0273-2289/98/70-72—0429$10.75

Production of Ethanol from Starch by Co-Immobilized *Zymomonas mobilis*–Glucoamylase in a Fluidized-Bed Reactor**

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ABSTRACT

The production of ethanol from starch was studied in a fluidized-bed reactor (FBR) using co-immobilized *Zymomonas mobilis* and glucoamylase. The FBR was a glass column of 2.54 cm in diameter and 120 cm in length. The *Z. mobilis* and glucoamylase were co-immobilized within small uniform beads (1.2–2.5 mm diameter) of κ-carrageenan. The substrate for ethanol production was a soluble starch. Light steep water was used as the complex nutrient source. The experiments were performed at 35°C and pH range of 4.0–5.5. The substrate concentrations ranged from 40 to 185 g/L, and the feed rates from 10 to 37 mL/min. Under relaxed sterility conditions, the FBR was successfully operated for a period of 22 d, during which no contamination or structural failure of the biocatalyst beads was observed. Volumetric productivity as high as 38 g ethanol/(Lh), which was 74% of the maximum expected value, was obtained. Typical ethanol volumetric productivity was in the range of 15–20 g/(Lh). The average yield was 0.49 g ethanol/g substrate consumed, which was 90% of the theoretical yield. Very low levels of glucose were observed in the reactor, indicating that starch hydrolysis was the rate-limiting step.

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Index Entries: Ethanol; starch; *Zymomonas mobilis;* glucoamylase; simultaneous saccharification and fermentation; fluidized-bed reactor.

INTRODUCTION

Despite the fact that ethanol fermentation is a very old process, attempts have constantly been made to improve it. Many of these efforts have aimed at the development of a continuous process. A highly efficient continuous fermentation process requires some form of cell retention to prevent wash-out and maintain sufficient cell concentrations for high substrate conversion rates. One method of cell retention is immobilization. *Zymomonas mobilis* immobilized in κ-carrageenan beads has been used in a fluidized-bed reactor (FBR) to produce ethanol from glucose at productivity as high as 120 g/(Lh) *(1)*. This was a significant improvement over other continuous systems for ethanol production, for example, 2–5 g/(Lh) in typical batch and fed-batch processes, 6–8 g/(Lh) for a free-cell continuous stirred tank reactor (CSTR), 10–16 g/(Lh) for an immobilized-cell CSTR, 10–30 g/(Lh) for a hollow-fiber reactor, and 16–40 g/(Lh) for a vertical packed bed with immobilized cells *(2)*. An FBR has distinct advantages over a mixed reactor. It allows a faster approach to reaction completion, thanks to its plug-flow characteristic, which maintains high substrate concentrations throughout most of the reactor and localization of product inhibition to the section near the exit. An FBR also offers minimization to mass transfer restrictions caused by channeling and CO_2 build-up, which are the two common problems of packed-bed reactor (PBR).

Starch is the most abundant renewable carbon source and has been used extensively in ethanol production. Unfortunately, the two best ethanol-producing organisms, *Saccharomyces cerevisiae* and *Z. mobilis,* cannot use starch. Therefore, the conversion of starch to ethanol normally requires two stages: hydrolysis of starch to glucose by acid or enzyme, and its subsequent conversion to ethanol. Siginificant savings on capital costs can be realized if both steps are carried out simultaneously in a single reactor. This has been the focus of research on starch-to-ethanol fermentation. One approach involved the integration of a gene encoding the enzyme glucoamylase (GA) into the chromosome of an ethanol-producing strain. The recombinant organism had the capability of converting soluble starch directly to ethanol *(3)*. Another approach involved the co-immobilization of GA, or an organism possessing that enzymatic function, and an ethanol-producing organism in small beads. The biocatalyst then was used to convert soluble starch to ethanol in a single reactor *(4–7)*. In addition to savings on capital costs, the co-immobilization system offers savings on enzyme costs, since, in this system, GA is retained in the reactor for repeated uses.

In a previous study, the performance of a GA-*S. cerevisiae* system was evaluated in an FBR *(6)*. In the present study, an FBR also was used to

evaluate the performance of a GA–*Z. mobilis* system. The intial results are reported in this paper.

MATERIALS AND METHODS

Microorganism

Z. mobilis NRRL-B-14023 was used. The stock culture was maintained in 25% glycerol and kept at −70°C. To provide the cells for the preparation of the biocatalyst beads, *Z. mobilis* was grown in a 75-L fermenter (New Brunswick, Edison, NJ). The inoculum was prepared in a 3-L fernbach containing 2 L medium. The fernbach medium contained 50 g/L glucose, 5 g/L Tastetone 900AG yeast extract (Red Star, Juneau, WI), and 6 g/L KH_2PO_4. The medium was adjusted to pH 5.0 with concentrated phosphoric acid, sterilized by autoclaving at 121°C for 20 min, and allowed to cool to ambient temperature prior to inoculation. One stock vial containing 1.5 mL culture was thawed and used to inoculate the fernbach. The fernbach was incubated at 30°C, with gentle mixing, for 36 h before its entire contents was used to inoculate the fermenter. The fermenter medium had the same composition as the fernbach medium. The fermenter was maintained at 30°C. The pH was maintained at 5.0 using 2 *N* NaOH. After 20 h, when 90% of the glucose in the medium had been consumed, the cells were recovered by centrifugation (Sharples Super-Centrifuge AS26 NF). The concentrated biomass was stored at 4°C until its immobilization was carried out.

Enzyme

GA immobilized on a solid matrix having average particle diameter of 1.0–1.5 mm was provided by Genencor. Since the particle size was too large for the preparation of the co-immobilization of the enzyme with the *Z. mobilis* cells (*see* Preparation of Biocatalyst Beads), the immobilized GA was ground in a ceramic mortar placed in an ice bath, until the particle diameter was less than 0.1 mm. After the grinding process was complete, a small sample was taken and centrifuged. The supernatant was tested for activity at 35°C, using maltodextrin at 80 g/L as substrate. In this test, no glucose was released, which indicated that no enzyme was lost from the support during the grinding process. The activity of the ground, immobilized GA was measured at 35°C and pH 5.0. One hundred mL buffer (6 g/L KH_2PO_4 adjusted to pH 5.0) was used to wash 2 mL ground GA into a 250-mL flask. The flask then was placed in a 35°C water bath and its contents was mixed by a small stir bar. When the temperature in the flask was the same as the temperature of the water bath, 100 mL of a 80 g/L maltodextrin in the same buffer, which had been kept at 35°C, was quickly added to the flask. Samples were taken at intervals, quickly centrifuged to separate the immobilized enzyme, and the glucose concentration in the

supernatant was measured with a YSI glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH). The specific activity of the ground, immobilized GA was calculated as 1.16 g glucose/mL enzyme-h.

Preparation of Biocatalyst Beads

To prepare the biocatalyst beads, 40 g κ-carrageenan (Type NSAL 798 from FMC) was slowly dissolved in about 600 mL de-ionized water kept in a water bath at 35°C. Mixing was provided by a stir bar. Then 40 g wet wt *Z. mobilis* cell paste was added, followed by 150 mL ground, immobilized GA. Finally, de-ionized water was added to the final volume of 1 L. In the original procedure for the preparation of immobilized *Z. mobilis* in κ-carrageenan, Fe_2O_3 was added to the mixture to increase the specific density of the biocatalyst beads, to prevent them from floating *(1)*. In this work, the addition of Fe_2O_3 was not necessary, and therefore omitted, because the solid matrix onto which the GA was immobilized by the manufacturer already made the specific density of the beads sufficiently high to allow them to sink. The slurry was pumped through a 100-mL pipet tip to form small droplets, which were allowed to drop into a solution of 0.3 *M* KCl. This fixing solution was stirred gently to prevent contact between droplets before they solidified. The beads were recovered, screened to remove those bigger than 2.5 mm in diameter, and stored in 0.3 *M* KCl at 4°C until ready for use. The total volume of the beads recovered was 1 L.

Fermentation Procedure

The FBR was a jacketed glass column of 2.54 cm id and 120 cm in length. The total working volume was 0.6 L. It consisted of four sections joined together. A schematic diagram of the FBR is shown in Fig. 1. Sample ports were installed at 0, 3, 9, and 12 cm. Temperature of the reactor contents was maintained at 35°C by circulation of water from a water bath through the jacket. The pH of the medium in the reactor was not controlled.

The feed solutions contained StarDri 100 starch (A.E. Staley, Decatur, IL) at various concentrations, from 40 to 185 g/L, 0.05 *M* KCl, 0.1% (w/v) Antifoam B (Dow Corning, Midland, MI), and 25% (v/v) light steep water (LSW), which was provided by the A. E. Staley corn processing plant in Loudon, TN). Feeds were sterilized by autoclaving at 121°C for 1 h.

Prior to the loading of the biocatalyst beads, the reactor was decontaminated by rinsing with hot water and 75% ethanol. The beads then were placed into the reactor. The volume occupied by the beads was 350 mL. The rest of the reactor was reserved for expansion during its operation. Feeds were pumped into the reactor at flow rates varied from 10 to 37 mL/min. Minimal efforts were made to mitigate contaminant growth. In addition to sterilization of the feed solutions and the cleaning of the reactor prior to biocatalyst loading, the feed lines and containers were changed

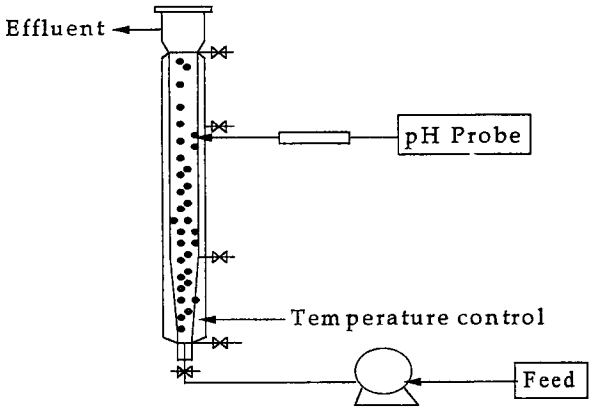

Fig. 1. FBR schematic. The reactor consisted of an expanded 30-cm inlet section (1.27–2.5 cm id), three 30-cm sections of 2.5 cm id jacketed glass pipe, and a-10 cm disengagement section of 9 cm id with a screened sidearm for disengagement of beads from the reactor effluent. One pH probe was inserted at 90 cm position for pH observing.

with each new charge of feedstocks. Previous experience demonstrated that sterile operation was not necessary *(6)*. For each set of experimental conditions (substrate concentration and feed flow rate), at least six residence times were allowed before samples were taken for analyses of starch, glucose, and ethanol. These analyses were performed with a high-performance liquid chromatography (HPLC) system consisting of a Waters 410 differential refractometer detector, a Waters 717Plus Autosampler autoinjector and an Alltech 425 HPLC pump. The column was an Aminex HPX-87H (BioRad, Hercules, CA) column using a 5 m*M* H_2SO_4 solution as the mobile phase. Data acquisition and analysis were performed with the Waters Millenium software.

RESULTS AND DISCUSSION

The operability of the reactor was good throughout the course of the experiments. The biocatalyst beads were used continuously for 22 d in the FBR without the need of recharging. There was no noticeable loss of biocatalyst from the reactor. There also were no obvious signs of structural failure of the beads.

Inside the biocatalyst matrix, starch was hydrolyzed by GA to glucose; the glucose then produced was converted to ethanol and CO_2 by *Z. mobilis*. Fig. 2 shows an example of concentration profiles of starch, glucose, and ethanol along the reactor. Starch concentration decreased with vertical position in the reactor as GA conversion proceeded. In this particular experiment, 65% conversion of starch was obtained. Glucose was an

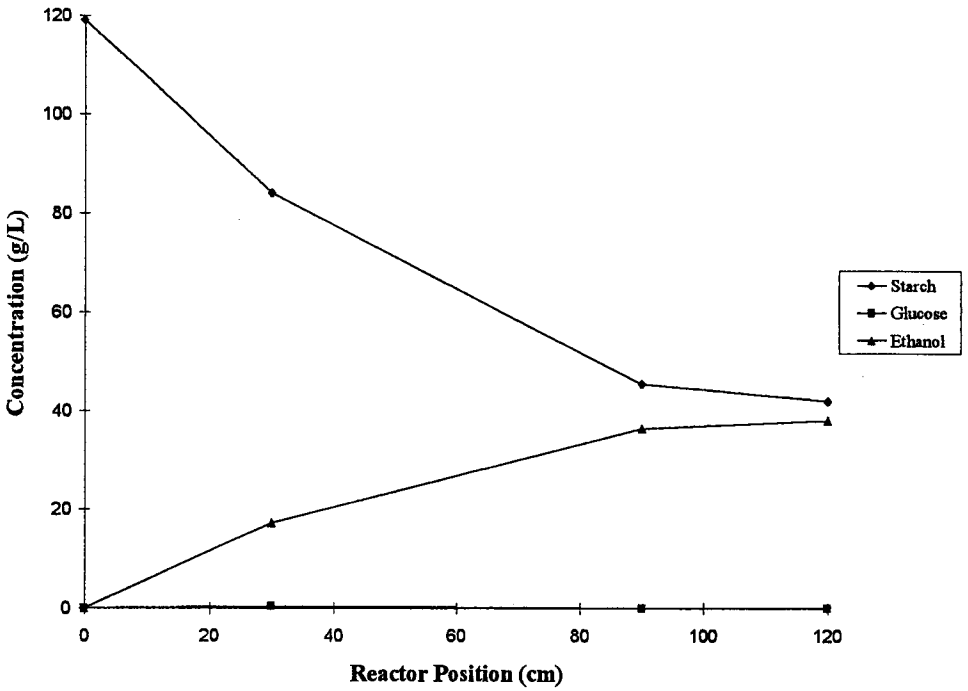

Fig. 2. Example of concentration profiles of starch, glucose, and ethanol. Starch concentration decreased with reactor position as GA conversion proceeded. Glucose was an intermediate between the GA and *Z. mobilis* reactions, and its concentrations were very low in the reactor. Ethanol concentration increased as the glucose released was converted.

intermediate of the series of two consecutive reactions, and therefore, its concentration was a function of both GA and *Z. mobilis* conversion rates. Glucose concentrations were extremely low (near zero) at all positions in the reactor. This indicated that glucose production rate was much slower than its consumption rate. In other words, the hydrolysis of starch by GA was the rate-limiting step. Glucose was converted to ethanol and CO_2 immediately after it was produced, and therefore did not accumulate in the reactor. These results were different from those obtained with the GA–*S. cerevisiae* system in a previous study *(6)*. In the GA–*S. cerevisiae* system, starch conversion rate was much faster than glucose consumption rate, and glucose accumulated in the middle section of the reactor. Starch diffusion accounted for this difference. In the GA–*S. cerevisiae* system, GA was in its free form before it was co-immobilized with the yeast. In the current system, the enzyme was immobilized on a solid matrix before it was co-immobilized with *Z. mobilis*. This double immobilization increased the restriction of starch diffusion into the active sites of the enzyme. Consequently, the concentrations of starch at these locations were low, and, therefore, its conversion rate to glucose was slow.

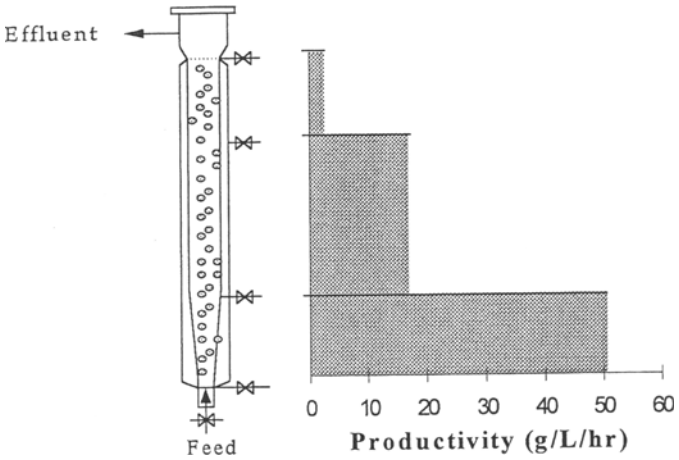

Fig. 3. Ethanol volumetric productivity at different sections of the reactor. The first section (1–30 cm) of the reactor had highest productivity. The third section had lowest productivity.

Ethanol volumetric productivity was calculated for the three sections of the reactor. The results are shown in Fig. 3. In the bottom section of the reactor, the ethanol volumetric productivity was 50 g/(Lh). The productivity decreased to 17 and 3 g/(Lh) for the middle and the top sections, respectively. This decrease was related to fluid dynamics, phase hold-up, and reaction kinetics in the reactor. The fluidization of the biocatalyst bed changed rapidly with the axial position because of significant changes in fluid flow rates and physical properties *(8,9)*. At the entrance to the reactor, the biocatalyst beads were fluidized by the liquid, and in the middle section by both liquid and the CO_2 gas. The uppermost section was characterized by high gas hold-up. The reaction rates in the reactor were a strong function of the biocatalyst concentration. This concentration was reduced in the upper part of the reactor by larger gas hold-up and liquid dispersion. The bottom section had the highest biocatalyst concentration, and consequently had the highest productivity. An example of the pH profile along the reactor is shown in Fig. 4. The pH gradually decreased from 5.5 at the reactor entrance to 4.0 at the exit. The production of ethanol also generated CO_2, some of which dissolved in the liquid and decreased the pH. Since the feed solutions were not buffered, the decrease of the liquid pH along the reactor was directly proportional to the quantities of CO_2 dissolved in it. In the bottom section of the reactor, where the rates of ethanol and CO_2 production were highest, the decreasing rate of pH also was fastest. For the same reason, the decreasing rate of pH in the middle section was faster than that in the top section. Although the pH dropped more than 1 U in the reactor, at the exit, the pH still was within the range suitable for ethanol production by *Z. mobilis (10)*.

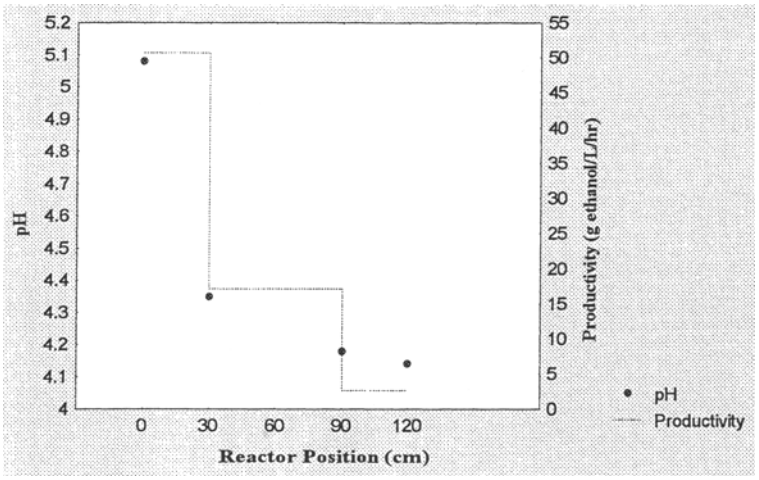

Fig. 4. pH and productivity changes along the reactor position. In the first section of the reactor, the pH decreased faster than in other sections. The rates of pH decrease was directly related to the rates of production of ethanol and CO_2.

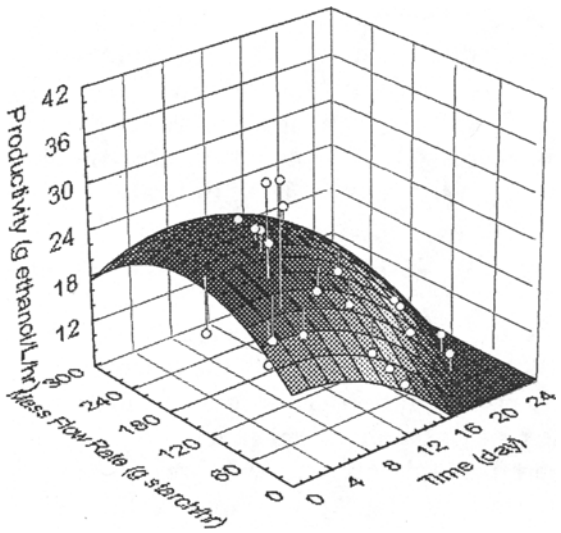

Fig. 5. The influence of starch mass feed rate and time of operation on productivity. Productivity decreased with time.

Figure 5 illustrates the effects of starch mass loading and operation time on the reactor performance. The surface was generated with the Statistica software (StatSoft, Tulsa, OK) using a method similar to that of McLain *(11)*. The results showed that the ethanol volumetric productivity decreased with time. For example, the ethanol volumetric productivity obtained on d 4, at a mass flow rate of 71.4 g/h, was 38.5 g/(Lh); it dropped

to 17.3 g/(Lh) on d 16, at a similar mass flow rate of 66 g/h. During the course of the experiments, no significant loss of the biocatalyst beads was observed. Therefore, the decrease of productivity could only be caused by lower activities of the beads. In all of the experiments, glucose concentrations stayed very close to zero in the reactor. Therefore, under the conditions of decreasing volumetric productivity, starch hydrolysis still was the rate-limiting step. The decrease in the rate of starch hydrolysis with time was probably the result of cell growth. At the beginning of the experiment, the cell concentrations inside the beads were low, and starch molecules could easily diffuse to the active sites of the immobilized GA. When the cells grew, they covered some of the active sites of the enzyme inside the beads. They could also form an outer layer, which would then severely restrict the diffusion of starch molecules into the beads. It has been shown by mathematical modeling *(12)* that even without a surface layer of microbial cells, starch concentration decreased rapidly along the radial direction, toward the center of the beads. Near the surface of the beads, more starch was available, which resulted in higher glucose formation and more cell growth. In the opposite, near the center of the beads, starch concentration dropped to zero and no glucose was produced. The highest volumetric producitivity of ethanol obtained was 38.5 g/(Lh). Since 150 mL ground, immobilized GA, having a specific activity of 1.16 g glucose/mL-h, was used to prepare 1 L of biocatalyst beads, and 350 mL of beads was placed in the reactor, having a working volume of 0.6 L, the ethanol volumetric productivity that could be expected in the reactor would be 51.8 g/(Lh). This was calculated assuming a yield of 0.51 g ethanol/g glucose consumed, and that the co-immobilization of the ground, immobilized GA with Z. *mobilis* did not increase restriction to starch diffusion into the biocatalyst beads. The highest ethanol volumetric productivity obtained, therefore, was 74% of the calculated value. This highest volumetric productivity obtained was slightly lower than the productivity obtained for co-immobilized GA-Z. *mobilis* in Na-alginate in a PBR, but significantly higher than the producitivity obtained with the same system co-immobilized in κ-carrageenan *(5)*. Figure 6 is a contour plot of the starch mass feed rate, operation time, and ethanol volumetric productivity. It can be seen that the optimum mass feed rate was between 120 and 180 g/h.

Figure 7 shows a plot of ethanol production vs starch consumption. The slope of the best-fit line gives an average yield of 0.49 g ethanol/g starch consumed. HPLC analysis of the substrate indicated that it contained 95 + % maltotetraose. The theoretical yield would be 0.55 g ethanol/g starch consumed. The average yield obtained, therefore, was 89% of the theoretical value. This was slightly lower than the yields obtained in other studies using immobilized Z. *mobilis* with glucose as substrate in an FBR (96% of the theoretical value) *(1)*.

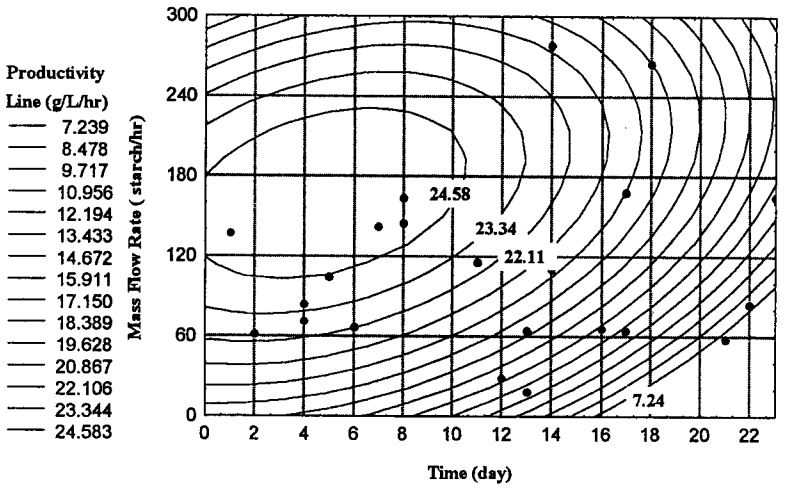

Fig. 6. The contour plot of productivity vs starch mass feed rate and time. The optimum mass feed rate was 120–180 g starch/h.

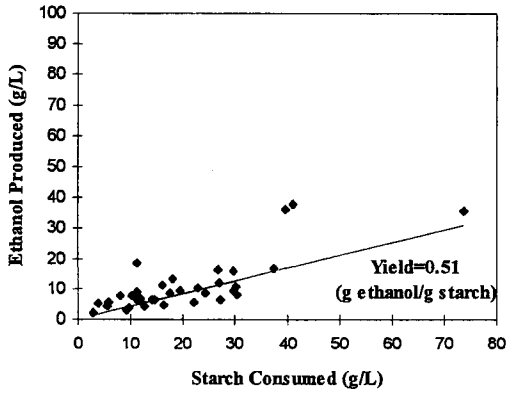

Fig. 7. Determination of average ethanol yield.

CONCLUSIONS

It has been demonstrated that co-immobilized GA and Z. *mobilis* could be used for ethanol production from starch in a single reactor. The FBR used was easy to operate and required no pH control. Under relaxed sterile conditions, no contamination was observed. The biocatalyst beads were quite structurally stable over the 22-d period of operation. Between the two consecutive conversion steps, starch hydrolysis was shown to be the rate-limiting one. The application of the FBR technology with co-immobilized GA–Z. *mobilis* will be extended to the use of real industrial feedstocks such as hydrolyzed dry-milled corn flour.

ACKNOWLEDGMENT

This research was supported by the Office of Transportation Technologies of the US Department of Energy under contract DE-AC05-96OR22464 with Lockheed Martin Energy Research. Immobilized GA and light steep water were generously donated by Genencor International Indiana, and A. E. Staley corn processing plant in Loudon, TN, respectively. The inputs from Bruce J. Jordan of Morris Ag-Energy Company, and O. J. Lantero of Genencor International Indiana, are greatly appreciated. The authors also would like to thank Bruce E. Suttle, Tommy L. Metheney, and Maria M. Blanco-Rivera for technical assistance.

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