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Ethanol Production from Glucose and Xylose by Immobilized *Zymomonas mobilis* CP4(pZB5)

Mahesh S. Krishnan, Maria Blanco, Christopher K. Shattuck, Nhuan P. Nghiem,* and Brian H. Davison

Bioprocessing Research and Development Center, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6226, E-mail: ndp@ornl.gov

Abstract

Fermentation of glucose-xylose mixtures to ethanol was investigated in batch and continuous experiments using immobilized recombinant *Zymomonas mobilis* CP4(pZB5). This microorganism was immobilized by entrapment in κ-carrageenan beads having a diameter of 1.5–2.5 mm. Batch experiments showed that the immobilized cells cofermented glucose and xylose to ethanol and that the presence of glucose improved the xylose utilization rate. Batch fermentation of rice straw hydrolysate containing 76 g/L of glucose and 33.8 g/L of xylose gave an ethanol concentration of 44.3 g/L after 24 h, corresponding to a yield of 0.46 g of ethanol/g of sugars. Comparable results were achieved with a synthetic sugar control. Continuous fermentation experiments were performed in a laboratory-scale fluidized-bed bioreactor (FBR). Glucose-xylose feed mixtures were pumped through the FBR at residence times of 2–4 h. Glucose conversion to ethanol was maintained above 98% in all experiments. Xylose conversion to ethanol was highest at 91.5% for a feed containing 50 g/L of glucose and 13 g/L of xylose at a dilution rate of 0.24/h. The xylose conversion to ethanol decreased with increasing feed xylose concentration, dilution rate, and age of the immobilized cells. Volumetric ethanol productivities in the range of 6.5–15.3 g/L·h were obtained. The improved productivities achieved in the FBR compared to other bioreactor systems can help in reducing the production costs of fuel ethanol from lignocellulosic sugars.

*Author to whom all correspondence and reprint requests should be addressed.

Index Entries: Ethanol; recombinant *Zymomonas mobilis*; xylose fermentation; immobilization; fluidized-bed bioreactor.

Introduction

Lignocellulosic biomass is a low-cost renewable resource that can be used to meet the projected increase in fuel ethanol demand. These feedstocks comprise cellulose, hemicellulose, and lignin. Lignocellulosic hydrolysates produced either chemically or enzymatically contain both pentoses and hexoses. Glucose derived from cellulose is the major hexose sugar, and the pentoses derived from hemicellulose comprise xylose and arabinose. Whereas glucose can be fermented to ethanol by a number of microorganisms, the conversion of xylose and arabinose to ethanol is more difficult. Since pentoses, which predominantly comprise xylose, can account for 8–28% of the raw material *(1)*, their efficient conversion to ethanol is necessary for an economical process. Advances in metabolic engineering have led to the construction of several xylose-fermenting microorganisms. These include recombinant bacterial strains of *Escherichia coli (2–4)*, *Klebsiella oxytoca (5,6)*, *Zymomonas mobilis (7)*, and a recombinant *Saccharomyces* yeast *(8)*.

Current batch processes for ethanol production from glucose and xylose using these free-cell biocatalysts have a low volumetric productivity and require long fermentation times. The development of high-productivity processes and reactors can potentially reduce capital costs for commercial fuel ethanol production. The use of continuous systems having high biocatalyst loading along with some form of biocatalyst retention mechanism can improve ethanol productivities compared to traditional batch systems. These methods include cell recycle by filtration, sedimentation, entrapment in membranes, or entrapment in gels. With glucose as the sole fermentation substrate, the volumetric ethanol productivities for continuous systems with high conversion have been compared *(9)*. The fluidized-bed bioreactor (FBR) was shown to achieve higher productivities compared to a free-cell continuous stirred-tank reactor (CSTR), immobilized cell CSTR, hollow-fiber reactor, and a packed-bed reactor with immobilized cells *(9)*. Table 1 presents the volumetric ethanol productivities for different continuous systems for bioreactor feeds containing xylose or mixtures of glucose and xylose. For xylose fermentation, species of the naturally occurring yeast *Pichia stipitis* have been immobilized and used in different bioreactor configurations. Since the fermentation rate of xylose to ethanol is significantly lower than the rate of glucose fermentation to ethanol, correspondingly lower volumetric ethanol productivities are achieved in comparison to feeds containing only glucose. To date, there are no reports in the literature dealing with the application of an FBR using an immobilized recombinant strain for ethanol production from glucose and xylose feed mixtures.

An FBR shows plug flow or multistage characteristics and has advantages over a well-mixed reactor. A higher reaction rate is maintained along

Table 1
Volumetric Ethanol Productivities from Xylose and Glucose-Xylose Mixtures Using Different Bioreactor Configurations

Bioreactor	Microorganism	Feed	Volumetric ethanol productivity (g/[L·h])	Reference
Free-cell CSTR	Recombinant *Z. mobilis* 39676(pZB4L)	8 g/L glucose, 40 g/L xylose	1–2	*10*
High cell density CSTR	Coculture of *Saccharomyces diastaticus* and *P. stipitis*	35 g/L glucose, 15 g/L xylose	1–4	*11*
Immobilized cell CSTR	*P. stipitis*	50 g/L xylose	2–3	*12*
Packed bed with immobilized cells	*P. stipitis*	50 g/L xylose	2–4	*13*
Pulsed packed bed with immobilized cells	*P. stipitis*	50 g/L xylose	2–4	*14*

the reactor length because of the overall higher substrate concentration and localization of product inhibition to the exit section. An FBR also provides effective mass transport compared to packed-bed reactors by overcoming channeling and carbon dioxide buildup.

The FBR has been used for ethanol production from glucose *(9)* and industrial dry-milled corn starch *(15)*. The economic impact of the use of FBR for ethanol production from glucose has been estimated to be 6 ¢/gal *(16)*. For the dry-milled corn starch to ethanol process using the FBR process technology, a cost savings up to 3 ¢/gal has been estimated *(17)*. In both cases, cost savings were realized owing to higher ethanol yields, lower operating costs, and lower capital costs for the continuous FBR process with an immobilized *Z. mobilis* biocatalyst compared to a conventional yeast batch process. These potential cost savings by using the FBR have provided the incentive to extend this bioreactor configuration to ethanol production from lignocellulosic feedstocks.

In this study, a recombinant *Z. mobilis* strain was immobilized by entrapment in κ-carrageenan and used in batch and continuous experiments for the conversion of glucose-xylose mixtures to ethanol. Batch fermentation kinetics using these immobilized cells on synthetic glucose-xylose mixtures and on lignocellulosic hydrolysates are presented. Results of continuous fermentation runs in the FBR are also reported. The impact of glucose to xylose feed ratio, residence time in the FBR, and long-term use of the biocatalyst are discussed.

Materials and Methods

Microorganism

Z. mobilis CP4(pZB5), which contains the *E. coli* genes for xylose assimilation (xylose isomerase, xylulokinase) and pentose metabolism (transketolase, transaldolase) on the plasmid pZB5 *(7)*, was obtained from the National Renewable Energy Laboratory, Golden, CO. The stock culture was maintained in medium containing 10 g/L of yeast extract, 2 g/L of KH_2PO_4, 10 mg/L of tetracycline, and 25% (w/w) glycerol at –70°C.

Preparation of Immobilized Biocatalyst

Cells were grown in a 75-L fermentor (New Brunswick, Edison, NJ) at 30°C and pH 5.5 (maintained using 2.5 *M* NaOH). A working volume of 50 L was used. The seed culture (4 L) was prepared in two fernbachs. The seed culture medium consisted of 50 g/L of glucose, 10 g/L of xylose, 10 g/L of yeast extract, 2 g/L of KH_2PO_4, and 10 mg/L of tetracycline. The medium without tetracycline was sterilized at 121°C for 20 min and allowed to cool before the antibiotic was added. Each fernbach was inoculated with 1.5 mL of stock culture. The seed culture was incubated at 30°C and 50 rpm for 36 h before it was used to inoculate the fermentor. The composition of the fermentation medium was the same as that of the seed medium. The cells were allowed to reach their late exponential growth

phase and then harvested using a Sharples centrifuge (Sharples, Philadelphia, PA). The cell pastes were stored at 4°C until ready for use in the immobilization step.

Bead preparation was initiated by dissolving 40 g of κ-carrageenan (FMC, Rockland, ME) in 600 mL of deionized water at 75°C. The dissolved gel was then placed in a water bath at 35°C. To this was added 40 g (wet wt) of recombinant *Z. mobilis* CP4(pZB5) cell paste. The final solution volume was brought up to 1 L with deionized water. To increase the density of the beads, 30 g of Fe_2O_3 was added to the gel solution. Bead formation was achieved using a previously developed technique in which the heated gel material was forced through a small nozzle using a peristaltic pump *(18)*. A vibration transducer was attached to the flexible delivery tube. By observing the nozzle exit stream under stroboscopic light, the vibrational frequency was tuned to produce monodispersed droplets having a diameter of 1.5–2.5 mm. The gel beads were collected in a stirred vessel containing 0.3 *M* KCl and were allowed to cure for 24 h at 4°C before use.

Batch Fermentation

The immobilized *Z. mobilis* CP4(pZB5) biocatalyst beads were used in batch fermentation studies on synthetic media containing glucose and xylose, and on lignocellulosic hydrolysate supplied by Arkenol (Mission Viejo, CA). This hydrolysate was obtained by the concentrated acid hydrolysis of rice straw by the patented Arkenol process *(19)*. Comparison of the high-performance liquid chromatography (HPLC) chromatograms of the hydrolysate solution and synthetic media did not indicate the presence of any new peaks for the hydrolysate, thereby indicating that there were no detectable inhibitors. All batch experiments were conducted in 125-mL shake flasks containing 60 mL of fermentation media and 20 mL of beads. The initial cell loading in the beads was determined to be 3 g of dry cell weight/L of beads. The temperature was maintained at 30°C and the agitation speed at 75 rpm.

The synthetic media contained different glucose and xylose concentrations, 10 g/L of yeast extract, 2 g/L of KH_2PO_4, 3.73 g/L of KCl, and 10 mg/L of tetracycline. The concentrations of glucose and xylose in the hydrolysate were 107 and 37 g/L, respectively. The hydrolysate was supplemented with 10 g/L of yeast extract, 2 g/L of KH_2PO_4, 3.73 g/L of KCl, and 10 mg/L of tetracycline. The synthetic medium was sterilized by autoclaving at 121°C. To avoid thermal decomposition of the hydrolysate, it was filter sterilized though a 0.2-μ membrane filter. The initial pH of both the synthetic sugar and hydrolysate media was 5.8.

Fluidized-Bed Bioreactor

The FBR, as shown in Fig. 1, was a jacketed glass column with an i.d. of 5.1 cm and a length of 47 cm. The volume of the FBR was 0.9 L. The FBR was sterilized at 121°C for 20 min before the biocatalyst beads were loaded. The bulk volume occupied by the beads was 550 mL. The pH in the upper

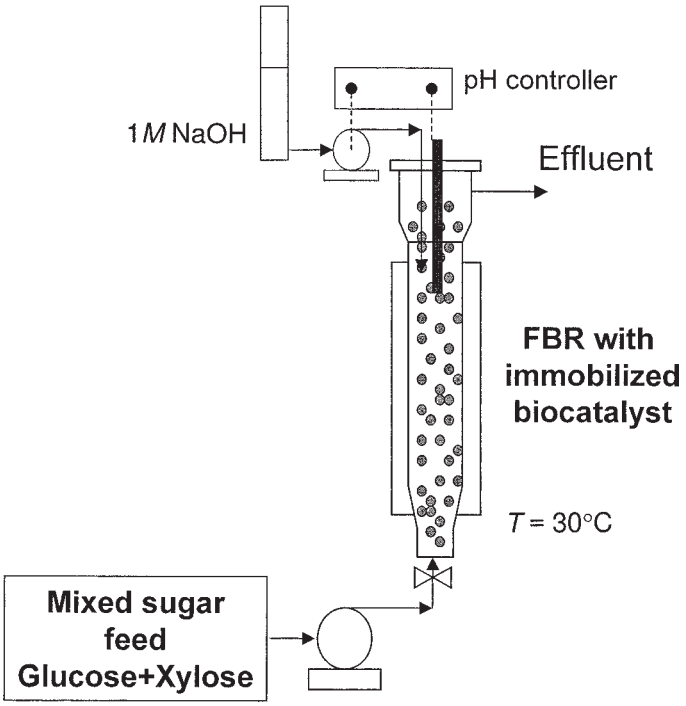

Fig. 1. Schematic of experimental setup for continuous FBR runs.

part of the FBR was controlled at 5.0 using 1 *M* NaOH. The base injection was placed very close to the pH probe in order to avoid pH overshoot. The temperature of the FBR was maintained at 30°C.

Recombinant *Z. mobilis* CP4(pZB5) grew within the beads for the first 2 to 3 d. For the continuous experiment starting with a 50 g/L glucose and 10 g/L xylose feed, this was accomplished by pumping feed solutions containing 50 g/L of glucose (A. E. Staley, Decatur, IL), 10 g/L of xylose (Sigma, St. Louis, MO), 3.73 g/L of KCl, 2 g/L of KH_2PO_4, 5 g/L of Difco (Detroit, MI) yeast extract, and 10 mg/L of tetracycline through the FBR at residence times of 3 to 4 h. For the continuous experiment starting with a 40 g/L glucose and 20 g/L xylose feed, the FBR was started by pumping feed solutions containing these sugar concentrations. The feed was sterilized by autoclaving at 121°C for 1 h. Following initial growth within the biocatalyst beads by the microorganism, feed solutions containing appropriate concentrations of glucose and xylose were pumped through the FBR to give residence times in the range of 2–4 h. Other components of these feed solutions were the same as those just described. These feeds also were sterilized by autoclaving at 121°C for 1 h. All the feed solutions contained 3.73 g/L of KCl for stabilization of the biocatalyst beads. The feed lines were changed when the empty feed reservoir was replaced. For each set of experimental conditions, at least six residence times were allowed for the FBR to reach steady state before samples were analyzed for glucose, xylose, ethanol, acetic acid, and lactic acid.

Analytical Methods

Glucose, xylose, ethanol, acetic acid, and lactic acid were analyzed using an HPLC system consisting of a Waters 410 RI detector (Waters, Milford, MA), a Waters 717 Plus Autosampler, and an Alltech 425 HPLC pump (Alltech, Deerfield, IL). The column was an Aminex HPX-87H (Bio-Rad, Hercules, CA) column. The mobile phase was 5 m*M* H_2SO_4 pumped at a flow rate of 0.6 mL/min. Data acquisition and analysis were performed using the Waters Millennium software. Glucose and lactic acid were also analyzed with a YSI Biochemistry Analyzer (YSI, Yellow Springs, OH).

To determine the cell loading in the biocatalyst beads, a known volume of beads was dissolved in 5% (w/v) sodium citrate solution with stirring. After the beads were completely dissolved, the solution was filtered through a 0.2-μm Millipore filter. The filter was dried in an 80°C oven and weighed to calculate the cell dry wt. The protein content of the beads was determined using the Peterson's *(20)* modification of the Lowry method.

Results and Discussion

Batch Fermentation Studies on Synthetic Sugar Medium

Fermentation of 32 g/L of glucose (Fig. 2A) was completed in approx 10 h, and an ethanol concentration of 16 g/L was obtained, giving a yield of 0.50 g of ethanol/g of glucose, corresponding to 98% of the theoretical yield. Fermentation of 20.4 g/L of xylose (Fig. 2B) was slower, and an ethanol concentration of 7.6 g/L was achieved after 48 h. The residual xylose concentration was 3.68 g/L. Based on the consumed sugar, the ethanol yield was calculated to be 0.45 g of ethanol/g of xylose, corresponding to 88% of the theoretical yield. Figure 2C shows the batch fermentation of a mixture of 17.1 g/L of glucose and 22.2 g/L of xylose. Cofermentation of the sugars to ethanol was observed, although the average rate of glucose utilization (2.86 g/[L·h]) was about three times higher than the average rate of xylose utilization (0.92 g/[L·h]). An ethanol concentration of 18.2 g/L was obtained after 24 h, giving a yield of 0.47 g of ethanol/g of sugars, corresponding to 92% of the theoretical yield. Comparing the xylose utilization profile with that in Fig. 2B, it is clear that the presence of glucose in the medium improves the xylose utilization rate. This observation has also been made in free-cell experiments *(7)*. During xylose fermentation alone, the average xylose utilization rate was 0.37 g/L·h. During fermentation of the glucose-xylose mixture, a significantly higher average xylose utilization rate of 0.92 g/L·h was obtained. Table 2 summarizes the batch fermentation results on individual sugars and different glucose-xylose mixtures. Ethanol yields from the glucose-xylose mixtures are in the range of 0.46–0.48 g/g, corresponding to 90–94% of the theoretical yield (0.51 g/g). The cofermentation pattern and the enhancement of xylose utilization in the presence of glucose was also observed during the batch fermentation of the other sugar mixtures shown in Table 2.

Table 2
Batch Fermentation Results on Glucose-Xylose Mixtures Using Immobilized *Z. mobilis* CP4(pZB5) Biocatalyst Beads

Glucose (g/L)	Xylose (g/L)	Total sugars (g/L)	Ethanol (g/L)	Residual xylose (g/L)	Xylose conversion (%)	Yield (g/g)	Fermentation time (h)	Productivity (g/[L·h])
32.0	0	32.0	16.0	—	—	0.50	10	1.60
0	20.4	20.4	7.6	3.7	81.9	0.45	48	0.16
0	43.0	43.0	10.4	18.2	57.7	0.42	72	0.14
28.2	10.7	38.9	17.7	0.6	94.4	0.46	24	0.74
17.1	22.2	39.3	18.2	0.8	96.4	0.47	24	0.76
6.9	34.5	41.4	18.2	2.6	92.5	0.47	48	0.38
53.7	21.8	75.5	35.1	2.7	87.6	0.48	48	0.73
33.6	43.9	77.5	31.8	9.6	78.1	0.47	48	0.66

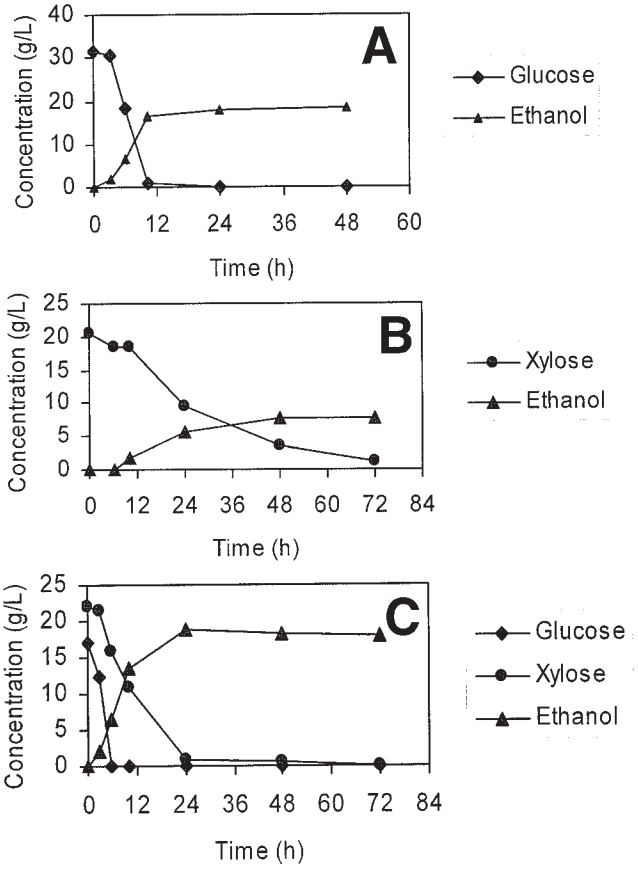

Fig. 2. Batch fermentation of **(A)** glucose, **(B)** xylose, and **(C)** glucose-xylose mixture by immobilized *Z. mobilis* CP4(pZB5).

Ethanol Inhibition of Xylose Fermentation

To evaluate the extent of ethanol inhibition on xylose fermentation, batch experiments with different initial ethanol concentrations in the fermentation media were conducted using the immobilized *Z. mobilis* CP4(pZB5) beads. The initial xylose concentration in all cases was 20 g/L. Xylose utilization profiles as a function of time and initial ethanol concentration are plotted in Fig. 3. Figure 3 shows that the rate of xylose utilization declined with increasing ethanol concentrations. When there was no initial ethanol present in the medium, the xylose utilization rate was 0.44 g/L·h. This is not significantly different from the xylose utilization rate in the batch fermentation experiment reported in Fig. 2B, because these are average rates and these differences can also be attributed to analytical errors (up to 5%) in HPLC. At an initial ethanol concentration of 52.8 g/L, the biocatalyst beads were still able to utilize xylose, although at a slower rate of 0.20 g/L·h.

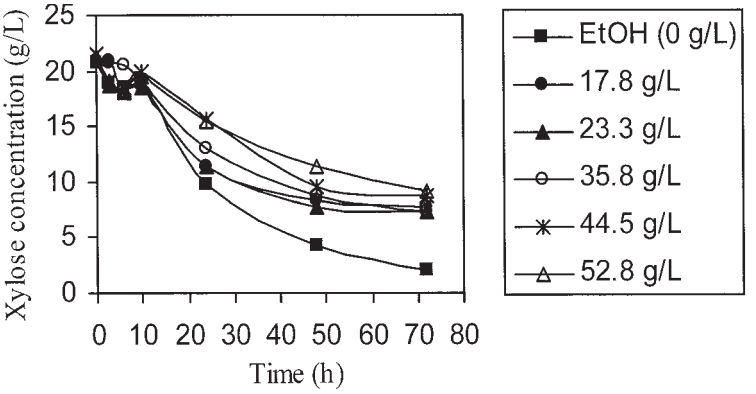

Fig. 3. Xylose concentration profiles as functions of initial ethanol concentration during batch fermentation studies with immobilized *Z. mobilis* CP4(pZB5).

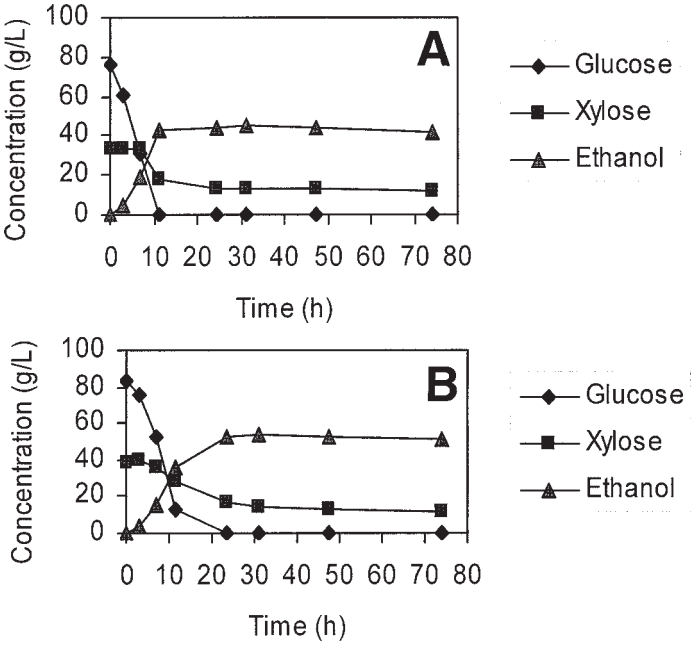

Fig. 4. Batch fermentation results on **(A)** Arkenol hydrolysate and **(B)** sugar control using immobilized *Z. mobilis* CP4(pZB5).

Batch Fermentation Studies on Lignocellulosic Hydrolysate

Batch studies were also conducted with the immobilized *Z. mobilis* CP4(pZB5) beads on lignocellulosic hydrolysate. The results were compared with those obtained in experiments in which synthetic solutions of glucose and xylose at similar concentrations were used. Figure 4A shows the results on hydrolysate containing 76 g/L of glucose and 33.8 g/L of xylose. An ethanol concentration of 44.3 g/L was achieved after 24 h,

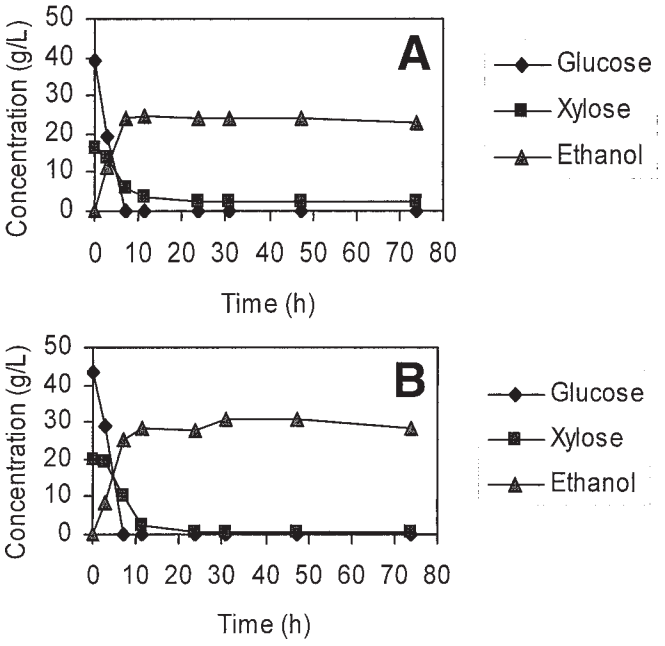

Fig. 5. Batch fermentation results on diluted **(A)** Arkenol hydrolysate and **(B)** sugar control using immobilized *Z. mobilis* CP4(pZB5).

corresponding to a yield of 0.46 g of ethanol/g of sugars. Glucose was completely consumed while the residual xylose was 13.3 g/L, giving a xylose conversion of 60.7%. Samples taken after 24 h showed that the rate of xylose utilization became very slow and that its concentration dropped to 12 g/L after 74 h. Figure 4B shows the results obtained in an experiment using a synthetic solution of 82.7 g/L of glucose and 39.5 g/L of xylose. Since the initial sugar concentrations were higher than those in the hydrolysate, a higher ethanol concentration of 52.1 g/L was obtained after 24 h, corresponding to a yield of 0.49 g/g. The residual xylose concentration was 16.8 g/L, giving a conversion of 57.5%. In this case also, xylose utilization almost ceased after 24 h. The pH of the fermentation media in both of these experiments dropped to 4.3 at the end of the experiment. However, the pH in the initial phase of the experiments (up to 24 h) was close to 5.0. Therefore, the decrease in xylose utilization rates was clearly owing to ethanol inhibition, similar to the observation discussed previously. Thus, at these sugar concentrations, comparable results were obtained on the hydrolysate and synthetic sugar medium. It can also be concluded from these results that there was no inhibition of the ethanol fermentation by components in the hydrolysate.

An experiment was also performed at lower initial sugar concentration by a 50% dilution of the hydrolysate and synthetic sugar medium. Figure 5A shows the results obtained on hydrolysate media containing 39 g/L of glucose and 16.5 g/L of xylose. An ethanol concentration of

24 g/L was achieved after 7 h, corresponding to a yield of 0.49 g/g. The residual xylose after 7 h was 6.3 g/L. After 24 h, the xylose concentration dropped to 2.6 g/L, thereby giving a xylose conversion of 84.2%. The pH of the medium dropped from an initial pH of 5.8 to 3.8 at the end of the experiment, and hence could be the cause for incomplete xylose utilization. Figure 5B shows the results on the synthetic sugar medium containing 43.4 g/L of glucose and 19.7 g/L of xylose. In this case, an ethanol concentration of 25.2 g/L was obtained after 7 h, corresponding to a yield of 0.48 g/g. Ethanol concentration increased to 28 g/L after 24 h, at which time the residual xylose dropped to 0.7 g/L, giving a xylose conversion of 96%. In this case, the pH of the medium only dropped from an initial value of 5.8 to 4.2 at the end of the experiment.

Continuous Fermentation Studies in the FBR

Different compositions of glucose-xylose mixtures were fed continuously through the FBR at residence times between 2 and 4 h. The feed compositions and the steady-state effluent concentrations of glucose, xylose, and ethanol are shown in Table 3. Based on daily base consumption, the dilution of effluent by base addition was in the range of 1–3%. These base dilution effects have been corrected for in Table 3. However, the concentration profiles shown in Figs. 6 and 7 are real-time data and have not been corrected for base dilution.

Figure 6 shows the concentration profiles of glucose, xylose, and ethanol during a continuous fermentation starting with a 50 g/L glucose and 13 g/L xylose feed. At a dilution rate of 0.24/h, an average steady-state ethanol concentration of 26.9 g/L was obtained at a yield of 0.44 g of ethanol/g of sugars (or 86% of theoretical yield). Glucose and xylose conversions were 99.8 and 91.5%, respectively. At a dilution rate of 0.36/h, glucose conversion was maintained at 99% whereas xylose conversion dropped to 79.4%. Ethanol concentration achieved in this case was 26.3 g/L at 88% of theoretical yield. At a higher dilution rate of 0.49/h, glucose conversion was essentially complete at 98.4% and xylose conversion was 81%. The ethanol concentration dropped slightly to 25.4 g/L at 86% of theoretical yield. Thus, maximum utilization of xylose occurred at the lowest dilution rate of 0.24/h. After 11 d of continuous operation, the dilution rate of 0.24/h was repeated. The steady-state effluent xylose concentration was 2.72 g/L as compared to 1.1 g/L achieved in the early stages of the experiment. Therefore, the xylose conversion dropped from 91.5 to 78.4%. However, the glucose conversion was maintained at 99.8%. This result indicates that there was activity loss toward xylose fermentation.

The concentration profiles for a new continuous FBR run started with a 37 g/L glucose and 22 g/L xylose feed are shown in Fig. 7. At a dilution rate of 0.25/h, an average steady-state ethanol concentration of 25.8 g/L was obtained at 90% of theoretical yield. Glucose and xylose conversions were 99.5 and 88%, respectively. Thus, at a similar residence time in the FBR, the xylose conversion dropped when the feed xylose concentration

Table 3
Initial Steady-State Data from Continuous FBR Runs with Immobilized *Z. mobilis* CP4(pZB5) and Glucose-Xylose Feed Mixtures

Feed glucose (g/L)	Feed xylose (g/L)	Dilution rate (1/h)	Effluent glucose (g/L)	Effluent xylose (g/L)	Effluent ethanol (g/L)	Yield (g/g)	Productivity (g EtOH/[L·h])	Xylose conversion (%)	Glucose conversion (%)
49.9	12.9	0.24	0.1	1.1	26.9	0.44	6.5	91.5	99.8
49.1	12.6	0.36	0.6	2.6	26.3	0.45	9.5	79.4	98.8
49.1	12.6	0.49	0.8	2.4	25.4	0.44	12.4	81.0	98.4
37.0	22.2	0.25	0.2	2.7	25.8	0.46	6.5	87.8	99.5
35.4	22.4	0.39	0.6	6.2	22.2	0.44	8.7	72.3	98.3
35.4	22.4	0.49	0.6	12.1	19.6	0.44	9.6	45.7	98.3
68.8	23.1	0.25	0.7	14.7	34.5	0.45	8.6	36.4	99.0
74.6	20.9	0.39	1.2	18.9	33.2	0.44	12.9	9.6	98.4
68.8	23.1	0.50	1.3	17.8	30.5	0.42	15.3	22.9	98.1

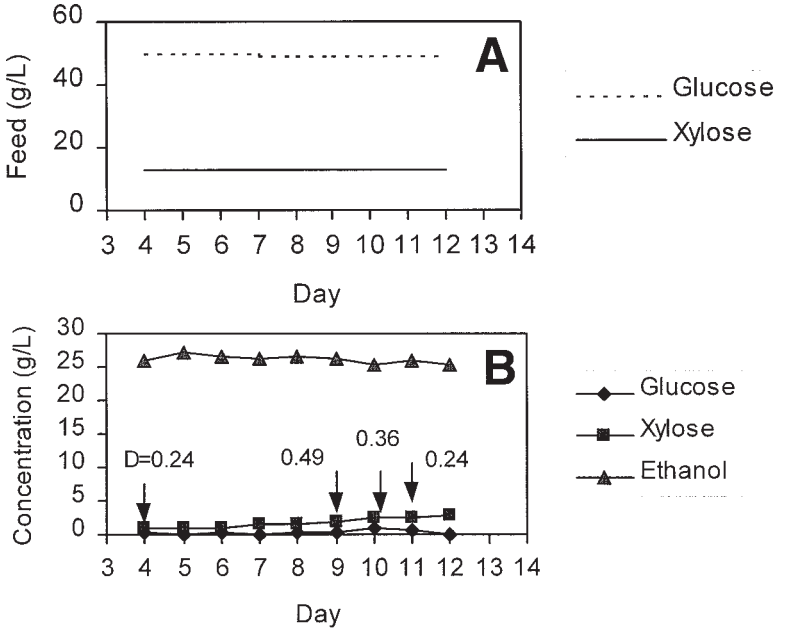

Fig. 6. Continuous fermentation results in FBR with immobilized *Z. mobilis* CP4(pZB5) starting with a 50 g/L glucose, 13 g/L xylose feed. Dilution rates (1/h) are indicated above the arrows.

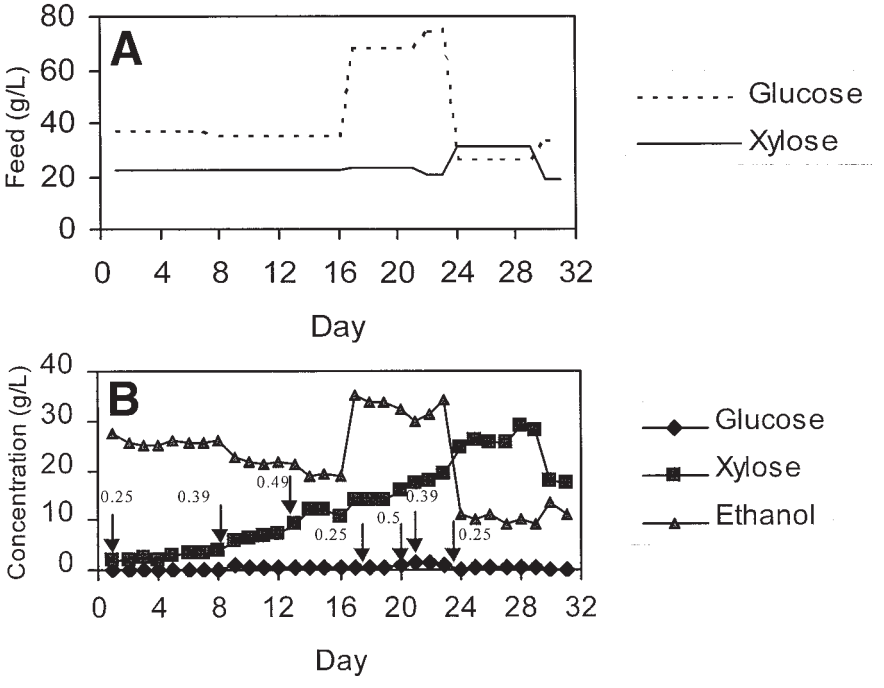

Fig. 7. Continuous fermentation results in FBR with immobilized *Z. mobilis* CP4(pZB5) starting with a 37 g/L glucose, 22 g/L xylose feed. Dilution rates (1/h) are indicated above the arrows.

was increased. At higher dilution rates of 0.39/h and 0.49/h, xylose conversions decreased to 72.3 and 45.7%, respectively. The corresponding glucose conversions were 98.3% in both cases.

After completing the experiments with the foregoing feed, the run was continued by switching the feed to 68.8 g/L of glucose and 23.1 g/L of xylose. At a dilution rate of 0.25/h, an average steady-state ethanol concentration of 34.5 g/L was obtained at 88% of theoretical yield. Glucose conversion was 99%, but xylose conversion fell to 36.4%. At higher dilution rates of 0.5/h and 0.39/h, the xylose conversion dropped to 22.9 and 9.6%, respectively. Since the feed glucose concentration was higher in this run, the steady-state ethanol concentration in the effluent stream was also correspondingly higher. This higher ethanol concentration could decrease the rate of xylose utilization in the FBR, as indicated by results of batch ethanol inhibition experiments. To obtain a higher xylose conversion, a longer residence time in the FBR would be necessary. This can be achieved by using a longer column. Results of the preceding continuous runs are summarized in Table 3. Volumetric ethanol productivities achieved in the continuous FBR runs were in the range of 6.5–15.3 g/L·h. Lactic acid and acetic acid were detected as minor fermentation byproducts in the FBR effluent during these runs at concentrations up to 4 and 0.3 g/L, respectively.

The feed was then switched to 26.4 g/L of glucose and 31.1 g/L of xylose and run at a dilution rate of 0.25/h for 5 d. Glucose conversion was again high at 98.5%, but xylose conversion was only 15.2%. These results again indicate that there was activity loss toward xylose fermentation either owing to these dilution rates or owing to microbial activity loss. To verify this, we returned to the initial feed condition of a 34.2 g/L glucose and 18.4 g/L xylose mixture, and the results were compared to the initial results. Glucose conversion remained >99%, but xylose conversion dropped from 87.8 to 1.6%. The cell loading in the biocatalyst beads was 3 g (dry wt)/L of beads at the start of this continuous FBR run. At the end of this month-long run, the cell loading was found to increase more than 10-fold and determined to be 38 g (dry wt)/L of beads. The increase in cell loading in the beads was confirmed by a protein assay. The protein content in the fresh beads and beads at the end of the continuous FBR run was determined to be 1.02 and 9.9 g/L in the protein solution, respectively. On a weight basis, the protein percentage of the cells for the fresh and used beads was determined to be 51 and 39%, respectively.

The feed used for these continuous FBR runs contained 10 mg/L of tetracycline for maintenance of the plasmid. However, plasmid instability may not be ruled out as the cause for the decline in xylose utilization as a function of time. In previous FBR experiments for ethanol production from glucose using immobilized *Z. mobilis (9)*, it was observed that the cells grew within the beads rapidly during the first week of operation. Following this growth, the cell density within the beads reached a steady-state value. Therefore, during long-term operation of the FBR, the cells were presumably in a nongrowth phase. Under these conditions, it was possible that the

decline in xylose utilization rates was owing to the instability of the xylose metabolism enzymes in this recombinant strain under nongrowth conditions. The batch fermentation data indicate that this effect is not directly owing to immobilization itself. This phenomenon is presently under investigation. If confirmed, this effect will negatively impact the use of this strain for long-term, nongrowth, immobilized cofermentation. However, it will not significantly impact its use in a simultaneous saccharification and cofermentation process in which the cells continue to grow. The testing of recombinant *Z. mobilis* strains ATCC 31821(pZB5) and ATCC 39676(pZB4L) for xylose fermentation under immobilized nongrowth conditions is currently in progress. FBR tests of other recombinant xylose-fermenting organisms are also planned.

Conclusion

Batch and continuous fermentation studies of lignocellulosic sugars to ethanol were conducted using immobilized recombinant *Z. mobilis* CP4(pZB5). Cofermentation of glucose and xylose was observed in batch experiments. The results showed that the presence of glucose in the fermentation medium improved the xylose utilization rate. Ethanol inhibition was observed to limit the xylose utilization rate during batch fermentation of the Arkenol rice straw hydrolysate and synthetic sugar media. Continuous fermentation studies conducted in the FBR with different glucose and xylose feed mixtures showed that while the glucose conversion to ethanol was maintained above 98% in all runs, the xylose conversion decreased with increasing feed xylose concentration, dilution rate, effluent ethanol concentration, and age of the immobilized cells. Long-term continuous FBR runs are being planned with other recombinant xylose-fermenting organisms.

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