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Recent advances in ABE fermentation: hyper-butanol producing *Clostridium beijerinckii* BA101

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This is an overview of the mutant strain *Clostridium beijerinckii* BA101 which produces solvents (acetone–butanol–ethanol, ABE) at elevated levels. This organism expresses high levels of amylases when grown on starch. *C. beijerinckii* BA101 hydrolyzes starch effectively and produces solvent in the concentration range of 27–29 g l^{-1}. *C. beijerinckii* BA101 has been characterized for both substrate and butanol inhibition. Supplementing the fermentation medium (MP2) with sodium acetate enhances solvent production to 33 g l^{-1}. The results of studies utilizing commercial fermentation medium and pilot plant-scale reactors are consistent with the results using small-scale reactors. Pervaporation, a technique to recover solvents, has been applied to fed-batch reactors containing *C. beijerinckii* BA101, and solvent production as high as 165 g l^{-1} has been achieved. Immobilization of *C. beijerinckii* BA101 by adsorption and use in a continuous reactor resulted in reactor productivity of 15.8 g l^{-1} h^{-1}. Recent economic studies employing *C. beijerinckii* BA101 suggested that butanol can be produced at US$0.20–0.25 lb^{-1} by employing batch fermentation and distillative recovery. Application of new technologies such as pervaporation, fed-batch culture, and immobilized cell reactors is expected to further reduce these prices. *Journal of Industrial Microbiology & Biotechnology* (2001) **27**, 287–291.

Keywords: *Clostridium beijerinckii* BA101; butanol; ABE; pilot plant; amylases; glucoamylases; pervaporation; economics

Introduction

The acetone–butanol–ethanol (ABE) fermentation is one of the oldest fermentation processes (second to ethanol) [11]. During the early part of the 20th century, this fermentation was commercially viable. However, between the 1950s and 1960s, fermentatively produced ABE was unable to compete economically with petrochemically produced ABE. This resulted in the virtual elimination of this fermentation. However, oil price increases in early 1970s resulted in a revival of research activities on this fermentation. As a result, the following factors which severely effect the economics of butanol fermentation were identified: (i) igh cost of substrate; (ii) low product concentration (<20 g l^{-1}); (iii) low reactor productivities (<0.3 g l^{-1} h^{-1}); (iv) low ABE yields (0.28–0.33); and (v) an escalated cost of butanol recovery by distillation which was the only technique for recovery at that time. Additional factors such as bioreactor costs, interest rate on the borrowed capital, and rate of return on the investment were also identified as factors which effect the price of fuels derived from renewable resources [14]. In order to reduce the cost of production, attempts were made to utilize cheaper substrates such as molasses, whey permeate, and corn depending upon availability and the region of the world where they are produced. A discussion of low product concentration, low reactor productivities, and low ABE yield will be presented later in this paper.

Starch, as a major component of agricultural crops, is an important substrate for chemical and enzyme production. Results from this laboratory demonstrated incomplete hydrolysis of corn starch by *Clostridium beijerinckii* NCIMB 8052 (formerly *C. acetobutylicum* 8052) and a lengthened time period for growth and solvent production relative to when glucose was the carbohydrate source. Lin and Blaschek [12] reported that higher starch utilization by different strains of *C. beijerinckii* NCIMB 8052 correlated with higher α-amylase activity and butanol production. They also suggested that the α-amylase associated with *C. beijerinckii* NCIMB 8052 was in need of amplification. Results from several laboratories suggested that amylolytic enzyme biosynthesis in various clostridia is subject to catabolite repression by glucose and induction by starch [2,7,8,12,15].

The cost of recovery of butanol is high due to the fact that its concentration in the fermentation broth is low because of product inhibition. The usual concentration of total solvents in the fermentation broth is 20 g l^{-1} of which butanol is only about 13 g l^{-1}. Phillips and Humphrey [19] evaluated the economics of butanol removal from the fermentation broth using distillation and demonstrated that as the concentration of butanol increased from 10 to 40 g l^{-1}, energy saving by a factor of several times is achieved. At 10 g l^{-1} feed butanol, the ratio of tons of oil used for fuel to ratio of tons of 100% recovered butanol is 1.5, while at 40 g l^{-1} feed butanol concentration, this ratio is 0.25. This suggests that a tremendous amount of energy savings can be achieved if the butanol concentration in the fermentation broth is increased from 10 to 25 g l^{-1}.

Hence, the objectives of our investigation became two-fold: (i) to enhance the capability of the *C. beijerinckii* NCIMB 8052 culture to utilize corn starch more effectively (we were interested in producing butanol from corn in the US); and (ii) to increase solvent production. As a result of this investigation, we were able to develop a new strain called *C. beijerinckii* BA101 which

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produced significant amounts of amylase and glucoamylase enzymes. The *C. beijerinckii* BA101 culture was able to hydrolyze starch effectively and the hydrolysis of starch to glucose was no longer a limiting step in the fermentation and resulted in a reduced lag phase for the culture. In addition to the increase in the level of amylases/glucoamylases, the *C. beijerinckii* BA101 culture also demonstrated enhanced solvent yield and total solvent production.

Isolation of hyperamylolytic *C. beijerinckii* BA101

C. beijerinckii BA101 was isolated using *N*-methyl-*N'*-*N*-nitrosoguanidine together with selective enrichment on the glucose analog, 2-deoxyglucose. *C. beijerinckii* BA101 is hyperamylolytic and produces 1.8-fold higher enzyme than the parental strain (*C. beijerinckii* NCIMB 8052) [1] when grown on starch. When grown in P2 medium containing either dextrin or maltose, *C. beijerinckii* BA101 demonstrated up to a two-fold increase in total amylolytic activity over that seen with *C. beijerinckii* NCIMB 8052. Localization studies demonstrated that the amylolytic activities of *C. beijerinckii* BA101 were primarily extracellular on all carbohydrates tested. Since starch cannot penetrate the bacterial cell wall, the lengthened lag phase is a reflection of the time needed to hydrolyze the starch to metabolizable subunits. It has been proposed that this may be achieved by the continual secretion of a low basal level of hydrolyzing enzymes [9,20]. When starch is present in the environment, it is degraded by these enzymes with the release of low-molecular-weight molecules, which in turn enter the cell and further effect induction of the amylolytic enzymes [9,20]. The amylolytic activities of *C. beijerinckii* BA101 were primarily extracellular on all carbohydrate sources tested. It has been reported that an increase in secretion of the amylolytic enzymes into the growth medium by *Clostridium* sp. strain EM1 grown on starch was accompanied by ultrastructural changes in the cell envelope that caused the cells to be leaky [3,13].

Strain characterization: butanol production by *C. beijerinckii* BA101

Batch and free-cell continuous culture

Hyperamylolytic *C. beijerinckii* BA101 produced dramatically elevated levels of butanol and acetone relative to the *C. beijerinckii* 8052 parental strain. The cultures were grown in semi-defined P2 medium containing either 60 g l^{-1} glucose or STAR-DRI 5 maltodextrin. *C. beijerinckii* BA101 consistently produced on the order of 19 g l^{-1} butanol in 20-l batch fermentation. This represented a greater than 100% increase in butanol concentration by the *C. beijerinckii* BA101 strain compared to the parental *C. beijerinckii* NCIMB 8052 strain. In addition, *C. beijerinckii* BA101 produced 9 g l^{-1} acetone, resulting in a total solvent production of 29 g l^{-1}. The parental strain produced 9 g l^{-1} butanol and 4 g l^{-1} acetone. The kinetics of butanol production over time also indicated a more rapid rate of butanol production by *C. beijerinckii* BA101. Lower levels of acetic and butyric acids were produced, which may have been utilized during the late stages of the fermentation [10]. In continuous culture employing *C. beijerinckii* BA101, a volumetric productivity of 1.74 g l^{-1} h^{-1} was achieved (dilution rate 0.20 h^{-1}) compared to 0.58 g l^{-1} h^{-1} for the batch culture. No drift towards acid synthesis was observed during continuous culture for up to 100 h.

Substrate and butanol inhibition

A high substrate concentration was toxic to *C. beijerinckii* BA101 [24]. The maximum cell concentration (1.74 g l^{-1}) was achieved at a 60–102 g l^{-1} glucose concentration. Cell growth was severely inhibited above 158 g l^{-1} glucose. At 200 g l^{-1} glucose, the maximum cell concentration achieved was 0.35 g l^{-1}. The rate of cell growth was not calculated at this substrate concentration. The lag phase for cell growth was 6 h at 60 g l^{-1}, while it was 100 h at the 200 g l^{-1} glucose concentration (Table 1).

For butanol inhibition studies, various amounts of butanol were added to the culture after 24 h of cell growth. At the end of the fermentation, butanol productivity was measured. The *C. beijerinckii* BA101 strain exhibited reactor productivities of 0.42 g l^{-1} h^{-1} (at 7.5 g l^{-1} added butanol) to 0.10 g l^{-1} h^{-1} (at 20 g l^{-1} added butanol) (Table 1). The parental strain (*C. beijerinckii* NCIMB 8052) demonstrated reactor productivities of 0.26 g l^{-1} h^{-1} (at 7.5 g l^{-1} added butanol) to 0.0 g l^{-1} h^{-1} (at 15 g l^{-1} added butanol). This suggested that *C. beijerinckii* BA101 is inhibited less by butanol than the *C. beijerinckii* NCIMB 8052 strain. Butanol production by *C. beijerinckii* BA101 is totally inhibited by 23 g l^{-1}, while it is inhibited at 11 g l^{-1} for *C. beijerinckii* 8052 [22].

Physical and molecular factors involved in solvent production by *C. beijerinckii* BA101

The mRNA expression levels of various genes and activities of the fermentation-associated enzymes in *C. beijerinckii* BA101 were examined in order to identify alterations in gene expression and enzyme activities that may be responsible for enhanced solvent production. The specific activities and mRNA expression levels associated with coenzyme A transferase (CoAT), acetoacetate decarboxylase (AADC), and butyralde-

Table 1 Substrate and butanol inhibition data for *C. beijerinckii* BA101/ *C. beijerinckii* NCIMB 8052

Substrate inhibition (BA101)	Substrate concentration (g l^{-1})			
	60	102	158	200
Average rate of cell growth (h^{-1})	0.04	0.04	0.02	–
Lag time for cell growth (h)	6	15	23	100
Maximum cell concentration (g l^{-1})	1.70	1.70	1.60	0.35

Butanol inhibition C. beijerinckii BA101	Added butanol concentration (g l^{-1})				
	0	7.5	10	15	20
Butanol productivity (g l^{-1} h^{-1})	0.66	0.42	0.40	0.23	0.10
C. beijerinckii NCIMB 8052 Butanol productivity (g l^{-1} h^{-1})	0.40	0.26	0.12	0.00	0.00

hyde dehydrogenase (BADH) were evaluated in the hyper-solvent-producing *C. beijerinckii* BA101 during the exponential growth phase. There were dramatic increases in the specific activities of CoAT, AADC, and BADH and at least a two-fold increase in the mRNA expression levels of the *sol* operon compared to the *C. beijerinckii* NCIMB 8052 parent strain when the organism was grown in P2 medium [4]. These results suggest that the increases in the CoAT, AADC, and BADH activities in *C. beijerinckii* BA101 may have been the result of an increase in *sol* operon expression in *C. beijerinckii* BA101 relative to the *C. beijerinckii* 8052 parental strain. The increase in expression of the *sol* operon and the associated enzyme activities may be responsible for enhanced solvent production by *C. beijerinckii* BA101 [4].

C. beijerinckii BA101 not only produced more solvents at a faster rate than *C. beijerinckii* NCIMB 8052, but also utilized glucose and reassimilated acids more completely [10]. An increase in acid reassimilation due to elevated CoAT and AADC activities may contribute to enhanced solvent production by *C. beijerinckii* BA101 since acetate uptake may enhance the glycolytic rate and, consequently, may increase glucose utilization.

Effect of acetate on solvent production and strain degeneration

Addition of sodium acetate to chemically defined MP2 medium increased and stabilized solvent production by *C. beijerinckii* BA101 and *C. beijerinckii* 8052. *C. beijerinckii* BA101 demonstrated a greater increase in solvent production than the *C. beijerinckii* 8052 parental strain when sodium acetate was added to MP2 medium [5]. In 1L batch fermentations, *C. beijerinckii* BA101 produced 32.6 g l^{-1} total solvents with butanol at 21 g l^{-1} when grown in MP2 medium containing 4.9 g l^{-1} sodium acetate and 80 g l^{-1} glucose. Addition of sodium acetate to chemically defined MP2 medium may prevent strain degeneration by ensuring expression of the *sol* operon in *C. beijerinckii* NCIMB 8052 [6]. This may also be the case for *C. beijerinckii* BA101 since sodium acetate is able to stabilize solvent production by *C. beijerinckii* BA101.

Use of a low-cost commercial medium

Optimization of commercial medium and pilot plant studies

To economize butanol production using *C. beijerinckii* BA101, a cost-effective medium containing corn steep liquor (CSL) was developed [18]. Performance of the *C. beijerinckii* BA101 and *C. beijerinckii* 8052 parental strains was evaluated and it was concluded that unsupplemented CSL was not a suitable substrate, whereas the addition of glucose supported growth and butanol production by both strains. In a batch culture containing 60 g l^{-1} glucose and 16 g l^{-1} CSL solids, 16.5 g l^{-1} butanol was produced compared to 10.7 g l^{-1} butanol when using the *C. beijerinckii* 8052 parental strain. In this medium, *C. beijerinckii* BA101 produced 22 g l^{-1} total solvent compared to 14 g l^{-1} by the *C. beijerinckii* 8052 parental strain. CSL can serve as an economic source of nitrogen, vitamins, amino acids, minerals, and other nutrients. In a similar study, Parekh and Blaschek [16] investigated butanol production using various pentoses/hexoses and CSL medium.

Parekh *et al* [17] compared the fermentation characterization of *C. beijerinckii* 8052 and *C. beijerinckii* BA101 in a pilot plant scale fermentor. In a 20-l fermentation using 60 g l^{-1} glucose–CSL medium, *C. beijerinckii* 8052 produced 14 g l^{-1} total solvents, while *C. beijerinckii* BA101 produced 24.5 g l^{-1} solvents (acetone 7.5 g l^{-1}, butanol 16 g l^{-1}, and ethanol 1 g l^{-1}). Further studies were carried out on a larger scale using an optimized 60 g l^{-1} glucose–CSL medium. In a 200-l fermentor, the *C. beijerinckii* BA101 strain produced 17.8 g l^{-1} butanol, 5.5 g l^{-1} acetone, and 1 g l^{-1} ethanol. The *C. beijerinckii* 8052 parental strain produced 12.7 g l^{-1} butanol and 6 g l^{-1} acetone, resulting in total solvent production of 18.7 g l^{-1}. These results represent a 40% increase in final butanol concentration by the *C. beijerinckii* BA101 mutant strain when compared to the 8052 parent strain. The total solvent (ABE) produced by *C. beijerinckii* BA101 was 24.3 g l^{-1} in the 200-l pilot plant fermentor using glucose and CSL. Parekh *et al* [18] compared the cost of semi-synthetic fermentation medium (P2) [23] and the cost of CSL medium. For the 200-l fermentation medium, the cost of CSL medium was 78% that of the P2 medium.

Product recovery and process integration

Batch culture

Since butanol is toxic to *C. beijerinckii* BA101, studies with *in situ* recovery of butanol were conducted. A pervaporation membrane was used to remove butanol and acetone from the batch fermentation [24]. The membrane used offer selectivities up to 68 and total flux up to 400 g m^{-2} h^{-1} depending upon the butanol concentration and type of solution (model solution or fermentation broth) when using sweep gas. Under vacuum, this membrane is likely to offer flux as high as 5 l m^{-2} h^{-1}. The fermentation and pervaporative butanol recovery were integrated in order to achieve a high productivity and a concentrated butanol stream. In the integrated process, *C. beijerinckii* BA101 produced 51.5 g l^{-1} total solvents compared to a non-integrated batch reactor where 24.2 g l^{-1} total solvents were produced. In this process, a sugar concentration as high as 150 g l^{-1} could be used compared to 60 g l^{-1} in a non-integrated batch reactor. The butanol permeate concentration (in the recovered stream) was 26.4–95.4 g l^{-1} depending upon butanol concentration in the fermentation broth. The *C. beijerinckii* BA101 strain was not negatively affected by the pervaporative conditions. In the integrated process, acids were utilized by the culture to produce ABE. Butanol fermentation and recovery experiments resulted in an increase in productivities of 200% compared to a non-integrated batch reactor. A schematic diagram of pervaporation and an integrated process of fermentation and recovery is shown in Figure 1.

Fed-batch culture

A high sugar concentration is toxic to the culture [24]. Fed-batch fermentation is an industrial technique where concentrated sugar solution can be fed into the reactor while keeping sugar concentration inside the reactor below toxic levels. Such a system was applied to the fermentation–product recovery technique. Solvents were removed by pervaporation from the reactor. In the fed-batch reactor, 165.1 g l^{-1} total solvents were produced compared to the batch culture of 25.3 g l^{-1} [22]. In the integrated process, solvent productivity increased from 0.35 to 0.98 g l^{-1}

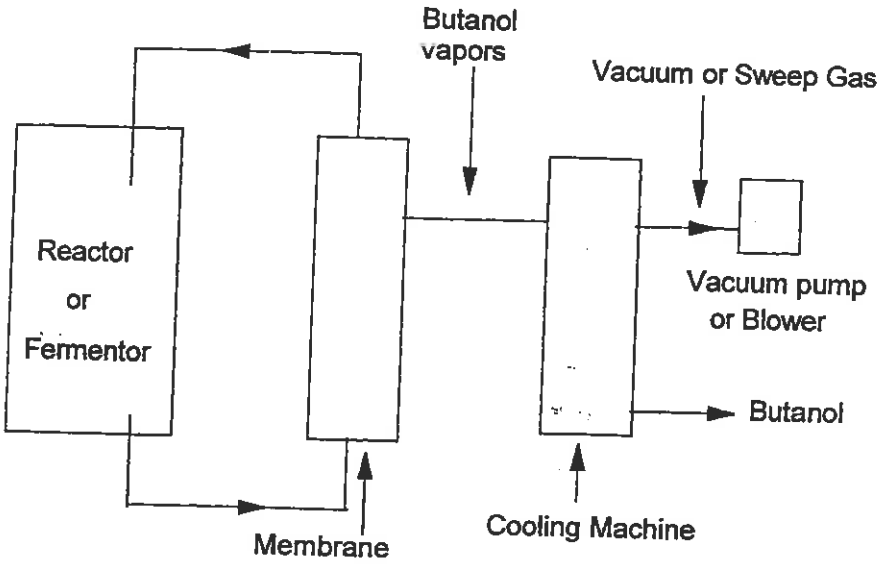

Figure 1 A schematic diagram of butanol removal from model solution or fermentation broth by pervaporation.

h^{-1}. The reactor was fed with 500 g l^{-1} glucose-based P2 medium and this reduced waste and process streams.

Continuous fermentation with immobilized cells

In a batch fermentation reactor, productivity is on the order of 0.35–0.40 g l^{-1} h^{-1}. Such a low reactor productivity requires large reactor volumes, which result in high capital and operational costs. In order to reduce the cost of production, high reactor productivity is desirable, which can be achieved using immobilized cell reactors or membrane cell recycle reactors. Since immobilized cell reactors are cheaper and have simple operation, we employed this technique to achieve a high reactor productivity. *C. beijerinckii* BA101 cells were adsorbed onto crushed brick which had been packed into a vertical reactor. The reactor was fed with sugar solution at the bottom and the product recovered at the top. The immobilized material we used is cheap and easily available and the process of immobilization is simple. Our immobilized cell reactor resulted in a solvent productivity of 15.8 g l^{-1} h^{-1} at a dilution rate of 2.0 h^{-1} [25]. This productivity is about 40 times that of a batch reactor.

Characterization and utilization of the CAK1 virus-like particle

An examination of the replication origin and stability determinant associated with the CAK1 filamentous virus-like particle recovered from *C. beijerinckii* NCIMB 6444 was carried out. Seven deletion derivatives — pCKE, pCEP1, pDT5, pCKP, pDTH102, pYL102E, and pYL102 — were constructed and electroplated into *C. beijerinckii* NCIMB 8052. The successful transformation of pCKE, pDT5, pCKP, pDTH102, pYL102E into *C. beijerinckii* 8052, together with the corresponding recovery of single-stranded DNA from *Escherichia coli*, indicated that the double- and single-stranded replication origins are located on a 0.4-kb CAK1 DNA fragment. Sequence analysis of the putative 0.4-kb replication origin region of CAK1 reveals a nick site containing 22 bp that has homology with plasmids pC194 and pUB110 and suggests the presence of a 2.0-kb DNA region involved in stability. The putative Rep protein of CAK1 contains three conserved motifs and three essential residues of the catalytic site in agreement with Rep proteins associated with the pC194 family. The developed CAK1-derived phagemid designated pYL102E was used successfully to bring about (i) the *manA* gene derived from *Thermoanaerobacterium polysaccharolyticum* in *E. coli* and *C. beijerinckii* NCIMB 8052, and (ii) the *sol* operon derived from *C. acetobutylicum* DSM 792 in *C. beijerinckii* SA-2. The pYL102E vector is currently being used in order to develop a "second generation hyperbutanol-producing" *C. beijerinckii* BA101 strain by using it to deliver various genes important in solventogenesis.

Economics

An economic assessment of butanol production from corn using the newly developed hyperbutanol-producing strain of *C. beijerinckii* BA101 has been completed [21]. For this exercise, butanol is produced in batch reactors and recovered by distillation. The ABE productivity is assumed to be 0.38–0.39 g l^{-1} h^{-1}. For a plant with 150,000 metric tons of acetone and butanol production capacity, the production equipment cost and total working capital cost are US$33.2×10^6 and US$109.6×10^6, respectively. Based on an ABE yield of 0.42, and a corn price of US$1.80 $bushel^{-1}$, butanol production cost is projected to be US$0.25 lb^{-1}. Further improvements such as increased yield of 0.45 and savings in electricity and steam will reduce this price to US$0.20. It was assumed that this plant be located in the midwestern region of the US where corn is easily available.

In general, *C. beijerinckii* BA101 results in improved solvent concentration (20 *vs.* 33 g l^{-1}), solvent yield (0.30 *vs.* 0.50), and reactor productivity (0.30 *vs.* 1.74 g l^{-1} h^{-1}). Reactor productivities as high as 15.8 g l^{-1} h^{-1} have also been achieved in immobilized cell reactors.

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