* (1989), in conditions of high aeration, a smaller amount of xylitol is accumulated in the medium, because the readiness of oxygen promotes a higher  $NAD^+/NADH$  proportion and, consequently, favors the xylitol oxidation to xylulose.

Table I shows that the Ca-alginate presented low cell retention capacity in both flasks. The concentration of free cells in the fermentation medium was of the order of  $10^7$  cells/ml after 24 hours of fermentation, probably because the sodium alginate concentration used was insufficient to form a relatively closed mesh to retain the cells. Domínguez (1998), using concentration of this compound same to 3% verified an insignificant loss of cells for the fermentation medium.

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