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Cell Immobilization and Xylitol Production Using Sugarcane Bagasse as Raw Material

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Abstract

Sugarcane bagasse pretreated by three different procedures (with 2% [v/v] polyethyleneimine (PEI), with 2% [w/v] NaOH, or with a sequence of NaOH and PEI) was used as cell immobilization carrier for xylitol production by *Candida guilliermondii* yeast. Fermentations using these pretreated carriers were performed in semidefined medium and in a hydrolysate medium produced from sugarcane bagasse hemicellulose. Sugarcane bagasse pretreated with NaOH was the best carrier obtained with respect to immobilization efficiency, because it was able to immobilize a major quantity of cells (0.30 g of cells/g of bagasse). Fermentation in semidefined medium using the NaOH-pretreated carrier attained a high efficiency of xylose-to-xylitol bioconversion (96% of the theoretical value). From hydrolysate medium, the bioconversion efficiency was lower (63%), probably owing to the presence of other substances in the medium that caused an inadequate mass transfer to the cells. In this fermentation medium, better results with relation to xylitol production were obtained by using PEI-pretreated carrier (xylose-to-xylitol bioconversion of 81% of the theoretical and volumetric productivity of 0.43 g/[L·h]). The results showed that sugarcane bagasse is a low-cost material with great potential for use as cell immobilization carrier in the fermentative process for xylitol production.

Index Entries: Sugarcane bagasse; immobilized cells; hemicellulosic hydrolysate; fermentation; xylitol.

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Introduction

Sugarcane bagasse, the residue obtained after crushing the cane to extract the broth, is the most abundant lignocellulosic residue in Brazil, with a generation of about 103 million t/yr. Although most of the bagasse has been employed in the sugarcane industry for generation of energy, there is a surplus of this agroindustrial residue and several alternatives for its utilization have been evaluated, including the production of xylitol, ethanol, paper, enzymes *(1)*. Among these alternatives, xylitol production appears to be very attractive, because xylitol is a high-value-added compound that can be used in a large number of industries, including the food, odontologic, cosmetic, and pharmaceutical industries *(2)*.

Xylitol is industrially produced by a chemical process, but it can also be produced by a biotechnological route, which is based on the fermentation of xylose into xylitol by microorganisms (mainly yeasts) *(3)*. Nevertheless, several factors, such as the culture conditions (pH, temperature, oxygen supply, and others) and the kind of process (continuous or batch, in flasks or other bioreactors), affect xylitol production by microorganisms. For this reason, many studies have been carried out aiming to establish the best conditions to perform the biotechnological process with high yield. Recently, the use of cell immobilization systems has been proposed *(4–7)*.

Several advantages have been reported for the use of immobilized cells, including a high cell concentration in the fermentation medium and, consequently, an increase in the process efficiency and productivity *(8)*. Moreover, through immobilized cell systems, the cells can be easily recovered for later use in repeated-batch operations *(9–11)*. However, good performance of immobilized cell systems mainly depends on the correct selection of immobilization carrier. On the whole, the carrier must be able to maintain its physical integrity, being insoluble and stable to the chemical and thermal conditions of the bioprocess. In addition, it must be resistant to microbial degradation it cannot react with the substrates and products present in the fermentation medium, and it must be available in large quantity *(12)*. Furthermore, for industrial purposes, an important criterion is the carrier cost, which, combined with the interest in the reuse of byproducts, led to an increased search for cheap and available potential cell carriers. For this reason, the use of lignocellulosic materials as cell carriers in fermentative processes has been explored. In this case, pretreatments are usually aimed at increasing the affinity of the biocatalyst to the lignocellulosic materials *(13)*.

In a previous work, sugarcane bagasse was used as raw material and cell immobilization carrier for xylitol production *(7)*. In that work, the treatment of the immobilization carrier with epichlorohydrin and hexamethylene diamine resulted in higher adhesion of the cells on the carrier surface when compared to untreated sugarcane bagasse. Nevertheless, pretreatment of bagasse with these substances is not recommended for

xylitol production, owing to their toxicity. This problem motivated the search for other strategies for pretreatment of bagasse prior to its use as cell carrier. In the present work, NaOH and polyethyleneimine (PEI) were evaluated for carrier pretreatment. PEI is a nontoxic substance and the US Food and Drug Administration has permitted its use as a secondary direct food additive by the Federal Food, Drug and Cosmetic Act *(14)*. A combination of the two pretreatments was also evaluated.

A point of great importance that must be considered in immobilized cell systems is that correct evaluation of immobilization efficiency depends on the use of an adequate method for immobilized cell quantification. Until now, there has not been an ideal method for immobilized cell quantification when adsorption immobilization techniques were used. Thus, before evaluation of the sugarcane bagasse pretreatments, two different methodologies for quantification of the immobilized cells were tested. Sugarcane bagasse was also used as raw material for obtaining hemicellulosic hydrolysate, the main component of the fermentation medium for xylitol production.

Materials and Methods

Sugarcane Bagasse Hemicellulosic Hydrolysate

Sugarcane bagasse was supplied by Usina Guarany (Olímpia, SP, Brazil). Hemicellulosic hydrolysate was prepared in a 350-L stainless steel reactor, which was loaded with sugarcane bagasse and a sulfuric acid solution in a ratio of 100 mg of acid/g of dry matter, and a solid/liquid ratio of 1/10 g/g. The reaction was maintained at 121°C for 10 min. Afterward, the resulting solid material was separated by centrifugation, and the liquid fraction (hemicellulosic hydrolysate) was concentrated under vacuum in a 30-L stainless steel evaporator at 70°C, in order to obtain a xylose concentration of approx 50 g/L. To minimize the concentration of the main fermentation inhibitors, the concentrated hydrolysate was detoxified with activated charcoal according to the methodology described by Alves et al. *(15)*, and finally autoclaved under manometric pressure of 0.5 atm for 15 min. After this treatment, the hydrolysate presented the following composition: 46.3 g/L of xylose, 2.8 g/L of glucose, 3.8 g/L of arabinose, and 2.2 g/L of acetic acid.

Microorganism and Inoculum

Candida guilliermondii FTI 20037 was the microorganism employed in the experiments. For preparation of inoculum, cells of this yeast maintained at 4°C on malt extract agar slants were transferred to 125-mL Erlenmeyer flasks containing 50 mL of medium composed of 30.0 g/L of xylose, 3.0 g/L of $(NH_4)_2SO_4$, 0.10 g/L of $CaCl_2{\cdot}2H_2O$, and 10% (v/v) rice bran extract. The flasks were maintained in a rotary shaker at 200 rpm and 30°C for 24 h. Afterward, the cells were recovered by centrifugation

(2000*g*, 20 min) and washed and resuspended in sterile distilled water. Suitable volumes of this solution were employed as inoculum to obtain an initial cell concentration of 1 g/L at the beginning of fermentations. To obtain the rice bran extract, an aqueous suspension of rice bran (200 g/L) was sterilized for 20 min under 1.0 atm of manometric pressure. After cooling, the liquid fraction was separated by centrifugation (2000*g*, 20 min) and used in the experiments.

Preparation of Carrier

To be used as cell immobilization carrier, sugarcane bagasse was initially ground so that it could be passed through a 14-mesh and retained in a 35-mesh sieve (standard Tyler sieves). The ground bagasse was washed with distilled water and dried at 100°C until constant mass, then subsequently added to 125-mL Erlenmeyer flasks and sterilized (1.0 atm, 20 min). The bagasse was treated (under aseptic conditions) by three different procedures, described next.

Treatment with PEI

A solution containing 2% (v/v) PEI was prepared in sterile distilled water (1.0 atm, 20 min) and added to the flasks containing the sterilized bagasse (0.5 g dry mass) in a sufficient quantity to soak the solids. The pH of the medium was adjusted to 7.0 by the addition of HCl, and the flasks were incubated at 200 rpm for 24 h. Subsequently, the material was washed with sterile distilled water (50 mL) and maintained at 60°C for 24 h, to be used in the fermentation.

Treatment with NaOH

An aqueous solution of 2% (w/v) NaOH was prepared, sterilized (1.0 atm, 20 min), and added to the flasks containing the sterilized bagasse (0.5 g dry mass) in a sufficient quantity to soak the solids. The flasks were maintained at 120 rpm for 24 h. Subsequently, the material was washed with sterile distilled water (125 mL) and dried at 100°C for 24 h, to be used in the fermentation.

Treatment with NaOH and PEI

A sequence of the two previously described procedures was used.

Fermentation Conditions

Fermentations were performed in semidefined medium and in a medium composed of the sugarcane bagasse hemicellulosic hydrolysate. The semidefined medium contained the same composition used for preparation of inoculum, with xylose (30 g/L) as the only carbon source. Hydrolysate-based medium was composed of the detoxified hydrolysate supplemented with the same nutrients used for preparation of inoculum and semidefined medium (3.0 g/L of $[NH_4]_2SO_4$, 0.10 g/L of $CaCl_2{\cdot}2H_2O$, and 10% [v/v] rice bran extract) and with an initial xylose concentration of 30 g/L. Fermentations were

performed in 125-mL Erlenmeyer flasks containing 50 mL of the medium and the pretreated carrier (the resultant mass from the 0.5 g pretreated). The flasks were inoculated with a cell concentration of 1 g/L and maintained in a rotary shaker at 200 rpm and 30°C. The cells were immobilized *in situ* by natural adsorption through their direct contact with the carrier at the beginning of each run. The final fermentation time was considered when at least 90% xylose was consumed, and for determining this time, xylose consumption was monitored by sampling the medium at each 12 h. At the final time (24 h for semidefined medium and 48 h for hydrolysate-based medium), cell growth, xylose and glucose uptake, and xylitol production were measured.

Control assays, under the same fermentation conditions used for semidefined and hydrolysate media but with no cell inoculation, were performed to determine whether other substances present in these media could also be adsorbed in the bagasse. The obtained values were subtracted for calculation of the real values of cells adsorbed in the bagasse.

The methodology for the quantification of the immobilized cells was evaluated in a fermentation experiment carried out for 24 h using semidefined medium and the PEI-pretreated carrier. This assay was performed in triplicate under the same conditions as previously described.

Analytical Methods

Free cell concentration in the media was determined at 640 nm using a Beckman model DU 640B spectrophotometer (Fullerton, CA), by means of a dry weight vs optical density calibration curve.

To determine the mass of cells immobilized in the carrier, two different procedures were tested. In the first one, the carrier was separated from the fermented broth by filtration in a 60-mesh sieve previously tared, washed with distilled water (50 mL), and dried at 105°C until constant mass (approx 24 h). The difference between carrier masses before and after fermentation was assumed to represent the dry mass of immobilized cells in the sample and was employed for the quantification of immobilized cell mass. The initial carrier mass in the fermentation medium was considered to be the dry mass added to the flasks (0.5 g) minus the mass lost during the carrier treatment, which was determined in preliminary experiments.

In the second procedure, the carrier was also separated from the fermented broth by filtration in a 60-mesh sieve previously tared, washed with distilled water (50 mL), and dried at 105°C until constant mass. A 0.5 *M* NaOH solution was added to the dried material and the suspension was maintained at 120 rpm, 30°C for 24 h. Subsequently, the carrier was washed with distilled water (50 mL) and dried at 105°C until constant mass. The dry mass of immobilized cells was calculated by the difference of the carrier mass before and after the NaOH treatment. This value was employed to calculate the immobilized cell mass.

Xylose and xylitol concentrations were measured with a model LC-10-AD Shimadzu high-performance liquid chromatograph (Tokyo, Japan) equipped with a Bio-Rad (Hercules, CA) Aminex HPX-87H (300 X 7.8 mm) column

and a refractive index RID 6A detector. Samples were previously filtered through a Sep Pak C18 filter and injected in the chromatograph under the following conditions: column temperature of 45°C, 0.01 *N* H_2SO_4 as the mobile phase used at a flow rate of 0.6 mL/min, injection volume of 20 μL.

Micrography of carrier surface was obtained by using a LEO1450VP scanning electron microscope (Schott Zeiss do Brasil Ltda, São Paulo/SP, Brazil).

Kinetic Parameters and Yields of Fermentations

The kinetic parameters and yields of fermentations were calculated at the end of the runs. Xylitol volumetric productivity (Q_P, g/[L·h]) was calculated as the ratio between the xylitol concentration (P_f, g/L) and the fermentation time (h). Xylitol yield factor ($Y_{P/S}$, g/g) was defined as the ratio between P_f and xylose consumed (g/L). Cell yield factor ($Y_{X/S}$, g/g) was defined as the ratio between total formed cells (free + immobilized, X_T, g/L) and xylose consumed (g/L). The efficiency of xylose-to-xylitol bioconversion (η, dimensionless) was determined as the ratio between $Y_{P/S}$ (g/g) and the theoretical value (0.917 g/g) of this parameter proposed by Barbosa et al. *(16)*. Cell retention in the bagasse (λ, g/g) was calculated as the ratio between the mass of cells immobilized in the carrier (g) and carrier mass (g). The concentration of immobilized cells (X_i, g/L) was calculated as the ratio between the mass of cells immobilized in the carrier (g) and the medium volume (L). Immobilization efficiency (η_i, dimensionless) was calculated as the ratio between immobilized cells (X_i, g/L) and total formed cells (X_T, g/L).

Results and Discussion

Quantification of Immobilized Cells

One of the main problems related to cell-immobilized systems is the correct quantification of the cells immobilized in the carrier, mainly when adsorption methods are used. For this reason, two different methodologies were tested for quantification of these values. The first one was based on the difference between the mass of the immobilized system and the carrier mass at the beginning of the fermentation. The second one was based on the desorption of the immobilized cells by using NaOH, as described by Almeida et al. *(13)* for determination of cells immobilized in spent grains.

As shown in Fig. 1, the two evaluated methodologies quantified similarly the cells immobilized in sugarcane bagasse. This suggests that such methodologies can be efficiently employed for quantification of cells immobilized in sugarcane bagasse. Nevertheless, some observations must be made regarding the use of each of them. The first methodology, which is based on the difference of the carrier mass before and after fermentation, is easier and quicker than the second one, rendering lower variability of the results. However, it requires the use of all the mass employed for fermentation, thus being more suitable for experiments performed in flasks or on small scale. On the other hand, the second procedure, which is based on

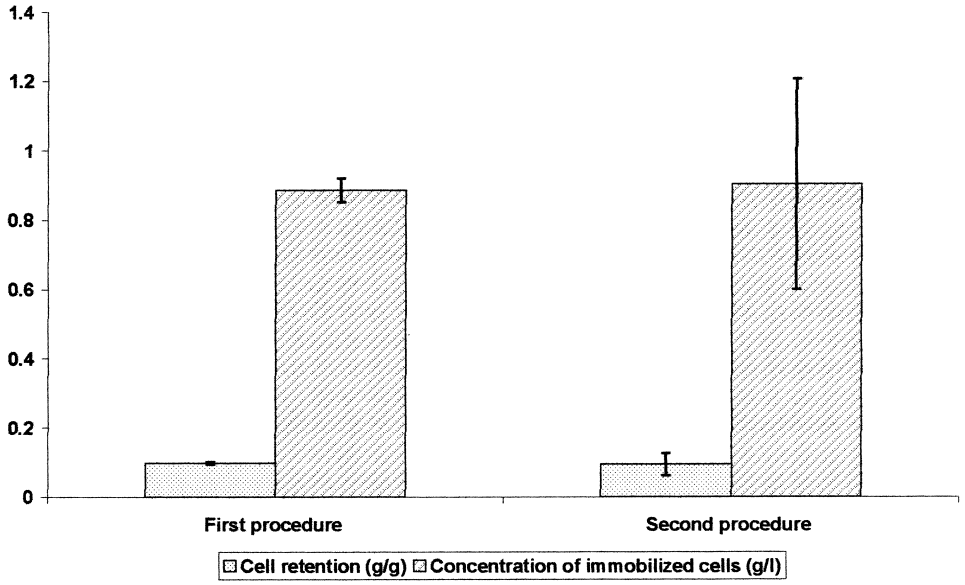

Fig. 1. Quantification of immobilized cells by difference of carrier mass before and after fermentation (first procedure), and before and after NaOH treatment (second procedure). Bars represent the SD.

the difference of the carrier mass before and after NaOH treatment, is slower than the first one, resulting in higher variability of the results. However, it does not require the use of all the mass employed for fermentation, because only a sample of the carrier can be used for analysis. This is advantageous, because it permits the quantification of cells immobilized during the fermentations performed in large-scale bioreactors. In the present work, both methodologies were suitable for quantification of immobilized cells, because the assays were performed in Erlenmeyer flasks and, thus, the first methodology was chosen to be used in the other experiments.

Assays in Semidefined Medium

Initially, the efficiency of the pretreated carriers on cell immobilization was evaluated in fermentations using semidefined medium. Table 1 shows the fermentative parameters obtained at the end of these fermentations, and Fig. 2 shows the correspondent cell growth parameters. The best xylitol production was achieved by using the NaOH-pretreated carrier. Although the fermentation using carrier pretreated with PEI promoted a similar yield ($Y_{P/S}$), the productivity (Q_P) values showed that the bioconversion process was faster when the NaOH-pretreated carrier was used.

As observed in Fig. 2, all of the systems were at the end of the runs a mix of free and immobilized cells. From semidefined medium, only about 17 to 18% of the total cells was in the immobilized form when the carrier was pretreated with NaOH or with PEI. However, in these cases, the

Table 1
Fermentative Parameters Obtained During Xylitol Production
Using Cells Immobilized in Sugarcane Bagasse Pretreated by Different Procedures[a]

	Semidefined medium			Hydrolysate medium		
Pretreatment	$Y_{P/S}$ (g/g)	Q_P (g/[L·h])	η (%)	$Y_{P/S}$ (g/g)	Q_P (g/[L·h])	η (%)
PEI	0.90 ± 0.10	0.38 ± 0.04	98.15 ± 10.13	0.74 ± 0.01	0.43 ± 0.04	80.70 ± 1.63
NaOH	0.88 ± 0.10	0.60 ± 0.04	95.97 ± 15.42	0.56 ± 0.04	0.30 ± 0.01	60.97 ± 4.45
NaOH + PEI	0.56 ± 0.04	0.53 ± 0.02	61.07 ± 4.46	0.50 ± 0.01	0.29 ± 0.01	54.53 ± 1.01

[a]The data given are the results ± the SD.

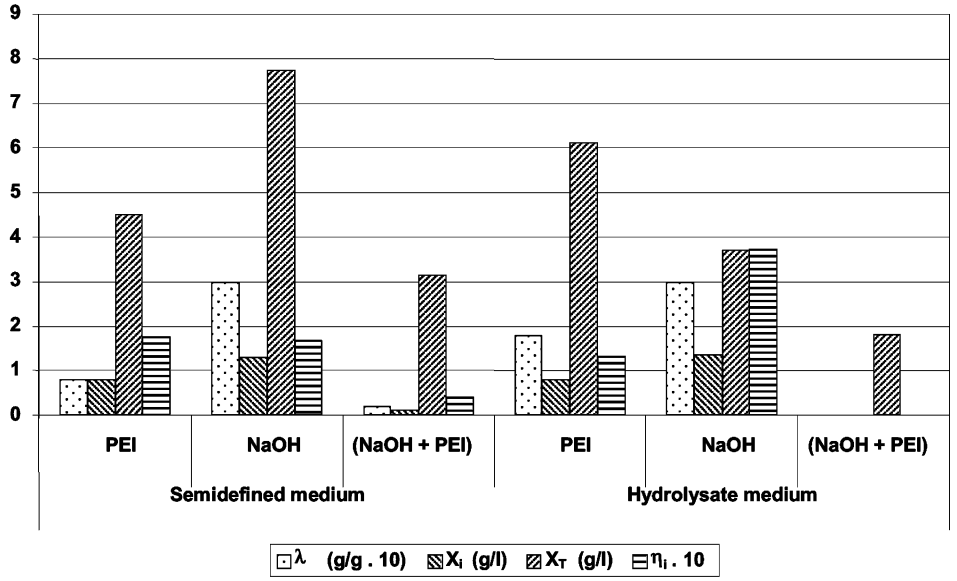

Fig. 2. Cell growth parameters obtained during fermentations using cells immobilized in sugarcane bagasse pretreated by different procedures. λ is the cell retention in the bagasse, X_i is the concentration of immobilized cells, X_T is the total formed cells (free + immobilized), and η_i is the immobilization efficiency.

immobilization can be useful for cell reutilization, because the immobilized cell concentration ($X_i > 0.8$) is sufficient to be used, e.g., as inoculum in a new fermentation batch.

Figure 2 also shows that the quantity of cells immobilized per carrier mass (cell retention, λ) was higher when the carrier was pretreated with NaOH. According to Mussatto et al. *(17)*, the NaOH solution causes a partial removal of the lignin from the material surface, modifying its fibrous structure, which becomes more porous. For this reason, the cells were able to adhere to the bagasse surface. Figure 3 shows a nonhomogeneous cell distribution over the carrier surface, with local cell accumulations. This confirms the existence of preferred attachment sites, probably crevices, pores, and tangled carrier particles. Similar behavior was observed during the immobilization of brewing yeast into spent grains pretreated with NaOH *(18)*.

The carrier obtained through PEI pretreatment was also able to immobilize cells, but with cell retention approximately fourfold lower than that observed for the NaOH-pretreated carrier. Unlike NaOH, the main objective of PEI is not to increase the material porosity, but to recover the material surface with positive loads *(19)*. Because the cells have a negatively loaded surface owing to the presence of phosphate in their phospholipidic membrane *(20)*, they are attracted to the material surface. Nevertheless, the obtained results suggest that the cells were immobilized in greater quantity by adsorption in porous structures than by electrostatic interactions.

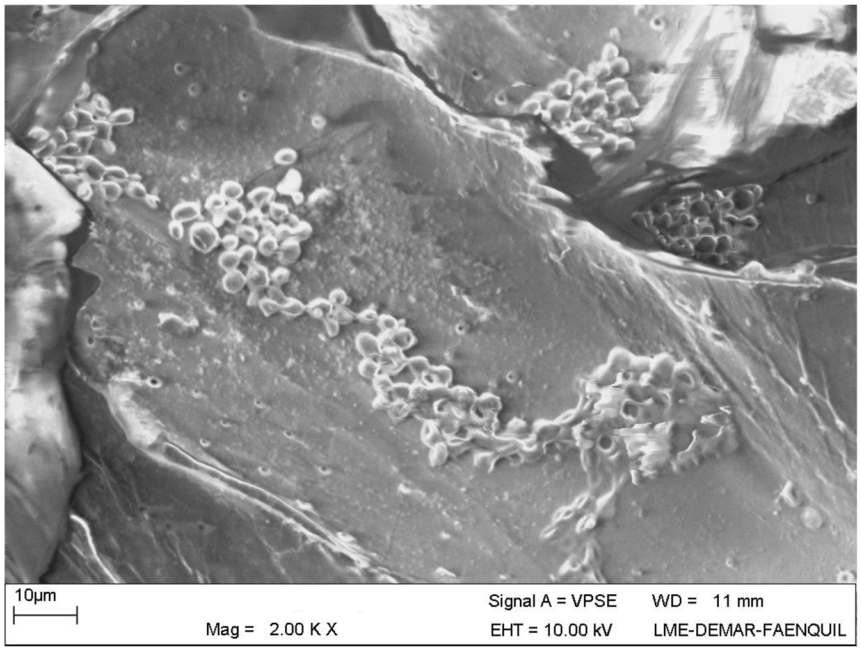

Fig. 3. Micrography of cells immobilized in sugarcane bagasse pretreated with NaOH, by scanning electron microscopy (magnification: 2000-fold; semidefined medium; 24-h fermentation time).

The worst immobilization results were observed with the carrier pretreated with a sequence of NaOH and PEI. The low cell adherence to this carrier can be attributed to the effect caused by the PEI. When NaOH was used, there was an increase in the porosity of the bagasse surface. Nevertheless, the sequential use of PEI recovered the porous, decreasing the surface available for adsorption, resulting in a smaller area for cell immobilization. As a consequence of the small quantity of immobilized cells, the fermentative process efficiency was strongly affected when this carrier was used, attaining only 61% of the maximum theoretical value (0.917 g/g), whereas 96% fermentation efficiency was obtained using the NaOH-pretreated carrier (Table 1).

The higher productivity obtained by using the NaOH-pretreated carrier (Table 1) can be attributed to the larger quantity of immobilized cells obtained under this condition (Fig. 2). The justification for this is that the pretreatment with NaOH resulted in a minor mass of carrier at the beginning of the fermentations. This smaller solid quantity with larger mass of adsorbed cell probably resulted in a more adequate microenvironment for xylitol production, because the oxygen uptake, e.g., was certainly minor under these conditions, and several investigators have reported that xylitol production is favored under semianaerobic conditions *(21,22)*.

Assays in Hemicellulosic Hydrolysate Medium

After evaluation of the carriers' potential for cell immobilization in semidefined medium, assays were performed in hemicellulosic hydrolysate medium to compare the performance of the carriers in a more complex substrate. Generally, when compared with the semidefined medium assays, all the fermentations from hemicellulosic hydrolysate presented a worse performance (Table 1). In addition, fermentations in semidefined medium lasted 24 h, while in the hydrolysate-based medium 48 h were necessary. These results were probably owing to the presence of toxic substances in the hydrolysate. It is known that hemicellulosic hydrolysates contain, besides sugars, several compounds that act as inhibitors of microbial metabolism *(23)*. According to Alves et al. *(15)*, the detoxification procedure used in the present work is able to partially remove these compounds from the hydrolysate. Consequently, some of their residue was still present in the fermentation medium and could have contributed to the drop in bioprocess efficiency and xylose consumption rate.

On the other hand, the data concerning the concentration of immobilized cells and cell retention (Fig. 2) were similar for both hydrolysate and semidefined media, with a higher X_i value obtained using hydrolysate-based medium and the PEI-pretreated carrier. This means that there was not any significant loss in cell immobilization owing to the change from the semidefined to a more complex fermentation medium. This is very interesting, because the hydrolysate contains several other substances that are not present in the semidefined medium and that could compete with the cells for the adsorption sites on the carrier surface, making immobilization difficult. However, this effect was not observed.

It can also be observed in Fig. 2 that the fermentation of hydrolysate-based medium using the NaOH-pretreated carrier resulted in higher immobilization efficiency ($\eta_i = 37\%$) than the fermentation of semidefined medium. This behavior was expected, because the carrier was saturated with the same cell retention and the total cell concentration was lower when the hydrolysate was used as the base of the medium. In fact, the presence of toxic compounds in the medium resulted in lower cell growth in this case.

When analyzing only the fermentations from sugarcane bagasse hydrolysate with the different carriers, it was observed that, in contrast to the semidefined medium, the best results concerning xylitol production were not obtained by using the NaOH-pretreated carrier, but by using the PEI-pretreated carrier (Table 1). These results suggest that problems with mass transfer to the immobilized cells occurred in the hydrolysate medium, probably owing to the presence of a large quantity of compounds in this medium, thus resulting in lower xylitol production. These mass transfer problems mainly occurred for the cells adsorbed in the porous structure of the NaOH-pretreated carrier. Thus, by using this carrier, a larger quantity of immobilized cells would be affected by the mass transfer limitations than

using the PEI-pretreated carrier. Perhaps the use of alternative systems that can promote better mass transfer conditions would result in higher xylitol production with cells immobilized in NaOH-pretreated sugarcane bagasse and cultured in hydrolysate-based medium. Moreover, the low cost of NaOH is an important advantage over PEI.

Conclusion

This work reveals a new technology for xylitol bioproduction using cells immobilized in sugarcane bagasse, an abundant and low-cost lignocellulosic material. Treatment of the bagasse with PEI resulted in better xylose-to-xylitol yield and volumetric productivity. However, with relation to immobilization efficiency, treatment with 2% (w/v) NaOH was more efficient than with 2% (v/v) PEI, because it made possible the immobilization of a major quantity of cells in the carrier surface. For this reason and because of the low cost, 2% (w/v) NaOH must be used in future works for pretreatment of sugarcane bagasse with the aim of using it as cell immobilization carrier. It is important to note that the larger the quantity of immobilized cells, the greater the possibility of reusing the cells in other fermentation batches, in continuous or repeated-batch processes, which is very interesting from an industrial viewpoint.

Acknowledgments

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