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Fed-batch acetone-butanol-ethanol fermentation using immobilized *Clostridium acetobutylicum* in calcium alginate beads

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Abstract−Butanol fermentation has garnered renewed interest due to the search for renewable sources of energy. Although acetone-butanol-ethanol (ABE) fermentation has been studied for a long time, the methods utilized yield either a high butanol/ABE productivity and low titer or high butanol/ABE titer at a low productivity. In this work, we report the utilization of a highly dense calcium alginate immobilized *Clostridium acetobutylicum* cells in combination with fed-batch mode of fermentation to attain a high butanol/ABE productivity and concentration. A butanol concentration of up to 21.6 g/L was attained with a productivity of 0.40 g/L·hr, which is a 65% and 192% improvement to the conventional batch fermentation.

Keywords: Butanol, ABE Fermentation, Fed-batch Fermentation, Immobilized *C. acetobutylicum* Cells

INTRODUCTION

n-Butanol has been widely used as a solvent, additive, and intermediate for the production of valuable chemicals such as butyl acrylate and butyl methacrylate [1-3]. It is also being considered as a potential alternative liquid fuel or liquid fuel additive due to its desirable properties and compatibility with current infrastructures for production, distribution, and use [2-4]. Current *n*-butanol production is mainly through chemical synthesis [2-4]. However, with the need to produce chemicals and fuels in a sustainable manner, direct production of *n*-butanol via fermentation has gained renewed interest [5,6].

Fermentative *n*-butanol production naturally occurs in solventogenic *Clostridia*, with *Clostridium acetobutylicum* as one of the most utilized and studied [3,7]. In *C. acetobutylicum*, the fermentation process undergoes an acidogenic phase and solventogenic phase that leads to the production of the solvents acetone, *n*-butanol, and ethanol (ABE) [7]. Solvent formation ceases at *n*-butanol concentration of ~13 g/L [1] and ~20 g/L [3,7] of total solvents after about four days of batch fermentation [3]. The low product titer and productivity are the main drawbacks of this process, which is mainly attributed to the toxicity of the solvents to the production strain [3,7]. Consequently, recovery of butanol from its diluted fermentation broth by the conventional distillation process is highly energy intensive [8,9]. To overcome energy intensive conventional distillation, various separation methods, such as adsorption, liquid-liquid extraction and pervaporation, have been investigated [10]. Various novel metabolic engineering [3,11-13] and integrated in-situ butanol recovery [9,13-15] approaches have been developed to circumvent the toxic effects of the solvents and improve solvent titers [3, 11-13] as well as productivity [9,13].

Various cell immobilization methods have also shown to improve productivity by use of high cell density and/or in continuous mode of fermentation [3,13], while entrapment of *C. acetobutylicum* in calcium alginate increased butanol titer from 15.7 g/L to 20.5 g/L [16,17]. Although the use of continuous mode of fermentation greatly enhances productivity, it also results in lower solvent titer as compared to butanol production via batch or fed-batch fermentation [13,18]. In this study, we explored the combination of high cell density *C. acetobutylicum* entrapment in calcium alginate and fed-batch fermentation to improve both the solvent titer and productivity.

MATERIALS AND METHODS

1. Microorganism and Media

The *n*-butanol producing *Clostridium acetobutylicum* ATCC 824 was obtained from Korean culture center of microorganisms (KCCM, Korea). The media used in all batch cultures were prepared by dissolving the following components in 1 L of distilled water: K_2HPO_4 (0.75 g), KH_2PO_4 (0.75 g), $MgSO_4$ (0.4 g), $FeSO_4 \cdot 7H_2O$ (0.01 g) $MnSO_4 \cdot H_2O$ (0.01 g), Cyseine·HCl (0.5 g), asparagine·H_2O (2.0 g), yeast extract (5.0 g), $(NH_4)_2SO_4$ (2.0 g), glucose (60.0 g) [19]. The media for additional feeding in fed-batch was the same as above except for the amounts of $(NH_4)_2SO_4$ and yeast extract were doubled, while glucose concentration was increased by four to five times. The pH of the media was adjusted to 6.2 by use of 2 N NaOH prior to sterilization by autoclave at 121 °C and 15 psi for 30 minutes.

2. Batch Fermentation

Batch fermentation experiments with free *C. acetobutylicum* were initiated by adding 10% (v/v) of cells inoculated for 24 hours in 500 mL Erlenmeyer flask with a working volume of 250 mL under nitrogen environment.

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3. Fed-batch Fermentation

Fed-batch fermentation was used for both free *C. acetobutylicum* and calcium alginate immobilized *C. acetobutylicum* cells in 1 L fermenters (Biotron, Korea) under anaerobic condition. The starting media and immobilized cell volume used was 400 mL and 200 mL, respectively. Additions of 100 mL fresh media containing 200-300 g/L of glucose were done after fermentation slowed down or ceased. Anaerobic condition was maintained by sparging filter sterilized nitrogen gas whenever an aliquot sample was taken out from the reactor or fresh media was introduced into the bioreactor. The state of fermentation was monitored through its pH, product concentrations and glucose concentration.

4. Immobilization by Calcium Alginate

C. acetobutylicum cells were cultured using the media mentioned in 2.1 for two to three days and harvested by centrifugation at 14,500 rpm and 4 °C for ten minutes. After the supernatant was discarded, the remaining cell paste recovered from one liter of culture was resuspended in 40 mL of supernatant liquid. The resuspended cells were mixed with 60 mL alginate solution (5% w/v). The alginate solution was prepared by dissolving 5 g of sodium alginate (Sigma, USA) to 100 mL of sterilized media.

Gel formation was accomplished by extruding the cell and alginate mixture with the use of 1 mL hypodermic syringe (Kovax, Korea) into the cross-linking solution. Cross-linking solution was prepared by dissolving 5.5 g of $CaCl_2$, 5 g of yeast extract and 10 g of glucose in 1 L of distilled water and then sterilized by autoclave at 121 °C and 15 psi for 30 minutes. The gel beads formed were allowed to harden for 24 hours. The immobilized cells were then pre-activated in the fermentation media for 24 hours prior to actual fermentation experiment.

5. Analysis

The glucose concentration was determined by DNS method [20]. The concentration of reducing sugar was calculated from its absorbance at 540 nm.

The concentrations of acetone, butanol, ethanol, acetic acid, and butyric acid were quantified by use of gas chromatography. A gas chromatograph (CP 9001, Chrompack, Netherlands) with a DB-23 capillary column (30 m in length, 0.25 mm internal diameter, and 0.25 mm film thickness, J&W Scientific, USA) as stationary phase and pure nitrogen gas as mobile phase was used for the GC analysis. The oven temperature was maintained at 35 °C for three minutes, then raised to 125 °C at rate of 40 °C per minute, and then maintained at this temperature for two minutes. The temperatures of the injector and detector were set at 220 °C and 275 °C, respectively [21,22]. The internal standard used was 2-propanol.

RESULTS AND DISCUSSION

1. Batch Fermentation

Two trials of batch ABE fermentation with free *Clostridium acetobutylicum* were carried out in flasks and used as a control for the succeeding fermentation studies. As shown in Fig. 1, the production of ABE experienced a long lag phase of about 36 hours and reached its maximum butanol concentration after about 96 hours. The highest butanol concentration achieved was about 13 g/L, which is consistent with that reported in literature [3].

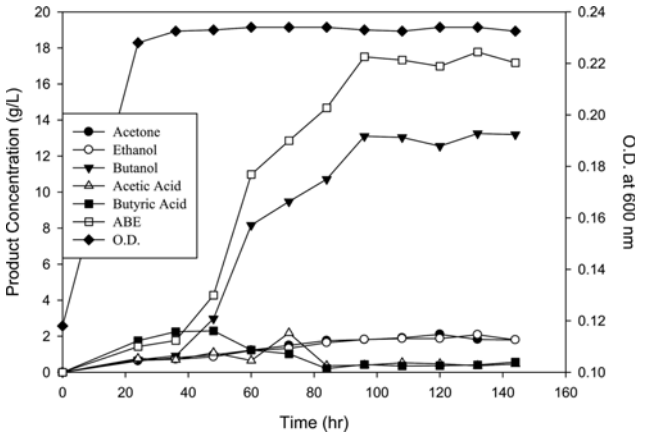

Fig. 1. Batch fermentation by free *C. acetobutylicum* in 500 mL flasks with a working volume of 250 mL at 36 °C.

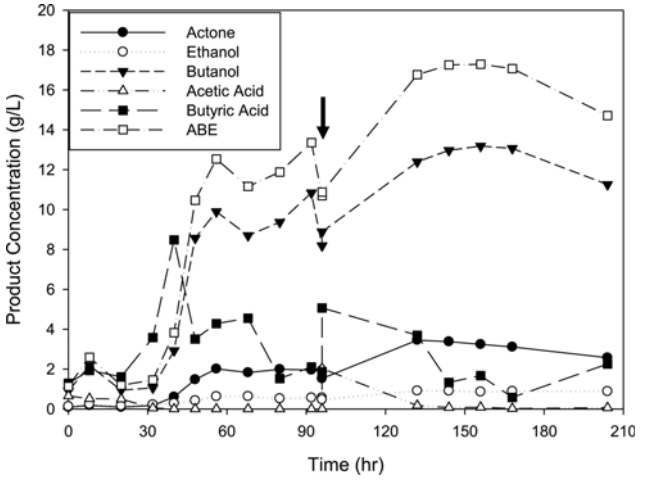

Fig. 2. Fed-batch fermentation by free *C. acetobutylicum* cells in 1 L fermenter. Conditions: initial volume=400 mL, pH control (5.00>), T=36.0±0.5 °C, agitation=100 rpm. Symbol ↓ represents addition of 100 mL of media containing 240 g/L glucose.

2. Fed-batch Fermentation

Fed-batch fermentation was also done for free *C. acetobutylicum* in a 1 L bioreactor. As shown in Fig. 2, the initial batch fermentation (96 hours) produced lower titer of *n*-butanol (8.9 g/L) and a higher concentration of butyric acid (5.0 g/L) as compared to the batch fermentation in flasks. This could be due to the agitation provided in the bioreactor, which was observed to promote acid formation and inhibition of alcohol formation [23-25]. At 96 hours, additional media (100 mL) containing 240 g/L of glucose was supplied to the bioreactor, which resulted in further production of *n*-butanol of up to 13.0 g/L. The additional glucose may have provided the energy necessary to produce more *n*-butanol [25]. However, even with such high excess glucose provided, the maximum butanol titer obtained in the fed-batch fermentation was just the same as the titer obtained with batch fermentation in flasks. This would suggest that the observed maximum butanol concentration in the batch fermentation is the limit for this strain,

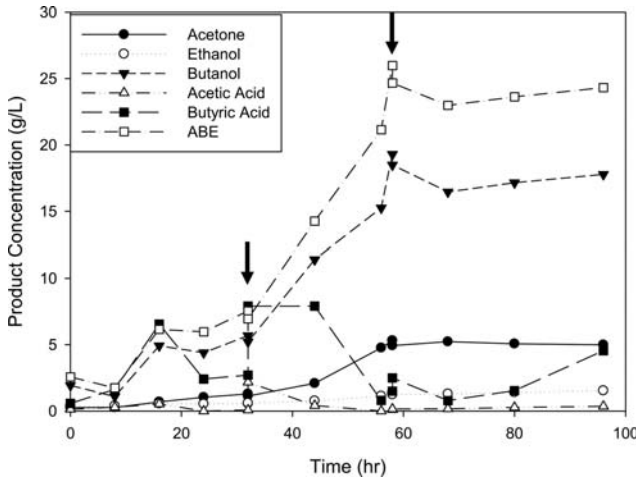

Fig. 3. Fed-batch fermentation by calcium alginate immobilized *C. acetobutylicum* cells cultured for 2 days prior to immobilization in 1 L fermenter. Conditions: initial volume=400 mL, immobilized cell to media volume ratio=1 : 2 (initial), pH control (5.00>), T=36.0±0.5 °C, agitation=100 rpm. Symbol ↓ represents addition of 100 mL of media containing 240 g/L glucose.

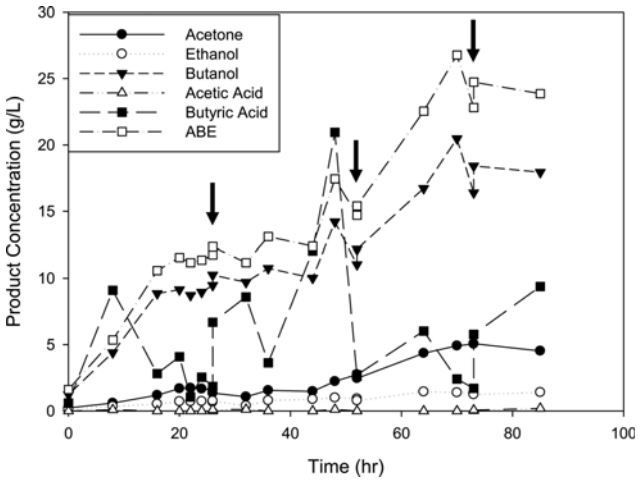

Fig. 4. Fed-batch fermentation by calcium alginate immobilized *C. acetobutylicum* cells cultured for 3 days prior to immobilization in 1 L fermenter. Conditions: initial volume=400 mL, immobilized cell to media volume ratio=1 : 2 (initial), pH control (5.00>), T=36.0±0.5 °C, agitation=100 rpm. Symbol ↓ represents addition of 100 mL of media containing 240 g/L glucose.

which is likely due to product inhibition rather than depletion of nutrients in the broth [3,7].

3. Fermentation with Calcium Alginate Immobilized *C. acetobutylicum* Cells

The cells *C. acetobutylicum* were cultured for two or three days in flasks prior to immobilization. The culture period was chosen in the stationary phase with rapid production of *n*-butanol [26]. With reference to batch *n*-butanol fermentation in flasks (Fig. 1), the two-day and three-day cultured cells should yield mostly vegetative and clostridial form cells, respectively [27]. The maximum *n*-butanol concentration attainable of the two or three day cultured cells entrapped in calcium alginate were determined through fed-batch fermentation [28]. As shown in Figs. 3 and 4, initial batch fermentation of calcium alginate immobilized *C. acetobutylicum* resembles the *n*-butanol production profile of the batch fermentation of free *C. acetobutylicum* in a bioreactor (Fig. 2), except that the calcium alginate immobilized *C. acetobutylicum* cells rapidly produced solvents but ceased after 16-20 hours. To check whether the solvent titer had reached its limit, 100 mL of fresh media containing 240 g/L of glucose was supplied every time solvent production stopped until no further increase in solvent titer was observed. The maximum *n*-butanol titer for *C. acetobutylicum* immobilized cells cultured for two and three days prior to immobilization was 19.3 and 20.5 g/L, respectively. These also resulted in the production of *n*-butanol at a rate of 0.33 and 0.29 g/L·h, respectively (Table 1).

As the *C. acetobutylicum* cells grown for three days prior to immobilization produced slightly higher *n*-butanol titer, the increase in its productivity in a fed-batch fermentation with immobilized *C. acetobutylicum* cells was attempted with a few adjustments. These included reduction of additional fresh media glucose concentration from 240 g/L to 120 g/L, reduction of agitation from 100 rpm to 15 rpm, and introduction of fresh media when the glucose concentration approached to 0 (Fig. 5). The highest *n*-butanol titer observed reached 21.6 g/L at an *n*-butanol production rate of 0.40 g/L·h (Table 1). These corresponded to about 65% and 192% increase in *n*-butanol titer and productivity with respect to the batch

Table 1. Summary of fermentation experiment results

Fermentation trial	Fermentation time (hr)	Product titers (g/L)						Productivity (g/L·hr)	
		Acetone (A)	Ethanol (E)	Butanol (B)	Acetic acid	Butyric acid	Total ABE	B	ABE
Batch free cells (Fig. 1)	96	2.6	1.85	13.1	0.4	0.5	17.55	0.14	0.18
Fed-batch free cells (Fig. 2)	156	3.25	0.86	13.2	0.7	0.4	17.3	0.08	0.11
Fed-batch immobilized cells (2 days culture, Fig. 3)	58	5.3	1.4	19.3	0.1	0.5	26.0	0.33	0.45
Fed-batch immobilized cells (3 days culture, Fig. 4)	70	4.9	1.4	20.5	0.4	0.45	26.8	0.29	0.38
Fed-batch immobilized cells (3 days culture, adjusted, Fig. 5)	54	3.75	0.88	21.55	0.51	2.42	26.2	0.40	0.49

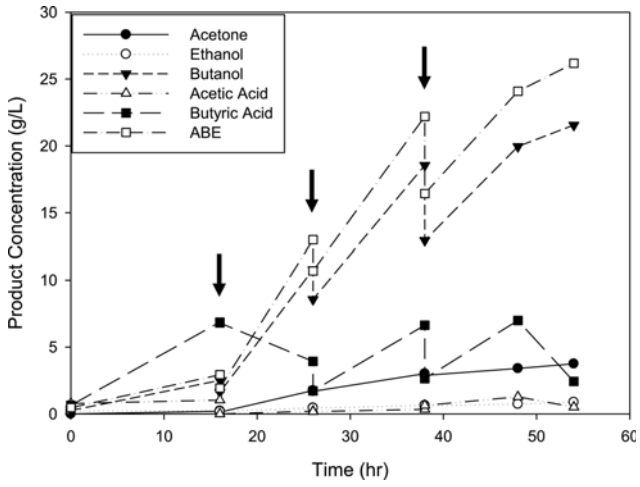

Fig. 5. Fed-batch fermentation by calcium alginate immobilized *C. acetobutylicum* cells cultured for 3 days prior to immobilization in 1 L fermenter with reduced glucose concentration in additional media and reduced agitation. Conditions: initial volume=400 mL, immobilized cell to media volume ratio= 1 : 2 (initial), pH control (5.00>), T=36.0±0.5 °C, agitation= 15 rpm. Symbol ↓ represents addition of 100 mL of media containing 120 g/L glucose.

fermentation with free *C. acetobutylicum*.

The rapid production of solvents can be attributed to the high cell density entrapped into calcium alginate and the state at which the cells were grown prior to immobilization [27]. At these states, the cells are expected to have increased expression of the genes necessary for solvent production, including the enzymes involved in converting glucose to acetone, butanol, and ethanol [26]. Thus, it leads to rapid solvent production with little or no observed lag phase. Improvement in the solvent titer due to fed-batch fermentation of immobilized cells in comparison with the free cells was clearly due to the entrapment of *C. acetobutylicum* cells as similar results were previously reported [16,17]. Alginates are known to be biocompatible, which makes them a suitable carrier for whole cells [29,30]. The hydrophilic nature of calcium alginate may have also aided in the improvement of *n*-butanol titer by about 50% (Table 1) [31]. Wherein, its hydrophilic nature would prevent the hydrophobic solvents released from the immobilized system to reenter the calcium alginate beads. Thus, the solvent concentration within the gel matrix where the cells are immobilized would be lower than the bulk concentration (broth concentration) in a reactor. This would allow the cells to continue solvent production, even though the bulk concentration already exceeded the observed maximum *n*-butanol titer in free *C. acetobutylicum* cells. Aside from *n*-butanol and solvent titer, the cell immobilization by calcium alginate combined with fed-batch fermentation also improved the productivity by at least 100% for both *n*-butanol and ABE (Table 1).

Aside from the timely addition of fresh feed, further improvements in *n*-butanol titer and productivity observed for fed-batch fermentation of calcium alginate immobilized *C. acetobutylicum* (Fig. 5) after adjustments in agitation and fresh feed glucose concentration may have resulted from the reduced agitation rate and glucose concentration of fresh feed. Vigorous agitation has been shown to inhibit *n*-butanol production [23,25] and reduce dissolved hydrogen [24], which aids *n*-butanol production [23,32]. The reduction in agitation speed from 100 to 15 rpm may have lessened the inhibition of *n*-butanol production and increased dissolved hydrogen, resulting in further *n*-butanol production and higher *n*-butanol titer. On the other hand, high substrate concentration has also been known to negatively affect *n*-butanol production due to substrate [33,34]. Thus, adjusting the glucose content to a lower concentration for the fresh feed may have alleviated the substrate inhibitory effect and contributed to the observed slight improvement in n-butanol production.

To the best of our knowledge, the 21.6 g/L of butanol obtained in this work is one of the highest (if not the highest) *n*-butanol titers in the fermentation broth reported in literature [8,11,16,35-37]. Although the *n*-butanol titer achieved in this work greatly reduces the energy required for the recovery of *n*-butanol [8], further improvements in *n*-butanol titer and productivity could be attained if the best *n*-butanol producing strains developed [11,35, 36] were to be used for immobilization in calcium alginate. Considering that almost 65% improvement in *n*-butanol titer was achieved in this work, the use of the best strains could potentially lead to accumulation of 20-32 g/L of *n*-butanol. Moreover, combining in-situ recovery of *n*-butanol at these *n*-butanol titers could lead to further reduction in the energy required for *n*-butanol recovery and further improve its productivity [8,9].

CONCLUSIONS

Low *n*-butanol titre and productivity achieved in the conventional batch butanol fermentations has always been a major drawback that hinders its reutilization as an industrial process to produce *n*-butanol. The strategy of combining cell immobilization in calcium alginate and the use of fed-batch fermentation greatly improved both the *n*-butanol titer and productivity. This led to an *n*-butanol titer of 21.6 g/L and *n*-butanol productivity of 0.40 g/L·hr, which is an improvement of 65% and 192%, respectively.

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