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Enhanced Butanol Production by *Clostridium beijerinckii* BA101 Grown in Semidefined P2 Medium Containing 6 Percent Maltodextrin or Glucose

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Dramatically elevated levels of butanol and acetone resulted in higher butanol and total solvent yields for hyperamylolytic *Clostridium beijerinckii* BA101 relative to the NCIMB 8052 parent strain grown in semidefined P2 medium containing either 6% glucose or STAR-DRI 5 maltodextrin. *C. beijerinckii* BA101 consistently produced on the order of 19 g of butanol per liter in 20-liter batch fermentations. This represents a greater than 100% increase in butanol concentration by the BA101 strain compared to the parent NCIMB 8052 strain. The kinetics of butanol production over time also indicate a more rapid rate of butanol production by BA101 in semidefined P2 medium containing glucose or maltodextrin. The lower levels of butyric and acetic acids produced over the course of the fermentation carried out by BA101 are consistent with an enhanced capacity for uptake and recycling of these acids. *C. beijerinckii* BA101 appears to more completely utilize carbohydrate compared to the 8052 strain. Carbon balance following fermentation by *C. beijerinckii* 8052 and BA101 indicates that sufficient carbon is available for the twofold increase in butanol concentration observed during BA101 fermentations. *C. beijerinckii* BA101 also has superior solvent production capacity during continuous culture fermentation in P2 medium containing 6% glucose. Volumetric solvent yields of 0.78 and 1.74 g/liter/h for BA101 and 0.34 and 1.17 g/liter/h for NCIMB 8052 were obtained at dilution rates of 0.05 and 0.20 h^{-1}, respectively. No drift towards acid synthesis (strain degeneration) was observed for up to 200 h ($d = 0.05\ h^{-1}$) and 100 h ($d = 0.20\ h^{-1}$).

The fermentation of carbohydrates to acetone, butanol, and ethanol (ABE) by the solventogenic clostridia is well known (13, 19). Currently, this value-added fermentation process is attractive for several economic and environmental reasons (6). Prominent among the economic factors is the current surplus of agricultural wastes or by-products that can be utilized as inexpensive fermentation substrates. One specific example is mycotoxin-contaminated corn, which is unsuitable for use as animal feed and is difficult to dispose of.

Butanol is an important industrial chemical. Compared to the currently popular fuel additive, ethanol, butanol is more miscible with gasoline and diesel fuel, has a lower vapor pressure, and is less miscible with water, all of which make it a superior fuel extender. Butanol is currently used as a feedstock chemical in the plastic industry and as a food-grade extractant in the food and flavor industry. Because of the potential for carcinogen carryover, the use of petroleum-derived butanol is not desirable for food applications. It is expected that the food and flavor industries would support a higher-than-normal price and be independent of market conditions for petroleum-derived butanol. At present, high recovery costs due to the rather low final concentration of butanol in the fermentation broth are a limiting factor. However, a doubling to 2% final concentration was projected to lower the energy requirements for distillative recovery of butanol by 62% (18). Such an increase in butanol concentration would help to make this fermentation an economically viable process.

Approximately 4.5 billion kg of butanol was produced by petrochemical processes in 1989. If this amount of butanol were produced by fermentation using solventogenic clostridia and corn as the substrate, this would translate into 136 million additional bushels of corn being utilized. This additional nonfood market for corn would have an obvious price-stabilizing effect on this commodity. In addition to 3.5 kg of butanol per bushel of corn, the ABE fermentative process also produces 1.7 kg of acetone and 0.9 kg of ethanol. Modeling studies based on butanol at $0.66/kg, corn at $3.00/bushel, and propylene at $0.44/kg suggested that butanol production from cornstarch is economically competitive with current petrochemical processes (16). These studies were dependent on the use of a stable, high-yielding strain of *Clostridium acetobutylicum.*

The genetic amplification of the extracellular amylases produced by the clostridia is seen as a way to improve the substrate-to-solvent conversion efficiency as well as to allow for simultaneous saccharification and fermentation of starch-based biomass. A rate-limiting step in the fermentative route to butanol is the efficient hydrolysis of starch, which affects the final concentration of butanol in the fermentation broth. Lin and Blaschek (15) reported that an increase in starch utilization by butanol-tolerant *C. acetobutylicum* SA-1 correlated with an increase in butanol production. It was suggested that further amplification of amylase activity may lead to enhanced butanol production. The hyperamylolytic *Clostridium beijerinckii* BA101 mutant was generated by using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) together with selective enrichment on the nonmetabolizable glucose analog 2-deoxyglucose (3). Amylolytic enzyme production by *C. beijerinckii* BA101 was 1.8- and 2.5-fold higher than that of the parent strain grown in starch and glucose, respectively. Preliminary small-scale batch studies in P2 medium containing 6% soluble starch and 0.3% yeast

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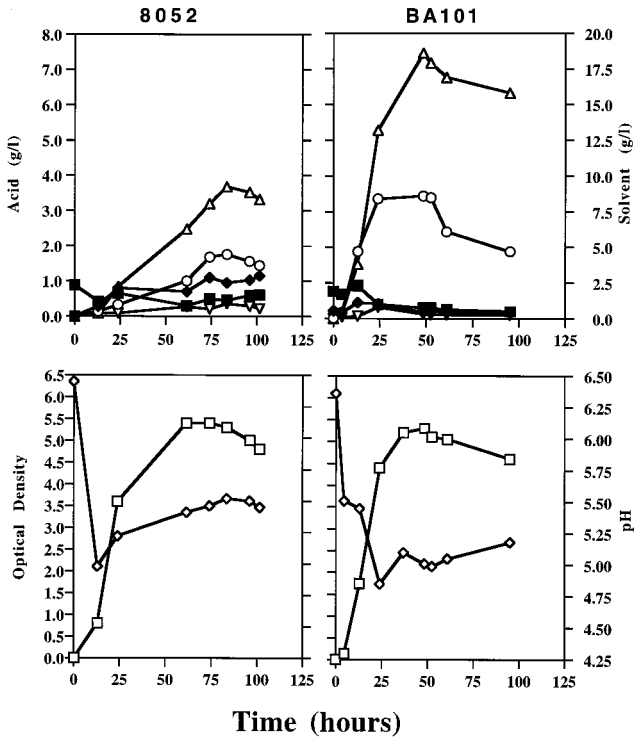

FIG. 1. Solvent, acid production, optical density, and pH values during batch fermentation by *C. beijerinckii* NCIMB 8052 and BA101 during growth in semidefined P2 medium containing 6% glucose. Symbols: △, butanol; ○, acetone; ▽, ethanol; ◆, butyric acid; ■, acetic acid; ◇, pH; □, optical density.

extract suggested that the hyperamylolytic BA101 strain produced ca. 70% more butanol than the wild type (1).

The objective of this study was to examine *C. beijerinckii* NCIMB 8052 and BA101 with respect to their ability to produce butanol during pilot-scale batch fermentations and in continuous culture with semidefined P2 medium containing either 6% maltodextrin or 6% glucose as the carbohydrate source.

MATERIALS AND METHODS

Strains, culture maintenance, and fermentation conditions. The *C. beijerinckii* BA101 mutant was generated with NTG together with selective enrichment on the nonmetabolizable glucose analog 2-deoxyglucose (3). Transverse alternating-field electrophoretic analysis of chromosomal DNA suggested that our laboratory stock, *C. acetobutylicum* 824, which was originally obtained from the American Type Culture Collection, was not the ATCC 824 type strain. The laboratory 824 stock was subsequently reidentified as *C. beijerinckii* NCIMB 8052 based on DNA similarity studies using the S1 nuclease method (7). Furthermore, Norah Goldfine at the University of Pennsylvania independently confirmed this finding by thin-layer chromatographic analysis of the lipid extract. These findings were further confirmed by ribotyping analysis carried out in our laboratories (data not shown). As a consequence, reference to our laboratory 824 stock has been changed to *C. beijerinckii* 8052, which is the parent strain of *C. beijerinckii* BA101. Consistent with this change, Johnson and Chen (12) recently indicated that *C. acetobutylicum* NCIMB 8052 should be designated *C. beijerinckii* 8052.

Laboratory stocks of *C. beijerinckii* NCIMB 8052 and BA101 were routinely maintained under anaerobic conditions as spore suspensions in ddH_2O at room temperature. Spores were heat shocked at 80°C for 10 min and inoculated into Trypticase-glucose-yeast extract (TGY) medium (2). After overnight growth, cultures were plated out on TGY agar plates, and single colony isolates were picked and inoculated into 10 ml of TGY medium. The culture was incubated anaerobically overnight at 37°C until an optical density at 600 nm of 1.0 to 1.5 was achieved as measured by a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, N.Y.). P2 medium (2) containing 0.1% yeast extract was prepared with either 6% glucose or 6% maltodextrin (STAR-DRI 5; A. E. Staley Manufacturing Co., Decatur, Ill.) as a carbohydrate source. STAR-DRI maltodextrins are a series of products which are produced from cornstarch. STAR-DRI 5 has a dextrose equivalent of 5 and a saccharide profile which suggests that it is mainly (87.8%) composed of higher saccharides (degree of polymerization [DP], 11+). Semidefined P2 medium (pH 6.5; 100 ml) was inoculated with 5 ml of TGY medium culture and incubated anaerobically for 18 to 20 h at ca. 30°C. The culture was decanted into 1 liter of semidefined P2 medium and incubated anaerobically for 16 to 18 h at ca. 30°C until the optical density at 600 nm was 1.0 to 1.5. Batch fermentations were performed with a 42-liter Braun fermentor (B. Braun Biotech International GmbH, Melsungen, Germany) located at the University of Illinois Biotechnology Center Fermentation Facility. Semidefined P2 medium was sterilized in the fermentor, agitated, and sparged with nitrogen overnight prior to inoculation.

A 5% inoculum of *C. beijerinckii* was routinely used for the batch fermentation experiments. Twenty-liter batch fermentations were performed at 33°C in the absence of agitation and pH control. Sterilized nitrogen gas was sparged (1,950 ml/min) through the fermentor to aid mixing and to exclude oxygen. During the course of the fermentation, temperature, pH, and percent oxygen were measured continuously. Optical density was monitored by spectrophotometric analysis of culture broth as described above.

Continuous cultivation of *C. beijerinckii* 8052 and BA101 was carried out in P2 medium plus 6% glucose with a Braun Biostat 2-liter continuous culture apparatus (B. Braun Biotech International GmbH) set at 35°C and at a 50-rpm stirring rate. P2 medium containing 6% glucose was flushed with nitrogen and inoculated with 100 ml of 18 to 20-h-old culture. Growth was initiated in the batch mode by waiting for 18 to 22 h before medium addition was started. The dilution rate was set at 0.05 (h^{-1}) or 0.20 (h^{-1}). After attainment of steady-state conditions, samples (1 ml) were routinely removed for solvent analysis. Volumetric solvent production rate was calculated as grams per liter per hour.

Analytical procedures. ABE production was measured by a gas chromatograph (5710A; Hewlett-Packard Co., Avondale, Pa.) equipped with a flame ionization detector and a glass column (1.83 m by 2 mm [inner diameter]) packed with 80/100 Carbopack C–0.1% SP-1000 (Supelco, Inc., Bellefonte, Pa.). Butyric and acetic acids were determined with a Hewlett-Packard 5890 series II gas chromatograph and a column packed with Supelco GP 10% SP-1200–1% H_3PO_4 on Chromosorb WAW. Run conditions consisted of a 175°C injector temperature, a 180°C detector temperature, and a 125°C oven temperature and a nitrogen carrier gas set at a flow rate of 72 ml/min. Total residual carbohydrate was determined by the phenol-sulfuric acid method (9). Product yield was calculated by dividing the grams of solvent produced by the grams of carbohydrate consumed. Carbon recovery following fermentation by *C. beijerinckii* 8052 and BA101 grown in semidefined P2 medium containing 6% carbohydrate was examined by determining the moles of substrate carbon utilized and the moles of product carbon produced as described by Gottschalk (11) for the ABE fermentation.

RESULTS

Solvent, acid production, and the corresponding optical density and pH values during batch fermentation by *C. beijerinckii* NCIMB 8052 and BA101 grown in semidefined P2 medium containing 6% glucose can be seen in Fig. 1. The batch fermentation data presented herein represents the average of duplicate determinations. *C. beijerinckii* BA101 produced substantially higher levels of butanol (18.6 versus 9.2 g/liter) and acetone

TABLE 1. Comparison of the production of various fermentation products by *C. beijerinckii* 8052 and BA101 grown in semidefined P2 medium containing 6% glucose

Characteristic or product	Value for strain:	
	NCIMB 8052	BA101
Fermentation time (h)[a]	83.5	48.5
Acetone (g/liter)	4.4	8.6
Butanol (g/liter)	9.2	18.6
Ethanol (g/liter)	0.9	0.3
Total solvent (g/liter)	14.5	27.5
Acetate (g/liter)	0.5	0.3
Butyrate (g/liter)	1.0	0.2
Glucose utilized (g/liter)	52.6	57.3
Butanol yield[b]	0.17	0.32
Solvent yield[b]	0.28	0.48

[a] Fermentation time at which the highest butanol concentration was recorded and all other values were determined.

[b] Yield was determined by dividing grams of butanol or total solvent produced by grams of glucose utilized.

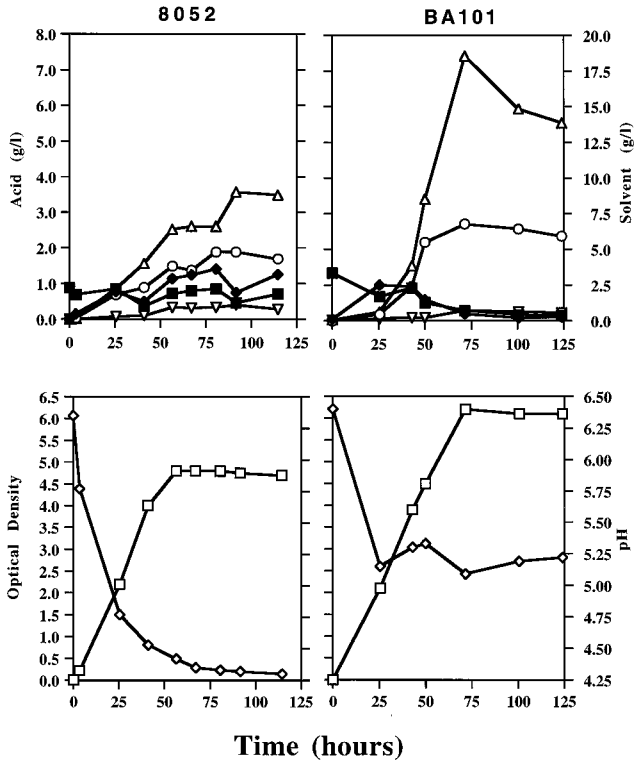

FIG. 2. Solvent, acid production, optical density, and pH values during batch fermentation by *C. beijerinckii* NCIMB 8052 and BA101 during growth in semidefined P2 medium containing 6% maltodextrin. Symbols: △, butanol; ○, acetone; ▽, ethanol; ◆, butyric acid; ■, acetic acid; ◇, pH; □, optical density.

(8.6 versus 4.4 g/liter) than did the wild-type strain (Table 1). The growth rate as well as the rate of solvent production is also more rapid for BA101 grown in medium containing 6% glucose. Solvent, acid production, and the corresponding optical density and pH values during batch fermentation by *C. beijerinckii* NCIMB 8052 and BA101 grown in semidefined P2 medium containing 6% maltodextrin can be seen in Fig. 2. Once again, *C. beijerinckii* BA101 produced substantially higher levels of butanol (18.6 versus 8.9 g/liter) and acetone (6.8 versus 4.7 g/liter) than did the wild-type strain (Table 2). The growth

TABLE 2. Comparison of the production of various fermentation products by *C. beijerinckii* 8052 and BA101 grown in semidefined P2 medium containing 6% STAR-DRI 5 maltodextrins

Characteristic or product	Value for strain:	
	NCIMB 8052	BA101
Fermentation time (h)[a]	91.5	71.5
Acetone (g/liter)	4.7	6.8
Butanol (g/liter)	8.9	18.6
Ethanol (g/liter)	1.0	0.7
Total solvent (g/liter)	14.6	26.1
Acetate (g/liter)	0.5	0.3
Butyrate (g/liter)	0.7	0.2
Maltodextrin utilized (g/liter)	48.1	52.7
Butanol yield[b]	0.19	0.35
Solvent yield[b]	0.30	0.50

[a] Fermentation time at which the highest butanol concentration was recorded and all other values were determined.

[b] Yield was determined by dividing grams of butanol or total solvent produced by grams of glucose utilized.

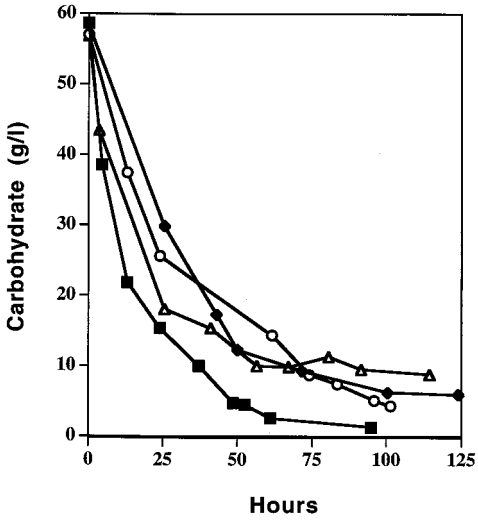

FIG. 3. Carbohydrate utilization during the course of batch fermentation by *C. beijerinckii* 8052 and BA101 in semidefined P2 medium containing either 6% glucose or 6% maltodextrin. Symbols: ○, *C. beijerinckii* NCIMB 8052 on glucose; ■, *C. beijerinckii* BA101 on glucose; △, *C. beijerinckii* NCIMB 8052 on maltodextrin; ◆, *C. beijerinckii* BA101 on maltodextrin.

rate of 8052 and BA101 was slower in maltodextrin relative to that in glucose, although the BA101 strain exhibited a higher final optical density. Furthermore, although initial levels of butyric and acetic acids were similar, these levels decreased dramatically as the BA101 fermentation progressed when either glucose or maltodextrin was the carbohydrate source. This observation suggests an enhanced capacity for uptake and recycling of these acids by the BA101 mutant strain. It is interesting to note that butanol production by BA101 appears to coincide with active growth of the microorganism, in contrast to what is observed for 8052. Rapid growth of BA101 in glucose corresponded with the early production of butanol. The decreased rate of growth when BA101 is grown on maltodextrin corresponded with a delay in production of butanol.

A comparison of the production of various fermentation products by *C. beijerinckii* 8052 and BA101 grown in semidefined P2 medium containing either 6% glucose or 6% maltodextrin can be seen in Tables 1 and 2, respectively. Dramatically elevated levels of butanol and acetone resulted in higher butanol and total solvent yields for BA101 relative to the 8052 strain grown in semidefined P2 medium containing either glucose or maltodextrin. The solvent yield for BA101 in glucose was 0.48, while that for the 8052 strain was 0.28 (Table 1). When maltodextrin was used as the carbohydrate source (Table 2), the solvent yield for BA101 was 0.50, while that for 8052 was 0.30.

Carbohydrate utilization during the course of batch fermentation by *C. beijerinckii* 8052 and BA101 in semidefined P2 medium containing either 6% glucose or 6% maltodextrin can be seen in Fig. 3. The highest rate of utilization as well as the most complete utilization of carbohydrate was seen in the case of *C. beijerinckii* BA101 grown on P2 medium containing glucose. The *C. beijerinckii* BA101 mutant appeared to more completely utilize carbohydrate in P2 medium containing 6% carbohydrate compared to 8052. This corresponds with the higher total butanol and solvent production by the BA101 strain grown in either carbohydrate source.

Carbon recovery following 20-liter batch fermentation by *C. beijerinckii* 8052 and BA101 grown in semidefined P2 medium

TABLE 3. Carbon recovery following fermentation by *C. beijerinckii* 8052 and BA101 grown in semidefined P2 medium containing 6% carbohydrate

Substrate or product	Total carbon (mol/liter) for strain with carbohydrate[a]			
	NCIMB 8052		BA101	
	Glucose	Maltodextrin	Glucose	Maltodextrin
Substrates				
Glucose utilized	1.75	1.05	1.91	1.75
Other carbon[b]	0.09	0.09	0.09	0.09
Products				
Butyrate	0.04	0.03	0.01	0.01
Acetate	0.02	0.02	0.01	0.01
Ethanol	0.04	0.04	0.01	0.04
Butanol	0.50	0.48	1.00	1.00
Acetone	0.23	0.24	0.44	0.35
Total product	0.83	0.81	1.47	1.41
Total substrate	1.84	1.14	2.00	1.84
% Carbon recovery[c]	45.1	71.1	73.5	76.6
% Carbon recovery[d]	76.7	102	106	108

[a] Total carbon was determined by multiplying the number of carbons for each compound by moles of each compound; units are based on moles of carbon per liter.
[b] Estimated carbohydrate carryover from P2 medium inoculum, 0.1% yeast extract, and acetate present in semidefined P2 medium.
[c] Total product carbon divided by total substrate carbon multiplied by 100.
[d] Total product carbon (including theoretical CO_2 value) divided by total substrate carbon multiplied by 100.

containing either 6% glucose or 6% maltodextrin can be seen in Table 3. Percent carbon recovery was defined as total product carbon divided by total substrate carbon multiplied by 100. The results suggest that there is sufficient carbon available for the observed twofold increase in butanol concentration during glucose- or maltodextrin-based BA101 fermentations. Since CO_2 was not directly measured, percent carbon recovery was also calculated by using a value for CO_2 which was estimated as 33% of total carbon (11).

Solvent production by *C. beijerinckii* 8052 and BA101 during continuous culture fermentation in P2 medium containing 6% glucose can be seen in Table 4. The results show that BA101 has superior solvent production capability compared to the 8052 wild type. Total solvent concentration was 15.63 and 8.71 g/liter for BA101 and 6.76 and 5.87 g/liter for 8052 at dilution rates of 0.05 and 0.20 h^{-1}, respectively. This results in a volumetric solvent yield of 0.78 and 1.74 g/liter/h for BA101 and 0.34 and 1.17 g/liter/h for NCIMB 8052 at dilution rates of 0.05 and 0.20 h^{-1}, respectively. The values for volumetric solvent yield are 2.3- and 1.5-fold higher for the mutant than for the wild type at the two dilution rates used. In these experiments, no drift towards acid synthesis was observed for up to 200 h ($d = 0.05$ h^{-1}) and 100 h ($d = 0.20$ h^{-1}). These results suggest that no strain degeneration occurred with *C. beijerinckii* BA101 and that stable solvent production can be maintained in single-stage continuous culture.

DISCUSSION

Dramatically elevated levels of butanol and acetone resulted in higher butanol and total solvent yields for hyperamylolytic *C. beijerinckii* BA101 relative to the NCIMB 8052 parent strain grown in semidefined P2 medium containing either 6% glucose or 6% STAR-DRI 5 maltodextrin. A plot of carbohydrate utilization by *C. beijerinckii* NCIMB 8052 and hyperamylolytic strain BA101 demonstrates that the BA101 strain does utilize carbohydrate more completely (Fig. 3).

The observation that BA101 also produces more butanol when grown in glucose-based medium suggests that the hyperamylolytic activity is not the reason for this strain producing elevated levels of butanol. Some other factor(s) must be involved. However, at this time it is not known why the BA101 strain produces elevated levels of butanol and acetone. More complete fermentation and physiological data analysis plus enzyme assays for critical pathways involved is expected to reveal the basis for the interesting changes in this mutant relative to the NCIMB 8052 wild-type strain.

It is likely that the acid-to-solvent switching mechanism has been altered in the BA101 mutant, as butanol production by BA101 was observed to occur during the active growth phase. This differs from the traditional ABE fermentation process in which solventogenesis occurs later in growth as the cells are approaching stationary phase (19). Although the initial levels of acids produced by BA101 were comparable to those observed for the 8052 strain, these values decreased dramatically over the course of the fermentation. The BA101 mutant may be a superrecycler of butyric and acetic acids, thereby detoxifying the cellular environment and contributing additional carbon to the production of neutral solvents.

The low value for percent carbon recovery for *C. beijerinckii* NCIMB 8052 grown in glucose (Table 3) may be explained as a consequence of rapid growth of this microbe and concurrent disposal of reducing equivalents during the formation of lactate from pyruvate. Lactate and CO_2, which were not directly measured in this study, may account for the low percent carbon recovery observed.

Several studies have shown that *C. acetobutylicum* has a limited ability to convert glucose to acetone and butanol for extended periods in single-stage continuous culture. Successful production of acetone-butanol in single-stage continuous culture with high product concentration has been reported elsewhere but only at very low dilution rates of 0.025 to 0.030 h^{-1} (4, 17). Other workers have reported periodic oscillations and metabolic drift to higher acid production at low (0.035 h^{-1} [8]) and high (0.16 h^{-1} [5]) dilution rates. Leung and Wang (14) reported successful production of high levels of solvents (14.9 g/liter) at a high dilution rate (0.18 h^{-1}). Recently, Gapes et al. (10) reported on the continuous cultivation of *C. beijerinckii* NRRL B592 with a two-stage chemostat with on-line solvent removal. This strain maintained the ability to produce solvents at an overall dilution rate of 0.13 h^{-1} and achieved an overall solvent productivity of 1.24 g/liter/h for more than 100 retention times. This compares to a volumetric solvent production rate of 1.74 g/liter/h for *C. beijerinckii* BA101 (a 40% increase) grown in a single-stage chemostat at a dilution rate of 0.20 h^{-1}.

TABLE 4. Solvent production by *C. beijerinckii* NCIMB 8052 and BA101 during continuous culture fermentation in P2 medium containing 6% glucose

Strain	Dilution rate (h^{-1})	pH	Solvent concn (g/liter)			Total solvent production (g/liter/h)[a]
			Ethanol	Acetone	Butanol	
8052	0.05	5.29	0.13	2.41	4.21	0.34
	0.20	5.38	0.08	0.41	5.38	1.17
BA101	0.05	5.03	1.25	5.58	8.80	0.78
	0.20	4.96	1.12	2.38	5.22	1.74

[a] Volumetric production rate.

The above results obtained with a 20-liter batch as well as continuous culture conditions suggest that *C. beijerinckii* BA101 is stable and can be used in a commercial fermentation process for producing butanol. Typically, *C. beijerinckii* produces on the order of 10 g of butanol per liter from cornstarch. From a process economics standpoint, the dramatic increase in butanol concentration by BA101 is very encouraging, since the most expensive aspect of this fermentation is the distillative recovery of butanol from the fermentor broth (18). Because of the increase to nearly 2% final concentration of butanol in the fermentor, significantly less energy must be used for product recovery, which in effect makes this a much more economically viable process.

Work is currently under way to characterize fermentative enzyme activities and mRNA expression levels, as well as to analyze the amylase gene associated with the *C. beijerinckii* BA101 mutant in order to understand the basis for the interesting changes in this mutant compared to NCIMB 8052.

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