0273-2289/04/115/0887–0898/$25.00

Continuous Production of Butanol by *Clostridium acetobutylicum* Immobilized in a Fibrous Bed Bioreactor

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Abstract

We explored the influence of dilution rate and pH in continuous cultures of *Clostridium acetobutylicum*. A 200-mL fibrous bed bioreactor was used to produce high cell density and butyrate concentrations at pH 5.4 and 35°C. By feeding glucose and butyrate as a cosubstrate, the fermentation was maintained in the solventogenesis phase, and the optimal butanol productivity of 4.6 g/(L h) and a yield of 0.42 g/g were obtained at a dilution rate of 0.9 h^{-1} and pH 4.3. Compared to the conventional acetone-butanol-ethanol fermentation, the new fermentation process greatly improved butanol yield, making butanol production from corn an attractive alternative to ethanol fermentation.

Index Entries: ABE fermentation; butanol; *Clostridium acetobutylicum*; fibrous bed bioreactor; dilution rate.

Introduction

Fermentation processes using anaerobic microorganisms provide a promising path for converting biomass and agricultural wastes into chemicals and fuels *(1)*. Acetone-butanol-ethanol (ABE) fermentation with the strict anaerobic bacterium *Clostridium acetobutylicum* was once (1917–1955) one of the largest fermentation processes ever developed in industry. However, with a few exceptions, anaerobic fermentation processes for production of fuels and chemicals, including ABE fermentation, usually suffer from a number of serious limitations including low yields, low productivity, and low final product concentrations *(2,3)*. It is unlikely that the fer-

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mentation route will become competitive with petroleum-based solvent synthesis unless some of these limitations can be overcome *(4)*. However, U.S. legislation to produce strategic chemicals, fuels, and energy from domestic renewable resources and the need to lessen the dependence on the diminishing petroleum supplies have resulted in the renaissance of the fermentation process as a possible source of solvents *(5–7)*.

Butanol has many characteristics that make it a better fuel extender than ethanol, now used in the formulation of gasohol *(8)*. It can solve many problems associated with the use of ethanol. Butanol has the following advantages over ethanol:

1. It has 25% more British thermal units per gallon.
2. It is less evaporative/explosive with a Reid vapor pressure 7.5 times lower than ethanol.
3. It is safer than ethanol because of its higher flash point and lower vapor pressure.
4. It has a higher octane rating *(9)*.
5. It is more miscible with gasoline and diesel fuel but less miscible with water *(10)*.

Petroleum-derived butanol is currently used in food and cosmetic industries as an extractant *(11)*, but there are concerns about its carcinogenic aspects associated with the residual petroleum components. Many new uses will occur in these fields as "green" butanol becomes available to the market. Other uses include current industrial applications in solvents, rubber monomers, and break fluids. Butanol has the propensity to solve some infrastructure problems associated with fuel cell use. Dispersed through existing pipelines and filling stations and then reformed onboard the fuel cell vehicle, butanol offers a safer fuel with more hydrogen.

The present study on butanol fermentation has been focused primarily on the effects of pH and dilution rate *(D)* in continuous cultures of the mutant strain from *C. acetobutylicum* ATCC 55025. To overcome the problems of low productivity and yield of butanol, cell immobilization in a convoluted fibrous bed bioreactor (FBB) and feeding with dextrose and butyric acid as cosubstrates to produce butanol and reduce production of ancillary byproducts were used in the fermentation. By changing the dilution rate from 0.1 to 1.2 h^{-1} at pH 4.3 and varying the pH from 3.5 to 5.5 at the dilution rate of 0.6 h^{-1}, the optimal conditions for high productivity and butanol yield were investigated.

Materials and Methods

Bacterial Culture and Medium

C. acetobutylicum ATCC 55025 was cultured in 38 g/L of Reinforced Clostridial Medium (Oxoid CM149; Oxoid Limited, Hampshire, England) supplemented with 20 g/L of glucose (Sigma, St. Louis, MO).

Oxoid Reinforced Clostridial Medium has the following typical formula: 3.0 g/L of yeast extract, 10.0 g/L of "Lab-Lemco" powder, 10.0 g/L

of peptone, 1.0 g/L of soluble starch, 5.0 g/L of glucose, 0.5 g/L of cysteine hydrochloride, 5.0 g/L of sodium chloride, 3.0 g/L of sodium acetate, and 0.5 g/L of agar.

The medium was autoclaved at 121°C and 15 psig for 20 min. A butyric acid medium was used in the fermentation study. The butyric acid medium was prepared in an 18-L vessel (Kimax) and consisted of 7.0 g/L of CM149, 50–67 g/L of dextrose, and 3 to 4 g/L of butyric acid. The solution of dextrose and CM149 were autoclaved separately to prevent caramelization, a browning reaction, and then blended and sealed shortly after autoclaving to reduce oxygen contamination. After cooling, sterile nitrogen was added to break the vacuum formed during cooling. Reagent-grade butyric acid at 3 to 4 g/L (Aldrich) and 1 mL of 1 *N* Na_2S solution were added aseptically via filter membrane. The medium in the vessel was kept anaerobic by initially sweeping filtered oxygen-free N_2 across the surface and then by continuously keeping a low head pressure using a nitrogen-filled elastic bladder, which prevented vacuum formation as the medium was transferred out during the continuous fermentation study. The pH of the medium was adjusted between 3.5 and 5.5, depending on the fermentation conditions studied, by adding 12 *N* HCl or 5 *N* NaOH.

Fibrous Bed Bioreactor

Figure 1 shows the experimental setup of the continuous FBB system with medium recirculation. The FBB was made of a jacketed glass column (total volume: 800 mL) packed with a spiral wound fibrous matrix (packed volume: 200 mL) *(12)*. Before use, the reactor was sterilized at 121°C and 15 psig for 30 min and then cooled to room temperature while purging with sterile filtered nitrogen for 1 h to ensure that anaerobic conditions were attained inside the reactor. The reactor was then filled with 700 mL of sterile medium containing 20 g/L of dextrose. The medium of the pH was adjusted to 5.4 using 1 *N* NaOH or HCl, and the reactor temperature was controlled at 35°C. The medium was continuously circulated at 20–50 mL/min via a peristaltic pump and flushed with filtered nitrogen to ensure anaerobiosis. The reactor was then inoculated with the cells by injecting 3 mL of a stock culture, and approx 2 to 3 d were allowed for cell growth and immobilization in the fibrous matrix. Once solventogenesis occurred, the butyric acid medium described earlier was fed into the FBB continuously.

Fermentation Kinetics Study

The fermentation kinetics, mainly in solventogenesis phase, was studied with the FBB fed continuously with the butyric acid medium under strict anaerobiosis. The effects of pH (3.5–5.5) and dilution rate (0.1–1.2) were studied at 35°C with two FBBs. These two FBBs, reactor L and R, were used in parallel experiments to ensure the reproducibility of the data. The reactor performance at various dilution rates was first studied at pH 4.3. The effect of pH was then studied at a fixed dilution rate of 0.6 h^{-1}. For each condition studied, the reactor was operated under a constant feed condi-

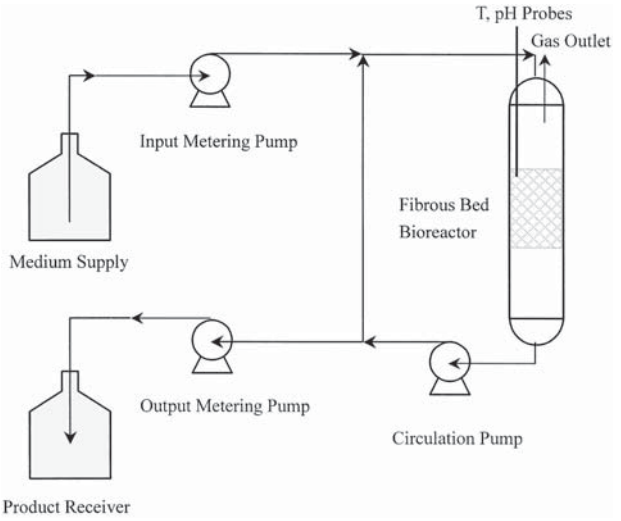

Fig 1. Continuous FBB system for ABE fermentation.

tion for ~100 h to allow the reactor to reach pseudo steady state before changing to the next feed condition. The dilution rate was estimated based on the reactor packed volume of 200 mL. With continuous medium recirculation and bubbling gas production in the fermentation, the reactor was sufficiently mixed and can be assumed to be well mixed.

Analytical Methods

At specified time intervals, 1 mL of sample was drawn aseptically. The cell density (data not shown) in the fermentation broth was measured by optical density (OD) at a 600-nm wavelength with a spectrophotometer (Model 3400; Sequoia-Turner, Mountain View, CA). The pH was measured with a pH/ORP Controller (model 5652-10; Cole Parmer, Vernon Hills, IL).

After removing the bacterial cells by centrifuging at 16,100*g* for 5 min, the clear fermentation broth was subjected to analysis for residual glucose and product concentrations. Glucose concentration was measured using a YSI model 2700 Select Biochemistry Analyzer (Yellow Springs, OH).

Acetone, butanol, ethanol, acetic acid, and butyric acid were determined using a gas chromatograph (Varian 3400 GC) equipped with a flame ionization detector and an SP4270 integrator. The glass column (2 m × 2 mm) (Supelco, Bellefonte, PA) was packed with 80/120 Carbopack BAW/6.6% Carbowax 20M. The oven temperature was programmed from 100°C to 185°C at a rate of 15°C/min after an initial holding time of 1 min. The injector and detector temperatures were set at 200 and 225°C, respectively. Nitrogen was the carrier gas, set at a flow rate of 20 mL/min.

Results

Effects of Dilution Rate

Figure 2 shows the concentration profiles of the outlet streams from the reactors operated at various dilution rates with the feed medium at pH 4.3. After the initial growth phase and the feed medium had been changed to butyric acid medium, reactor L was operated by gradually increasing the dilution rate from 0.1 to 0.3 h^{-1} at 102 h, 0.5 h^{-1} at 201 h, and 1.0 h^{-1} at 311 h, and then dropped to 0.8 h^{-1} at 407 h before increasing again to 1.2 h^{-1} at 503 h. By contrast, reactor R was initially operated at 0.9 h^{-1} and then gradually decreased to 0.6 h^{-1} at 109 h, 0.4 h^{-1} at 205 h, and 0.2 h^{-1} at 301 h. The dilution rate was then increased to 0.7 h^{-1} at 407 h and again to 1.1 h^{-1} at 500 h. As can be seen in Fig. 2, the outlet concentrations of substrates and products changed as the dilution rate changed and in general, they fluctuated before approaching a pseudo steady state toward the end of each operating condition period.

Although a clear steady state was not always achieved, there were clear trends in solvent production and substrate utilization as affected by the dilution rate. In general, as the dilution rate increased from 0.1 to 1.2 h^{-1}, the outlet concentrations of glucose, butyrate, and acetate also increased, whereas the concentrations of acetone, ethanol, and butanol decreased (reactor L). Consistent trends in reactor responses to decreasing the dilution rate were also observed (reactor R). These were expected results since at a higher dilution rate, the medium had shorter contact time with cells and, therefore, less substrates were consumed with less products formed in the fermentation. The highest concentration of butanol produced in the fermentation was 12.5 g/L at a dilution rate of 0.1 h^{-1}, whereas a low butanol concentration of ~3.5 g/L was obtained at higher dilution rates of 0.8–1.2 h^{-1}. The acetone concentration was approximately half that of butanol on average, consistent with the reported product ratio of 3:6:1 for ABE fermentation. On the other hand, ethanol production was relatively low compared to the other products. Note that the butyrate concentration in the reactor outlet was always lower than that in the feed medium, indicating that butyrate also was used as a carbon source in the solventogenic fermentation. However, a significant amount of acetate was still produced in the fermentation, especially at high dilution rates when there was significant cell growth or acidogenesis. As the dilution rate decreased, acetate concentration also decreased because the acetate produced in acidogenesis was reconsumed in solventogenesis.

The reactor volumetric productivities and product yields at pH 4.3 and various dilution rates were estimated based on the time course data in the pseudo steady state and are shown in Fig. 3. In general, production rates for butanol and acetate increased with an increase in the dilution rate from 0.1 to 1.2 h^{-1}, whereas acetone and ethanol remained relatively unaffected by the dilution rate except at 0.1 h^{-1}. A maximum butanol productivity of 4.6 g/(L·h) at a dilution rate of 0.9 h^{-1} was obtained with reactor R at the beginning of the solventogenic fermentation, whereas a lower productivity of

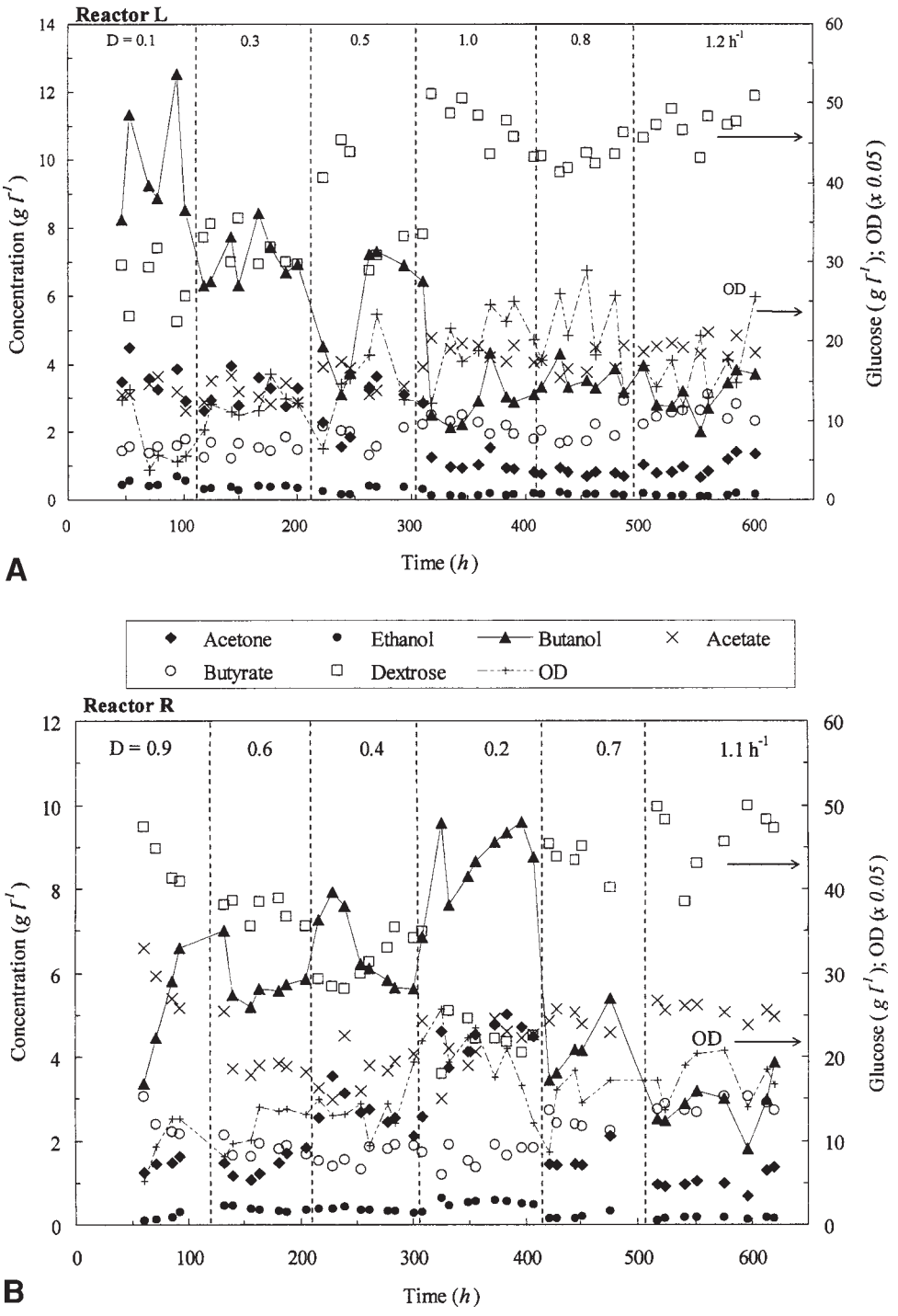

Fig. 2. Kinetics of continuous ABE fermentation at various dilution rates and pH 4.3. Glucose and OD are on the right axis; acetone, butanol, ethanol, acetate, and butyrate are on the left axis. **(A)** Reactor L; **(B)** reactor R.

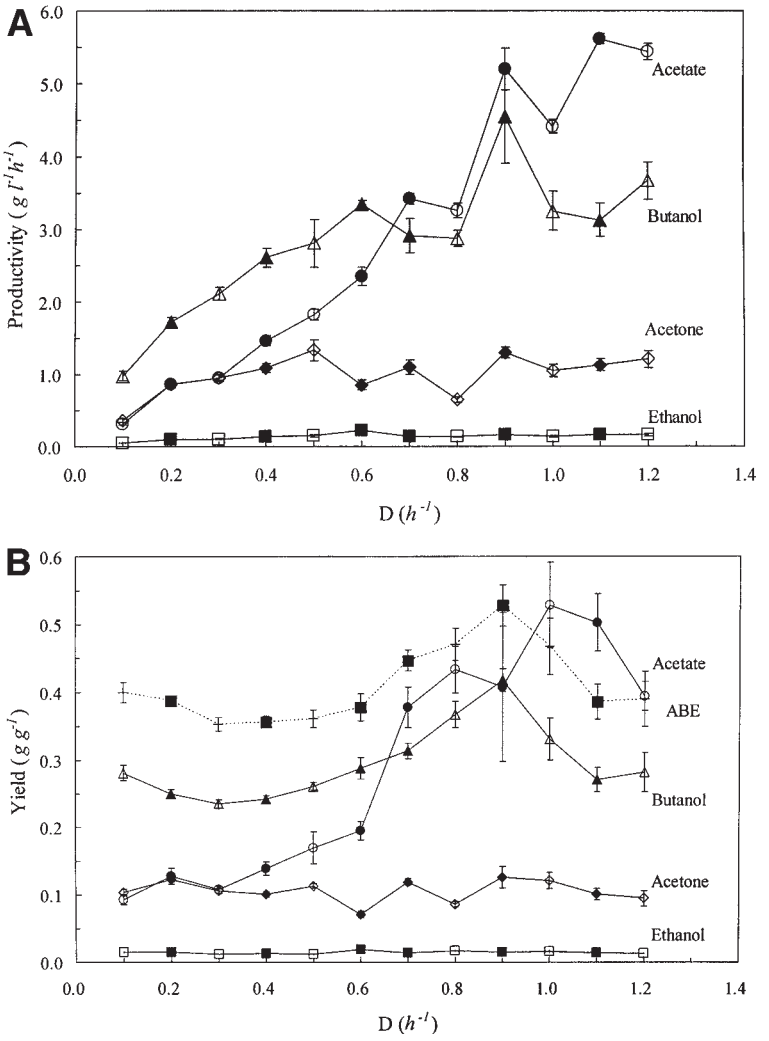

Fig. 3. Effects of dilution rate on productivities and product yields at pH 4.3. Open symbols: reactor L; solid symbols: reactor R. **(A)** Productivity; **(B)** yield.

~3.3 g/(L·h) at higher dilution rates was obtained after the reactor had been operated for a long period. It is not clear if the large difference was attributed to the effect of dilution rate or the culture age. Butanol is a strong inhibitor to the fermentation. A lower butanol productivity at a lower dilution rate where the butanol concentration was higher is therefore expected. The increased acetate productivity with an increase in the dilution rate was consistent with the higher cell growth rate because acetate is the main product in the acidogenic phase during which cell growth also occurs.

The product yields from glucose in the fermentation were based on the total consumption of glucose and butyrate, and each gram of butyric acid consumed was considered as 2 g of glucose in the feed since the butyric acid yield from glucose in butyric acid fermentation was ~50% *(12)*. As can be seen in Fig. 3B at pH 4.3, the butanol yield decreased slightly as the dilution rate increased from 0.1 to 0.3 h^{-1} but then increased with an increase in the dilution rate until reaching the maximum value of 0.42 g/g at 0.9 h^{-1}. The butanol yield was lower as the dilution rate continued to increase to 1.2 h^{-1}. By contrast, acetone and ethanol yields appeared to be not significantly affected by the dilution rate. The overall yield of solvents (acetone, butanol, and ethanol) was ~0.4 g/g, with a maximum value of 0.53 g/g obtained at 1.0 h^{-1}. The average product yields at all dilution rates studied were found to be 0.27, 0.12, and 0.015 g/g for butanol, acetone, and ethanol, respectively.

Effects of pH

After 606 h, the dilution rate in reactor L was decreased from 1.2 to 0.6 h^{-1}. When the pH was adjusted to 3.5 at 623 h, the concentrations of acetone, butanol, and ethanol decreased dramatically and reduced to almost 0 g/L by 700 h (*see* Fig. 4A). In the meantime, the concentrations of glucose and butyric acid increased with time to reach almost their feed concentrations. Coupled with the low OD value, these findings make it apparent that the fermentation had almost ceased at this low pH value.

However, the trends were reversed and the reactor started to recover after the feed pH was increased to 3.8 at 726 h. The product concentrations (ABE) continued to increase while substrates decreased as the feed pH was increased to 4.0 at 887 h. For reactor R, after reducing the dilution rate from 1.1 to 0.6 h^{-1} and increasing the feed pH from 4.3 to 5.3 at 661 h, the concentration of butanol increased rapidly and the concentrations of glucose and butyrate reduced (*see* Fig. 4B). This trend appeared to continue with time, although it slowed down as the pH changed to 4.7 at 733 h, even though the pH decreased to 4.5 at 795 h and then increased to 5.1 at 901 h, reaching a significantly higher butanol concentration of ~7.7 g/L at 956 h. The observed effects probably were mainly attributed to the change in the dilution rate and much less to the pH in the range studied (4.5–5.3). However, a further increase in the feed pH to 5.5 at 1028 h abruptly decreased butanol production in the fermentation, and the concentration of butanol dropped to below 4 g/L, with a corresponding increase in the glucose concentration. For both reactors, the concentration of acetic acid appeared to increase somewhat with an increase in the pH, consistent with the fact that acidogenesis is generally favored at a higher pH.

Although steady state might not have been reached in certain operating pH conditions studied, the productivities and product yields at the dilution rate of 0.6 h^{-1} and various feed pH values were estimated and are shown in Fig. 5. In general, the reactor productivity for butanol increased with an increase in the pH until reaching a maximum value of 4.6 g/(L·h)

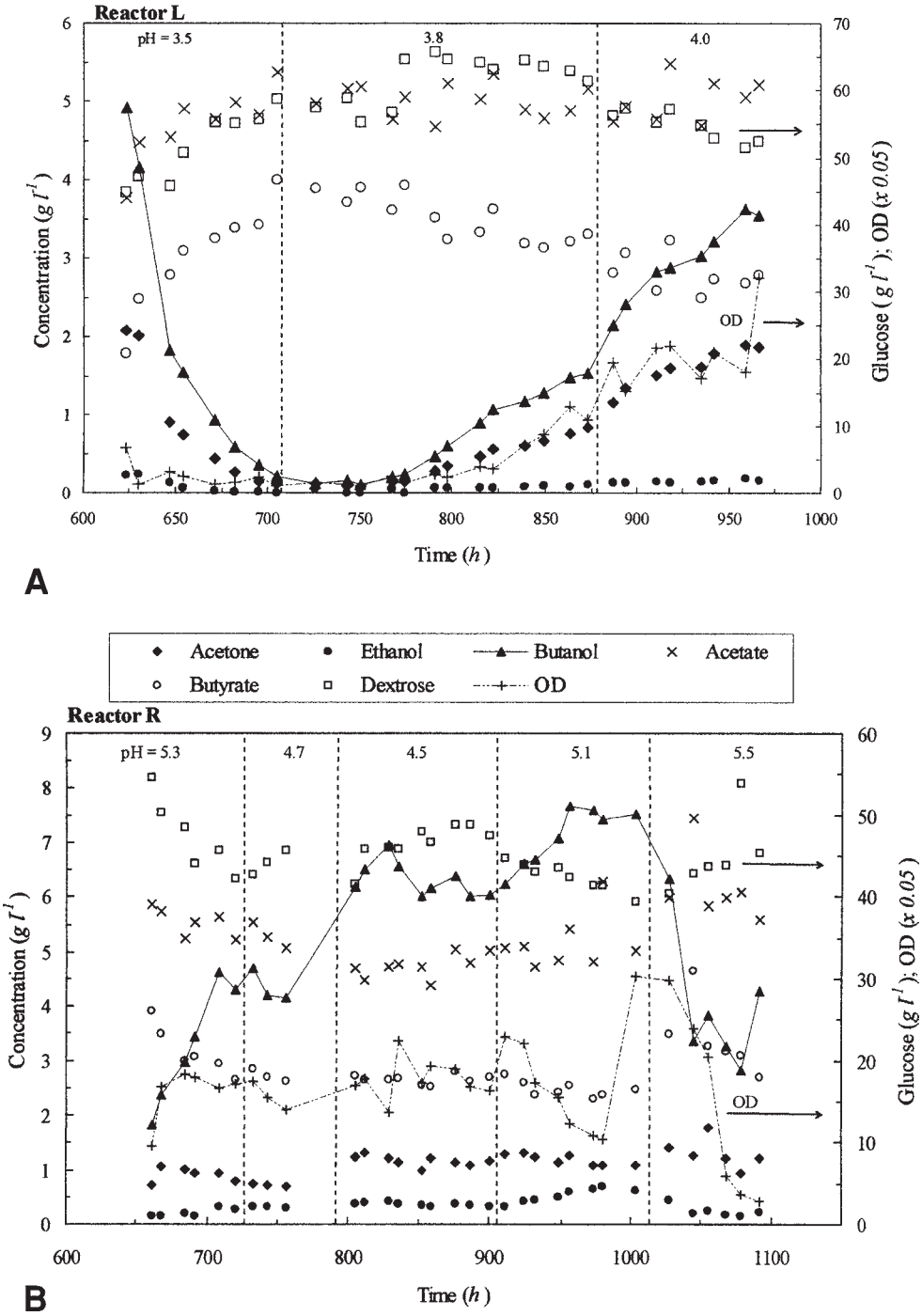

Fig. 4. Kinetics of continuous ABE fermentation at various pH values and 0.6 h^{-1} dilution rate. Glucose and OD are on the right axis; acetone, butanol, ethanol, acetate, and butyrate are on the left axis. **(A)** Reactor L; **(B)** reactor R.

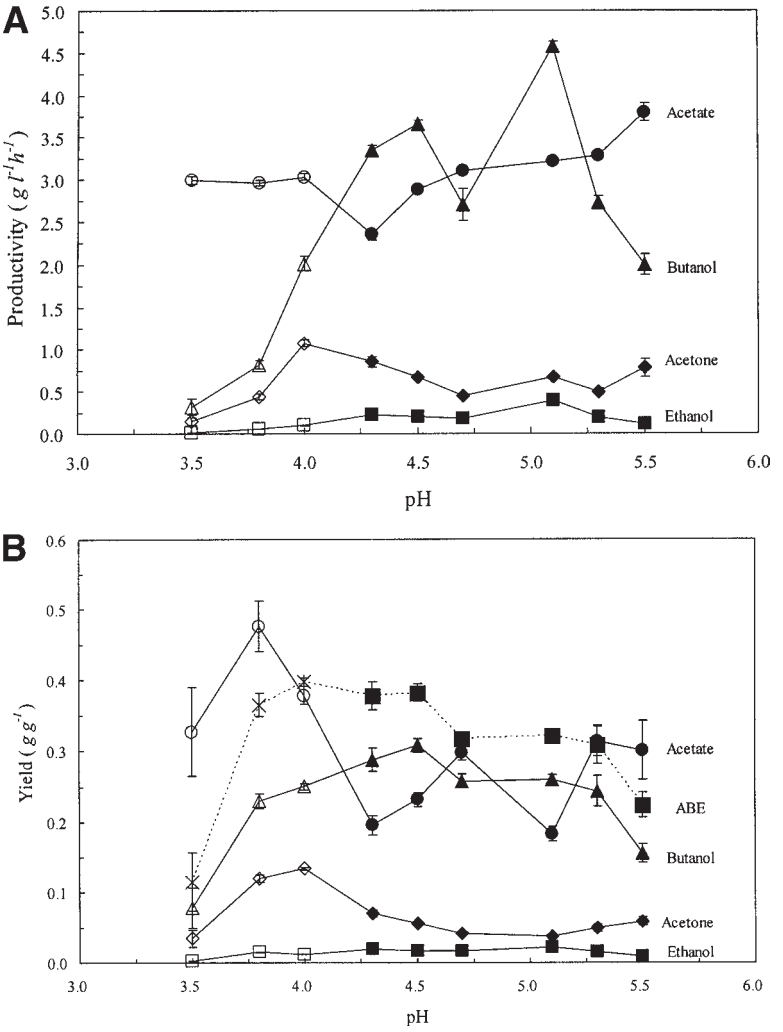

Fig. 5. Effects of pH on productivities and product yields at D = 0.6 h^{-1}. Open symbols: reactor L; solid symbols: reactor R. **(A)** Productivity; **(B)** yield.

at pH 5.1, but then it decreased as the pH increased further to 5.5. The effect of pH on the butanol yield appeared to be small in the range of 3.8–5.3, although the maximum value was found at pH 4.5. Butanol yield was significantly lower at pH 5.5, which is usually considered to be the pH value more favorable to acidogenesis. Both the productivity and butanol yield were low at pH 3.5, which appeared to be the lower limit for ABE fermentation. Acetone and ethanol production appeared to be much less sensitive to the pH in the range studied.

Discussion

Optimizing the ABE fermentation process has long been the aspiration of more than a century of research. Conventionally, the profitability of fermentation is influenced by the type and concentration of substrate, dilution rate, pH, culture medium, and product recovery. Even using cell recycle, cell immobilization, or extractive fermentation to increase cell density and productivity, the yield of the combined ABE production never exceeded 0.44 g/g *(13–15)*.

The production of butanol is usually associated with the uptake of various acids. In the present, we developed a continuous fermentation that improved the uptake of butyric acid and glucose by *C. acetobutylicum* in the FBB. The production of butanol via butyric acid converted from carbohydrates was efficient owing to the high density of viable cells maintained in the FBB through continual cell renewal *(16)*. An optimal butanol yield of 0.42 g/g and a productivity of 4.6 g/(L·h) were obtained in a 200-mL FBB at a dilution rate of 0.9 h^{-1}, pH 4.3, and 35°C with 54 g/L of glucose and 3.6 g/L of butyric acid in the feed stream. The concentration of butanol was 5.1 g/L on average. The conversions of glucose and butyric acid were 0.19 and 0.31, respectively. The optimum solvent (ABE) yield was 0.53 g/g under the same process conditions.

The higher butanol and total solvent yields obtained in our study can be attributed to feeding with butyrate as a cosubstrate, which greatly reduced acidogenesis and promoted solventogenesis. The increased butanol yield also could be owing to the dramatically reduced ethanol production. In addition, cell immobilization in the FBB allowed the bacteria to survive long in the solventogenic phase, allowing for long-term continuous solvent production without frequent cell regeneration.

Our study has shown that doubling the yield of butanol to approx 2.5 gal/bushel of corn (0.37 L/kg) in the conventional ABE fermentation can be achieved by converting carbohydrates into mainly butanol, which can make fermentation-derived butanol economically competitive with petrochemically derived butanol. Compared to the conventional ABE fermentation (the optimum butanol yield of 0.25 g/g and productivity of 4.5 g/(L·h), the FBB notably enhanced the yield of butanol and ABE (more than 68 and 20%, respectively) by *C. acetobutylicum*, making butanol production from renewable resource an attractive alternative to ethanol fermentation. Commercialization of this new technology has the propensity to reduce our the United States's dependence on foreign oil, protect its fuel generation grid from sudden disruption, develop its agricultural base, solve the hydrogen supply problem associated with fuel cells, and help reduce global warming.

Acknowledgments

We acknowledge Ying Zhu for helping to provide the information about butyric acid fermentation. This research was supported in part by a grant from the Department of Energy (DE-FG02-00ER86106).

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