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ETHANOL PRODUCTION FROM RICE-STRAW HYDROLYSATE USING *ZYMOMONAS MOBILIS* IN A CONTINUOUS FLUIDIZED-BED REACTOR (FBR)

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ABSTRACT

Rice-straw hydrolysate obtained by the Arkenol's concentrated acid hydrolysis process was fermented to ethanol using a recombinant *Zymomonas mobilis* strain capable of utilizing both glucose and xylose in a continuous fluidized-bed reactor (FBR). The parameters studied included biocatalyst stability with and without antibiotic, feed composition, and retention time. Xylose utilization in the presence of tetracycline remained stable for at least 17 days. This was a significant improvement over the old strain, Z. *mobilis* CP4 (pZB5), which started to lose xylose utilization capability after seven days. In the absence of tetracycline, the xylose utilization rate started to decrease almost immediately. With tetracycline in the feed for the first six days, stability of xylose utilization was maintained for four days after the antibiotic was removed from the feed. The xylose utilization rate started to decrease on day 11. In the presence of tetracycline using the Arkenol's hydrolysate diluted to 48 g/L glucose and 13 g/L xylose at a retention time of 4.5 h, 95% xylose conversion and complete glucose conversion occurred. The ethanol concentration was 29 g/L, which gave a yield of 0.48 g/g sugar consumed or 94% of the theoretical yield. Using the Arkenol's hydrolysate diluted to 83 g/L glucose and 28 g/L xylose, 92% xylose conversion and complete glucose conversion were obtained. The ethanol concentration was 48 g/L, which gave a yield of 0.45 g/g sugar consumed or 88% of the theoretical yield. Maximum productivity of 25.5 g/L-h was obtained at a retention time of 1.9 h. In this case, 84% xylose conversion was obtained.

INTRODUCTION

Ethanol is a fuel that can be produced from renewable resources such as lignocellulosic materials. These materials are polymers of cellulose, hemicellulose, and lignin, plus smaller quantities of other components (Strawn and Hinman, 1995). In addition to being a renewable fuel, ethanol has attracted strong interest since it is considered a clean fuel. It has smaller environmental impacts compared to fossil fuels (Ho et al., 1988). The lignocellulosic materials, which are used for ethanol production, can be produced for this purpose or collected from municipal wastes and agricultural residue (Ho et al., 1999). The first step in the production of ethanol from lignocellulosic materials is hydrolysis of these polymeric materials to liberate the free sugars (Kademi, A. & Baratti). The sugars are then fermented to ethanol by suitable strains of microorganisms.

Hydrolysis of lignocellulosic materials produces a solution of both five and six-carbon sugars, mostly xylose and glucose. The two most effective ethanol-producing organisms, *Saccharomyces cerevisiae* and *Zymomonas mobilis*, are able to ferment glucose to ethanol at high rates and yields but cannot utilize xylose. Genetic engineering has been used to create strains of *Saccharomyces* and *Z. mobilis* that can effectively utilize both of these sugars.

The present research focuses on a recombinant strain of *Z. mobilis*, which carries the genes for xylose metabolism on a plasmid. This plasmid also contains a marker tetracycline-resistant gene. During growth, recombinant organisms tend to lose the plasmid inserted in them. To ensure plasmid stability, tetracycline is normally included in the fermentation media. In the presence of the antibiotic, only cells that retain the plasmid survive. The microbial population consists of only plasmid-bearing cells, and valuable substrate will not be wasted on the metabolism of plasmid-free cells. However, the use of antibiotics in fermentation media adds to the production costs. In addition, it may create problems for the wastewater treatment operation. Therefore, in our research, we investigated the effect of removing tetracycline from the media on the stability of xylose metabolism by the *Z. mobilis* strain. Instead of using freely suspended cells, we used immobilized cells. The purpose was to determine if immobilization would improve plasmid stability in the absence of the antibiotic selection pressure by restricting cell growth.

Lignocellulosic materials can be hydrolyzed by mineral acids such as sulfuric acid. This hydrolysis can be carried out with either dilute or concentrated acid solutions. Arkenol, Inc. has recently developed a process using concentrated acid for hydrolysis of lignocellulosic materials (Anonymous, 1999). The product of this process is a solution of glucose and xylose in high concentrations. Previous studies in batch experiments at Oak Ridge National Laboratory (ORNL) have shown that this sugar solution was readily fermentable to ethanol by immobilized cells of the recombinant strain *Z. mobilis* CP4 (pZB5) (Krishnan et al., 1999). We extended the study to a continuous process using immobilized cells of a new recombinant strain of *Z. mobilis* with improved xylose metabolism in a fluidized-bed reactor (FBR). Synthetic sugar solutions were also used and the results compared with those obtained with the actual hydrolysate produced by the Arkenol process.
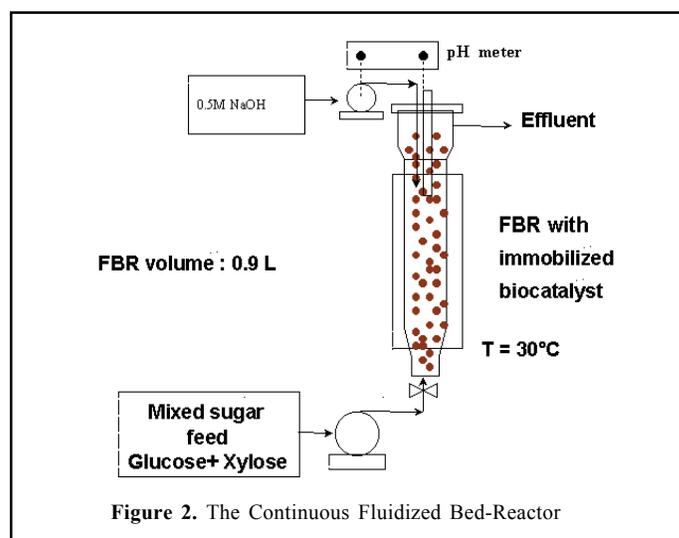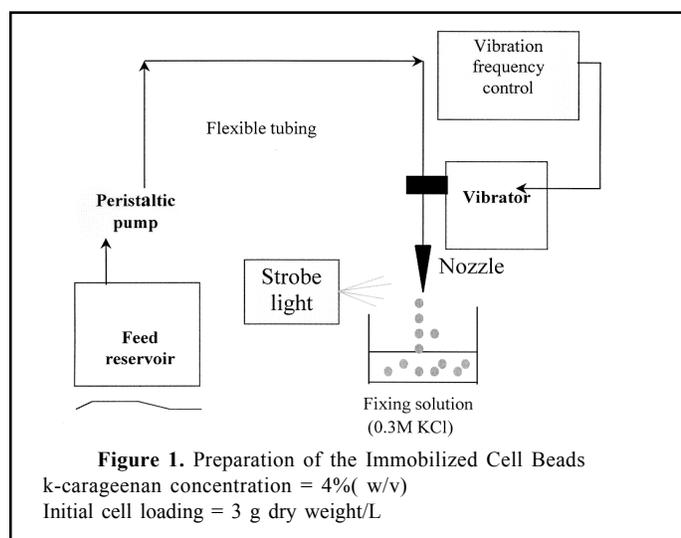
MATERIALS AND METHODS

The microorganism used in this research was *Z. mobilis* ATCC 31821 (pZB5). This strain was obtained from Dr. Min Zhang at the National Renewable Energy Laboratory (NREL) in Golden, Colorado. The stock culture was maintained in 33% (v/v) glycerol and kept at –70°C.

To obtain the cells for immobilization, a 10 liter bioreactor containing 8.35 liters growth medium was used. The growth medium contained 50 g/L glucose, 10 g/L xylose, 10 g/L Difco yeast extract, 2 g/L KH_2PO_4, and 10 mg/L tetracycline. All of the components except xylose and tetracycline at quantities needed for 8.35 liters of medium were dissolved in 7.6 liters of deionized water. The pH was adjusted to 6.0 with an 8 M NaOH solution. The medium was sterilized by autoclaving at 121°C for 20 minutes. A 200 g/L xylose solution was autoclaved separately. Upon cooling of the medium, 400 mL of the concentrated xylose solution and 5.3 mL of a tetracycline stock solution (15 mg/mL in 50% ethanol) were added. The inoculum medium had the same composition as that of the growth medium. All of the components except xylose and tetracycline were dissolved in 175 mL of deionized water. The pH was adjusted to 6.0 with 8 M NaOH and the medium was autoclaved at 121°C for 20 minutes. Upon cooling, 116 µL of the

tetracycline stock solution and 8.75 mL of the autoclaved 200 g/L xylose solution were added. Each flask was inoculated with 1.8 mL stock culture and incubated at 30°C and 100 rpm for 16 hours. The entire contents of the two flasks were used to inoculate the bioreactor. The reactor was placed in a water bath, which was maintained at 30±1°C. After 4 days, when all of the sugars in the medium had been consumed, the cells were recovered by centrifugation. The cell pastes were stored at 4°C for one day and were then used for immobilization.

The cells were immobilized by entrapment in κ-carrageenan gel beads for use in the FBR. Eighty grams of κ-carrageenan was dissolved by heating in 1.6 liters of deionized water. Sixty grams of Fe_2O_3 was added and the mixture was placed in a 35°C water bath. When the mixture cooled to 35°C, 80 g of the wet cell paste was added. The mixture was diluted to 2 liters with deionized water. A mechanical agitator was used to keep the cells and the Fe_2O_3 particles in suspension. The mixture was pumped through Masterflex size 14 tubing with a 100 µL pipet tip at the end. A device was used to provide vibration, which broke up the jet stream exiting the nozzle into small beads. The beads were collected in cold 0.3 M KCl. The average diameter of the beads was between 1 and 2 mm. The beads were allowed to cure overnight and then kept in the 0.3 M KCl solution at 4°C until they were used in the FBR. Previous experiments showed that the microbial activity in the beads was maintained for at least one month. The setup for preparation of the immobilized cell beads is shown schematically in Figure 1.

The FBR was a jacketed glass column with a diameter of 5.1 cm and 47 cm in length. The working volume of the FBR was 0.9 L, with a bead occupation volume of about 0.6 L. The temperature in the FBR was maintained at 30°C by circulating water from a 31°C water bath through the jacket. The pH was maintained at 5.0 with a 0.5 M NaOH solution. To avoid pH overshoot, the delivery point of the base solution was placed immediately below the tip of the pH probe. Feed solutions were pumped upward through the FBR at various flow rates. The upward fluid motion combined with the rising bubbles of carbon dioxide produced during the fermentation caused fluidization of the beads in the FBR. The FBR is shown in Figure 2. The feed solutions contained 3.73 g/L


Figure 1. Preparation of the Immobilized Cell Beads
k-carageenan concentration = 4%(w/v)
Initial cell loading = 3 g dry weight/L

Figure 2. The Continuous Fluidized Bed-Reactor

KCl, 2 g/L KH_2PO_4, 5 g/L yeast extract (Difco), and glucose and xylose at various concentrations. In each experiment, the cells on the surface and within the beads were allowed to grow for at least four days before the actual experimental conditions to be investigated were applied. The FBR was operated continuously and the feed flow rates were varied to study the effects of hydraulic retention time on the FBR performance. At each flow rate, at least six retention times were allowed to ensure steady state was reached before samples were taken for analysis. Samples were collected from the overflow of the FBR over a period of at least one hour. The volume collected was measured and the correct retention time was calculated. The collected sample was mixed thoroughly and a 1.5 mL sample was centrifuged on a microcentrifuge to remove cell debris. The supernatant was stored in a freezer for analysis. HPLC was used to determine the concentrations of glucose, xylose, ethanol, acetic acid, lactic acid, glycerol, and xylitol.

RESULTS

In the first experiment, the Arkenol's hydrolysate was used with tetracycline omitted from the feed solution. The progress of the experiment is shown in Figure 3, with measured retention times given. This experiment was started using a feed solution containing 49.1 g/L glucose and 11.8 g/L xylose. We tried to maintain a retention time of 4.5 h for the first five days before changing it to 3 h and then 2 h. The first feed solution was completely used after day 5, just before the retention time was changed to 3 h. The second feed solution contained 51.8 g/L glucose and 13.2 g/L xylose. It can be seen that complete conversion of glucose was achieved throughout the course of the experiment. An average of 80% conversion of xylose was observed for the first five days. Whereas complete conversion of glucose continued, xylose conversion decreased when the retention time was lowered. At the same time, ethanol concentration also decreased. To test the stability of the biocatalyst, the retention was brought to 4.9 h. Complete glucose conversion was still observed and xylose conversion stayed at 37% instead of increasing to near the level observed for 4.5 h retention time at the beginning of the experiment (80%). When the second feed solution was completely used on day 10, the third feed solution was used. We tried to maintain the same feed composition, but because of the non-uniformity of the concentrated Arkenol's hydrolysate, the third feed solution contained 40.3 g/L glucose and 13.5 g/L xylose. Figure 3 shows the xylose conversion continued to decline although the retention time was kept constant. In the second experiment, the Arkenol's hydrolysate was used and tetracycline was included in all feed solutions. The progress of this experiment is shown in Figure 4. It can be seen that a much higher conversion of xylose was observed. At the start of the experiment, a feed solution containing 48.3 g/L glucose and 12.6 g/L xylose was used. The retention times used were 4.5, 3, and 2 h. After steady state was achieved for the 2 h retention time, the feed solution was replaced with a new feed solution containing 82.8 g/L glucose and 27.8 g/L xylose. Even with the high xylose concentration in the second feed, xylose conversion was maintained at high levels. The steady-state results are summarized in Table 1. Complete conversion of glucose was observed at all
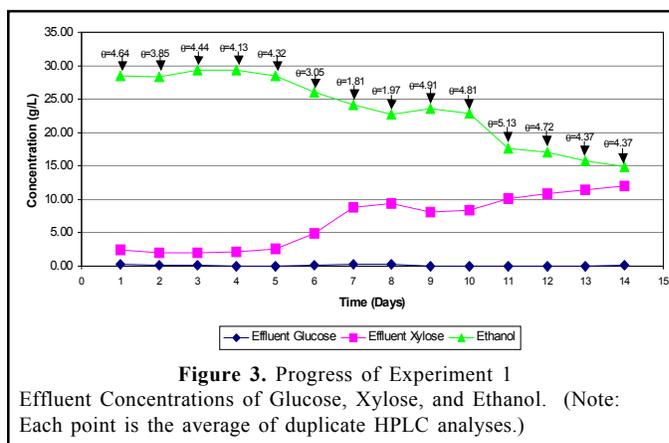
Figure 3. Progress of Experiment 1 Effluent Concentrations of Glucose, Xylose, and Ethanol. (Note: Each point is the average of duplicate HPLC analyses.)
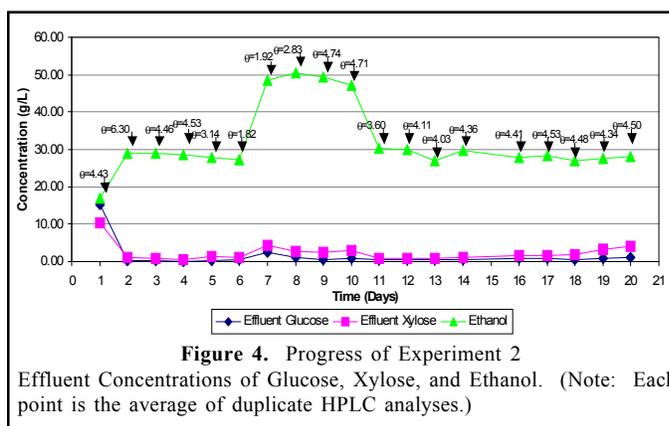
Figure 4. Progress of Experiment 2 Effluent Concentrations of Glucose, Xylose, and Ethanol. (Note: Each point is the average of duplicate HPLC analyses.)
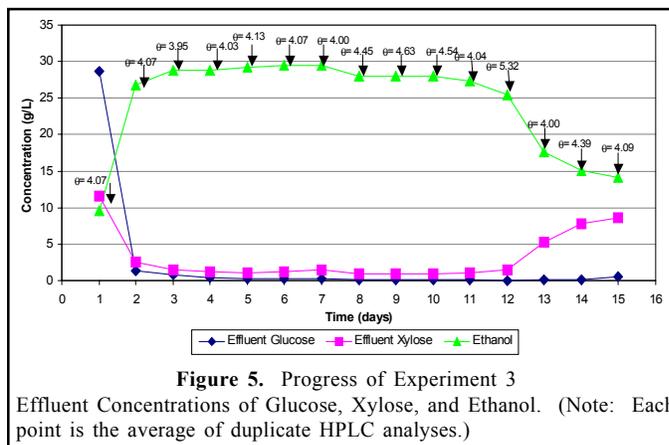
Figure 5. Progress of Experiment 3 Effluent Concentrations of Glucose, Xylose, and Ethanol. (Note: Each point is the average of duplicate HPLC analyses.)

conditions. At all three retention times used for the first feed solution, xylose conversion of >90% was achieved. When the second feed solution was used, even with a xylose concentration of more than two times that in the first feed solution, xylose conversion was still as high as 84% at the lowest retention time of 2 h. The productivity under these conditions was 25.5 g/L-h, which was more than ten times the typical productivity of ethanol production from a xylose/glucose mixture in a batch reactor. Good ethanol yield also was observed. The yield value under all the conditions employed was very close to the theoretical yield of 0.51 g ethanol/g sugar consumed. To test the stability of the biocatalyst, after needed data were collected for all three retention times using the second feed solution, a new feed solution having sugar composition similar to the first feed solution was used. The

Table 1. Steady-State Results of Experiment 2

Feed (g/L)		Retention time (h)	Effluent (g/L)		Conversion (%)		Ethanol (g/L)	Ethanol Yield (g/g)	Productivity (g/L-h)
Glucose	Xylose		Glucose	Xylose	Glucose	Xylose			
48.32	12.62	4.31	0.07	0.65	100.00	94.85	30.22	0.50	7.01
48.32	12.62	3.14	0.21	0.73	99.86	94.26	27.94	0.47	8.90
45.61	13.25	1.82	0.60	1.16	99.58	91.29	27.39	0.48	15.05
82.77	27.79	1.92	2.42	4.41	97.08	84.15	48.49	0.47	25.46
82.77	27.79	2.83	1.02	2.66	98.78	90.45	50.55	0.48	17.86
82.77	27.79	4.74	0.67	2.36	99.19	91.51	49.34	0.46	10.41

third feed solution, which contained 52.9 g/L glucose and 11.5 g/L xylose, was used for three days and then was replaced by the fourth feed solution, which contained 58.8 g/L glucose and 11.3 g/L xylose. The retention time was maintained at around 4.5 h for these two feed solutions. Figure 4 shows that conversion of xylose was maintained at a constant level of near 90% until day 18 when it started to decrease (as indicated by an increase in the effluent xylose concentration). In the third experiment, tetracycline was used in the first feed solution, which contained 48.3 g/L glucose and 12.1 g/L xylose. This feed solution lasted seven days. It was then replaced by the second feed solution, which contained 47.9 g/L glucose and 10.5 g/L xylose. The second feed solution did not have tetracycline. The retention time was kept constant throughout the course of this experiment. The progress of the third experiment is shown in Figure 5. It can be seen that xylose conversion was maintained at the initial level up to day 12.

DISCUSSION

The results obtained in this investigation indicated that the Arkenol's rice-straw hydrolysate was a suitable feedstock for ethanol production by immobilized *Z. mobilis* ATCC 31821 (pZB5) in a continuous FBR. However, the results also indicated the need for tetracycline. In the first experiment where tetracycline was not used, the capability of the organism to metabolize xylose was lost rapidly. At the end of the two-hour retention time condition, the retention time was reset to 4.5 h, which was used at the beginning of the experiment. The fact that xylose conversion did not return to the high level observed at the beginning of the experiment confirmed the loss of the capability to metabolize this sugar. The results also indicated immobilization did not improve plasmid stability of the particular strain tested in the absence of the selection pressure. When the antibiotic was first used in the feed solution but then removed from it, the results indicated some improvement in the stability of xylose metabolism. However, to maintain stability for longer periods, tetracycline in the feed was needed. In the experiment where tetracycline was used in all experimental conditions, the results indicated the superior performance of the FBR. At the highest sugar concentrations tested, i.e., 83 g/L glucose and 28 g/L xylose, and a retention time of 2 h, ethanol concentration of almost 50 g/L was obtained, a concentration at almost theoretical yield. This gave a productivity greater than 25 g/L-h, which was more than ten times the typical productivity in a batch reactor using glucose/xylose mixtures. The use of recombinant strains, in which the genes for xylose metabolism are integrated into the chromosome instead of being placed on a plasmid, will remove the need for the antibiotic. Testing of such strains will be the focus of future experiments.

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