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Membrane-Mediated Extractive Fermentation for Lactic Acid Production from Cellulosic Biomass

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ABSTRACT

Lactic acid production from cellulosic biomass by cellulase and *Lactobacillus delbrueckii* was studied in a fermenter-extractor employing a microporous hollow fiber membrane (MHF). This bioreactor system was operated under a fed-batch mode with continuous removal of lactic acid by an *in situ* extraction. A tertiary amine (Alamine 336) was used as an extractant for lactic acid. The extraction capacity of Alamine 336 is greatly enhanced by addition of alcohol. Long-chain alcohols serve well for this purpose since they are less toxic to micro-organism. Addition of kerosene, a diluent, was necessary to reduce the solvent viscosity. A solvent mixture of 20% Alamine 336, 40% oleyl alcohol, and 40% kerosene was found to be most effective in the extraction of lactic acid. Progressive change of pH from an initial value of 5.0 down to 4.3 has significantly improved the overall performance of the simultaneous saccharification and extractive fermentation over that of constant pH operation. The change of pH was applied to promote cell growth in the early phase, and extraction in the latter phase.

Index Entries: Hollow fiber membrane; lactic acid; SSF; extraction; *in situ* separation.

INTRODUCTION

Lactic acid production from corn by fermentation routes is an established industrial practice. The current demand of lactic acid as a monomer *(1)* and the market of it as polymers (polylactic acid) *(2,3)* is bringing its status from a specialty chemical to a commodity chemical. In view of this growing demand, the lignocellulosic materials are currently being regarded as impor-

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tant feedstocks for lactic acid production *(4,5)*. The process schemes for the production of lactic acid from cellulosic biomass can share with those proposed for production of ethanol from biomass. One such scheme is simultaneous saccharification and fermentation (SSF). SSF is a bioprocess capable of directly converting lignocellulosic materials to end product. It has been extensively investigated in connection with ethanol production from cellulosic biomass *(6,7)*. Recently it has also been brought up as a means of producing lactic acid *(8)*. There is one technical aspect in lactic acid fermentation that makes it particularly suitable for SSF operation. Many of the lactic acid producing bacteria are thermotolerant. The operating temperature of the SSF can thus be brought to the level close to the optimum of the cellulase enzymes, making the overall process more efficient, especially in the use of enzymes. On the other hand, as is the case with most organic acid fermentations, the lactic acid fermentation is strongly inhibited by the end product *(9)*. Therefore, it is necessary to separate lactic acid from broth during the fermentation.

In this work, we explored a method of removing lactic acid from the fermenter. This idea stems from recent research work concerning *in situ* removal of nonvolatile fermentation products. Various types of integrated fermentation-separation systems have indeed been developed and successfully applied for processes in which product inhibition is significant *(10,11)*. In the recovery of carboxylic acids, extraction has been found to be most suitable *(12)*. Kertes and King *(13)* have reviewed the extraction chemistry of carboxylic acids. The experimental data on extraction of citric acid using a tertiary amine has also been reported *(14)*. For extraction of carboxylic acid, tertiary amides have been reported to be more suitable than the primary and secondary amines *(15)*. All of these fermentation processes, however, dealt only with liquid substrates. With lactic acid production from cellulosic material, the traditional extraction methods become extremely difficult because of the emulsion formed when the particulate solid substrates come in contact with organic solvent. Use of hollow fiber membrane is, therefore, proposed here to resolve this problem. Insertion of a membrane between the two phases (broth and extractant) would certainly prevent solvent particle interaction. Proper choice of membrane can also prevent loss of the solvent as a result of partial dissolution. A clean and simple phase separation is therefore obtainable without having to deal with emulsion and coalescence. This investigation was undertaken to assess the technical feasibility of employing membrane-mediated extraction in the SSF to produce lactic acid from cellulosic biomass.

MATERIALS AND METHODS

Substrates

Two different substrates were employed in this study: α-cellulose (Sigma, St. Louis, MO) and switchgrass (Alamo species). The later was supplied by National Renewable Energy Laboratory (NREL) in the

form of fine particles (20–60 mesh). The switchgrass was pretreated in a 2-gal batch reactor with 0.07% sulfuric acid at 175°C for 30 min, prior to fermentation.

Enzyme

The cellulase enzyme, *Cytolase CL* (Lot No. 17-92262-09) was obtained from Environmental Biotechnologies, Santa Rosa, CA. It has filter paper cellulase activity of 95.9 FPU/mL, β-glucosidase activity of 80.6 p-NPGU/mL, and endo-glucanase activity of 613 CMCU/mL.

Micro-organism and Medium

Lactobacillus delbrueckii (NRRL-B445) was used for fermentation. Elliker broth (Difco) was used as the culture medium. The culture was grown at 37°C for 36 h, and stored at 4°C in agar slants made of Elliker broth and 5% tomato juice agar (Difco). The fermentation medium contained (per liter): solid substrate (10–30 g), yeast extract (30 g), NaOH (1.25 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).

Solvents

Alamine 336 was purchased from Henkel Co. (Tucson, AZ). Oleyl alcohol (technical grade) and n-dodecanol (technical grade) were from Aldrich. Other solvents were from Fisher Scientific.

Microporous Hollow Fiber Membrane (MHF) and its Mediated Extraction

Liqui-Cel 5PCM-106, Hoechst Celanese, Charlotte, NC, was used as the MHF. The manufacturer's specifications are: Celgard 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 0.23 m^2. The outer shell of the MHF was removed in order to bring the fibers in direct contact with the fermentation broth.

Experimental Setup

The experimental setup of the simultaneous saccharification and extractive fermentation (SSEF) is shown in Fig. 1. The SSEF system involved two processes, the SSF in the broth and the extraction across the MHF. The bioreactor (1.3-L working volume) is divided into two compartments separated by a partial glass wall, leaving openings at the top and bottom. The MHF unit is installed in one compartment, the extraction chamber. In the other fermentation chamber, an agitator is installed. The fermentation broth is driven downward by agitation in the fermentation chamber, and flows into the extraction chamber through the open area under the glass wall. It then flows upward in the extraction chamber, and returns to the fermentation chamber across the

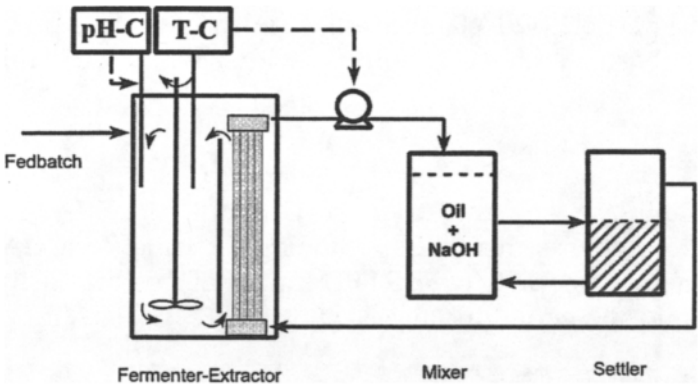

Fig. 1. Schematic of the SSEF.

top of the glass wall. The polypropylene membrane is hydrophobic. The organic solvent passes through the tube side of the fibers, and the broth is on the shell side. Since the membrane is hydrophobic the aqueous broth does not penetrate or wet the hollow fiber membrane pores. The organic solvent penetrates the pores and directly contacts the aqueous broth phase (Fig. 2). By applying a lower pressure on the solvent phase, a steady interface is formed at the pore entrances on the aqueous broth side. The temperature was controlled by a Proportional Temperature Controller (Cole-Parmer Versa-Therm). The pH was controlled by New Brunswick Model PH-22 pH controller. The pH of the fermentation broth was adjusted initially by adding NH_4OH (5 N) for promotion of cell growth. During the latter phase of the fermentation, the pH was controlled 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) initiates pumping of the extractant into the tube side of MHF. The extracted lactic acid was back-extracted by 5*N* NaOH. The mixed two phases were separated in a settler. The stripped solvent was then returned to the extractor.

Analytical Methods

The samples were analyzed for sugars, lactic acid, and acetic acid by HPLC (Water Associate), equipped with an RI detector. Bio-Rad's HPX-87H column 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

Solvent Selection

There are a number of constraints in the selection of solvent for the MHF-mediated extraction. A solvent should have high extractive capacity and selectivity for lactic acid, low viscosity, and low toxicity to the micro-

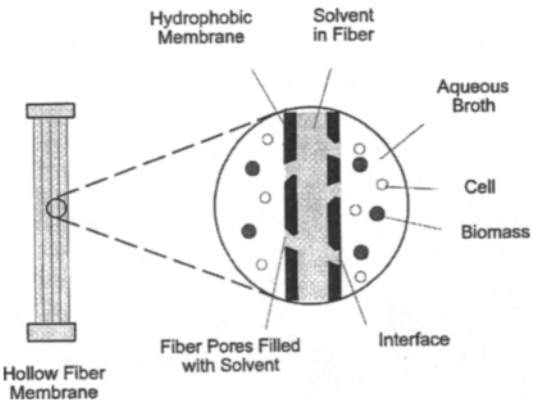

Fig. 2. Microporous hollow fiber mediated extractive fermentation.

organism. A series of extraction experiments was conducted to screen the solvent addressing this issue. High molecular weight aliphatic amines, such as Alamine 336 (trioctyl amine), have been reported to be effective in extraction of dilute carboxylic acids. Alamine 336 is a water-insoluble, saturated, straight-chain tertiary amine, with the alkyl groups of C_8–C_{10} *(16)*. Its extraction capacity can be greatly enhanced when it is mixed with a polar solvent such as water insoluble alcohols. Since Alamine 336 and water insoluble alcohols are extremely viscous, and inoperable in a hollow fiber membrane system, kerosene was introduced as a diluent. For 20 (v/v)% Alamine 336 dissolved in kerosene alone without alcohol, the distribution coefficient (m) is only 0.1. When it is supplemented with 20% oleyl alcohol, the distribution coefficient (m) rose to 0.7 (Fig. 3). This improvement is a result of the fact that alcohols can interact with the acid-amine complex through hydrogen bonding and make the complex more stable in the solvent phase *(17)*. Further experiments were carried out for final selection of alcohol. Four alcohols, 1-octanol, decyl alcohol, dodecanol, and oleyl alcohol, were examined for this purpose. As shown in Fig. 4, the order of the distribution coefficients from highest to lowest is 1-octanol→decyl alcohol→dodecanol→ oleyl alcohol. It is shown that the distribution coefficient decreases with the chain length of alcohols. For a given alcohol, the higher the level of concentration, the more the hydrogen bonds are available, therefore, the higher the distribution coefficient observed.

For the solvents to be used in extractive fermentation, they should also be biocompatible. The same organic solvent system was tested for toxicity to the micro-organism. The solvents were washed with culture medium to remove water soluble impurities and saturated with mineral components of the medium. An autoclaved culture medium was added with 10% inoculum. For each sample, 10 mL culture medium containing seed bacteria was mixed with 1 mL prewashed solvent in a 20-mL vial. They were incubated at 37°C for 12 h in a shaker bath. A control test was

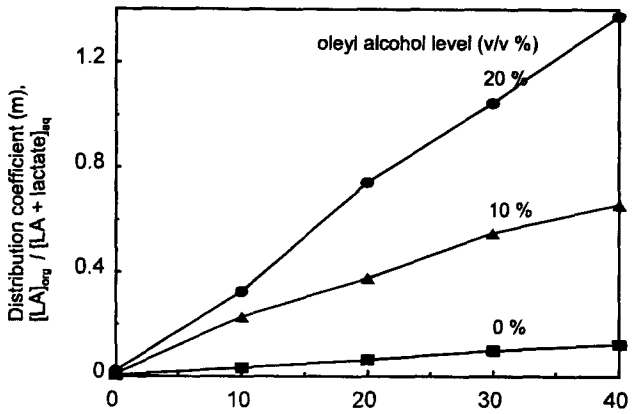

Fig. 3. Effect of alcohol modifier on distribution coefficient (solvent contains Alamine 336, alcohol, and kerosene. extraction conditions: solvent:lactic acid solution = 1:1, temperature = 26°C, initial pH = 2.3, and initial lactic acid conc. = 15.06).

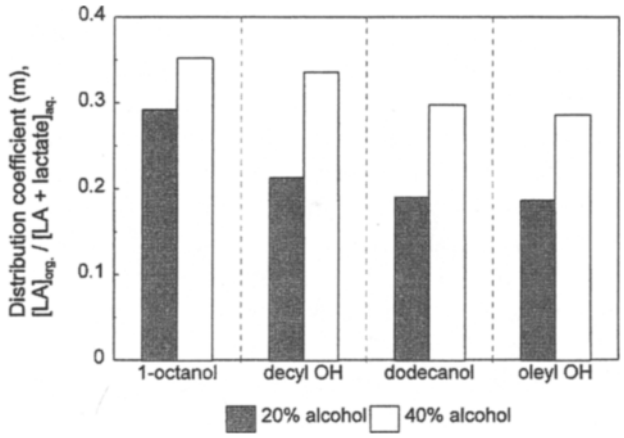

Fig. 4. Effect of alcohol type and concentration on distribution coefficient, (20% Alamine 336 + alcohol + balanced kerosene, extraction conditions: solvent:lactic acid solution = 1:1, temperature = 26°C, initial pH = 4.01, and initial lactic acid conc. = 15.86 g/L).

made with culture medium without solvent. Lactic acid was taken as the indicator in these tests. The results are summarized in Table 1. It is clearly shown that kerosene and oleyl alcohol are nontoxic to the micro-organism. From observation of the various mixtures, the solvent combination of 20% Alamine 336 + 40% oleyl alcohol + 40% kerosene was chosen for this work.

Effect of Temperature

The SSEF for lactic acid from cellulosic substrates involves three steps, saccharification, fermentation, and extraction. Unfortunately, the optimal temperature and pH differ significantly for the three processes.

Table 1
Biocompatibility of Solvent

Solvents		% of lactic acid produced
Control (medium only)		100
Pure solvents:		
Diluent:	n-decane	71
	n-dodecane	96
	kerosene	100
Modifier:	1-octanol	5
	decyl alcohol	16
	oleyl alcohol	100
Solvent mixtures:		
20% Alamine 336 + 60% kerosene +:		
	20% decyl alcohol	46
	20% oleyl alcohol	84
20% Alamine 336 + 40% kerosene +:		
	40% decyl alcohol	21
	40% dodecanol	35
	40% oleyl alcohol	92

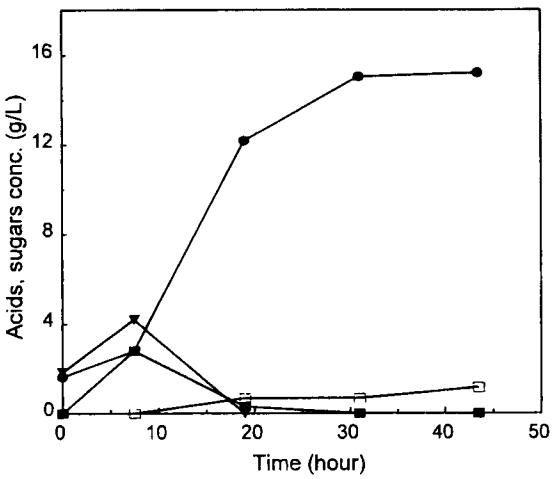

Fig. 5. Time course of a batch SSF (conditions: switchgrass 28.3 g/L, cellulase 25 IFPU/g substrate, pH 5.0, and temperature 46°C, symbol: ▼ glucose, ■ cellobiose, ● lactic acid, and □ acetic acid.)

Figure 5 shows a time course of an SSF run using pretreated switchgrass. Accumulation of glucose and cellobiose were seen in the initial phase. The fermentation then proceeded under glucose-limited conditions, indicating that the hydrolysis is the rate-limiting step in the SSF. The subsequent work in the SSF was, therefore, focused on the improvement of the hydrolysis process.

The optimum temperature for *Lactobacillus delbreuckii* is reported to be in the range of 42–60°C *(18)*. The optimum temperature for enzymatic hydrolysis is about 50°C. However, when a temperature above 47°C was applied in the SSF, we observed that the micro-organism activity was dramatically reduced and showed no lactic acid formation. The temperature of the SSF was chosen as the highest temperature the micro-organism can withstand. In this work, it was set at 46°C, one degree below the upper limit allowing a safety factor. The effect of temperature on extraction is shown in Fig. 6. The distribution coefficient (m) for lactic acid linearly decreases with temperature, low temperature favoring the extraction. The saccharification, fermentation, and extraction occur essentially in sequence. We have, therefore, chose the highest possible temperature as the optimum with the understanding that the enzymatic hydrolysis is the rate-limiting step in the SSEF.

Effect of pH

The effect of pH on enzymatic hydrolysis was studied. It has been reported that the optimum pH for lactic acid production by *L. delbreuckii* is between 5 and 6 *(18)*. The optimum pH of the enzyme has been found to be 4.5–5.0 in our study. If the two processes are combined (SSF), the optimum pH is 5.0. The effect of pH on extraction is shown in Fig. 7. The pH values were measured after extraction equilibrium was attained. The distribution coefficients (m) for the total lactic acid (lactate plus free lactic acid) were seen to linearly decrease as pH increases. It has been reported that Alamine 336 can extract the undissociated acid, but cannot extract organic acid under basic conditions *(19)*. To improve the extraction, it is desirable to raise the concentration of free lactic acid in the broth. This can be done by raising the total amount of lactic acid in the broth at a given pH. The concentration of the lactic acid, however, is limited by the micro-organism's tolerance. The only other option to increase free lactic acid in the broth is to reduce the pH. We have also observed that the lower limit of pH for lactic acid production is 4.1. From considerations of these findings we have chosen pH of 4.3 for the SSEF experiments.

However, employing a uniform pH of 4.3 for the entire SSF process resulted in low cell growth. The decreased cell activity was evidenced by low lactic acid production shown in Fig. 8. This indicates the process was limited by the fermentation. To overcome this problem, a progressive change of pH was attempted. The pH was first controlled at 5.0, an optimal pH for cell growth in the fermentation. It was maintained at this pH by addition of 5*N* NH_4OH solution. After sufficient bacterial growth, the alkali pump was stopped allowing pH to decrease to 4.3. The pH was then maintained at 4.3 for the remainder of the process. The results of the effect of pH on the SSF are shown in Fig. 8

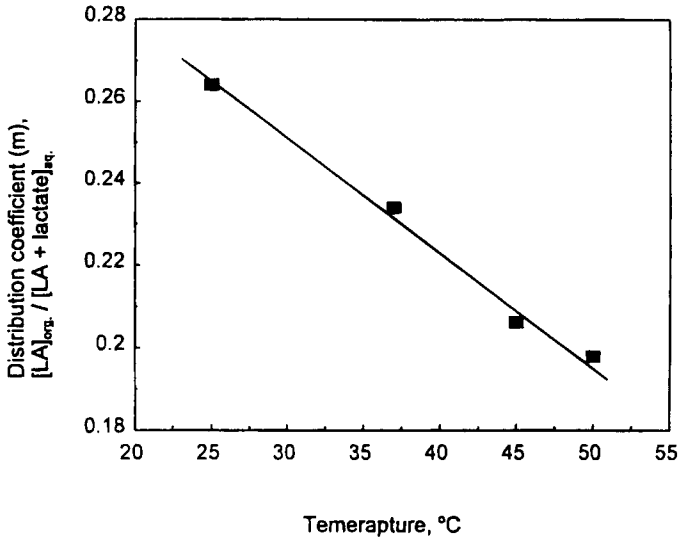

Fig. 6. Effect of temperature on distribution coefficient (extraction conditions: solvent: lactic acid solution = 1:1, initial aqueous pH = 4.09, and initial lactic acied conc. = 15.61 g/L).

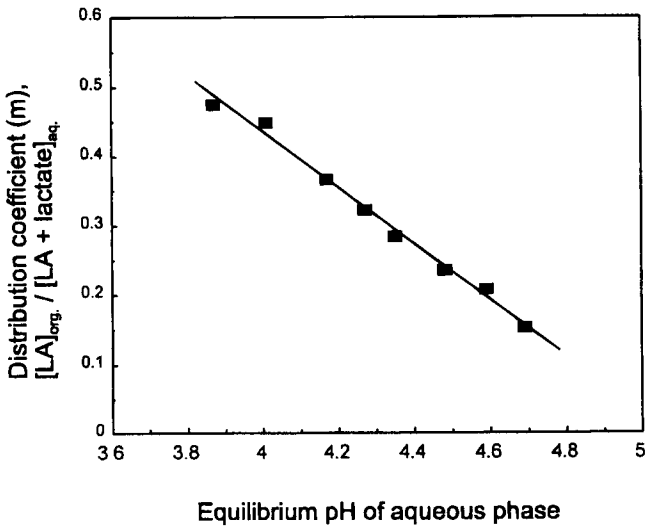

Fig. 7. Effect of pH on distribution coefficient (extraction conditions: solvent:lactic acid solution = 1:1, temperature 45°C, initial lactic acid conc. = 13.94 g/L).

for three different cases: constant pH at 4.3, constant pH at 5.0, and gradual change of pH from 5.0 to 4.3. With gradual change of pH (from 5.0 to 4.3), the lactic acid production was enhanced almost to the level of SSF at pH 5.0. There was no sugar accumulation in this run, another evidence of high cell activity.

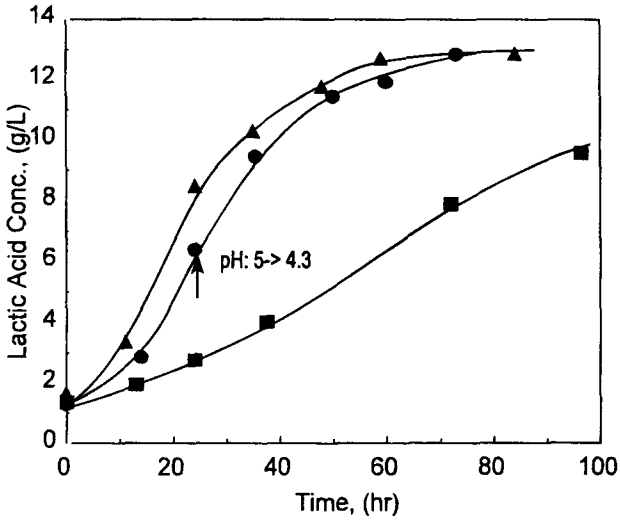

Fig. 8. Effect of pH on SSF (conditions: temperature 46°C, initial cellulose loading = 12.5 g/L, and enzyme load = 60 IFPU/g cellulose; symbols: ■ pH = 4.3, ▲pH = 5, and ● pH = 5 → 4.3).

Simultaneous Saccharification and Extractive Fermentation

The SSEF of this work is designed such that the fermentation products are continuously extracted through a membrane mediated extractor. The bioreactor system was sterilized with 0.1% NaOH solution for 2 h and washed with sterilized DI water. The solvent was prewashed with DI water to remove water soluble impurities and mixed with fermentation broth to saturate with minerals. For the extraction, 1300 mL solvent (20% Alamine 336, 40% oleyl alcohol, and 40% kerosene) was used, and 250 mL 5N NaOH for back extraction.

Two substrates, α-cellulose (76% glucan) and pretreated switchgrass (with 56.1% glucan and 26.2% lignin), were used as lignocellulosic biomass for lactic acid production. Fig. 9 shows the results of a SSEF run made in fed-batch mode using α-cellulose. The bioreactor was initially charged with 21.5 g/L substrate, 7% inoculum, 45 IFPU cellulase/g-substrate. Each additional feed in fed-batch operation contained 13 g α-cellulose with the same enzyme loading, and 6 g yeast extract. The SSEF was first run under the condition favoring the growth of the micro-organism. The pH was controlled at 5.0 with the use of 5*N* NH_4OH as a neutralizing agent for a period of 9 h. The pH was then reset to 4.4. When the pH reached 4.4, the extraction was initiated by the pH controller. This procedure of pH change is identical to that of SSF. The pH was then maintained by on–off control of an extraction recirculation pump. The glucose level in the broth dropped to almost zero when extraction began. This reaffirms that the hydrolysis is the rate-controlling step in the SSEF. Fed-batch feeding was applied twice at reaction times of 21.5 and 41 h. Glucose accumu-

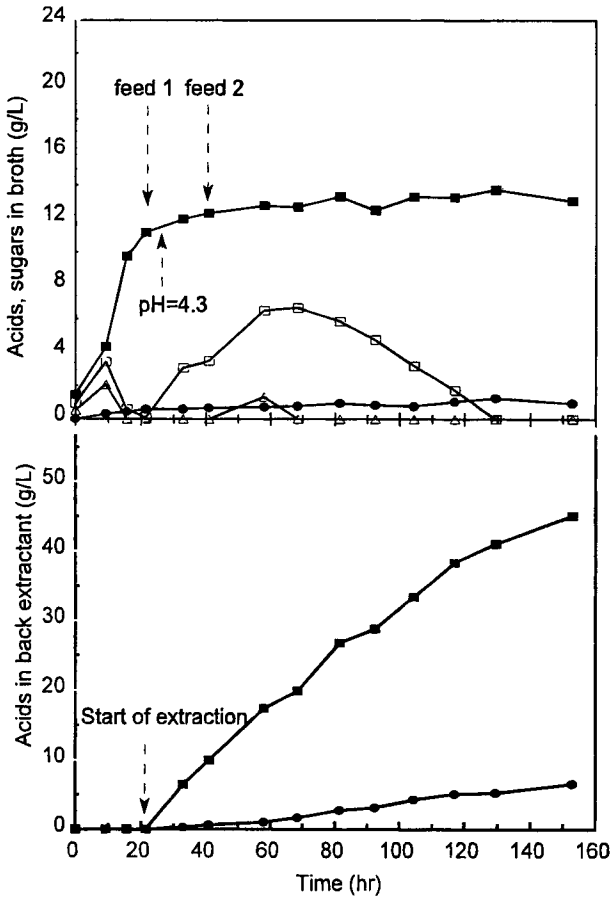

Fig. 9. SSEF of α-cellulose (conditions: fermentation volume = 1300 mL, temperature = 44°C, initial pH 5.0, pH 4.3 after start of extraction; inital loading: α-cellulose = 21.5 g/L, and enzyme = 45 IFPU/g sustrate; fed batch loading: α-cellulose = 13 g(dry), and enzyme = 45 IFPU/g substrate; symbols: ■ lactic acid, ● acetic acid, □ glucose, and △ cellobiose.)

lation was seen after fed-batch feeding (Fig. 9) during which the process was limited by the fermentation step. 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. After 130 h, glucose was depleted indicating that the process was again limited by hydrolysis. The lactic acid concentration in the back extractant is seen to increase steadily showing stable and active SSEF process for the entire duration. It has been reported that about 1.1 g/L lactic acid formed from 30 g/L yeast extract by fermentation, in a control test at 45°C and pH 5.0 without using cellulose substrate *(20)*. This lactic acid contribution from yeast extract was considered in our yield calculations. The observed overall yield of lactic acid (total of broth, extractant, and back extractant) in this run was calculated at 152 h, on the base of total substrate input, to be 67% of the theoretical maximum. At this point of the process, the bioreaction is incomplete leaving a significant

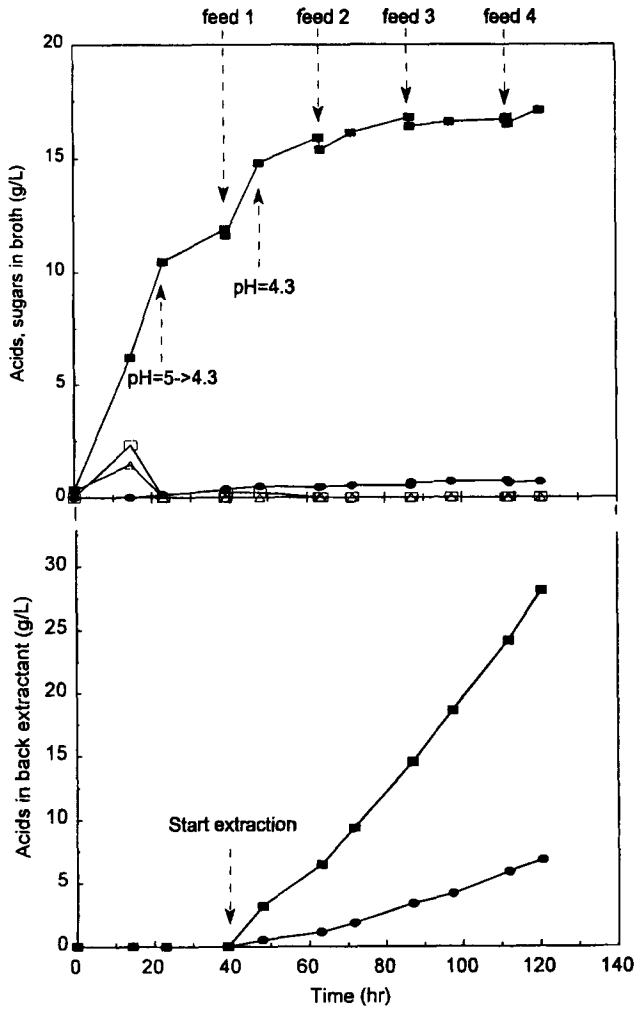

Fig. 10. SSEF of pretreated switchgrass (conditions: fermentation volume = 1300 mL, temperature = 43°C, initial pH 5.0, pH 4.3 after start of extraction; inital loading: switchgrass = 30 g/L, and enzyme = 25 IFPU/g sustrate; fed batch loading: switchgrass = 18 g(dry), and enzyme = 25 IFPU/g substrate; symbols: ■ lactic acid, ● acetic acid, □ glucose, and △ cellobiose.)

amount of unreacted substrate in the fermenter. Allowing more reaction time would therefore increase the overall yield.

The results of SSEF with pretreated switchgrass are shown in Fig. 10. The enzymatic digestibility of switchgrass after pretreatment was about 60%. The experiment was run with initial loading of 30 g/L substrate, 45 IFPU cellulase/g-glucan. Each additional fed-batch feed contained 18 g (dry base) substrate with 75% moisture content, 6 g yeast extract, and the same enzyme loading. The pH adjustment was same as that of α-cellulose run. Fed-batch feeding was applied four times at 24 h intervals. Hydrolysis is the rate-controlling step. The time-course data of Fig. 10 shows that lac-

tic acid in the broth stayed at a relatively constant level once the extraction was applied. The lactic acid level in the back extractant again showed steady increase. Acetic acid was also extracted from the broth for both substrates. The lactic acid production was lower in the switchgrass run than in the α-cellulose run mainly because of lower digestibility of switchgrass.

In general, the SSEF approach works well with certain substrates in the form of fine particles, such as switchgrass sawdust, ground corn cobs/stover mixture, which can flow easily with fermentation broth. For those substrates with big size or pulp sludge, further modifications are needed. Yeast extract loading is not studied in this work. Further work on this matter needs be addressed.

CONCLUSIONS

In this work we have demonstrated that the extractive fermentation for lactic acid from lignocellulosic substrates can sustain a stable operation in fed-batch mode. The solvent extraction mediated by the microporous membrane has proven to be an effective *in situ* product separation scheme in the SSEF. The extraction capacity of Alamine 336 is greatly enhanced by addition of alcohol. Long-chain alcohols are preferred over short chain alcohols because of their low toxicity. Addition of a diluent (kerosene) is essential in reducing solvent viscosity. The solvent combination of 20% Alamine 336, 40% oleyl alcohol, and 40% kerosene was found to be most effective in the SSEF. For SSF the optimum pH is 5.0. For SSEF, a progressive change of pH, from 5.0 to 4.3 provides a better performance than a uniform pH condition because of a mismatch of pH optima for SSF and extraction. The SSEF run with α-cellulose gave higher overall product yield and productivity than with those switchgrass primarily because of the difference in digestibility.

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