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Ethanol Production in Immobilized-Cell Bioreactors from Mixed Sugar Syrups and Enzymatic Hydrolysates of Steam-Exploded Biomass

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Abstract

We investigated ethanol production from mixed sugar syrups. Hydrolysates were prepared from enzymatic saccharification of steam-pretreated aspen chips. Syrups containing 45 g/L of glucose and 12 g/L of xylose were detoxified through two ion-exchange resins and then fermented with *Pichia stipitis* and *Saccharomyces cerevisiae* immobilized in Ca-alginate gel beads. Combinations of different gel fractions in the fermentation volume, amount of yeast cells, and ratios of *P. stipitis* vs *S. cerevisiae* within each bead were compared. In the best conditions, by using a total beads volume corresponding to 25% of the working volume, we obtained a yield of 0.39 $g_{ethanol}/g_{initial\ sugars}$. This amount of gel entrapped an initial cell concentration of 6×10^{12}cells/L with ratio of *S. cerevisiae*/*P. stipitis* of 0.25 g/g. Modified stirred-tank reactors were obtained either by adding marbles or by inserting a perforated metal cylinder, which reduced considerably the rupture of beads while visibly improving oxygenation of the medium.

Index Entries: Biomass; ethanol; coimmobilization; Ca-alginate; *Pichia stipitis*; steam explosion.

Introduction

The simultaneous bioconversion of mixed sugar syrups is one of the most ambitious challenges in the field of bioethanol production. Different productivities and ethanol-tolerance of the yeasts used in the fermentation of glucose and xylose (the most abundant biomass sugars) have led

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investigators to prefer process schemes in which hydrolysis and fermentation of cellulose and hemicellulose streams occur separately *(1)*. However, when the percentage of glucose in the feed biomass is much higher than xylose, cofermentation could be a more efficient approach since the cost of separate processes would be high.

The use of recombinant microorganisms for cofermentation is one of the most promising approaches in the field of bioethanol production, though their use for large-scale industrial processes still requires fine-tuning of the reliability of the entire process *(2)*. The technical hurdles of cofermentation increase when real biomass hydrolysates have to be fermented. In fact, whatever the biomass pretreatment, the formation of degradation byproducts that could inhibit the fermentation usually requires the addition of a further detoxification step. Therefore, the production of ethanol from hydrolysates should be considered in its entirety, from the optimal pretreatment to the choice of the proper fermentation process.

Among the most common hexose- and pentose-fermenting yeasts, *Saccharomyces cerevisiae* and *Pichia stipitis* are by far the most used *(3)*. However, cocultures of both yeasts do not always ensure the xylose conversion because of the diauxic behavior of *P. stipitis* (i.e., the preferential use of glucose when both substrates are available) *(4)*. To favor xylose consumption by *P. stipitis*, some researchers have proposed the use of a respiratory-deficient strain of *S. cerevisiae* while maintaining a specific oxygen uptake per cell during the fermentation process *(5)*. Nevertheless, the feasibility of this approach depends on the development of a system able to monitor and automatically adjust the oxygen level throughout the process.

Immobilization of yeasts into Ca-alginate beads is regarded as a valuable alternative to overcome the limitations of *P. stipitis (6)*. Using computer simulations, Grootjen et al *(6)* investigated the cofermentation of glucose and xylose mixtures with bead-immobilized *P. stipitis* in combination with either suspended or coimmobilized *S. cerevisiae*. They demonstrated that *P. stipitis* was not able to grow when it was coimmobilized with *S. cerevisiae*. It was also concluded that to make the process feasible, higher initial *P. stipitis* concentrations in the beads and/or a higher beads fraction relative to the fermentation volume would be required.

Although a preindustrial project of ethanol production with immobilized cells goes back to the 1980s *(8)*, its application is still limited because of technical problems related to the gel stability and even more to the mass-transfer resistance of the gel membrane *(9)*. Specifically, both substrate and product counterdiffusion (in addition to the presence of cells inside the beads) could reduce the diffusion coefficients of glucose and ethanol up to 13.7 and 28.1%, respectively *(10)*.

In a conventional stirred-tank bioreactor (STR) equipped with air bubbling, an increase in the agitation of the medium could improve oxygenation and circumvent the diffusion limitations. Conversely, the shearing effect of the impeller could somehow damage the beads, especially at high stirring rates. Alternatively, fluidized-bed bioreactors (FBR) allow

continuous movement of the beads, which ensures an efficient mass transfer. However, some design optimization is required to achieve the fluidization while preventing the cells from sloughing off the beads.

In the present study, the fermentation of glucose and xylose model solutions and hydrolysate samples from steam-pretreated aspen was investigated. After the steam explosion, the obtained slurry was suspended in water to a solid-to-liquid ratio of 200 g/L of dry matter (DM) and enzymatically hydrolyzed with a mixture of cellulolytic and hemicellulolytic enzymes. Subsequently, the hydrolysate was detoxified using a two-step treatment through cationic and anionic resins.

The fermentation tests were carried out in shake flasks, STR and FBR. The effect of the following parameters was investigated: the amount of gel and the total cell concentration in the bioreactor; the addition of hydrogen acceptor (acetone), instead of air, to activate the electron transport in the respiratory chain; and the use of Teflon-made filters as air diffusers to reduce air bubble dimension and increase oxygen solubility.

Furthermore, design modifications to the STR were also considered: equipping the STR with marbles to ensure a larger distribution of the gel beads inside the reactor while damping down the shearing effect of the Rushton impeller; and equipping the STR with a perforated stainless still cylinder that confines the beads in the external cavity between the reactor walls and the impeller zone. A description of the explained configurations is shown in Fig. 1.

The obtained results are intended to provide a preliminary assessment of the incidence of the investigated parameters on the process yields.

Materials and Methods

Pretreatment of Biomass

Steam explosion pretreatment was carried out in a continuous Staketech digester processing 150 kg/h of DM. Water was added to the chips to raise the intrinsic humidity to 50%. The severity of the steam explosion pretreatment was expressed through the logarithm of the semiempirical relation described in Eq. 1 *(11)*:

$$R_0 = t \times \exp\left(\frac{T - 100}{14.75}\right) \quad (1)$$

in which T is the steaming temperature (°C) and t is the residence time (min).

A mild severity parameter was chosen to reduce the hemicellulose degradation while ensuring an adequate cellulose fiberization. The steam pretreatment was therefore carried out at 215°C for 3 min corresponding to Log R_0 3.86.

Biomass was analyzed for carbohydrate, lignin, and ash content by the following standard procedures: carbohydrates and lignin by the Klason procedure (modified TAPPI T-13 m-54 and ASTM D1106), and ash by ASTM D1102. Details of the procedures adopted in our laboratory

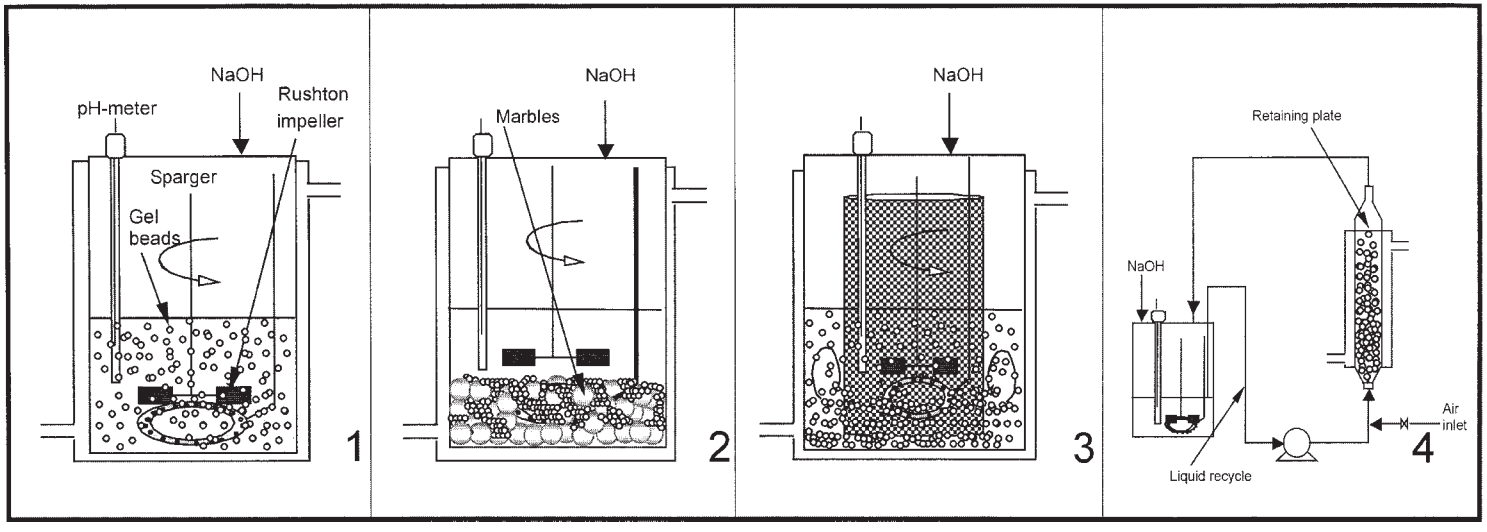

Fig. 1. Description of studied bioreactor configurations: 1, STR; 2) STR modified through addition of marbles; 3, STR modified through insertion of a metal perforated cylinder; 4, FBR.

are provided elsewhere *(12,13)*. The results reported here are the means of six values (two replicates for three samples).

Analysis of hydroxymethylfurfural (HMF), furfural, acetic acid, and formic acid was performed using the HP1100 system equipped with a diode array detector. Aliquots of a few milliliters were filtered (0.2 μm) and eluted through a Dionex AS1 column with a flow rate of 0.8 mL/min. A gradient of HCl and CH_3CN from 0.3 m*M* HCl and 1% (v/v) CH_3CN to 4 m*M* HCl and 15% (v/v) CH_3CN was used as the eluent. Quantification was carried out at two different wavelengths, 205 and 280 nm, for acids and aldehydes, respectively. The analytical method was tested over five calibration standards in the range of 3–140 ppm for HMF and furfural and 5-380 ppm for acetic and formic acid. The detector sensibility to HMF and furfural was 100-fold higher than that to acetic acid. Nevertheless, the analytical procedure showed a good linearity also for acetic acid.

Microorganisms and Maintenance

S. cerevisiae was Bakers Yeast Type II from Sigma. *P. stipitis* NRRL Y-11544 was obtained from DSMZ (Germany). The culture was maintained at 4°C on agar slants containing 20 g/L of D-xylose, 3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, and 15 g/L of agar. The culture medium contained 50 g/L of D-xylose, 5 g/L of D-glucose, 3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone (pH 5 ± 0.2). According to the procedure reported by Nigam *(14)*, the nutrients were sterilized by autoclaving at 120°C for 15 min, and D-xylose and D-glucose were sterilized at 110°C for 10 min.

To prepare the inoculum, 1000-mL Erlenmeyer flasks containing 200 mL of medium were inoculated from a fresh agar slant and incubated at 30 ± 2°C in a rotary shaker at 200 rpm. After 65 h, yeast cells were harvested by centrifuging at 2000 rpm for 15 min. The cells were washed twice and resuspended in saline solution (0.85% NaCl). The obtained suspensions were used for preparation of the gel beads.

To evaluate the cell mass concentration, a few milliliters of the suspension was filtered through 0.45-μm filters and dried at 60°C overnight until there was no weight change. The number of live cells was determined by counting in the samples the number of cells capable of forming colonies (CFU) on agar slants maintained at 30 ± 2°C. Cell viability was determined by using methylene blue.

Cell Immobilization

Cell suspensions containing one or both yeasts were mixed with alginate solution containing 2% (w/v) sodium alginate *(15)*. The suspension was pumped through a peristaltic pump in 0.2 *M* $CaCl_2$ solution. The alginate gel beads had diameters of 2 to 3 mm. After 30 min the beads were replaced with medium, washed twice, and stored in saline solution at 4°C.

The cell concentration was determined by microscopic counting (Olympus B-201) in a Bürker-type chamber on five beads dissolved in 10 mL of 1% sodium citrate solution and shaken for 10 min at 30°C. Although this method gives the total number of cells (dead and live), methylene blue dyeing revealed that most of the cells were alive.

The dosage of the yeasts during preparation of the beads was based on cell dry mass. However, the ratio by weight did not coincide with the ratio between *S. cerevisiae* and *P. stipitis* cell numbers; that is, 1 g of *P. stipitis* was roughly 10^{11} cells whereas 1 g of *S. cerevisiae* was approx 3×10^6 cells. Under the microscope, neither yeast showed appreciable difference in shape or dimension. Therefore, when referring to coimmobilized yeasts, the overall number of cells is reported. Details on the specific conditions of each test are provided when necessary.

Fermentation

The composition of the fermentation medium was 2 g/L of yeast extract, 5 g/L of KH_2PO_4, 0.4 g/L of $MgSO_4$, 2 g/L of $(NH_4)_2SO_4$, and 0.04 g/L of $CaCl_2$. $CaCl_2$ was added to prevent the alginate beads from dissolving owing to the phosphates. Before fermentation, the hydrolysate was sterilized by membrane filtration. The pH was adjusted to 5.5 and the temperature was kept at 30°C.

Batch fermentations with immobilized cells were carried out both in 500-mL Erlenmeyer flasks and in the 2-L Biostat B from BBInternational. All results are the mean of two duplicate experiments. For the shake-flask experiments, 100 mL of the culture medium supplemented with glucose and xylose or aspen hydrolysates was used. pH corrections were done discontinuously every 10 h.

The STR was equipped with temperature, pH, and P_{O_2} control units. Only one Rushton impeller was used to homogenize the system. Almost 1 L of the culture medium supplemented with the carbon sources was used during the test runs. In the configuration with marbles, the total volume occupied by marbles (average diameter of 16 mm) was roughly 350 mL. In the configuration with the metal cylinder around the stirrer axle, the volume available in the external cavity was 565 mL.

For FBR configuration, a column (73 × 3.2 cm) was packed with a volume of beads corresponding to half the volume of the column. A water jacket kept the temperature stable at 30°C. The culture medium was continuously pumped through a peristaltic pump from the reservoir into the packed column with a flow rate of 3–3.5 mL/min. pH adjustment was carried out in the reservoir tank.

Preparation of Hydrolysate Samples

Three enzymatic mixtures with predominantly cellulase and xylanase activity were used for the saccharification tests. To hydrolyse the cellulose, the mixtures Celluclast 1.5L and Novozym 188 from Novo Nordisk were

Table 1
Chemical Composition of Aspen Chips
Steam Exploded at Log R_0 3.86

Compound	Chemical composition (%)
Glucan	46 ± 2
Xylan	7.8 ± 0.3
Arabinan	0.48 ± 0.04
Glactan	0.30 ± 0.02
Ash	2.01 ± 0.01
Lignin	31 ± 1

added by using the internal dosage previously optimized *(13)*. The xylanase mixture, Multieffect xylanase, kindly provided by Genencor, was used to hydrolyze the hemicellulose fraction. From the data in the literature, the xylanase activity was 400 IU/mL *(16)*. Dosages of this enzyme mixture were based on the xylanase activity reported in the literature. Preliminary evaluations were carried out in shake flasks thermostated at 45°C and stirred at 180 rpm. The hydrolysate used in the detoxification and cofermentation tests was prepared in a 10-L bioreactor.

Detoxification of hydrolysates was accomplished in a continuous ion-exchange bench-scale unit. The resins used were Dowex 1 (50/100 mesh, density: 0.71 g/cm^3), weak basic anion exchanging, and Dowex-50W (50/100 mesh, density: 0.80 g/cm^3), strong acidic cation exchanging.

Results and Discussion

Hydrolysis

The steam-exploded aspen had an average DM of 63.5%; the chemical composition is described in Table 1. Poplar has been one of the most investigated biomass sources for ethanol production. The xylose/glucose ratio in the raw material is almost 0.3; that is, glucose is the most abundant component. After steam explosion, the residual xylose in the biomass could be recovered by posthydrolysis of the water-extracted hemicellulose or by enzymatic hydrolysis of the entire slurry. Through the former route, the maximum xylose concentration that we recovered by using an extracting ratio of 130 g/L (DM) was 6.94 g/L (68% of the xylose in the biomass). The solution also extracted degradation byproducts whose total amount was 3.6 g/L. Under these conditions, to ferment this stream a subsequent step of concentration in addition to an effective detoxification treatment would be required. A further optimization of the pretreatment (e.g., by adding acid catalysts) is likely to enhance the water-extracted hemicellulose, but this topic is beyond the scope of this article.

Preliminary evaluations of the enzymatic hydrolysis yields of the entire slurry (cellulose and hemicellulose) were carried out in shake flasks in order to evaluate the effect of concentrated slurries on the process yields. High contents of enzymatic proteins were used in order to overcome the inhibition of byproducts already ascertained in concentrated and nondetoxified suspensions *(13)*; the results are summarized in Table 2. The percentages of the recovered xylose ranged between 72 and 95%, higher than those obtained by the posthydrolysis of the water-extracted hemicellulose. The effect of doubling the biomass loading was remarkable, as shown by the decrease in both glucose and xylose hydrolysis yields. By contrast, no significant improvement could be observed by increasing the IU of xylanase. Therefore, hydrolysate samples used in the detoxification and cofermentation tests were prepared in a 10-L STR according to the conditions regarding the third case listed in Table 2. The obtained yields were comparable with those obtained in shake flasks. These results do not presume to be conclusive on the optimization of the enzymatic hydrolysis of nondetoxified slurries; they are simply a way to produce concentrated syrups containing 45 g/L of glucose and 12 g/L of xylose. Obviously, this approach could become more valuable depending on the research progresses concerning the production of more effective enzyme mixtures *(17)*.

Detoxification of Hydrolysate by Treatment with Cation- and Anion-Exchange Resins

To optimize the operating conditions for the detoxification of the hydrolysates with resins, experiments were carried out with synthetic solutions. Table 3 shows how the detoxifying efficiency of the anionic resin Dowex 1 diminished with increasing treated volume. Aliquots of the solution eluted through the column were sampled after 120, 230, and 250 mL. As a whole, after the early 600 mL, the column still had a fair cleaning efficiency for acids but a reduced removal efficiency toward the furan compounds: the data show that the cleaning efficiency of the column was 99% for formic acid, 87% for acetic acid, 40% for HMF, and 46% for furfural. An optimized resin/solution ratio of 0.14 g/g was thus extrapolated. The addition of a subsequent detoxification step with the cationic resin Dowex-50W left the concentrations of the acids almost unchanged whereas it further reduced those of HMF and furfural by 14 and 26%, respectively. The detoxification of the hydrolysate was performed on the basis of this optimized detoxification setup.

Table 4 summarizes the concentrations of the steam-exploded degradation byproducts and the most abundant monosaccharides released from the chips during hydrolysis. No meaningful differences in the concentrations of the byproducts were observed after 48 h. Only the concentrations at 24 h appeared slightly higher than they were later. This trend could be owing to a partial adsorption through the hydrolysis time of these mol-

Table 2
Monosaccharide Yields (%DM) from Bench-Scale Enzymatic Hydrolysis of Steam-Exploded Aspen Chips[a]

Xylanase (IU/g)	Biomass loading (DM) (g/L)	Glucose	Xylose
63	100	53 ± 4	95 ± 3
100	100	55 ± 6	92 ± 2
63	200	42 ± 3	72 ± 6
100	200	45 ± 1	79 ± 5

[a]The cellulase and β-glucosidase activities were 30 filter paper units/g of DM substrate and 47 IU/g of DM substrate, respectively.

Table 3
Variation of Detoxifying Efficiency of Anionic Resin DOWEX 1 at Increasing Eluted Volume: After 120 mL (I), After 230 mL (II), and After 250 mL (III)[a]

		Percentage of removed inhibitors		
Compound	Initial concentration (ppm)	I	II	III
Formic acid	1285	100.0	99.8	98.1
Acetic acid	10302	99.6	100.0	63.4
5HMF	56	100.0	100.0	6.9
Furfural	1178	76.5	36.3	19.6

[a]The column bed was made with 90.13 g of DM resin. The elution flow rate was 3 mL/min.

ecules onto the insoluble lignin fraction. Although not quantitative, the hydrolysis was already completed at 48 h and the syrup composition did not change after the stage of thermal deactivation of the enzyme. Through the use of the two ion-exchange columns arranged as described under Subheading "Detoxification of Hydrolysate by Treatment with Cation- and Anion-Exchange Resins," almost all the detected inhibitors were efficiently removed. The sugars composition remained unaffected in agreement with data reviewed by Parajó et al. *(18)* on the retention of carbohydrates of ion-exchange resins. Other aromatic compounds (e.g., lignin-derived compounds) were likely to be removed from this treatment since the treated solution appeared pale yellow while the hydrolysate was brown .

Fermentation of Standard Model Solutions of Glucose and Xylose

The fermentative performance of *P. stipitis* immobilized in Ca-alginate beads was first investigated using concentrated solutions of xylose. The higher ethanol yield and productivity obtained from the fermentation of 60 g/L of xylose working with a cell concentration of 2.34×10^9 cells/L were 0.46 $g_{ethanol}/g_{initial\ sugars}$ (g_e/g_s) and 0.40 g/(L·h), respectively. Only traces of secondary metabolites were detected.

Table 4
Concentrations (g/L) of Main Degradation Byproducts and Monosaccharides Obtained During Enzymatic Hydrolysis and After Detoxification with Ion-Exchange Resins

Compounds	Concentrations during hydrolysis			Concentrations after thermal deactivation of enzymes	Concentrations after detoxification
	24 h	48 h	72 h		
Formic acid	1.60 ± 0.06	1.30 ± 0.03	1.4 ± 0.04	1.39 ± 0.05	0.049 ± 0.008
Acetic acid	8.1 ± 0.4	7.26 ± 0.16	7.8 ± 0.4	8.0 ± 0.3	0.553 ± 0.041
5HMF	0.160 ± 0.001	0.146 ± 0.001	0.148 ± 0.008	0.139 ± 0.004	0.008 ± 0.001
Furfural	0.420 ± 0.003	0.356 ± 0.001	0.345 ± 0.001	0.337 ± 0.001	0.0160 ± 0.0005
Glucose	34 ± 3	49 ± 4	45 ± 4	43 ± 3	43 ± 3
Xylose	12 ± 1.0	12.2 ± 1.1	11 ± 1.0	12.0 ± 1.1	11.2 ± 1.0

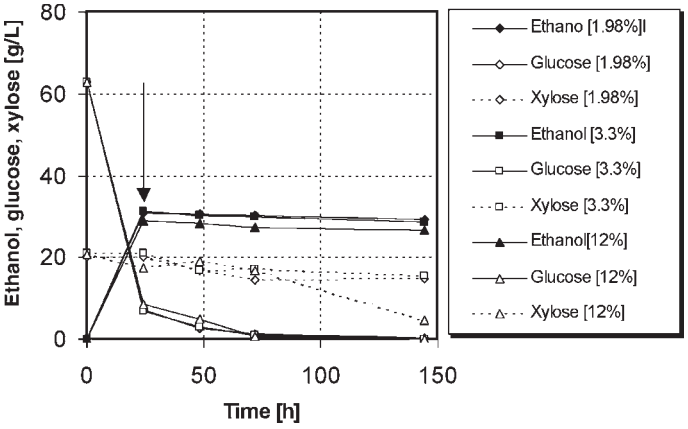

Fig. 2. Sequential fermentation of model solutions of glucose (60 g/L) and xylose (20 g/L) in shake flasks containing same amount of suspended *S. cerevisiae* (3 g/L) and different gel fractions (percentages in parenthesis) of immobilized *P. stipitis*. The initial cell concentrations of *P. stipitis* were (◆), 7.02×10^9; (■), 1.17×10^{10}; (▲), 4.10×10^{10} cells/L.

Several strategies were investigated to achieve the cofermentation of mixed sugar syrups containing glucose and xylose in concentrations similar to those recoverable from the hydrolysis of steam-exploded aspen chips. Since glucose causes the so-called xylose catabolism repression, beads of *P. stipitis* were introduced into the flasks after the glucose consumption from *S. cerevisiae* was almost completed. This approach is not exactly a cofermentation scheme but rather a sequential fermentation. The pH was not adjusted. Figure 2 shows the trend of ethanol production and sugar consumption obtained by varying the gel fractions. During the early 24 h almost all the glucose in the medium was converted into ethanol. At that time (arrow in Fig. 2) different fractions of beads containing *P. stipitis* were introduced into the three systems in order to convert the residual xylose. However, from that point the ethanol yield leveled off, and the diminution of the xylose concentration in the system was owing to the formation of xylitol. The nonregulation of the pH and/or the relatively high ethanol concentration (30 g/L) during the lag phase of *P. stipitis* could have inhibited the production of ethanol from this yeast. To ascertain the influence of pH, mixed sugar syrups were fermented using the same approach but with regular adjustment of the pH to the initial value of 5.5. Higher concentrations of *P. stipitis* were also loaded; Fig. 3 summarizes the results. The trends show that even when the pH was adjusted to 5.5, the xylose consumption did not correspond to the ethanol increase and that already 15 g/L could inhibit the fermentation from *P. stipitis* in a sequential fermentation approach. Isolated strains of ethanol-adapted *P. stipitis* could be necessary in this process scheme. Moreover, this result also confirms that the immobilization somehow stabilizes the fermentation capacity of yeasts in a wide range of pH values *(19)*.

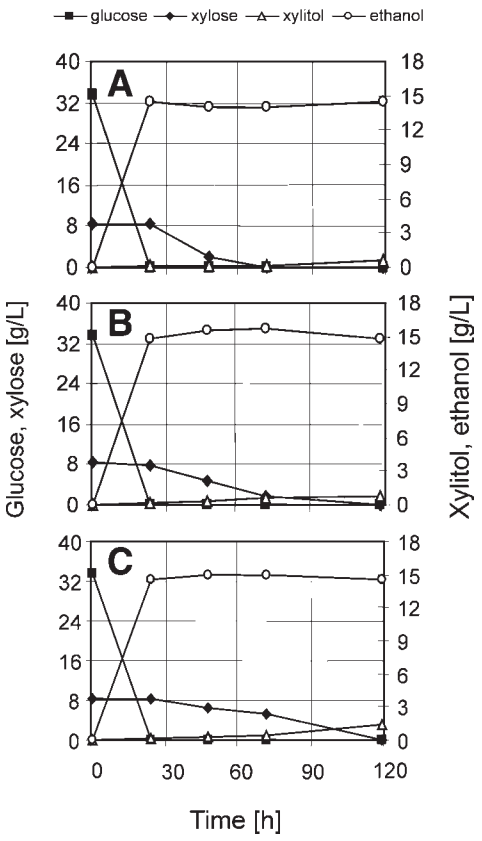

Fig. 3. Sequential fermentation of model solutions of glucose and xylose in shake flasks. Systems A, B, and C contained suspended *S. cerevisiae* and immobilized *P. stipitis* with a loading ratio of *P. stipitis*/*S. cerevisiae* of 1.4 g/g dry wt. The gel fractions were as follows: system A, 0.36; system B, 0.18; and system C, 0.09 g/g. The concentrations of *P. stipitis* cells were as follows: system A, 2.83×10^{12}; system B, 1.41×10^{12}; and system C 7.31×10^{11} cells/L.

The coimmobilization of both yeasts was therefore investigated. Taking into account the results from Grootjen et al. *(6,7)*, the amounts of *P. stipitis* loaded into the beads were higher than those of *S. cerevisiae*. Usually the internal ratio was fixed at 1:4 *S. cerevisiae*/*P. stipitis* dry cell mass.

Figure 4 presents data on the cofermentation of glucose and xylose. The production of ethanol still appeared to be at the expense of glucose consumption. Note that systems E and F did not show significant differences whereas the amount of ethanol in system D was roughly 25% higher. This could imply that a threshold cell concentration is necessary.

To ascertain whether different process configurations could have any effect on the process yields, the same cell loading of system D in Fig. 4 was

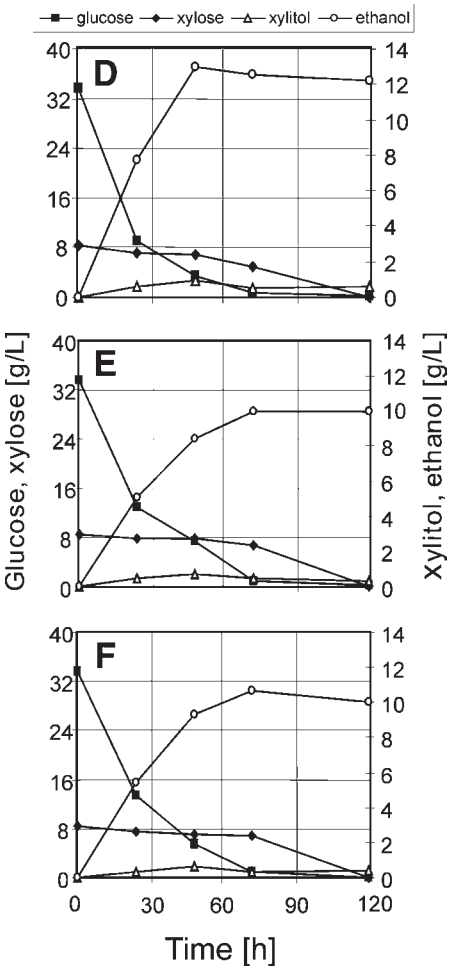

Fig. 4. Cofermentation of model solutions of glucose and xylose. Systems D, E, and F contained beads of *S. cerevisiae* and *P. stipitis* coimmobilized with a loading ratio of *P. stipitis*/*S. cerevisiae* of 4 g/g. The gel fractions were as follows: system D, 0.10; system E, 0.05; and system F, 0.025 g/g. The total cells concentrations were as follows: system D, 4.80×10^{11}; system E, 2.40×10^{11}; and system F, 1.20×10^{11} cells/L.

used in the FBR and STR. Moreover, to avoid rupture of the beads owing to the impeller agitation in the STR, experiments were conducted using modified STR configurations. An early attempt to achieve this purpose was made by adding marbles to the bioreactor volume. The resulting effect was a broader distribution of the gel beads throughout the bioreactor and the formation of a low shear environment. Specifically, the marbles protected the beads from shear and allowed them to overcome the diffusion limitations by settling at higher stirring rates.

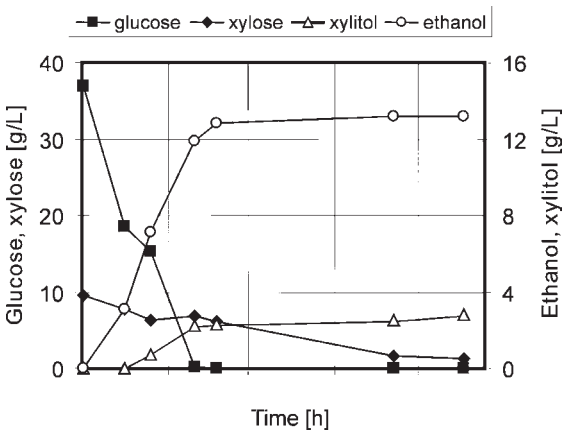

Fig. 5. Cofermentation of model solutions of glucose and xylose in FBR. The total initial cell concentration was 6.57×10^{11}cells/L and the gel fraction was 0.1 g/g. The ration of *P. stipitis*/*S. cerevisiae* was 4 g/g. The number of beads inside the column was 3286. The airflow in the column and inside the reservoir was 0.030 vvm.

Figure 5 shows the experimental results of the continuous glucose and xylose cofermentation in the FBR. The aeration conditions were chosen on the basis of the ranges reported in the literature for free-cell cultures *(5,20)*. The maximum amount of ethanol produced is comparable with that of system D in Fig. 4, and the xylose consumption is evidently the result of the formation of xylitol. Despite the air bubbled into the medium reservoir and at the bottom of the column, the oxygen concentration in the medium continuously dropped during the fermentation, reaching zero after 24 h. When the concentration of ethanol leveled off, the process was stopped, and the beads were washed with saline solution before being used in a second fermentation run with a fresh solution of sugars. After 24 h, the glucose concentration was almost zero while only 47% of xylose was consumed. In other words, the ethanol productivity in the second cycle was 49% higher than in the first cycle, whereas the rate of xylose consumption was still lower than that of glucose.

It is already known that under anaerobic conditions the xylose-fermenting yeasts do not produce ethanol because the electron transport in the respiratory chain is not activated *(20)*. Since either aeration or oxidizing compounds, such as acetone, could increase the ethanol yield, the effect of improving the oxygenation of the medium or adding oxidizers was investigated using an STR partially filled with marbles.

Perego et al. *(20)* found that during the fermentation of hemicellulose hydrolysates by *Candida shehatae*, the addition of 50 m*M* acetone resulted in ethanol yields comparable with those obtained under microaerophilic conditions. Thus, the addition of 50 m*M* acetone to the culture

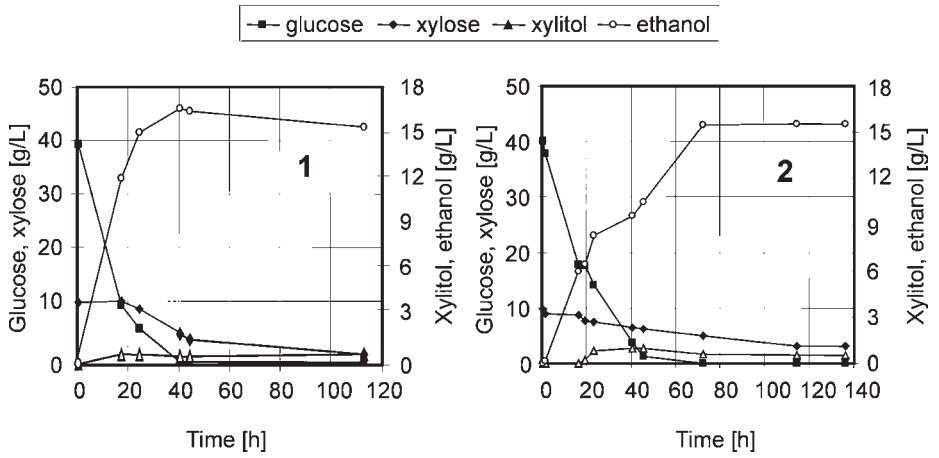

Fig. 6. Effect of aeration (0.030 vvm) through Teflon-made diffusers (system 1) and acetone addition (50 m*M*) (system 2) on cofermentation of synthetic solutions of glucose and xylose in STR containing marbles. In both cases, the beads were prepared with an internal ratio of *P. stipitis*/*S. cerevisiae* of 4 g/g, and the gel fraction was almost 0.1 g/g. The initial cell concentration in system 1 was 8.6×10^{11} cells/L while that in system 2 was 8.42×10^{11} cells/L.

broth was considered in place of aeration during the fermentation of synthetic solutions.

On the other hand, to improve oxygenation of the medium, two Teflon-made high-performance liquid chromatography filters were used as air diffusers. The dimension of the bubbles was then visibly smaller than that obtained with the metallic annular sparger and a slightly better dispersion of bubbles was possible with the Rushton impeller. The results obtained by improving the oxygenation and adding acetone are shown in Fig. 6 (system 1) and (system 2), respectively. Both trends show an increase in ethanol production, though in both cases a significant xylose consumption never started before 24 h, and the ethanol production appeared still dependent mainly on the glucose metabolism. In addition, the presence of acetone even slowed down the glucose consumption and therefore the ethanol productivity.

Based on the results discussed so far, it appears that under similar cell-loading conditions neither the reactor configuration nor the aeration conditions had a remarkable effect on the xylose conversion into ethanol, although they could affect the yield of ethanol from glucose and the concentration of secondary metabolites (xylitol and lactic acid). As a result, higher initial cell concentrations were considered. However, when beads were loaded with high amounts of yeast, the cells counted inside the beads after all the sugars were consumed did not differ significantly from the initial number.

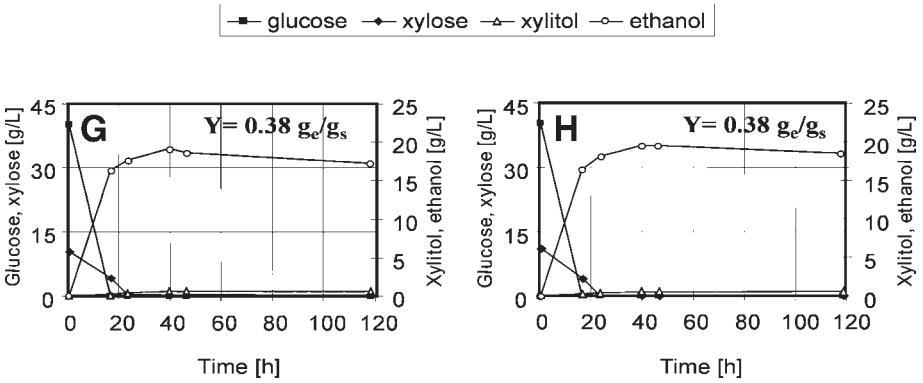

Fig. 7. Cofermentation of model solutions of glucose and xylose with *P. stipitis* and *S. cerevisiae* separately immobilized (system G) and coimmobilized (system H) in Ca-alginate beads. The gel fraction in system G was made of 0.20 g/g of beads containing *P. stipitis* and 0.05 g/g of beads containing *S. cerevisiae*. The initial concentrations of *P. stipitis* and *S. cerevisiae* cells were 5.64×10^{12} and 1.89×10^{11} cells/L, respectively. The gel fraction in system H was made of 0.25 g/g of beads containing *P. stipitis* and *S. cerevisiae* coimmobilized with a loading ratio of *P. stipitis*/*S. cerevisiae* of 4 g/g of dry cells. The total cells concentration was 6.01×10^{12} cells/L.

Moreover, it would seem that whatever the amount of yeast suspended in the alginate mixture before its gelling in the $CaCl_2$ solution, the beads would not entrap more than 6 to 7 $\times$ 10^8 cells/bead. On the whole, it could be inferred that the microenvironment of the beads has a saturating uptake and that an excessive yeast loading could produce a rapid diffusion of the cells out of the beads. In light of these results, higher initial cell concentrations were realized by loading a higher fraction of beads instead of higher cell concentrations inside the gel matrix. The maximum number of yeast cells that could be entrapped within the beads might be critical when yeasts with different growing rates are coimmobilized. In fact, the faster-growing yeast could reduce the available bead volume. To ascertain whether a different distribution of the yeasts in the gel phase could affect the process yield, cofermentation tests were conducted with two different gel phases: (1) a gel phase made of 80% beads entrapping *P. stipitis* and 20% beads entrapping *S. cerevisiae*; and (2) a gel phase made of beads coimmo-bilizing *P. stipitis* and *S. cerevisiae* with an internal ratio of 4:1 g/g, respectively. In the former case, the same yeast DM per gel was used for the preparation of the two stocks of beads; the results are displayed in Fig. 7. In both cases, glucose and xylose were consumed simultaneously. The ethanol yields after 40 h were very similar (0.38 g_e/g_s). This means that the cell concentration affected the process yields more than the way that the yeasts were distributed in the gel phase.

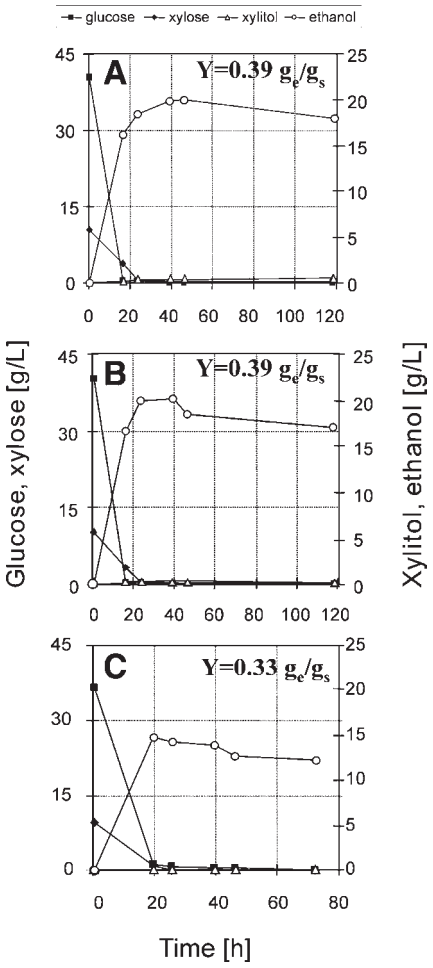

Fig. 8. Cofermentation of detoxified hydrolysates in shake flasks (A and B) and in STR modified with perforated cylinder. For the experimental conditions of graph A, refer to graph G in Fig. 7, and for those of graphs B and C refer to graph H in Fig. 7.

Fermentation of Hydrolysates

Considering the results obtained in the test runs of synthetic solutions, the cofermentation of detoxified hydrolysates was carried out at flask scale using the same experimental conditions as described in Fig. 7; the results are presented in Fig. 8. The ethanol yields were slightly higher than those obtained working with synthetic solutions, but they were still comparable with respect to the composition of the sugar syrup used (0.39 g_e/g_s). It is likely that in the absence of furfural, the residual amount of acetic acid in the detoxified hydrolysate could have enhanced ethanol production *(21,22)*.

Figure 8C illustrates the experimental results of the cofermentation of the hydrolysate in the STR modified with a perforated cylinder. The function of this cylinder was the same as previously discussed for marbles. The cylinder confined the beads in the cavity between the bioreactor and the impeller, thereby preventing the beads from knocking against the impeller. This allows higher stirring rates (up to 300 rpm) to be settled. Moreover, the radial agitation of the Rushton impeller, combined with the presence of the pierced cylinder, made the beads flow in a circular bottom-up direction. Glucose and xylose were consumed simultaneously and even faster than in shake flasks under similar experimental conditions. Nevertheless, the ethanol yield was $0.33 g_e/g_s$, lower than that obtained in shake flasks and a diminution of ethanol was observed soon after 20 h. This result was probably owing to nonoptimized aeration conditions.

Conclusion

The main purpose of this study was to investigate one of the possible process schemes for ethanol production from glucose and xylose of steam-exploded aspen chips. The process strategy provided the simultaneous hydrolysis of cellulose and hemicellulose followed by detoxification through resins and cofermentation with immobilized cells. In the best conditions, the cofermentation of 40 g/L of glucose and 10 g/L of xylose with coimmobilized *S. cerevisiae* and *P. stipitis* yielded 0.396 $g_{ethanol}/g_{sugars\ consumed}$ and a conversion of 0.995 $g_{consumed}/g_{initial}$. In other words, the process produced 77% of the theoretical ethanol yield. Attention was devoted to improvement of the performances of the STR by making some modifications in the original design. Although preliminary, our results suggest that insertion of marbles at the bottom of the reactor enables a high stirring rate (up to 300 rpm) without affecting the beads' lifetime. The same effect was observed using a perforated metal cylinder placed around the stirrer axle. In the latter case, a larger fluidization can be achieved. These results are not intended to be conclusive. Further investigations are still in progress to define the fermentation scheme and bioreactor configuration that will ensure higher productivities and yields without reducing the catalyst stability inside the gel carrier.

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