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# Use of Immobilized *Candida* Yeast Cells for Xylitol Production from Sugarcane Bagasse Hydrolysate

*Cell Immobilization Conditions*

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## Abstract

*Candida guilliermondii* cells were immobilized in Ca-alginate beads and used for xylitol production from concentrated sugarcane bagasse hydrolysate. A full factorial design was employed to determine whether variations in the immobilization conditions would have any effects on the beads, chemical stability and on the xylitol production rates. Duplicate fermentation runs were carried out in 125-mL Erlenmeyer flasks maintained in a rotatory shaker at 30°C and 200 rpm for 72 h. Samples were periodically analyzed to monitor xylose and acetic acid consumption, xylitol production, free cell growth, and bead solubilization. Concentrations of sodium alginate at 20.0 g/L and calcium chloride at 11.0 g/L and bead curing time of 24 h represented the most appropriate immobilization conditions within the range of conditions tested.

**Index Entries:** Xylitol; sugarcane bagasse hydrolysate; immobilized cells; immobilization conditions; factorial design.

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## Introduction

Xylitol, a special sweetener with outstanding organoleptic and anticariogenic properties, is an important component of oral hygiene products, diabetic foods, sugarless chewing gums, candies, and other products *(1)*.

Because xylitol is currently obtained by a chemical process involving high costs, its use is still limited to higher-end products *(2)*. The production of xylitol by biotechnologic means is an alternative way to reduce such costs, since the downstream processing is expected to be cheaper *(3)*. In fact, several researchers are pursuing the development of an economical and feasible technique for xylitol bioproduction from hemicellulosic hydrolysates derived from agricultural residues.

The outcome of the xylose-to-xylitol bioconversion significantly depends on the nature of the fermentation medium. Two types of medium can be used: synthetic xylose solutions and hemicellulosic hydrolysates. The advantage of hydrolysates made from lignocellulosic residues is that these materials are renewable, widespread, inexpensive, and contain a large proportion of xylose *(4)*. On the other hand, the bioconversion of the xylose contained in such hydrolysates is complicated by the presence of toxic compounds, which inhibit the microbial growth and the fermentative activity of the yeasts *(5)*. These compounds either are released from the lignocellulosic structure or originated from the sugar degradation during the hydrolytic process.

Several methods have been proposed to minimize the toxicity of these hydrolysates: adaptation and recycling of cells and hydrolysate treatment by solvent extraction, neutralization, overliming, ion-exchange resins, and active charcoal adsorption *(6)*. High initial cell concentrations have also been used for the same purpose *(5,7)*, with cell death caused by the assimilation or degradation of toxic compounds reduced as a consequence *(6)*.

High cell concentrations can be achieved with the use of immobilized cell systems *(8–10)*, which, in addition, increase the efficiency of the fermentation process by providing higher fermentation rates, permitting the reuse of the same biocatalysts for extended periods of time, facilitating the separation of the biocatalysts from the liquid phase where the desired products are present, improving the protection of the cells against inhibitors, and encouraging continuous processes that may lower capital costs *(10)*.

Cell entrapment within polymeric materials is by far the most suitable method for entrapping biomass in fermentation processes. Alginate is the most common support employed for this purpose, owing to its rapid gelation caused by calcium ions without drastic changes in temperature, pH, and osmotic pressure, which could affect the activity and viability of the microorganisms *(11,12)*. The main drawback of using Ca-alginate as the immobilization support is its chemical instability; thus, it is necessary to determine the immobilization conditions that could enhance the chemical stability of the beads without imposing mass-transfer limitations *(13)*.

Table 1
Composition of Sugar Cane Bagasse Hemicellulosic Hydrolysate Before and After Vacuum Concentration at 70°C

| Component (g/L) | Before concentration | After concentration |
|---|---|---|
| Glucose | 1.66 ± 0.01 | 4.51 ± 0.46 |
| Xylose | 20.15 ± 0.17 | 59.36 ± 2.15 |
| Arabinose | 1.57 ± 0.06 | 5.15 ± 0.43 |
| Acetic Acid | 2.47 ± 0.12 | 4.03 ± 0.33 |
| Furfural | 0.32 ± 0.02 | 0.06 ± 0.01 |
| Hydroxymethylfurfural | 0.05 ± 0.01 | 0.14 ± 0.01 |

In the present study, different cell immobilization conditions were investigated with a view to improving the efficiency and stability of the cell-gel beads during xylose-to-xylitol bioconversion in concentrated sugarcane bagasse hemicellulosic hydrolysate.

## Materials and Methods

### *Preparation and Treatment of Sugarcane Bagasse Hydrolysate*

Sugarcane bagasse from Usina Santa Helena (Rio das Pedras, S.P., Brazil) was hydrolyzed in a 250-L steel reactor at 121°C for 10 min, using 100 mg of sulfuric acid/g of bagasse (dry wt) at a solid:liquid ratio of 1:10. The liquid fraction of the hydrolysate was then separated by centrifugation and concentrated threefold at 70°C under vacuum. Table 1 lists the hydrolysate compositions before and after vacuum concentration.

To minimize the concentrations of the main fermentation inhibitors, 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 *(14)*. In all the treatments, the precipitate resulting from the pH adjustment and from the addition of activated charcoal was removed by vacuum filtration.

### *Cultivation of Microorganism and Inoculum*

*Candida guilliermondii* FTI 20037, described by Barbosa et al. *(15)*, was maintained on a malt extract agar slant at 4°C. A loopful of cells was transferred to 125-mL Erlenmeyer flasks containing 50 mL of medium consisting of xylose (30 g/L), ammonium sulfate (3 g/L), calcium chloride (0.1 g/L), and rice bran extract (20% [v/v]). The inoculum was cultivated in a rotatory shaker at 200 rpm and 30°C for 24 h. Afterward, the cells were collected by centrifugation (2000*g*, 15 min), rinsed thoroughly with sterile distilled water, centrifuged, and resuspended in sterile distilled water.

Table 2
Variable Levels Used According to $2^3$ Full Factorial Design

| Variable | Symbol | Level | | |
|---|---|---|---|---|
| | | – | 0 | + |
| Sodium alginate concentration (g/L) | AC | 10.0 | 15.0 | 20.0 |
| Calcium chloride concentration (g/L) | CC | 11.0 | 16.5 | 22.0 |
| Bead curing time (h) | CT | 12.0 | 18.0 | 24.0 |

### *Cell Immobilization*

The yeast cells were immobilized by entrapment in Ca-alginate beads. An adequate volume of the cellular suspension was added to a solution of sodium alginate (SG 800; Sanofi do Brasil Indústria e Comércio, Brazil) previously heated at 121°C for 15 min, with a final cell concentration of 3 g/L (dry wt). Cell-gel beads (2.8 mm in diameter) were produced by dripping this mixture into a gently stirred solution of calcium chloride. A 19-gage needle (1.5 in.) and a peristaltic pump were used to make the beads. The cell-gel beads were maintained in the calcium chloride solution at 4°C. Afterward, the beads were washed with sterile distilled water and introduced into the fermentation flasks. Table 2 gives the levels of the immobilization variables (concentrations of sodium alginate and calcium chloride and bead curing time) evaluated.

### *Medium and Fermentation Conditions*

The treated hydrolysate was heated to 110°C for 15 min and supplemented with ammonium sulfate (3 g/L), calcium chloride (0.1 g/L), and rice bran extract (20% [v/v]) before being used as the fermentation medium.

Erlenmeyer flasks (125-mL) containing 10 mL of immobilized biocatalysts (void volume neglected) and 40 mL of fermentation medium were maintained in a rotatory shaker at 200 rpm and 30°C for 72 h. Tables 2 and 3 provide the sodium alginate and calcium chloride concentrations and the bead curing time used for cell immobilization in each experiment.

### *Analytical Methods*

Xylose, xylitol, and acetic acid concentrations were determined by high-performance liquid chromatography (HPLC) with a refraction index detector and a Bio-Rad HPX87:H column using 0.01 *N* $H_2SO_4$ as the eluent, a flow rate of 0.6 mL/min, a column temperature of 45°C and a sample volume of 20 µL.

Furfural and hydroxymethylfurfural concentrations were determined by HPLC with a UV-VIS detector at 276 nm and a Hewlett Packard RP18 column using acetonitrile (1:8) with 1% acetic acid as the eluent, flow of 0.8 mL/min, column temperature of 25 ºC and sample volume of 20 µL.

Table 3
Values of Xylitol Volumetric Productivity ($Q_P$), Yield Factor ($Y_{P/S}$), and Ca-Alginate Bead Solubilization ($\phi$), After 48 h of Fermentation, According to $2^3$ Full Factorial Design

| | Coded variable | | | Response[a] | | |
|---|---|---|---|---|---|---|
| Assay | AC | CC | CT | $Q_P$ (g/L·h) | $Y_{P/S}$ (g/g) | $\phi$ (%) |
| 1 | –1 | –1 | –1 | 0.41 | 0.41 | 6.4 |
| 2 | +1 | –1 | –1 | 0.41 | 0.43 | 3.9 |
| 3 | –1 | +1 | –1 | 0.36 | 0.40 | 2.1 |
| 4 | +1 | +1 | –1 | 0.40 | 0.43 | 3.6 |
| 5 | –1 | –1 | +1 | 0.41 | 0.41 | 2.1 |
| 6 | +1 | –1 | +1 | 0.43 | 0.47 | 1.9 |
| 7 | –1 | +1 | +1 | 0.39 | 0.40 | 2.0 |
| 8 | +1 | +1 | +1 | 0.38 | 0.41 | 0 |
| 9 | 0 | 0 | 0 | 0.43 | 0.43 | 5.5 |
| 10 | 0 | 0 | 0 | 0.41 | 0.42 | 5.6 |
| 11 | 0 | 0 | 0 | 0.38 | 0.40 | 3.8 |

[a]$Q_P$, xylitol concentration/fermentation time; $Y_{P/S}$, xylitol produced/xylose consumed; $\phi$, [(initial mean diameter of Ca-alginate beads–final mean diameter of Ca-alginate beads)/initial mean diameter of Ca-alginate beads] × 100.

The cell concentrations in the immobilization step were determined by absorbance at 600 nm and correlated with the dry wt of the cells through a corresponding calibration curve. The suspended cell concentrations in the fermentation medium were determined by direct count in a Neubauer chamber.

The chemical stability of the Ca-alginate beads was estimated by monitoring their solubilization (reductions in the mean diameter, assuming a spherical geometry) during the batch fermentations.

### *Statistical Analyses*

A two-level full factorial design *(16)* was employed to identify the levels of the immobilization variables (sodium alginate concentration, calcium chloride concentration and beads curing time) that maximize the chemical stability of the Ca-alginate bead without imposing limitations on the transfer of substrates from the bulk fermentation medium to the immobilized cells. The effects of the immobilization variables on the bead chemical stability and on the xylose-to-xylitol bioconversion were simultaneously investigated according to statistical concepts using the Statgraphics program (version 6.0).

## Results and Discussion

As can be seen in Table 3, the xylitol volumetric productivity ($Q_P$) and yield factor ($Y_{P/S}$) varied slightly from 0.36 to 0.43 g/(L·h) and from 0.40 to

Table 4
Estimated Effects, SEs and student's *t*-Test Values for Xylitol Volumetric Productivity ($Q_P$), Yield Factor ($Y_{P/S}$), and Ca-Alginate Bead Solubilization ($\phi$) After 48 h of Fermentation

| | $Q_P$ (g/L·h) | | | $Y_{P/S}$ (g/g) | | | ($\phi$) (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable[a] | Effect | SE | *t* Value | Effect | SE | *t* Value | Effect | SE | *t* Value |
| Average | + 0.401 | ± 0.007 | — | + 0.419 | ± 0.005 | — | + 3.355 | ± 0.621 | — |
| AC | + 0.013 | ± 0.016 | 0.787[b] | + 0.030 | ± 0.011 | 2.727[d] | – 0.800 | ± 1.457 | 0.549[b] |
| CC | – 0.033 | ± 0.016 | 2.047[c] | – 0.020 | ± 0.011 | 1.818[c] | – 1.650 | ± 1.457 | 1.132[b] |
| CT | + 0.008 | ± 0.016 | 0.472[b] | + 0.005 | ± 0.011 | 0.455[b] | – 2.500 | ± 1.457 | 1.716[c] |
| AC x CC | + 0.003 | + 0.016 | 0.157[b] | – 0.010 | ± 0.011 | 0.909[b] | + 0.550 | ± 1.457 | 0.377[b] |
| AC x CT | – 0.008 | ± 0.016 | 0.472[b] | + 0.005 | ± 0.011 | 0.455[b] | – 0.300 | ± 1.457 | 0.206[b] |
| CC x CT | – 0.003 | ± 0.016 | 0.157[b] | – 0.015 | ± 0.011 | 1.364[c] | + 0.650 | ± 1.457 | 0.446[b] |

[a] AC, CC, CT: main effects; AC × CC, AC × CT, CC × CT: two-factor interactions.

[b] $p > 0.2$

[c] $0.1 < p < 0.2$

[d] $p < 0.05$

0.47 g/g, respectively, while the bead solubilization percentage (ϕ) varied from 0 to 6.4%. Table 4 summarizes the statistical analysis of each response variable evaluated.

The student's *t*-test revealed that the xylitol volumetric productivity ($Q_p$) was influenced only by the calcium chloride concentration ($0.1 < p < 0.2$). Higher values of productivity and xylitol concentration were obtained when using 11.0 g/L of calcium chloride. Using a low calcium chloride concentration during cell immobilization increased xylitol production from 18.5 to 19.9 g/L ($0.1 < p < 0.2$) (data not shown). The xylitol yield factor ($Y_{P/S}$) was influenced both by the sodium alginate ($p < 0.05$) and by the calcium chloride ($0.1 < p < 0.2$) concentrations. Higher yield values were obtained when using 11.0 g/L of calcium chloride and 20.0 g/L of sodium alginate. The percentage of Ca-alginate bead solubilization (ϕ) was influenced only by the bead curing time ($0.1 < p < 0.2$). Lower solubilization values were obtained after 24 h of bead curing time.

According to Ogbonna et al. *(13)*, incubating freshly prepared gel beads in calcium chloride solution increases their chemical stability. Consequently, the use of high sodium alginate concentrations during the immobilization step, which leads to beads with lower diffusion efficiency *(17, 18)*, becomes unnecessary. In a previous study, Domínguez et al. *(9)* reported the necessity of using high sodium alginate concentrations during the immobilization step, in order to increase the bead chemical stability to produce xylitol from wood hydrolysates without curing the cell-gel beads. The values of final concentration of xylitol, volumetric productivity, and yield factor obtained by these investigators were lower than those obtained in the present study, probably owing to mass-transfer limitations. Unfortunately, the xylitol production rates and yields obtained in the present study with the use of an immobilized cell system with lower mass-transfer limitations are still smaller than those observed in fermentations with free cells of several microorganisms, including *C. guilliermondii*, *Candida tropicalis*, *Debaryomyces hansenii*, and *Pachysolen tannophilus (6)*.

The concentrations of free cells suspended in the medium after 48 h of fermentation varied from $1.0 \times 10^8$ to $1.9 \times 10^8$ cells/mL. Yahashi et al. *(19)* also observed the presence of free cells in the fermentation medium while using Ca-alginate immobilized cells for xylitol production in synthetic xylose medium. It was verified that none of the variables studied influenced the final concentration of free cells significantly ($p < 0.2$). According to Corcoran *(20)*, the higher the sodium alginate concentration used in the immobilization step, the lower the cell leakage from the Ca-alginate beads. Since the concentrations of free cells in the fermentation medium were not influenced by the different sodium alginate concentrations used, the high final concentrations of suspended cells observed when the runs were finished could be owing to a specific growth rate of the free cells higher than that of the immobilized cells.

Acetic acid, which is known to inhibit the fermentation of lignocellulosic hydrolysates, was not detected by us in the medium on completion of

the experiments but was found by some researchers *(5,14,21)* in the medium with free cells of *C. guilliermondii* cultivated under similar conditions. Why the immobilized yeast cells consume this acid is still unknown. However, it is undoubtedly true that the fermentation of lignocellulosic hydrolysates is greatly favored by acid consumption, since the hemicellulosic fraction of such hydrolysates commonly contains acetyl groups.

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