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Butanol Production by *Clostridium beijerinckii* BA101 in an Immobilized Cell Biofilm Reactor

Increase in Sugar Utilization

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Abstract

Acetone butanol ethanol was produced in a continuous immobilized cell (biofilm) plug-flow reactor inoculated with *Clostridium beijerinckii* BA101. To achieve high reactor productivity, *C. beijerinckii* BA101 cells were immobilized by adsorption onto clay brick. The continuous plug-flow reactor offers high productivities owing to reduced butanol inhibition and increased cell concentration. Although high productivity was achieved, it was at the expense of low sugar utilization (30.3%). To increase sugar utilization, the reactor effluent was recycled. However, this approach is complicated by butanol toxicity. The effluent was recycled after removal of butanol by pervaporation to reduce butanol toxicity in the reactor. Recycling of butanol-free effluent resulted in a sugar utilization of 100.7% in addition to high productivity of 10.2 g/(L·h) at a dilution rate of 1.5 h^{-1}. A dilution rate of 2.0 h^{-1} resulted in a reactor productivity of 16. 2 g/(L·h) and sugar utilization of 101.4%. It is anticipated that this reactor-recovery system would be economical for butanol production when using *C. beijerinckii* BA101.

Index Entries: *Clostridium beijerinckii* BA101; butanol; immobilized cell biofilm reactor; pervaporative recovery; selectivity; flux.

Introduction

Recent increases in oil price (up to 300%), environmental concerns, and the need to lessen the reliance on the diminishing petroleum supplies

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have renewed interest in obtaining solvents from renewable resources such as corn, soy molasses, cane molasses, wood hydrolysate, and other agriculturally based products. However, utilization of inexpensive agricultural raw materials can only be realized when new technology is applied for the economic bioconversion processes. Butanol (acetone butanol ethanol [ABE]) in particular appears to be a good choice as a fuel additive to reduce exhaust smoke and particulates *(1)*. Highly toxic to the cells of *Clostridium acetobutylicum/C. beijerinckii* butanol, does not accumulate to more than 13–15 g/L in a batch reactor. As a result of toxicity, low productivities are achieved. Owing to the potential for reduced toxicity, continuous plug-flow immobilized cell culture is a technique to improve solvent productivity and reduce the cost of butanol production *(2)*.

Immobilized cell continuous biofilm reactors are more stable (fewer fluctuations), and operate longer in addition to being more productive *(3,4)*. Two major immobilization cell techniques—adsorption and entrapment—have been viewed as cost-effective approaches. Examples of cell immobilization by entrapment include carrageenan and chitosan *(5)*, and calcium alginate *(6–11)*, while that of adsorption include coke *(12)*, Beachwood shavings *(13)*, bonechar *(14)*, and clay brick *(15)*. Productivities as high as 15.8 g/(L·h) have been achieved in this type of adsorption immobilized cell reactor when compared with 0.3–0.5 g/(L·h) in batch reactors using *C. beijerinckii* BA101, a genetically altered mutant strain. The *C. beijerinckii* BA101 mutant strain produced up to 33 g/L of total solvents *(16)* with a typical ratio of ABE on the order of 3:16:1. The *C. beijerinckii* BA101 strain shuttles more carbon to butanol production than other cultures.

However, high reactor productivities are obtained at the expense of low ABE concentration and low sugar utilization (usually 30%). The reactor effluent cannot be recycled owing to the presence of butanol, which is toxic. To recycle reactor effluent and improve sugar utilization, butanol must be removed from the effluent prior to recycling. For this reason, we examined an alternative approach for removing butanol selectively by pervaporation. Pervaporation is a membrane-based process in which butanol (along with acetone and ethanol) selectively diffuses through the membrane and the diffused ABE is removed using either vacuum or sweep gas followed by condensation into a liquid. Recovery of butanol by pervaporation results in a concentrated product stream that requires less energy for further concentration and purification. Additionally, butanol-free effluent can be supplemented with concentrated sugar solution and recycled to the reactor for further ABE production.

The objective of the present study was to remove ABE from the effluent of an immobilized cell biofilm reactor and recycle the effluent (low in butanol) to achieve increased sugar utilization. Total sugar utilization is essential for economic production of butanol. An additional objective of this study was to investigate if the culture was adversely affected by the recycle stream.

Materials and Methods

Culture and Maintenance

Spores of *C. beijerinckii* BA101 were stored at 4°C in sterile distilled water. The spores were heat shocked at 80°C for 10 min in cooked meat medium (Difco, Detroit, MI) for preparation of the inoculum. This was followed by incubation at 35°C in an anaerobic chamber for 12–16 h.

Bioreactor, Cell Immobilization, and Pervaporative Recovery

C. beijerinckii BA101 cells were immobilized by adsorption onto clay brick and used in a continuous reactor to produce ABE *(15)*. The feed medium was kept anaerobic by sweeping oxygen-free N_2 gas across the surface of the medium in an airtight glass bottle. The feed medium contained 60 g/L of glucose, 1 g/L of yeast extract (Difco), and P2 medium ingredients (buffer, minerals, and vitamins) *(17)*. Glucose and yeast extract solution was sterilized at 121°C for 15 min followed by cooling to room temperature by sweeping oxygen-free N_2 gas across the surface. Filter-sterilized (0.2-µm) stock solutions, which were stored at 4°C, were added to the cooled medium aseptically. The immobilized cell reactor was run as a control (without recycle) at dilution rates of 1.0, 1.5, and 2.0 h^{-1}. Figure 1A is a schematic diagram of such a control experimental setup.

To remove ABE from the effluent of the reactor, approx 2 L of effluent was collected in a sterile anaerobic container. From the effluent, ABE was removed by pervaporation membrane at 35°C as described elsewhere *(18–20)*. A silicone tubing (id: 3.4 mm; wall thickness: 0.6 mm; area based on id: 0.08 m^2) was used as a membrane to remove (at 35°C) ABE from the reactor effluent. Oxygen-free N_2 gas (flow rate of 120 L/h) was used to sweep ABE vapors from the membrane surface. The vapors were condensed at 0 to –15 °C in a condenser (cooling area of 1292 cm^2) and collected in a receiver. Ethylene glycol (50%, [v/v]) was used as a coolant. Sterile distilled water was added to the broth to compensate for lost volume during ABE recovery. At the end of solvent removal, concentrated sterile sugar solution containing 1 g/L of yeast extract and P2 medium stock solution was added to the solvent-free effluent. The concentration of glucose in the solvent-free broth was 55–58 g/L. The effluent was recycled to the reactor at dilution rates of 1.0, 1.5, and 2.0 h^{-1} to compare sugar utilization and reactor productivities with those of the control experiment. Figure 1B is a schematic diagram of pervaporation.

Statistical Analyses

Solvents (ABE) and acids were determined by gas chromatography (GC) (Hewlett Packard Gas Chromatograph 6890) using a flame ionization detector and a capillary column (crosslinked FFAP; 30 m × 0.53 mm, film thickness of 1µm). Glucose was measured enzymatically using a Sigma Diagnostics Kit, Glucose HK (Sigma, St. Louis, MO). To determine glucose, absorbance was measured at 340 nm using a Beckman DU-40 spectropho-

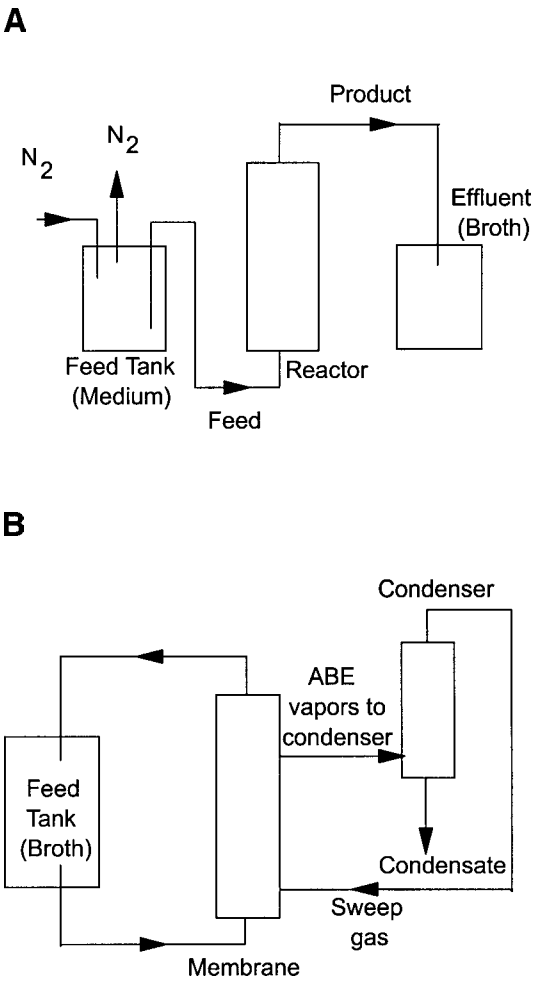

Fig. 1. Schematic diagram of butanol production by *C. beijerinckii* BA101 using continuous immobilized cell biofilm reactor. (**A**) Continuous immobilized cell biofilm reactor; (**B**) pervaporation.

tometer. The yield was calculated as the total ABE produced divided by the glucose utilized and is expressed as grams/gram. ABE productivity was calculated as the total ABE produced (g/L) multiplied by the dilution rate. The sugar utilization was calculated as the utilized sugar (g/L) divided by the added sugar (g/L). The reactor was monitored for ABE production by injecting samples into the GC. The dilution rate was altered after a steady state was achieved (20 residence times), in terms of both ABE production and glucose utilization. Selectivity (α) and flux have been defined as $[y/(1-y)]/[x/(1-x)]$, and W/Ah, respectively, in which x,y, W, A, and h are weight fraction of butanol in feed, weight fraction of butanol in condensate, weight of condensate (grams), membrane area (square meters), and time period (hours) during which condensate was collected, respectively *(20)*.

Table 1
Continuous Production of ABE in Immobilized Cell Column Reactor of *C. beijerinckii* BA101 at Dilution Rates of 1.0 and 1.5 h^{-1}.

Fermentation product/parameter	Dilution rate			
	1.0 h^{-1}		1.5 h^{-1}	
	Influent	Effluent	Influent	Effluent
Acetone (g/L)	0.0	2.1	0.0	2.2
Butanol (g/L)	0.0	4.3	0.0	4.4
Ethanol (g/L)	0.0	0.6	0.0	0.3
Acetic acid (g/L)	0.0	0.7	0.0	0.9
Butyric acid (g/L)	0.0	0.3	0.0	0.4
Total ABE (g/L)	0.0	7.0	0.0	6.9
Total acids (g/L)	0.0	1.0	0.0	1.3
ABE productivity (g/[L·h])	0.0	7.0	0.0	10.4
Yield (g/g)		0.38		0.38
Glucose (g/L)	61.0	42.5	60.1	41.9
Glucose utilization (%)		30.3		30.2

Results and Discussion

A control immobilized cell reactor was fed with 61.0 g/L of glucose-based P2 medium at a dilution rate of 1.0 h^{-1}. At this dilution rate the reactor produced 7.0 g/L of total ABE and resulted in a productivity of 7.0 g/(L·h) (Table 1). The reactor produced 2.1 g/L of acetone, 4.3 g/L of butanol and 0.6 g/L of ethanol. In addition to ABE, acetic acid and butyric acid at 0.3 and 0.7 g/L were produced. ABE yield of 0.38 was achieved. Although a high productivity was achieved, it was at the expense of low sugar utilization (30.3% of that available in the feed).

The effluent could not be recycled because of butanol toxicity as recycle with butanol in the stream is not beneficial. Hence, effluent was collected anaerobically from the reactor and ABE was removed by pervaporation. Prior to recovery the concentrations of ABE and acids were 8.4 g/L (3.0 g/L of acetone, 5.1 g/L of butanol, 0.3 g/L of ethanol) and 2.0 g/L (1.4 g/L of acetic acid, and 0.6 g/L of butyric acid), respectively. These concentrations of ABE and acids are higher than achieved at a dilution rate of 1.0 h^{-1} (Table 1), owing to continued fermentation by free cells during effluent collection.

At the end of pervaporative recovery, 0.5 g/L of total ABE (0 g/L of acetone, 0.3 g/L of butanol, 0.2 g/L of ethanol), and 1.0 g/L of total acids (1.0 g/L of acetic acid, 0 g/L of butyric acid) were present. The pervaporation was run until ABE concentration was reduced to the above level. The removal of ABE from the fermentation broth by pervaporation was efficient. Flux through the membrane was on the order of 20.3–28.1 g/(m^2·h). This flux is in the range we reported earlier *(20)*, and the membrane did not

Table 2
Acetic Acid and Butyric Acid Selectivities During
ABE Removal from Fermentation Broth by Pervaporation

Acetic acid concentration in pervaporate (g/L)	α = Acetic acid	Butyric acid concentration in pervaporate (g/L)	α = Butyric acid
0.7	1.65	0.6	1.66
0.0	0.0	0.3	1.74
0.0	0.0	0.4	3.38
0.0	0.0	0.3	5.93

foul. Note that during ABE removal, concentration of acids decreased from 2.0 g/L initially to 1.0 g/L at the end of pervaporation, suggesting that acids diffused through the membrane. Diffusion of acids through silicone pervaporation membranes has not been reported. However, polypropylene membranes allows acid diffusion *(21)*. While it is desirable that all the solvents be removed from the fermentation broth for economic reasons, removal of acids is not preferred. Retention of acids and their assimilation (when recycled) could improve ABE yield. Acetone selectivity varied from 7.73 to 0.5 depending on acetone concentration in the broth. Similarly, butanol selectivity varied from 5.74 to 17.81, and the concentration of butanol in pervaporate was 11.9 – 49.2 g/L. Selectivities for ethanol ranged from 1.51 to 3.63. Note that glucose did not diffuse through the membrane.

Acetic acid and butyric acid are reaction intermediates and should not be removed. Table 2 gives the selectivities for acetic acid and butyric acid. Diffusion of acetic acid was detected with a selectivity of 1.65, suggesting that the membrane was selective for acetic acid (a selectivity of higher than 1 means that the membrane is selective). The selectivities for butyric acid ranged from 1.66 to 5.93. The membrane allowed butyric acid to be diffused selectively. Diffusion of acids would result in reduced ABE yield.

Next, the treated effluent containing 0 g/L of acetone, 0.2 g/L of butanol, and 0.3 g/L of ethanol, was supplemented with glucose and nutrients and fed to the reactor (pH 6.5). The glucose concentration in the feed was 58.0 g/L. The reactor was operated at a dilution rate of 1.0 h^{-1} to compare its performance with the control experiment at the same dilution rate. The reactor productivity of this system was 7.2 g/(L·h) as compared with 7.0 g/(L·h) for the control, suggesting that recycle of butanol-free effluent did not negatively affect the reactor (Table 3). The ABE concentrations in the effluent were 2.3, 4.6, and 0.8 g/L, respectively. Acetic and butyric acid concentrations were 0.3 and 0.2 g/L, respectively. ABE yield was high at 0.45. Table 3 shows that acids were utilized by the culture to produce ABE as acid concentration decreased significantly. Interestingly, sugar utilization was 103.2% that of supplemented prior to feeding the reactor. This experiment demonstrated that high reactor productivities,

Table 3
Continuous Production of ABE in Immobilized Cell Biofilm Reactor of *C. beijerinckii* BA101 Using Recycle Stream as Feed

Fermentation product/parameter	Dilution rate 1.0 h^{-1} Influent	1.0 h^{-1} Effluent	1.5 h^{-1} Influent	1.5 h^{-1} Effluent
Acetone (g/L)	0.0	2.3	0.0	2.3
Butanol (g/L)	0.2	4.6	0.1	4.5
Ethanol (g/L)	0.3	0.8	0.2	0.3
Acetic acid (g/L)	2.6	0.3	2.4	0.3
Butyric acid (g/L)	1.6	0.2	1.3	0.3
Total ABE (g/L)	0.5	7.7	0.3	7.1
ABE produced (g/L)	—	7.2	—	6.8
Total acids (g/L)	4.2	0.5	3.7	0.6
ABE Productivity (g/[L·h])		7.2[b]		10.2[b]
Yield (g/g)		0.45	0.44	
Glucose (g/L)	58.0[a]		55.3[a]	
Glucose utilization (%)		103.2[c]		100.7[c]

[a] Includes added sugar.
[b] ABE produced × dilution rate.
[c] That of supplemented after ABE removal.

high ABE yield, and 100% sugar utilization are possible when recycling butanol-free effluent.

Often increased dilution rates result in increased productivity. Hence, we increased reactor dilution rate to 1.5 h^{-1} while recycling butanol-free effluent. The composition of feed and product streams is shown in Table 3. The reactor influent contained 0.3 g/L of ABE and 3.7 g/L of acids. Sugar concentration in the feed was 55.3 g/L. The effluent contained 7.1 g/L total of ABE and 0.6 g/L of acids. The concentration of ABE produced was 6.8 g/L, resulting in a reactor productivity of 10.2 g/(L·h). The reactor utilized acids present in the influent resulting in a yield of 0.44. Sugar utilization was 100.7% that of supplemented to the feed. The two productivities 10.4 (control) and 10.2 g/(L·h) (recycle) are comparable. A further increase in dilution rate to 2.0 h^{-1} resulted in a productivity of 16.2 g/(L·h) and sugar utilization of 101.4% (as compared with control reactor productivity of 14.9 g/[L·h], yield of 0.34, and sugar utilization of 36.2%) that of added after butanol recovery. Note that ABE yield in the control experiment was 0.38 (Table 1) and in the recycle experiment was 0.44 (Table 3). This was owing to the fact that acids were utilized by the culture.

In conclusion, the recycle stream did not negatively affect the *C. beijerinckii* BA101 culture. High sugar utilization (100.7–103.2% that of supplemented in the feed) was achieved in addition to high reactor productivities. The yield was also improved by 16% owing to acid utilization. In

this reactor system high reactor productivity of 16.2 g/(L·h) was achieved at a dilution rate of 2.0 h^{-1}. Recycling of the effluent reduced the waste stream in addition to bringing about the aforementioned benefits. Hence, it was concluded that recycling of the effluent after pervaporative recovery resulted in 100% sugar utilization (that of added after ABE removal).

Acknowledgments

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