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Xylitol Production from Wood Hydrolyzates by Entrapped *Debaryomyces hansenii* and *Candida guilliermondii* Cells

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Abstract

Debaryomyces hansenii cells were entrapped in Ca-alginate beads and used for producing xylitol from wood hydrolyzates. Batch experiments showed that bioconversion was severely hindered when Ca-alginate beads were hardened with Al^{3+} solutions. As an alternative to Al^{3+} hardening, the improvements in both mechanical stability of bioparticles and fermenting ability of the immobilized system derived from using increased concentrations of sodium alginate were assessed. The best results were obtained using a 4% (w/v) Na-alginate solution in the gelification step. This concentration was selected to perform continuous fermentations in a packed-bed reactor using raw or charcoal-treated hydrolyzates (15.5 g of xylose/L) with two different yeasts: *Candida guilliermondii* and *Debaryomyces hansenii*. With a final cell concentration of about 50 g of cells/L (0.075 g of cells/g of beads), the volumetric productivities reached with these yeasts in media made from charcoal-treated hydrolyzates were 0.58 and 0.91 g/L·h, respectively.

Index Entries: Ca-alginate; *Candida guilliermondii*; *Debaryomyces hansenii*; hemicellulose hydrolyzate; xylitol.

Introduction

Xylitol, a five-carbon polyol with high sweetening power, is tolerated by diabetics, has anticariogenic properties, and has been recommended for

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parenteral nutrition and use as a food ingredient in sugar-free confections, yogurts, jams, bakery products, and drinks *(1)*. Xylitol can be obtained by various technologies, including the following:

1. Extraction from some fruits and vegetables—this procedure is not economical owing to its small concentrations.
2. Chemical reduction of xylose—this process is relatively expensive because of the extensive purification and the separation steps required.
3. Biotechnological conversion of xylose solutions—this is a selective and promising process for xylitol production *(2)*.

Bioconversion can be carried out with fungi, bacteria, yeast, or purified enzymes from these microorganisms. The most studied xylitol producers are yeasts, with strains of the genus *Candida* and *Debaryomyces hansenii* being the best natural producers. The latter has been reported to produce xylitol with good yields from either synthetic sugar solutions *(3–7)* or hydrolyzates *(8–14)*.

Eucalyptus globulus wood, a cheap and widespread resource having xylan as the main constituent of its hemicellulosic fraction, has been subjected to acid treatments to obtain xylose-containing solutions *(8–14)*. The fermentation of hydrolyzates is hindered by inhibitors that can be present in the raw material or produced during the chemical processing. Inhibitors include sugar degradation products, lignin-derived compounds, and acetic acid derived from the acetyl groups of wood *(15,16)*. Several technologies have been employed for minimizing inhibition, including adaptation and recycling of yeasts, solvent extraction, neutralization and overliming, ion exchange, and charcoal adsorption *(9–15,17–21)*.

Increasing the biomass concentration resulted in both higher fermentation efficiency and limitation of inhibition by toxic substances present in hydrolyzates *(7,9,12,22,23)*. High cell densities can be achieved with immobilized systems *(24)*, but this technology requires selecting an immobilization method that will limit cell leakage *(25)*. Among the different immobilization methods, solid supports (polyacrylamide, ceramic, nonwoven fabrics or macroporous glass beads) *(25,26)* or gel entrapment (in alginate or carrageenan) *(25,27–32)* have been used. Ca-alginate is the support most used for this purpose owing to both its fast gelification and mild immobilization conditions *(31)*. Higher resistance of gel-entrapped microorganisms to inhibitors has also been reported *(33)*. The main drawback of gel immobilization is the low mechanical strength of beads, which limits the duration of the working period. Trivalent ions have been proposed and used with good results as hardening agents with the aim of improving the mechanical strength of the beads *(27–29,31,32)*. On the other hand, high gel concentrations also improve bead stability, even if diffusional limitations appear *(34)*.

Our work deals with the bioconversion of *Eucalyptus* wood hydrolyzates by *D. hansenii* or *Candida guilliermondii* cells immobilized in Ca-alginate

beads. To allow prolonged fermentation periods, hardening of beads with aluminum ions and utilization of a concentrated gel have been assayed as strategies for increasing the bead strength. The best conditions found in this step were selected to carry out continuous fermentations in a packed-bed bioreactor. Hydrolyzates were treated with charcoal for removing some inhibitory compounds and were used to make culture media for xylitol production with immobilized cells.

Materials and Methods

Raw Material

E. globulus wood chips obtained from a local pulp mill were milled to a particle size <1 mm, homogenized in a single lot, air-dried, stored, and used for experimentation. Xylan content of samples was 17.4 wt % of wood (oven-dry basis).

Acid Hydrolysis

Wood meal samples were treated in an autoclave at 130°C with 3% sulfuric acid for 1 h using a liquid/wood ratio = 8 g/g. The liquid phase from the acid hydrolysis was neutralized with $CaCO_3$ to a final pH of 6.5, and the $CaSO_4$ precipitate was separated from the supernatant by filtration. Neutralized hydrolyzates contained an average of 17.0 g of xylose/L, 3.6 g of glucose/L, 2.2 g of acetic acid/L, and <0.5 g of furfural/L.

Treatment with Activated Charcoal

Activated powdered charcoal (Probus, Madrid, Spain) was mixed with neutralized hydrolyzates for 1 h at a ratio of 1:20 g of charcoal:g of hydrolyzate as reported elsewhere *(9)*. Liquors were recovered by filtration and treated again for 1 h further with the same amount of charcoal. The 98.2% of the liquid phase was recovered by filtration and used for making culture media. The charcoal also was recovered in this step. After being regenerated it could be used again. Charcoal adsorption removed 88.8% of the phenolic compounds.

Microorganism

D. hansenii NRRL Y-7426 and *C. guilliermondii* NCR 5578 were used.

Culture and Fermentation Media

Freeze-dried cells were grown in a fermentation medium containing per liter the following: 10 g of pure xylose, 3 g of yeast extract, 3 g of malt extract, and 5 g of peptone. Microorganisms were maintained in agar slant tubes containing a medium formulated with the same components and concentrations plus 20 g of agar. Adapted yeasts (maintained in agar slants made from hydrolyzates instead of commercial xylose solutions) were used for fermentation of *Eucalyptus* hydrolyzates. Fermentation media were

made from neutralized hydrolyzates (with or without charcoal treatment), which were supplemented with 3 g/L of yeast extract, 3 g/L of malt extract, and 5 g of peptone/L as described by Parajó et al. *(9)* and sterilized in an autoclave.

Immobilization

A dense inoculum of cells grown in raw hydrolyzates was added to a solution of Na-alginate (Protanal lf 10/60, Protan, Norway) previously autoclaved for 15 min at 121°C, in proportions leading to a dry weight cell concentration of 3 g/L (gel). The final gel concentrations were: (1) 2% in the experiments with bioparticles hardened with Al^{3+}, and (2) within the range of 1–5% in experiments exploring the effects caused by the Na-alginate concentration. Gel beads (2 to 3 mm diameter) were obtained by dropping the suspension in a 50 m*M* $CaCl_2$ solution at 30°C. Beads were recovered after 30 min and washed with a 0.9% NaCl solution.

Batch Fermentation of Hydrolyzates

Batch fermentations were performed with free cells in an orbital shaker at 200 rpm and 30°C under microaerobic conditions, using 250-mL Erlenmeyer flasks with 50 mL of fermentation medium.

Experiments with immobilized yeasts were carried out with 20 g of beads in 50 mL of autoclaved fermentation medium giving a final cell concentration of about 30 g/L. This concentration was in the range of the ones used in experiments with free cells *(9)*.

Bioreactor

Continuous xylitol production was performed in an upflow packed-bed bioreactor (180 mL reaction volume and 2.3:1 length:diameter ratio). The top and bottom heads of the column was filled with glass spheres to minimize the dead volume, improving the fluid distribution and avoiding cell deposition. The bed contained 120 g of proliferated beads and the void fraction was 0.49. The bioreactor was jacketed to control the temperature at 30°C by means of a thermostatic bath. A peristaltic pump was used for feed delivery.

Analytical Methods

To obtain a semiquantitative estimation of the phenolics removal, the 276-nm absorbance of hydrolyzates was measured before and after charcoal adsorption *(15)*. At given fermentation times, samples from the fermentation media were taken, centrifuged, filtered through 0.45-µm membranes and analyzed by high-performance liquid chromatography using two Shodex SH columns (mobile phase, H_2SO_4 0.01 *M*; flow rate, 0.7 mL/min; infrared and ultraviolet detection). This method allowed the determination of glucose, xylose, arabinose, acetic acid, ethanol, xylitol, and furfural.

Results and Discussion

Hardening of Beads with Al^{3+} Solutions and Batch Fermentation of Hydrolyzates

D. hansenii cells were entrapped in Ca-alginate beads to carry out the bioconversion of the *E. globulus* wood hydrolyzates into xylitol. Gel beads were immersed in a 0.3 *M* Al^{3+} solution for both improving their mechanical strength and avoiding the leakage of cells to the medium. This technique has been previously applied to ethanol production with different yeasts *(27–29,31,32)*. Hardening of beads with aluminum ions resulted in both increased cellular retention and improved mechanical strength, leaving most of the cells in the living state; however, Al^{3+} severely hindered fermentation, as confirmed by performing successive batch cultures lasting 4 to 5 d. No xylose was consumed after the first two batches, whereas 50–70% of xylose was depleted between the third and the sixth ones. This latter value was not surpassed after eight to nine fermentation stages, and no xylitol was produced.

Contradictory effects about the toxicity of Al^{3+} are found in the literature depending on the yeast or the operational conditions. Sanromán et al. *(28)*, dealing with the alcoholic fermentation of xylose by *Pichia stipitis* cells entrapped in κ-carrageenan, reported an increase in volumetric productivity (from 0.713 g/L·h to 0.892 g/L·h) when bioparticles were contacted with a 0.05 *M* solution of hardening agent (Al^{3+}) for 5 min. Both longer contact times (15 min) and higher concentrations of hardening agent (0.15 *M*) inhibited bioconversion, decreasing the volumetric productivity to 0.591 g/L·h. Chamy et al. *(29)* reported that the volumetric ethanol productivity obtained with *Saccharomyces cerevisiae* entrapped in Ca-carrageenan increased from 1.34 g/L·h with untreated beads up to 2.04 g/L·h for bioparticles hardened with 0.1 *M* $Al(NO_3)_3$ for 15 min, whereas the volumetric productivity dropped to 0.91 g/L·h for a shorter exposure (5 min) to a more concentrated solution of aluminum ions (0.3 *M*). This fact suggests a detrimental effect of high concentrations of aluminum ions, which could be attributed to hindered diffusion, possibly with a viability-associated problem. On the contrary, Roca et al. *(31)*, using the same yeast entrapped in Ca-alginate, observed that beads hardened with Al^{3+} performed at least as well as those without treatment, with improvements in their mechanical properties. The volumetric productivity increased from 2.16 g/L·h for nonhardened beads up to 2.48 g/L·h for beads immersed for 15 min in a solution containing the highest Al^{3+} concentration assayed (0.3 *M*).

Evaluation of Inhibition: Experiments with Free Cells

To study the combined effects of the toxic compounds present in hydrolyzates and the inhibition by Al^{3+}, four batch fermentations with free cells were performed using media made with pure xylose; media made with raw hydrolyzates; media made with charcoal-treated hydrolyzates; and media made with charcoal-treated hydrolyzates supplemented with

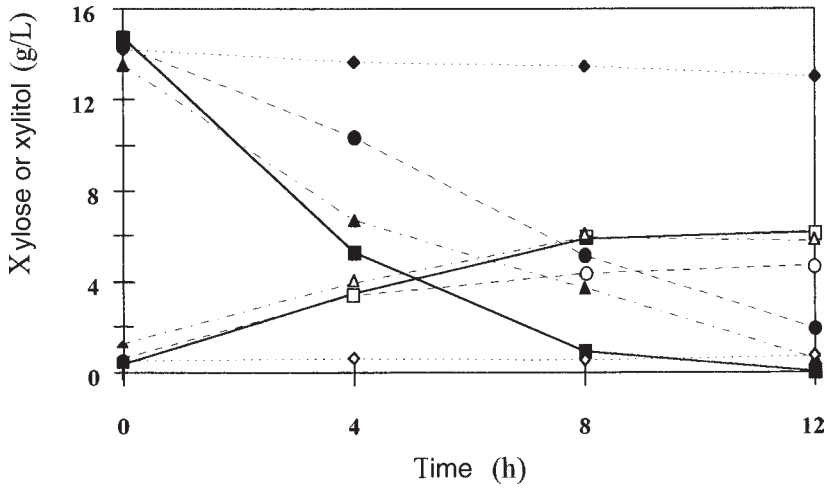

Fig. 1. Time course of xylose (solid symbols) and xylitol (open symbols) concentrations in batch cultures with free cells of *D. hansenii*. Culture media made from commercial xylose (—■—), raw hydrolyzates (—●—), or charcoal-treated hydrolyzates with added Al^{3+} (– –◆– –) or without added Al^{3+} (– · ▲ – ·).

0.3 *M* Al^{3+}. Figure 1 presents the experimental results. In the experiment made with media containing commercial xylose, more than 93% of the initial sugar was consumed after 8 h of fermentation, resulting in a xylitol volumetric productivity of 0.68 g/L·h and a xylitol yield of 0.39 g/g. In the fermentation using raw hydrolyzates, 14% of the initial xylose remained unconverted after 12 h of fermentation, and the xylitol productivity (0.46 g/L·h) was slightly lower in comparison than the one corresponding to the fermentation medium made with commercial xylose. This reduction in xylitol productivity can be justified by the presence of inhibitors such as acetic acid (liberated from naturally occurring acetylated hemicelluloses), furfural, and hydroxymethylfurfural (generated by pentose degradation), compounds of wood derived from the extractive fraction of wood and lignin degradation products *(9)*.

Charcoal adsorption has been successfully employed for overcoming the effects of inhibitors *(9–13,17–19)*. In our case, charcoal treatments increased both volumetric productivity (up to 0.58 g/L·h) and xylitol yield (up to 0.47 g/g).

The presence of 0.3 *M* Al^{3+} in media made from charcoal-treated hydrolyzates was observed to be highly inhibitory: The yeast was unable to consume xylose (94.7% of the initial substrate remained after 12 h of fermentation), and limited values of both xylitol volumetric productivity (0.01 g/L·h) and xylitol yield (0.07 g/g) were obtained. Biomass concentration was slightly increased, but the maximum concentration of xylitol was not improved (data not shown). This fact could be related to the inhibition of the xylose reductase system of *D. hansenii* that allows the reduction of xylose into xylitol. In this field, Roca et al. *(32)*, working with a recombinant *S. cerevisiae*

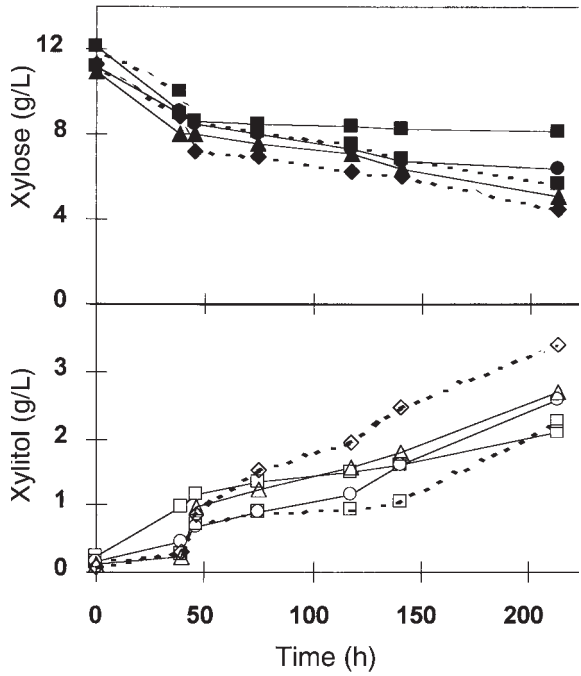

Fig. 2. Dynamics of xylose (solid symbols) and xylitol (open symbols) concentrations in fermentation experiments using immobilized *D. hansenii* cells entrapped in beads made with 1% (—■—), 2% (—●—), 3% (—▲—), 4% (– –◆– –), or 5% (– –■– –) w/v Na-alginate (culture media made from charcoal-treated hydrolyzates).

strain, pointed out that the xylose-reductase activity was much lower in cells immobilized in Ca-alginate beads hardened with Al^{3+} than in free cells.

Hardening of Beads: Effect of Ca-Alginate Concentration

As an alternative to the use of aluminum ions as a hardening agent of Ca-alginate beads, the possibility of using higher gel concentrations for increasing the bead strength was explored. This stage was performed with nonadapted yeasts, even if this decreased both yields and productivities. Gelification with higher Na-alginate concentrations allowed an improved stability of beads. This finding is in agreement with the results obtained by Jamuna et al. *(34)* in a study based on an experimental design. The gel concentration also affected the overall fermenting ability of the experimental system. Beads obtained with 1% w/v Na-alginate were unable to retain yeasts and lost their shape easily. When using gelification medium with 5% w/v Na-alginate, the high viscosity of gel made pumping difficult and the beads showed irregular shapes. Beads made in media containing 2-4% w/v Na-alginate presented both favorable handling properties and acceptable mechanical resistance after long periods, with the best results corresponding to beads made with 4% Na-alginate.

Figure 2 shows the dynamics of fermentations carried out with gel beads made from solutions containing Na-alginate concentrations in the

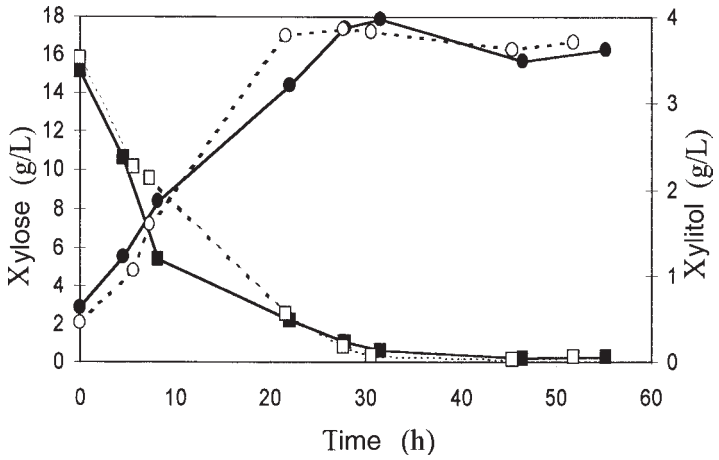

Fig. 3. Xylose consumption and xylitol production in batch fermentations using *C. guilliermondii* and *D. hansenii* cells immobilized in calcium alginate. (—■—), xylose *D. hansenii*; (– –□– –), xylose *C. guilliermondii*; (—●—), xylitol *D. hansenii*; (– –○– –), xylitol *C. guilliermondii*.

range explored. After inoculation, the initial xylose concentration was reduced to 11 to 12 g/L owing to the dilution effect provoked by the addition of 20 g of beads gelified in a $CaCl_2$ solution. These fermentations led to reduced maximum xylitol concentrations (in the range of 2.58–3.39 g/L) and volumetric productivities (in the range of 0.011–0.016 g/L·h). These values show a pattern similar to reported results, with decreased productivities in immobilized cells in comparison with fermentation with free cells for several microorganisms, including *C. tropicalis*, *Pachysolen tannophilus*, and *C. guilliermondii (25,35–37)*. However, the main interest of these results for us was to show 4% Na-alginate as the best concentration to be used in the gelification step.

Batch Experiments with Cells Immobilized Under Optimized Conditions

To assess the viability of carrying out the bioconversion of *E. globulus* wood hydrolyzates into xylitol using the best immobilization conditions selected in the previous step, batch fermentations were performed in media made from raw hydrolyzates with gel-entrapped, adapted cells of *D. hansenii* Y-7426 or *C. guilliermondii* NCR 5578. In both cases, adaptation to raw hydrolyzates was carried out by successive batch fermentations using inocula obtained from the previous experiment. Figure 3 shows the time course of xylose consumption and xylitol production. Ethanol, a fermentation by-product, was also detected in experiments. After 27.5 h of fermentation, 93 and 95% of the initial xylose was consumed by *D. hansenii* and *C. guilliermondii*, respectively. In both cases, the maximum xylitol concentration was 3.86 g/L and the volumetric productivities and xylitol yields reached 0.12 g/L·h and 0.23 g/g, respectively.

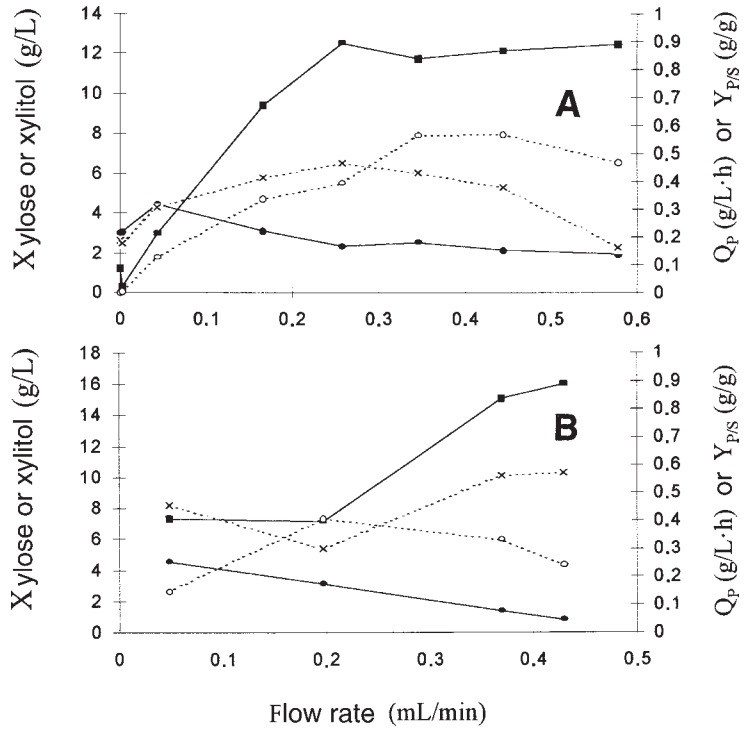

Fig. 4. Results obtained with raw hydrolyzates. **(A)** Time course of xylose and xylitol concentration, volumetric productivity (Q_p), and product yield ($Y_{p/s}$) vs volumetric flow rate using *C. guilliermondii* cells. **(B)** Times course of xylose and xylitol concentration, volumetric productivity, and product yield vs volumetric flow rate using *D. hansenii* cells. (—■—), xylose; (—●—), xylitol; (– –○– –), Q_p; (– –×– –), $Y_{p/s}$.

In comparison with the results obtained in the set of batch experiments made with nonadapted, immobilized cells, adaptation resulted in reduced fermentation time (and hence higher productivity), even when the productivity was still lower than that reached with free cells.

Because no differences were observed when comparing *D. hansenii* and *C. guilliermondii*, both yeasts were used to perform the bioconversion of the hydrolyzates into xylitol in a continuous bioreactor.

Continuous Fermentation with Immobilized Cells

The feeding flow rate is a parameter affecting the volumetric productivity and product yield of continuous bioconversion, its optimization being a crucial factor for finding the best fermentation conditions. Adapted yeasts from the batch fermentations were transferred to two similar upflow packed-bed bioreactors to perform the continuous xylitol production, using a feeding medium made from raw hydrolyzates. Figure 4 shows the effects of flow rate on the concentrations of xylose and xylitol as well as on the volumetric productivity and product yield. For both yeasts assayed, xylose was almost completely depleted at the lowest flow rates assayed, but

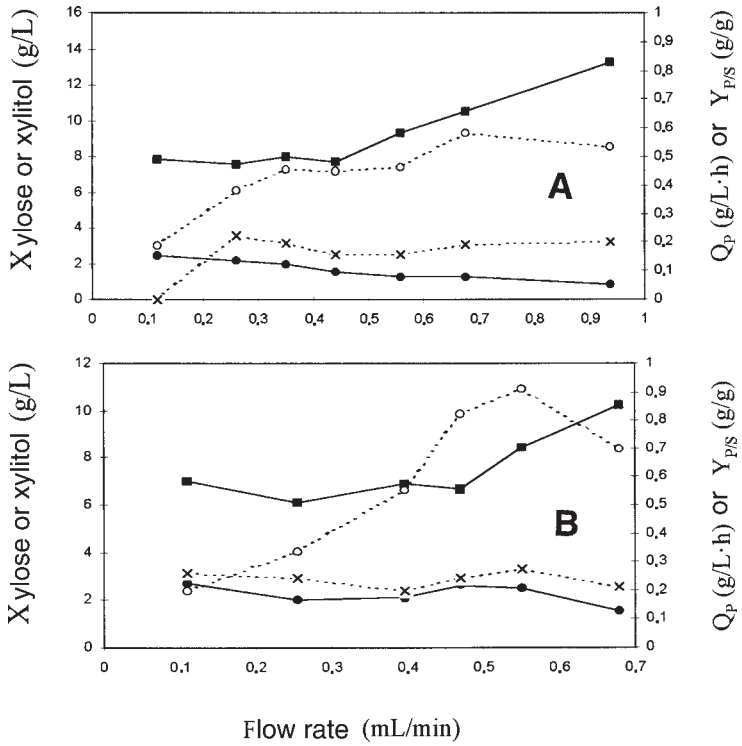

Fig. 5. Results obtained with charcoal-treated hydrolyzates. **(A)** Time course of xylose and xylitol concentration, volumetric productivity (Q_p), and product yield ($Y_{p/s}$) vs volumetric flow rate using *C. guilliermondii* cells. **(B)** Time course of xylose and xylitol concentration, volumetric productivity, and product yield vs volumetric flow rate using *D. hansenii* cells. (—■—), xylose; (—●—), xylitol; (– –○– –), Q_p; (– –×– –), $Y_{p/s}$.

the high residence times led to decreased volumetric productivities. At the highest flow rates utilized, yeasts were unable to metabolize the carbon source, leaving the packed-bed bioreactor with poor substrate conversion and low concentrations of xylitol. Intermediate flow rates provided the best results, as a compromise solution between extreme situations.

In the experiment with *C. guilliermondii* (Fig. 4A), low volumetric productivity ($1.1 \cdot 10^{-4}$ g/L·h) was observed at the lowest flow rate assayed ($5.46 \cdot 10^{-5}$ mL/min), but the productivity increased dramatically up to 0.47 g/L·h at the highest flow rate considered (0.58 mL/min). The maximum productivity (0.57 g/L·h) was obtained at flow rates of 0.44 mL/min. A similar pattern was observed for the product yield, with a maximum value (0.47 g/g) obtained with a flow rate of 0.26 mL/min.

A related behavior was observed for the yeast *D. hansenii* (Fig. 4B). The lowest productivity (0.15 g/L·h) and product yield (0.46 g/g) were reached working at the lowest flow rate (0.048 mL/min), whereas slightly higher values of these parameters (0.23 g/L·h and 0.57 g/g, respectively) were found with the highest flow rate tested (0.43 mL/min), with the optimum volumetric productivity (0.41 g/L·h) corresponding to a flow rate of 0.19 mL/min.

To reduce the inhibitory effect of some substances present in the fermentation medium, raw hydrolyzates were treated with charcoal. Charcoal adsorption decreases the concentration of both acetic acid and phenolic compounds derived from the acid-soluble lignin of wood *(9)*. Charcoal treatment did not significantly improve the fermentation with *C. guilliermondii* (Fig. 5A), the maximum productivity (0.58 g/L·h) being similar to that obtained in the case of raw hydrolyzates (0.57 g/L·h). Conversely, the yeast *D. hansenii* drastically increased the volumetric productivity up to 0.91 g/L·h (in comparison with the 0.41 g/L·h obtained using raw hydrolyzates; *see* Fig. 5B). This optimum value was achieved with a flow rate of 0.55 mL/min, a higher value than that leading to optimum productivity in the case of media made from raw hydrolyzates (0.19 mL/min).

Continuous fermentation with charcoal-treated hydrolyzates improved the volumetric productivity significantly in comparison with the 0.58 g/L·h achieved with free cells and charcoal-treated hydrolyzates. Moreover, since continuous fermentation can be done for prolonged periods without the addition of fresh inocula, the studied process shows interesting features for practical purposes.

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