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Butanol Production Using *Clostridium beijerinckii* BA101 Hyper-Butanol Producing Mutant Strain and Recovery by Pervaporation

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Abstract

Clostridium beijerinckii BA101 (mutant strain) and *C. beijerinckii* 8052 (wild type) were compared for substrate and butanol inhibition. The wild-type strain is more strongly inhibited by added butanol than is the mutant strain. Acetone and butanol were removed from a fed-batch reactor inoculated with *C. beijerinckii* BA101 by pervaporation using a silicone membrane. In the batch reactor, *C. beijerinckii* BA101 produced 25.3 g/L of total solvents, whereas in the fermentation-recovery experiment it produced 165.1 g/L of total solvents. Solvent productivity increased from 0.35 (batch reactor) to 0.98 g/L·h (fed-batch reactor). The fed-batch reactor was fed with 500 g/L of glucose-based P2 medium. Acetone selectivities ranged from 2 to 10 whereas butanol selectivities ranged from 7 to 19. Total flux varied from 26 to 31 g/m^2·h.

Index Entries: *Clostridium beijerinckii* BA101; acetone; butanol; fed-batch reactor; pervaporation.

Introduction

The production of acetone, butanol, ethanol (ABE) by fermentation is well known *(1–3)*. This fermentation was commercially viable during the first half of the twentieth century. The ABE fermentation continued to be used industrially until after the World War II, when rapid development of the petrochemical industry began. By the 1960s, efficient production of ABE by the oil industry, together with higher costs of carbohydrate substrate sources, saw the virtual elimination of industrial ABE fermentation. The current US market for petrochemically derived butanol stands at 3.5 billion lb, which is expected to grow 4% annually *(4)*. The major

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applications of butanol include its use as a feedstock chemical in the plastics industry, as a food-grade extractant in the food and flavor industry, and as a fuel extender. The oil price increase in early 1970s and the uncertainty of petroleum supplies have revived research efforts aimed at obtaining solvents from renewable resources. Since then, efforts have been focused on improving microbial cultures, and fermentation and downstream process development.

We have reported on *Clostridium beijerinckii* BA101 that was derived from the NCIMB 8052 wild-type strain *(5,6)*. The *C. beijerinckii* BA101 can produce up to 32.6 g/L of total solvents and 20.9 g/L of butanol in optimized P2 medium *(7)*. To reduce further the cost of butanol production, we examined corn steep water/corn steep liquor (CSW/CSL) as a nutrient/substrate for butanol production. CSW/CSL is a byproduct of the corn wet-milling industry. Currently, CSW/CSL is evaporated to 50% solids syrup that is utilized primarily as an animal feed supplement in the cattle industry at $55/t *(8)*. The size of the fermentors used for these studies ranged from 500 mL to 200 L *(4,9)*.

Butanol toxicity in the fermentation reactor limits the use of concentrated sugar solutions. The maximum solvent concentration that can be achieved in our reactors is limited to 26–32.6 g/L depending on the medium used. *In situ* removal of butanol using pervaporation is an approach for improving butanol production *(10)*. Pervaporation has been successfully applied to remove butanol from the fermentation broth *(3,11)*. The objectives of the present study were to investigate substrate and butanol inhibition of the *C. beijerinckii* BA101 mutant and to produce butanol in a fed-batch reactor using BA101 and recovery by pervaporation.

Materials and Methods

Culture Maintenance

Spores of *C. beijerinckii* BA101 and 8052 were stored at 4°C in sterile distilled water. The spores were heat shocked at 80°C for 10 min in cooked meat medium for inoculum preparation. This was followed by incubation at 35°C in an anaerobic chamber for 16–18 h. After growth, 5 mL of the culture was inoculated into 100 mL of P2 medium containing 60 g/L of glucose and 1 g/L of yeast extract (Difco, Detroit, MI) and allowed to grow at the foregoing conditions. Following growth, the culture was inoculated into P2 medium that also contained 60 g/L of glucose and 1 g/L of yeast extract.

Inhibition Studies

Inhibition studies were conducted in 125-mL screw-capped bottles containing 100 mL of P2 medium. For substrate inhibition, various concentrations of glucose (60, 100, 150, 200 g/L) were added into the P2 medium. P2 medium containing glucose and yeast extract was autoclaved at 121°C for 15 min in screw-capped bottles followed by cooling in an anaerobic

chamber. After inoculation, samples were taken intermittently and analyzed for butanol production. For butanol inhibition studies, P2 medium contained 60 g/L of glucose and 1 g/L of yeast extract in addition to minerals, vitamins, and citrate buffer. Following 20 h of growth, the medium was spiked with butanol to give a final butanol concentration of 7.5–17.5 g/L.

Membrane and Bioreactor

The details of the membrane, sterilization, and reactor operation have been given elsewhere *(12,13)*. The total volume of the reactor and the membrane was 1100 mL, and the volume of the fermentation broth was 800 mL. The membrane area was 0.16 m^2 based on inside diameter. The fed-batch reactor was initiated as a batch reactor with 60 g/L of glucose in P2 medium. Fermentation was allowed to proceed for 24 h using *C. beijerinckii* BA101 before pervaporative recovery of butanol was begun. Concentrated glucose (500 g/L) solution containing 1 g/L of yeast extract in P2 medium was added to the bioreactor to replace the utilized glucose. The feed medium was kept anaerobic using oxygen-free nitrogen gas, and stringent precautions were taken to keep the system anaerobic. Samples were taken from the bioreactor under sterile conditions. The temperature of the reactor was controlled at 36 ± 1°C. The volume in the reactor was kept constant by adding sterile distilled water.

Analyses

ABE and acids (acetic and butyric) were determined by gas chromatography (Hewlett Packard Gas Chromatograph 6890) using a flame ionization detector and a capillary column (crosslinked FFAP; 30 m × 0.53 mm and 1-μm film thickness). Glucose was measured enzymatically using a Sigma Diagnostics Kit, Glucose HK (Sigma, St. Louis, MO). For these determinations, absorbance was measured at 340 nm using a Beckman DU-40 spectrophotometer. The reactor was monitored and controlled by the simultaneous analyses of samples for ABE and glucose concentrations. Cell concentration was determined by measuring the optical density at 540 nm. The solvent productivity was calculated as the total solvent produced in the culture (grams/liter) divided by the total fermentation time (*see* Eq. 1). The yield was calculated as the total solvents produced divided by total glucose utilized (*see* Eq. 2). Acetone, butanol selectivity (α), and flux were calculated using Eqs. 3 and 4, respectively:

$$R_p = [C_{sfe}V_{fp} + \Sigma(C_{s1}V_{c1} + C_{s2}V_{c2} + \ldots + C_{sn}V_{cn})]/(V_{fe}T) \quad (1)$$

$$Y_{p/s} = [C_{sfe}V_{fp} + \Sigma(C_{s1}V_{c1} + C_{s2}V_{c2} + \ldots + C_{sn}V_{cn})]/\{[C_{g0}V_{f0} + \Sigma(C_{g1}V_{g1} + C_{g2}V_{g2} + \ldots + C_{gn}V_{gn})] - C_{ge}V_{fe}\} \quad (2)$$

$$\alpha = [y/(1-y)]/[x/(1-x)] \quad (3)$$

$$\text{Flux} = W/AH \quad (4)$$

Table 1
Batch Fermentation Parameters
for ABE Production Using *C. beijerinckii* BA101 and 8052

Parameter	BA101 (mutant)	8052 (wild type)
Butanol (g/L)	19.7	10.8
Acetone (g/L)	4.8	3.2
Ethanol (g/L)	0.8	0.8
Acetic acid (g/L)	0.0	3.8
Butyric acid (g/L)	0.2	0.5
Total solvent (g/L)	25.3	14.8
Total acids (g/L)	0.2	4.3
Initial glucose (g/L)	62.1	59.8
Final glucose (g/L)	4.6	0.8
ABE yield (g/g)	0.44	0.25
ABE productivity (g/L·h)	0.35	0.16
Total fermentation time (h)	72	90

Results and Discussion

To compare the two strains, batch fermentations of *C. beijerinckii* 8052 and BA101 were run; Table 1 presents the results. The *C. beijerinckii* BA101 strain produced 25.3 g/L of total solvent whereas the *C. beijerinckii* 8052 strain (the wild type) produced 14.8 g/L of total solvent. Interestingly, the solvent yield for the BA101 and 8052 strains was 0.44 and 0.25, respectively, suggesting that *C. beijerinckii* BA101 produced 1.76 times more solvent than the wild-type strain. *C. beijerinckii* BA101 produced 0.2 g/L of total acids whereas the wild-type strain produced 4.3 g/L of total acids. The course of fermentation for *C. beijerinckii* 8052 and BA101 is shown in Figs. 1 and 2, respectively.

Substrate inhibition studies demonstrated that neither of the strains grew above 175 g/L of glucose. Substrate inhibition studies for *C. beijerinckii* BA101 have been published elsewhere *(13)*. Figure 3 presents the data for the butanol inhibition studies for *C. beijerinckii* BA101 and 8052. At 7.5 g/L of added butanol, maximum butanol productivities for the two strains were 0.26 (8052) and 0.42 g/L·h (BA101). *C. beijerinckii* 8052 was severely inhibited above 12 g/L of added butanol, whereas *C. beijerinckii* BA101 was able to continue to produce butanol above 17.5 g/L of added butanol.

The *C. beijerinckii* BA101 batch fermentation was initiated using 60.1 g/L of glucose. The fermentation was allowed to proceed in a batch mode for 24 h, at which time total solvents were 7.2 g/L. At this time, pervaporative recovery of butanol was begun. Figure 4 shows a schematic diagram of fermentation and recovery. The unutilized glucose at 24 h was 42.1 g/L, which was increased to 60 g/L by adding concentrated glucose medium (Table 2). During the batch mode, a glucose utilization rate of 0.75 g/L·h and a solvent production rate of 0.28 g/L·h was achieved. As the removal of butanol continued, butanol in the reactor increased to 6.7 g/L

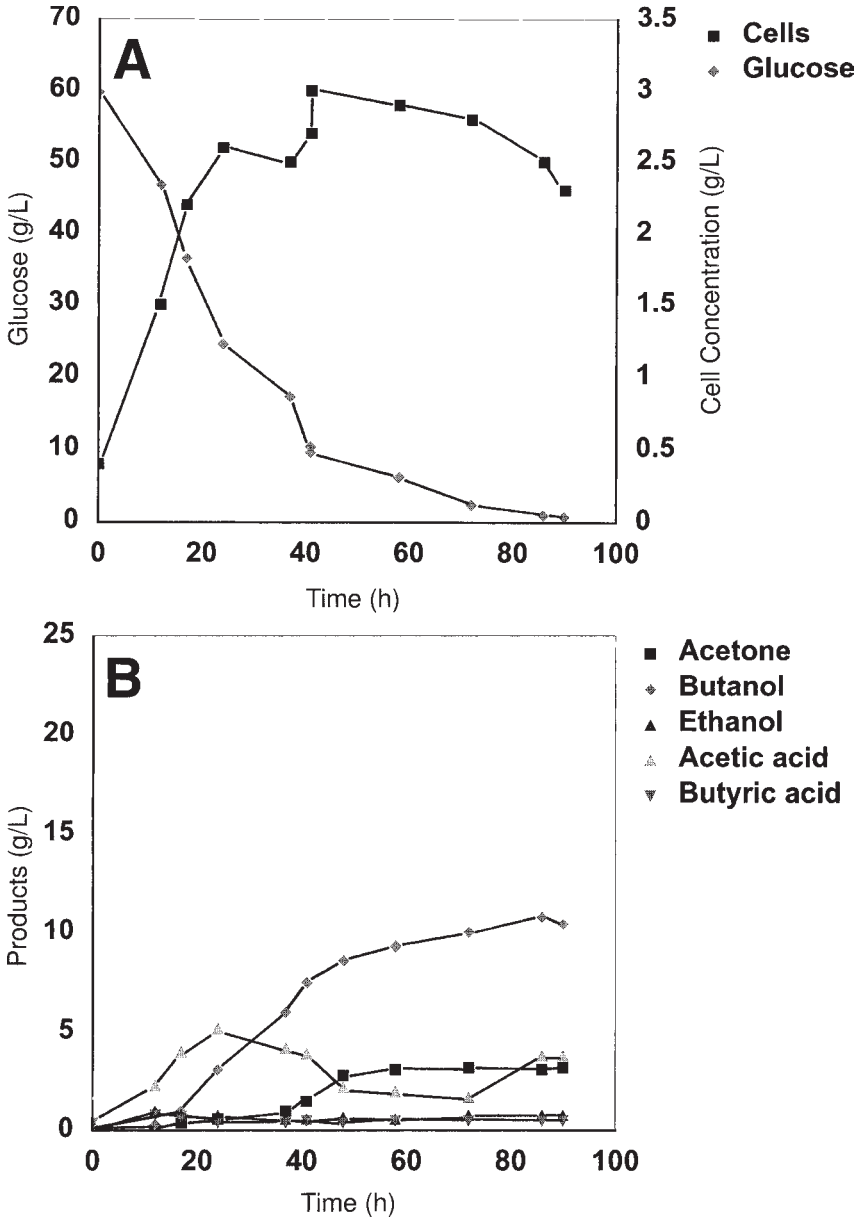

Fig. 1. Production of solvents using *C. beijerinckii* 8052 in a batch reactor. **(A)** Utilization of glucose and cell concentration over time; **(B)** production of solvents and acids (acetic acid and butyric acid) over time.

followed by intermittent decreases and increases (Fig. 5). The glucose concentration and the glucose utilization and solvent production rates given in Table 2 suggest that there was good solvent production during the fermentation. When compared to the batch glucose utilization rate of 0.75 g/L·h, a glucose utilization rate up to 3.41 g/L·h was achieved in the fed-batch

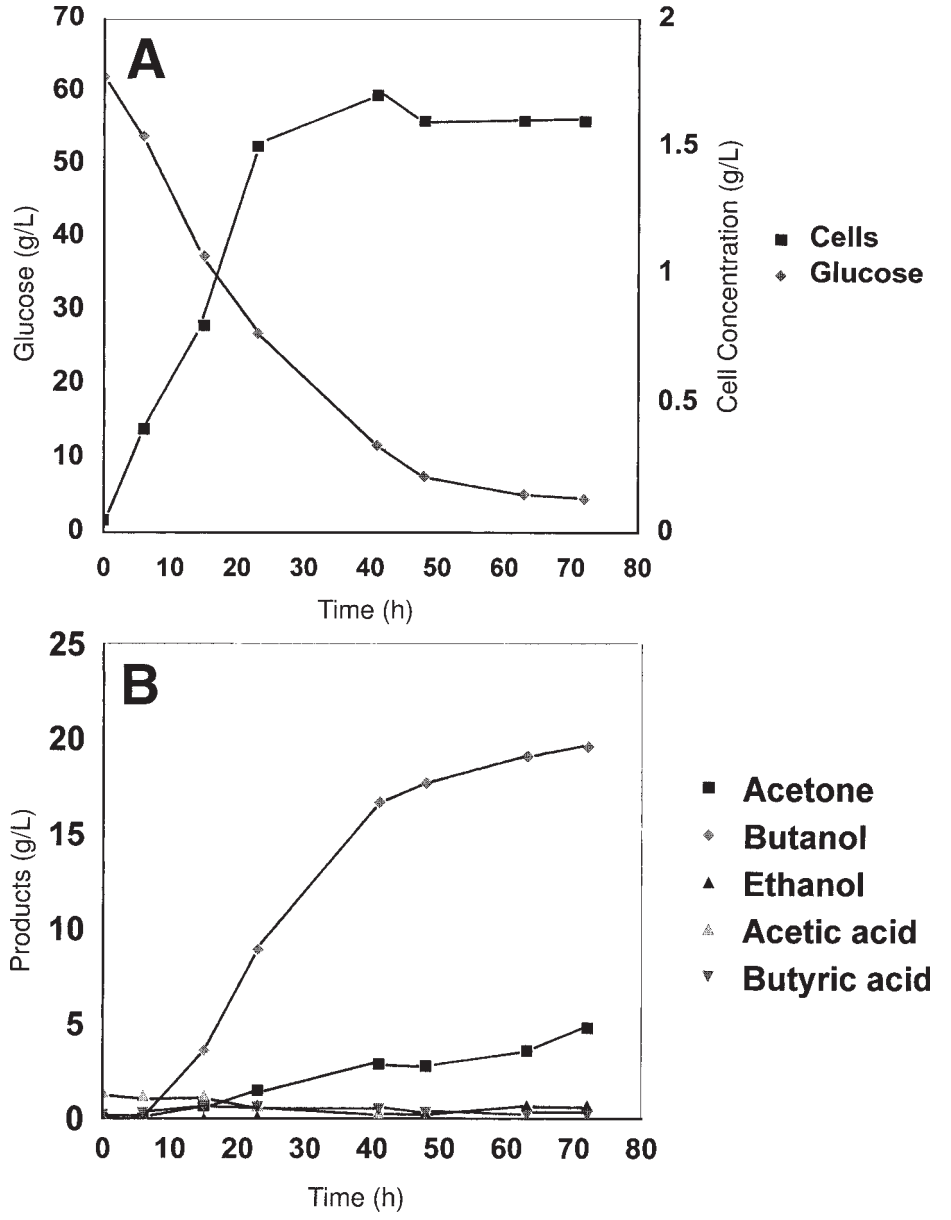

Fig. 2. Production of solvents and acids when using *C. beijerinckii* BA101 in a batch reactor. **(A)** Glucose utilization and cell concentration over time; **(B)** production of solvents and acids (acetic acid and butyric acid) over time.

fermentor. This was owing to the continuous removal of butanol. At the end of 148 h, the fermentation stopped; we do not know why the fermentation stopped. Possibilities for cessation of fermentation include the accumulation of unknown toxic components since there was no bleed from the reactor, a decrease in water activity, or diffusion of oxygen into the fermentor.

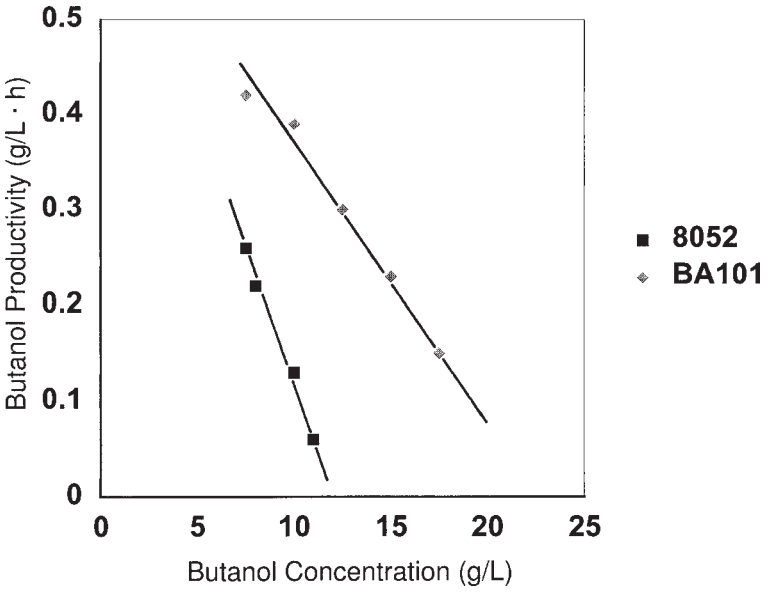

Fig. 3. Maximum butanol productivities for *C. beijerinckii* BA101 (◆) and 8052 (■) at various added butanol concentrations.

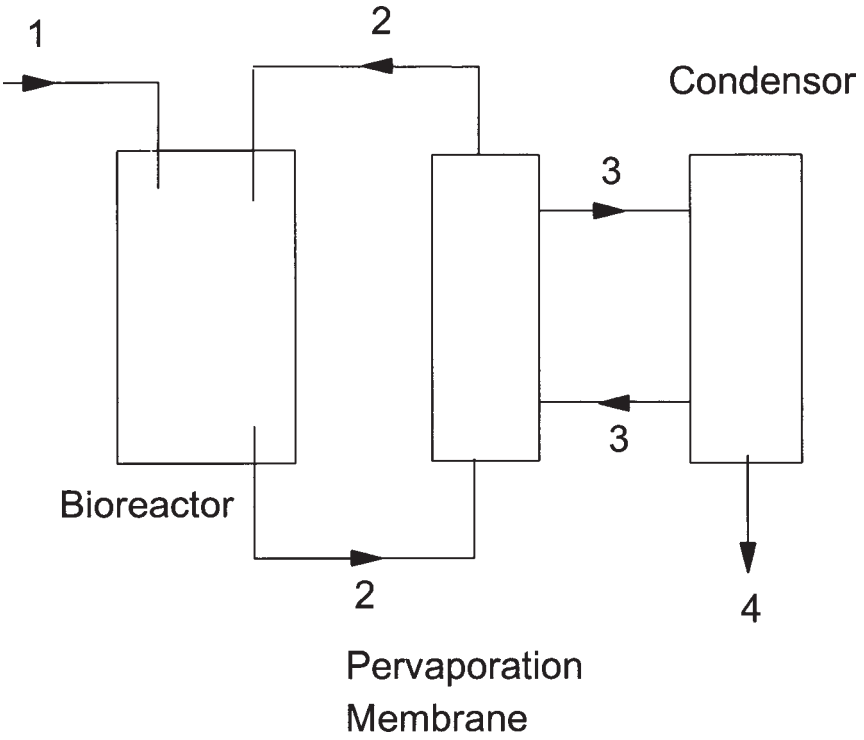

Fig. 4. A simple schematic diagram of ABE production and recovery by pervaporation. 1, Concentrated feed; 2, fermentation broth recycle; 3, gas recycle; 4, condensate.

Table 2
Glucose Concentration at Various Times
in the Reactor and Glucose Utilization and Solvent Production Rates
in Fed-Batch Fermentation of Glucose by *C. beijerinckii* BA101

Time (h)	Glucose concentration (g/L)		Glucose utilization rate (g/L·h)	Solvent production rate (g/L·h)
	Before adding medium[a]	After adding medium[a]		
0	60.1			
12	55.9			
24	42.1	60.0	0.75	0.28
37	41.4	61.3	1.43	0.60
48	26.5	63.0	3.16	1.36
58	44.4	44.4	1.86	0.80
72	19.4	59.1	1.79	0.79
85	14.8	61.5	3.41	1.50
96	24.8	65.0	3.24	1.50
111	16.2	60.1	3.25	1.40
120	30.8	61.4	3.26	1.43
132	40.1	64.2	1.78	0.80
148	35.1	59.3	1.82	0.80
168	58.1	FS[b]		

[a]P2 medium containing 500 g/L of glucose.
[b]Fermentation stopped.

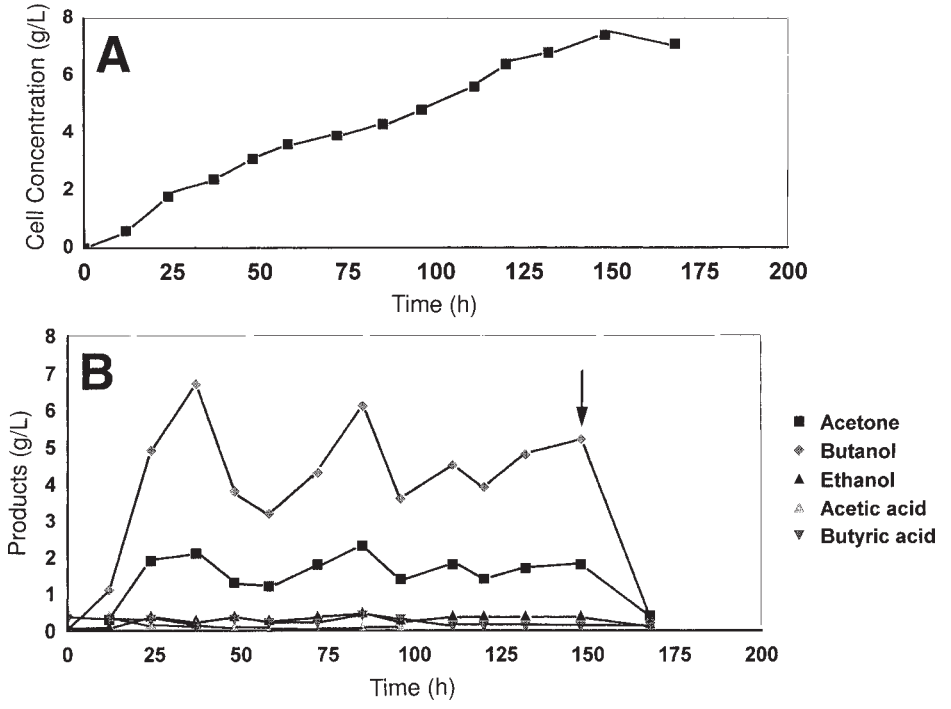

Fig. 5. Production of ABE using *C. beijerinckii* BA101 in a product removal fed-batch reactor. **(A)** Cell concentration over time; **(B)** production of solvents and acids over time. Arrow shows that fermentation stopped.

Table 3
Fermentation Parameters Obtained
in Fed-Batch Reactor Employing *C. beijerinckii* BA101

Fermentation parameter	Average value
Total solvent produced (g/L)	165.1
Total glucose utilized (g/L)	384.0
Solvent yield (g/g)	0.43
Overall productivity (g/L·h)	0.98
Total fermentation time (h)[a]	168
Maximum cell concentration (g/L)	7.5

[a]Fermentation stopped at 148 h.

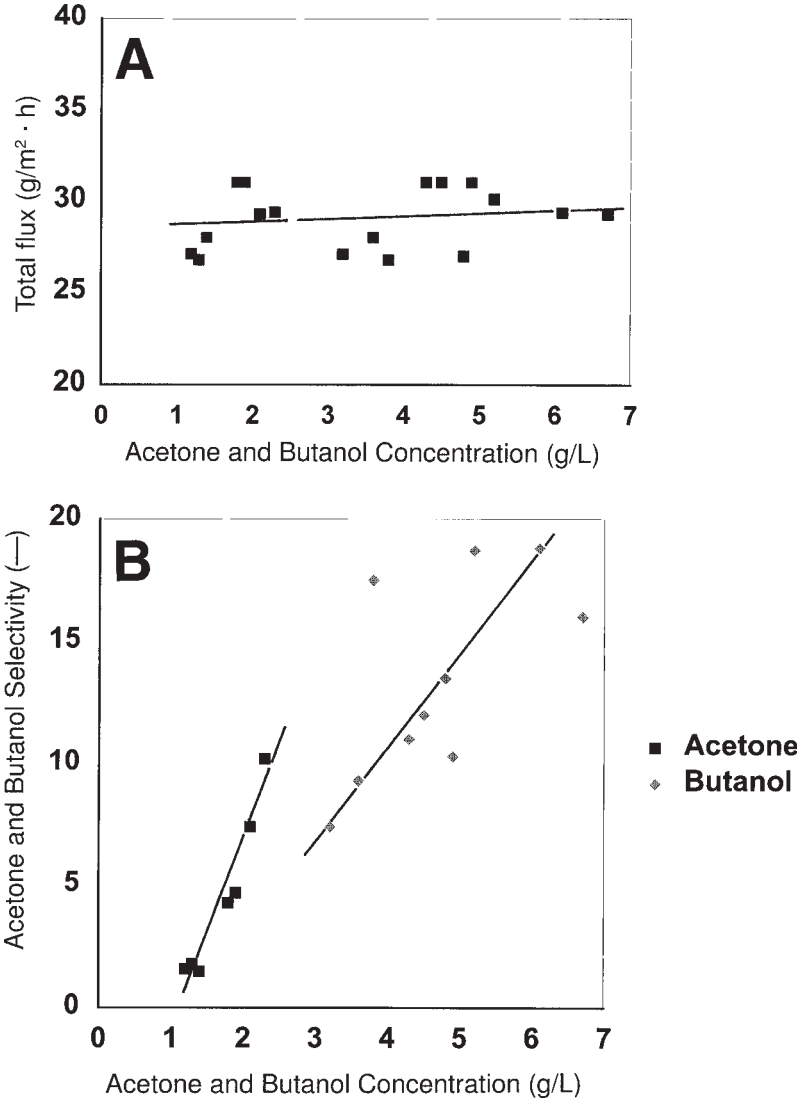

Fig. 6. Acetone (■) and butanol (◆) selectivities and flux at various acetone and butanol concentrations in the reactor. **(A)** Flux; **(B)** selectivities.

During the integrated fermentation and recovery experiment, 307.2 g of glucose was utilized and 132.1 g of solvents was produced, which corresponds to the production of 165.1 g/L of total solvents. A total of 384.0 g/L of glucose was utilized in the system. Table 3 presents the average values of the fermentation parameters in the fed-batch reactor. The overall solvent productivity was 0.98 g/L·h as compared to the batch reactor productivity of 0.35 g/L·h (Table 1). The increase in productivity was owing to butanol removal from the fermentation broth. Additionally, a concentrated sugar solution was fed to the reactor that would result in a reduction of process stream volume and waste disposal volume. The concentration of the recovered solvents was higher than the concentration in the reactor when calculated using the selectivity equation.

The solvents were recovered from the condensate collector intermittently. From solvents (acetone and butanol) concentration and condensate weights, selectivities and flux values were calculated; these are plotted in Fig. 6. Acetone selectivities ranged from 2 to 10 whereas butanol selectivities ranged from 7 to 19. A comparison of butanol selectivities using various pervaporation membranes has been published recently *(14)*. Total flux values varied from 26 to 31 g/m^2·h. These flux and selectivity values are lower than reported earlier *(12)*, which suggests that active fermentation broth may have fouled the membrane.

Conclusion

C. beijerinckii 8052 is more strongly inhibited by added butanol than is *C. beijerinckii* BA101. Above 175 g/L of glucose in the P2 medium, neither strain grows. The fermentation-recovery fed-batch reactor containing *C. beijerinckii* BA101 resulted in the production of 165.1 g/L of total solvents as compared to 25.3 g/L in the batch reactor; this is 6.55 times higher. The solvent productivity in the integrated system was 0.98 g/L·h as compared to 0.35 g/L·h in the batch reactor.

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Nomenclature

A = Pervaporation membrane area (m^2)

C_g = Glucose concentration in concentrated feed medium (C_{g1} and C_{g2} for first and second additions, respectively) (g/L)

C_{g0} = Glucose concentration in fermentation reactor at 0 time (g/L)

C_{ge} = Glucose concentration in fermentation broth at the end of fermentation (g/L)

C_{gn} = Glucose concentration in nth addition of concentrated feed medium (n = 1, 2, 3, . . . n) (g/L)

C_s = Solvent concentration in condensate (C_{s1} and C_{s2} for first and second condensates, respectively) (g/L)

C_{sfe} = Solvent concentration in fermentation broth at the end of pervaporation (g/L)

C_{sn} = Solvent concentration in nth condensate (n = 1, 2, 3, . . . n) (g/L)

H = Time period during which pervaporation membrane condensate was collected (h)

R_p = Reactor/solvent productivity (g/L·h)

T = Fermentation time (h)

V_c = Volume of pervaporation membrane condensate (V_{c1} and V_{c2} for first and second condensates, respectively) (L)

V_{cn} = Volume of nth condensate of pervaporation membrane (n = 1, 2, 3, . . . n) (L)

V_{f0} = Volume of fermentation broth at 0 time (L)

V_{fe} = Volume of fermentation broth at the end of fermentation (L)

V_{fp} = Volume of fermentation broth at the end of pervaporation (L)

V_g = Volume of concentrated feed medium added to the fed-batch reactor (L)

V_{gn} = Volume of nth addition of concentrated feed medium to the fed-batch reactor (n = 1, 2, 3, . . . n) (L)

W = Weight of condensate collected from the pervaporation membrane (g)

X = Weight fraction of butanol or total solvent in fermentation broth (–)

Y = Weight fraction of butanol or total solvent in the membrane permeate (condensate) (–)

$Y_{p/s}$ = Yield of solvents (g solvents/g glucose utilized)

α = Selectivity of acetone/butanol or total solvents (–)

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