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Simultaneous Saccharification and Extractive Fermentation of Lignocellulosic Materials into Lactic Acid in a Two-Zone Fermentor-Extractor System

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Abstract

Simultaneous saccharification and extractive fermentation of lignocellulosic materials into lactic acid was investigated using a two-zone bioreactor. The system is composed of an immobilized cell reactor, a separate column reactor containing the lignocellulosic substrate and a hollow-fiber membrane. It is operated by recirculating the cell free enzyme (cellulase) solution from the immobilized cell reactor to the column reactor through the membrane. The enzyme and microbial reactions thus occur at separate locations, yet simultaneously. This design provides flexibility in reactor operation as it allows easy separation of the solid substrate from the microorganism, *in situ* removal of the product and, if desired, different temperatures in the two reactor sections. This reactor system was tested using pretreated switchgrass as the substrate. It was operated under a fed-batch mode with continuous removal of lactic acid by solvent extraction. The overall lactic acid yield obtainable from this bioreactor system is 77% of the theoretical.

Index Entries: Lactic acid; SSF; cell immobilization; switchgrass; *in situ* extraction.

Introduction

Lactic acid is a specialty chemical widely used in the food, chemical, and pharmaceutical industries *(1)*. It is produced by a synthetic or a fermentation route *(2)*. Because of the recent upsurge in demand for naturally produced lactic acid, biological production now accounts for more than 50% of the world's production *(3)*. The current feedstock for fermentative production of lactic acid is glucose or sucrose. The process is currently done in batch mode in which strong product inhibition and the substrate inhibition

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effects have been cited *(4,5)*. This in turn causes dilute product concentration. It is estimated that lactic-acid purification accounts for 50% of the total production cost *(6)*.

Lignocellulosic materials are viable alternative feedstock for biological production of lactic acid *(7)*. In utilization of lignocellulosics, its degradation to sugars is often a critical element in the overall process because it is done enzymatically. The research along these lines has yielded an efficient bioprocess scheme termed as simultaneous saccharification and fermentation (SSF).

The SSF of lignocellulosic materials has its advantages over separate hydrolysis and fermentation (SHF) *(8,9)*. Glucose released by *Cellulase* enzyme is simultaneously converted to the end product by the microorganism, thus eliminating glucose inhibition on the enzyme. There is one technical aspect in lactic-acid fermentation that makes it particularly suitable for the SSF operation. Many of the lactic-acid producing bacteria are thermo-tolerant. The operating temperature of the SSF can thus be brought to the level close to the optimum of the cellulase enzyme, making the overall process more efficient, especially in the use of enzymes. However, as is the case with most organic-acid fermentation, the lactic-acid fermentation is strongly inhibited by the end-product. Lactic acid also inhibits the enzymatic hydrolysis *(10)*. The inhibition on fermentation is so severe *(11)* that the rate-controlling step in the SSF can actually shift from saccharification to fermentation when lactic-acid concentration is higher than 90 g/L. For efficient operation of the SSF, it becomes necessary to separate lactic acid from the broth during the fermentation.

Various types of integrated fermentor-separator systems based on extraction have been applied for the processes in which product inhibition is significant. All of the known processes, however, dealt only with liquid substrates *(12,13)*. The presence of solid particles in the SSF makes it extremely difficult to incorporate *in situ* separation. Particularly troublesome is the interaction of the solid particles with the emulsions of the organic solvent. In order to overcome these limitations, we introduce a novel fermentor-separator system composed of two separate reactor zones and a membrane-mediated extractor. This investigation was undertaken to assess the performance of this fermentor-separator as it applies to production of lactic acid from lignocellulosic biomass.

Materials and Methods

Materials

Switchgrass obtained from the National Renewable Energy Laboratories (NREL) was used as the primary feedstock. The composition of it is shown in Table 1. It was pretreated with aqueous ammonia in a percolation reactor. The details of this process is shown elsewhere *(14)*. The composition of the feedstock after pretreatment is also shown in Table 1. α-cellulose

Table 1
Composition of Switchgrass Before and After Pretreatment

Components identified[a]	Percentage	
	Untreated	Pretreated[b]
Glucan	34.2	62.7
Xylan	20.2	21.5
Arabinan	3.8	1.5
Galactan	1.9	
Mannan	3.4	
Klason lignin	19.8[c]	8.1
Acid-soluble lignin	3.3	
Ash	6.1	4.8
Extractives	6.2	

[a]Based on oven-dry substrate.
[b]Switchgrass pretreated with ammonia in percolation, 175°C, 1 mL/min, 30 min, 10 wt% ammonia *(14)*.
[c]For Klason lignin determination, biomass was treated with 95% ethanol to remove extractives prior to analysis.

and yeast extract were purchased from Sigma Chemical (St. Louis, MO). The cellulase enzyme, Spezyme-CP (Lot No. 41-95034-004) was obtained from Environmental Biotechnologies Inc. (Menlo Park, CA). The specific activity of the enzyme as determined by the supplier is as follows: filter paper activity = 64.5 FPU/mL, β-glucosidase activity = 57.6 p-NPGU/mL. Hollow fiber membrane (Liqui-Cel 5PCM-102) was obtained from Hoechst Celanese (Charlotte, NC). The manufacturer's membrane specifications are: Celgrad X-10 microporous polypropylene hollow fiber, 2100 fibers, fiber internal diameter 240 μm, fiber-wall thickness 30 μm, effective pore size 0.05 μm, porosity 30%, and effective surface area 1.02 m^2.

Microorganism and Media

The microorganism employed in this work was *L. delbreuckii* (NRRL-B445). The culture was grown at 37°C for 48 h in the agar slants made of 5% Elliker broth (Difco, Detroit, MI) and 5% tomato juice agar (Difco) and stored at 4°C. The fermentation medium contained (per L): Yeast extract 15 g, K_2HPO_4 (0.2 g), KH_2PO_4 (0.2 g), $MgSO_4 \cdot 7H_2O$ (0.6 g), $MnSO_4 \cdot H_2O$ (0.03 g), and $FeSO_4 \cdot 7H_2O$ (0.03 g). The inoculum for fermentation was prepared by growing the culture anaerobically in a flask containing 5% Elliker broth at 37°C for 36 h. The average cell concentration in the inoculum was 0.7 g/L (dry cell mass).

Cell Immobilization

For preparation of the immobilized cell beads, a procedure described elsewhere *(15)* was followed. Briefly, the cells are grown in a 5% Elliker broth at 37°C for 36 h and harvested using a centrifuge. The cells are then

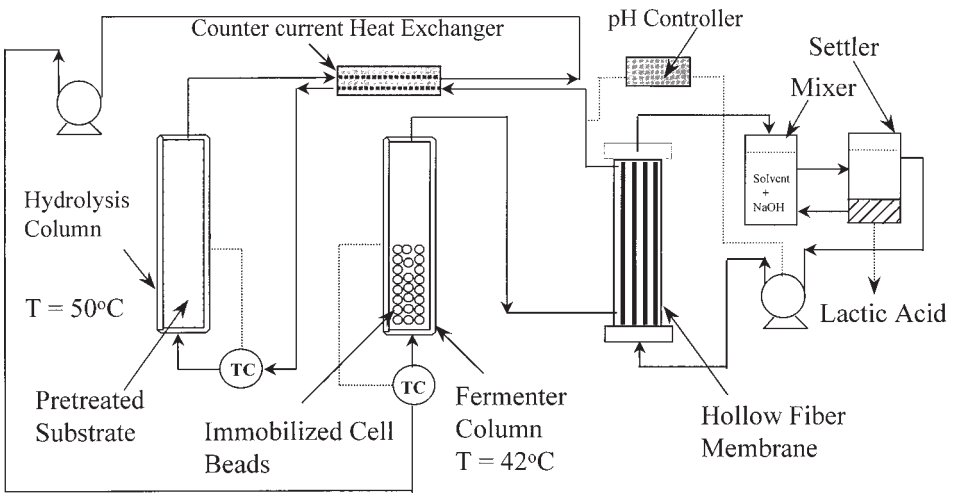

Fig. 1. Schematics of two-zone fermentor-extractor system.

washed and resuspended in distilled water and then mixed with 5% (w/v) sodium alginate solution of the same volume. The mixture is dripped through a Pasteur pipet using a peristaltic pump (Masterflex, Cole Parmer) into a 3% $CaCl_2 \cdot 2H_2O$ solution. Gel beads of about 2-mm diameter are obtained. Beads were hardened in the same solution for about 4 h before washing and incubated at 37°C for 48 h in a 5% Elliker broth before use. Average weight of cells/mL of bead was determined to be 0.13 g.

Experimental Set Up and Operation

Two-Zone SSF Bioreactor

A schematic diagram of the experimental set up is shown in Fig. 1. The system is composed of an immobilized cell reactor 1.25" I.D. × 19" length (325 mL internal volume), a hydrolysis reactor 1" I.D. × 14" length (150 mL internal volume) and a microporous hollow-fiber membrane. Jacketed glass columns were used as reactors. The hydrolysis reactor containing the lignocellulosic substrate was maintained at 50°C, while the immobilized cell reactor was maintained at 42°C by a recirculating water bath. If desired, a countercurrent heat exchanger can be used to maintain the temperature difference. Substrate equivalent to 1% (w/v) glucan was packed in the hydrolysis reactor. The fermentation reactor was packed with 5% (v/v) of immobilized cell beads. Enzyme loading equivalent to 25 IFPU/g cellulose was applied to the reactor system. The cell-free solution containing the enzyme was pumped through the membrane into the hydrolysis reactor in which the pretreated biomass was hydrolyzed. The liquid stream coming out of the hydrolysis reactor is fed to the fermentor where the microorganism consumes the sugar. The aqueous fermentation broth is then recycled back to the hydrolysis reactor through the mem-

brane. The working volume of the total system was 600 mL. Periodically, the direction of flow was reversed to prevent clogging and channeling. When the substrate in the column was near depletion, a fed-batch operation ensued, where additional substrate equivalent to 1% (w/v) of cellulose and cellulase enzyme equivalent to 25 IFPU/g cellulose were put into the column every 36 h.

Extractor

The aqueous fermentation broth passed through the shell side of the hollow-fiber membrane module as described earlier. An extractant composed of tertiary amine (Alamine 336), oleyl alcohol (modifier), and kerosene (diluent) was passed through the tube side of the hydrophobic membrane *(16)*. By applying a lower pressure on the solvent phase, a steady interface was formed at the pore entrances on the aqueous-broth side. The organic solvent penetrated the pores and directly contacted the aqueous-broth phase. The pH was controlled in such a way that the production of lactic acid (downward shift of pH) initiates pumping of the extractant into the tube side of the membrane. The extracted lactic acid was back-extracted by 5 *N* NaOH. The two mixed phases were separated in a settler.

Analytical

The fermentation broth samples and back-extracted samples were taken every 12 h and were analyzed for sugar, lactic acid, and acetic acid by HPLC (Water Associates) equipped with a RI detector. Bio-Rad's HPX-87H column (Bio-Rad, Hercules, CA) was used at 65°C with 0.005 *M* H_2SO_4 as mobile phase. The flow rate was set at 0.6 mL/min.

Results and Discussion

Effect of Recirculation Flow Rate

Flow rate in the packed-bed column reactor influences the fluid dynamic conditions and the mass-transfer characteristics. It may play a significant role in the reaction, especially the enzymatic hydrolysis. The effect of flow rate on the reactor performance was therefore investigated over the range of 4–20 mL/min. Figure 2 shows the glucose profile during enzymatic hydrolysis of α-cellulose at varying flow rates. The rate of hydrolysis increases with flow rates over the range of 4–20 mL/min. Although the hydrolysis rate was higher at flow rates beyond 12 mL/min, the glucose yield at 72 h (67%) at flow rate of 16 mL/min was actually lower than that at the flow rate of 12 mL/min (72%). Above the flow rate of 12 mL/min, channeling was noticeable causing poor solid/liquid contact in the column, a plausible explanation for the early leveling-off in the hydrolysis curve. High flow rate also caused occasional clogging in the column. Hence, a flow rate of 12 mL/min was applied in all of the subsequent runs.

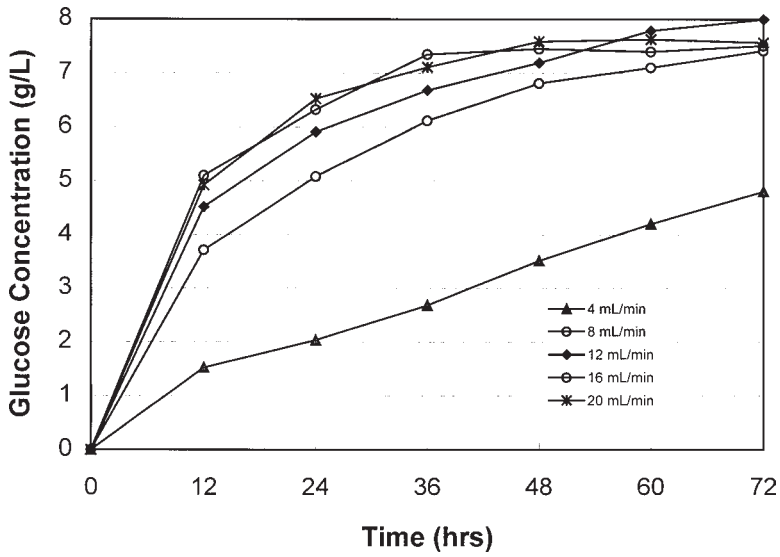

Fig. 2. Effect of flow rate of recirculation fluid on enzymatic hydrolysis of cellulose in a two-zone bioreactor. Hydrolysis conditions: 50°C at hydrolysis column, 42°C at fermentor column without beads, 1% (w/v) α-cellulose, pH 4.8, enzyme loading of 25 IFPU/g cellulose.

Comparison of Batch and Column Hydrolysis

A preliminary test in our laboratory has shown that the upper-limit in operable temperature for *L. delbreuckii* is about 46°C in free-cell form. A batch enzymatic digestibility test was therefore conducted at 46°C using α-cellulose as the substrate. The results were then compared to those of the column hydrolysis conducted with temperature difference being maintained in the two-zone bioreactor. The hydrolysis column was kept at 50°C (optimum for cellulase enzyme) and the fermentor column at 42°C. The fermentor column temperature was lowered from 46°C (the normal optimum) to 42°C because of low heat stability of the calcium alginate beads at high temperatures. Enzyme loading of 25 IFPU/g cellulose was employed. Under these set conditions, a dry run of SSF was made without the microorganism present in the alginate beads. A comparison of glucose formation by enzymatic hydrolysis between the batch and the column run was made. As shown in Fig. 3, the rate of hydrolysis was slightly lower in the column run than in the batch run. The same figure also shows the glucose profile in the enzymatic hydrolysis of the ARP treated switchgrass in the two-zone bioreactor without the microorganism. In this run, the 72-h glucose yield was 80.37%, substantially higher than the yield obtainable from α-cellulose (72%) over the same period.

Column Hydrolysis in Presence of Beads

In the preliminary experiments using the two-zone bioreactor, we observed an erratic behavior, which indicates that there might be an inter-

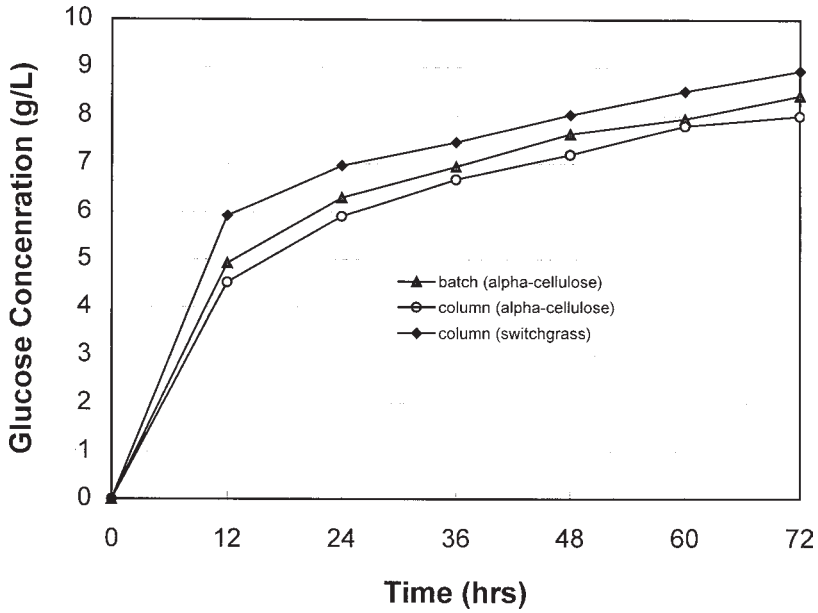

Fig. 3. Cellulose enzymatic hydrolysis in batch and two-zone bioreactor. Hydrolysis conditions: 1% (w/v) α-cellulose, pH 4.8, enzyme loading 25 IFPU/g cellulose, 46°C for batch and for the two-zone bioreactor, 50°C at hydrolysis column, and 42°C at fermentor column without beads.

action with enzyme and the alginate beads. This brought about further investigation. For this purpose, an enzymatic hydrolysis in the bioreactor was conducted with and without the presence of cell-free beads. As shown in Fig. 4, the glucose concentration was much lower in presence of beads than without. It was unclear at that point whether enzymatic hydrolysis was somehow affected or glucose released was diffusing into the beads. Follow-up batch experiments were thus conducted to clarify this point. A 1% (w/v) glucose solution was mixed with 10% (v/v) air-dried beads and agitated at 42°C. It was then noticed that the concentration of glucose decreased from 10 to 8.8 g/L during the first 12 h and remained constant thereafter. It is thus concluded that the lower glucose during column enzymatic hydrolysis is at least partly owing to diffusion of glucose into the alginate beads. The enzyme activity was also tested with the presence of beads. An enzyme solution of 5 IFPU/mL was mixed with air-dried beads equivalent to 10% (v/v) and agitated at 42°C. The supernatant was tested for enzyme activity. Separately the enzyme solution of 5 IFPU/mL not exposed to beads was collected as the control. Batch hydrolysis experiments were then performed using 1% (w/v) α-cellulose at pH 4.8 and 50°C. Enzyme solution from bead supernatant and the control was used to achieve the desired enzyme loading. As shown in Fig. 5, the glucose profile was much lower in the case of the supernatant enzyme exposed to the alginate beads. This is a clear indication that the

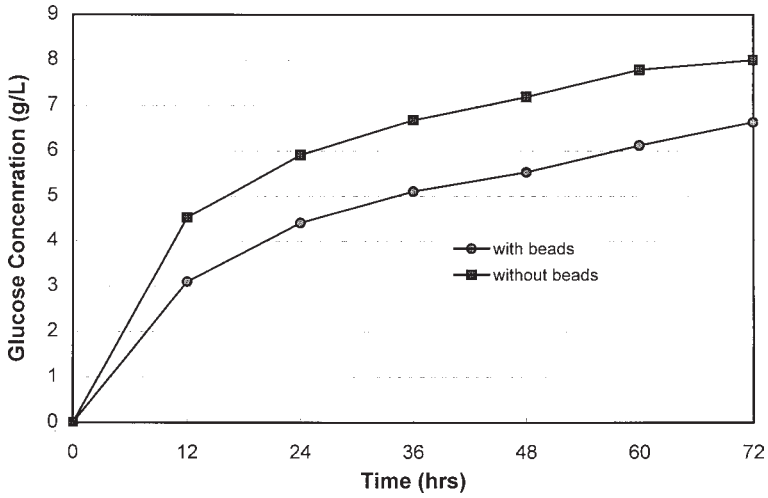

Fig. 4. Cellulose enzymatic hydrolysis in two-zone bioreactor containing unloaded beads. Hydrolysis conditions: 1% (w/v) α-cellulose, pH 4.8, enzyme loading 25 IFPU/g cellulose, bead loading 10% (v/v), 50°C at hydrolysis column, and 42°C at fermentor column.

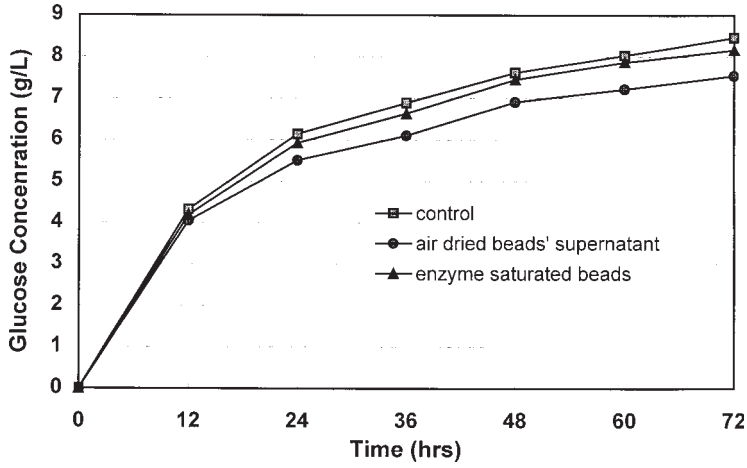

Fig. 5. Cellulose enzymatic hydrolysis in batch using enzyme solution from the bead supernatant and in presence of enzyme-saturated beads. Hydrolysis conditions: 1% (w/v) α-cellulose, pH 4.8, enzyme loading 25 IFPU/g cellulose, 46°C.

enzyme is actually adsorbed into the alginate beads. To further verify this, the same test was repeated with one modification: the cell-free alginate beads were presoaked in 5 IFPU/mL enzyme solution. With this modification, no discernible difference was seen in glucose profile between the two cases (Fig. 5). Evidently, once the gel beads are saturated with enzyme, no further adsorption occurs.

Simultaneous Saccharification and Extractive Fermentation (SSEF)

SSEF of pretreated switchgrass was conducted in the two-zone bioreactor. A substrate equivalent to 1% (w/v) was initially packed in the hydrolysis column. An enzyme loading of 25 IFPU/g glucan was employed. A fed-batch operation was attempted by adding additional substrate equivalent to 1% (w/v) of glucan and cellulase enzyme equivalent to 25 IFPU/g glucan every 36 h. The system was therefore operated without any change in microbial section. The cell-free fermentor fluid was recirculated but retained in the system at all times without being replenished. Lactic acid was continuously extracted through a membrane-mediated extractor. A solvent mixture of 20% Alamine 336, 40% oleyl alcohol, and 40% kerosene previously found most effective in the extraction of lactic acid in our previous study was used *(16)*. For extraction, 1800 mL of the solvent mixture was used, whereas for back extraction, 100 mL 5 *N* NaOH was employed. The fermentation broth was recirculated at the flow rate of 12 mL/min while the solvent flow rate was kept at 6 mL/min during extraction. After back extraction with 5 *N* NaOH every 4 h, the regenerated solvent was recycled back to the solvent tank for subsequent operation.

Figure 6 shows the results of a SSEF operated in a fed-batch mode. The pH was controlled at 4.5 by on-off operation of lactic-acid extraction. The pH was controlled in such a way that the production of lactic acid (downward shift of pH) initiated pumping of the extractant into the tube side of the hollow-fiber membrane. The glucose concentration in the broth initially increased to 3.1 g/L and then dropped to near zero after 24 h and stayed at that level throughout. This reaffirms that the hydrolysis is the rate-controlling step in the SSEF. Lactic-acid concentration in the broth increased to 8.6 g/L and stabilized at that level after first feed addition. The near constant level of lactic acid in the broth indicates that the extraction is not the controlling step at any time during the SSEF. The slight dip in the lactic-acid concentration observed on each feed addition was owing to the dilution effect caused by the moisture in the feed substrate. The lactic-acid concentration in the back extractant is seen to increase steadily, showing stable and active SSEF process for the entire duration. Acetic acid was also extracted from the broth. The dashed line shows the concentration of lactic acid that would be obtained in absence of *in situ* extraction and dilution effects caused by feed addition. The overall lactic-acid yield at any time can be estimated from this curve.

The cumulative total substrate addition in the fed-batch operation was equivalent to 3% (w/v) glucan. The overall yield (% of theoretical) based on total glucan fed was calculated to be 77% at 108-h point. The analysis of the substrate residue at the end of the run (collected from the hydrolysis reactor) revealed that about 5% of the initial glucan remained unconverted. It is well known that a certain fraction of a cellulosic substrate is not accessible to the enzyme action even after a pretreatment. Also, the nonideal behavior in the column, especially channeling, may have further contributed to incomplete hydrolysis in the column.

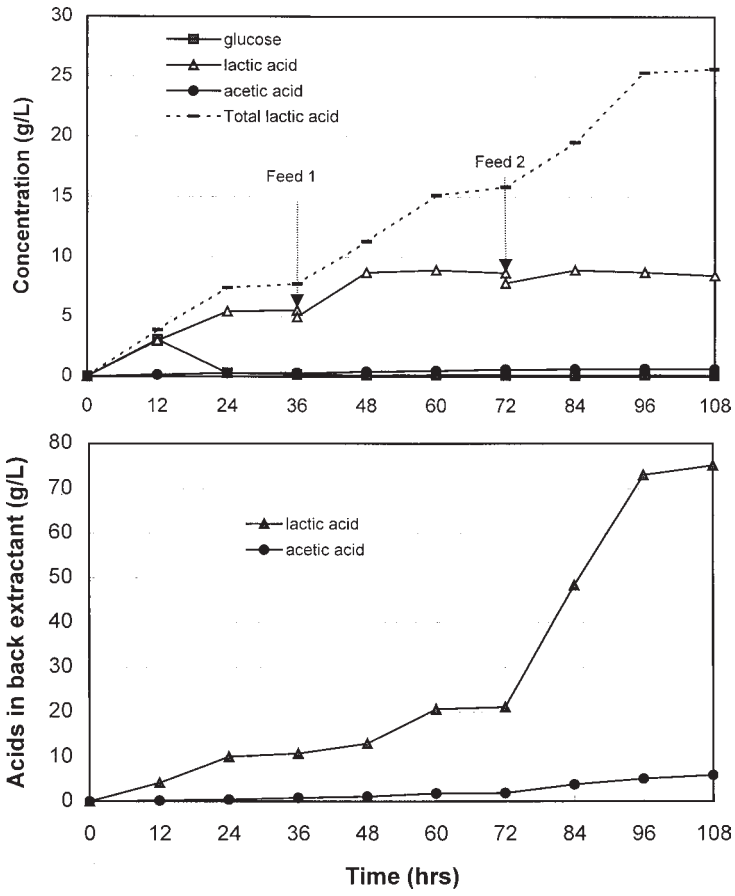

Fig. 6. SSEF of pretreated switchgrass in a two-zone bioreactor. Fermentation conditions: 50°C at hydrolysis column, 42°C at fermentor column, 5% (v/v) immobilized beads, pH 4.5, initial loading: 1% (w/v) glucan, and 25 IFPU/g glucan enzyme; feed 1 = feed 2 = 1% (w/v) glucan, and 25 IFPU/g glucan enzyme.

Conclusions

The proposed two-zone bioreactor employing immobilized cell and a separate biomass column can sustain a stable operation in fed-batch mode. The calcium alginate bead becomes an interfering factor with the SSF system at the initial phase owing to adsorption of enzyme and glucose. The system, however, stabilizes and no ill effect was seen in the long-term operation. The SSEF in a two-zone bioreactor offers additional advantages. It simplifies the separation of the solid substrate from the microorganism. It makes a fed-batch or continuous operation feasible. Most importantly, the simultaneous product removal of inhibitory lactic acid can be easily achieved in this system because the product stream is free of microorganism and solid substrate. *In situ* product removal is especially meaningful in this bioprocess because lactic acid is a strong inhibitor even with pH con-

trol. The solvent extraction mediated by the microporous hollow-fiber membrane has proven to be an effective *in situ* product separation scheme in the SSEF. The SSEF in a two-zone bioreactor is a versatile bioconversion process most efficient for a bioprocess that combines microbial and enzyme reactions and produce inhibitory products.

Acknowledgments

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References

1. Vick-Roy, T. B. (1985), in *Comprehensive Biotechnology: Lactic Acid*, vol. 3, Moo-Young, M., ed., Pergamon, Oxford, UK, pp. 716–774.
2. Holten, C., Muller, H. A., and Rehbinder, D. (1971), *Properties and Chemistry of Lactic Acid and Derivatives*, Verlag Chemie, Weinheim.
3. Litchfield, J. H. (1996), *Adv. Appl. Microbiol*. **42,** 45–96.
4. Luedeking, R. and Piret, E. L. (1959), *J. Biochem. Microbial. Technol. Eng*. **1,** 431–459.
5. Goncalves, L. M. D., Xavier, A. M. R. B., and Almeida, J. S. (1991), *Enzyme Microb. Technol.* **13,** 311–319.
6. Evangelista, R. L., Mangold, A. J., and Nikolov, Z. L. (1994), *Appl. Biochem. Biotechnol*. **45,** 131–144.
7. Bassham, J. A. (1975), *Biotechnol. Bioeng. Symp*. **5,** 9.
8. Takagi, M., Abe, S., Suzuki, S., Emert, G. H., and Yata, N. (1977), in *Proceedings of the Bioconversion Symposium*, Ghose, T. K., ed., IIT, Delhi, 551.
9. Ghosh, P., Pamment, N. B., and Martin, W. R. B. (1982), *Enzyme Microb. Technol*. **4,** 425.
10. Takagi, M. (1984), *Biotechnol. Bioeng.* **16,** 1506–1507.
11. Yeh, P. L. H., Bajpai, R. K., and Ionnotti, E. L. (1991), *J. Fermentation Bioeng*. **71,** 75–77.
12. Daugulis, A. J. (1988), *Biotechnol. Prog*. **4,** 113.
13. Lewis, P. V. and Yang, S. (1992), *Biotechnol. Prog*. **8,** 104.
14. Iyer, P. V., Wu, Z. W., Kim, S. B., and Lee, Y. Y. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 121–132.
15. Scott, C. D. (1987), *Ann. NY Acad. Sci.* **501,** 487–493.
16. Chen, R. F. and Lee, Y. Y. (1997), *Appl. Biochem. Biotechnol*. **63/65,** 435–448.
17. Kaufman, E. N., Cooper, S. P., Clement, S. L., and Little, M. H. (1995), *Appl. Biochem. Biotechnol.* **51,** 605–620.