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Biobutanol Production Using Fed-Batch High-Cell Density Extractive Fermentation

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Abstract. Butanol, as a product with specific toxicity for its producer, is necessary to be maintained in low concentration during fermentation. In-situ integrated recovery using extensive volume extraction was used in high-cell-density fermentation to prevent the lag phase in the prime condition. *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564was employed to ferment glucose in extractive fermentation with oleyl alcohol as an extractant. As the results of fed-batch cultures using high cell density with different extractant to broth volume ratios (Ve/Vb), 0.8 g/l butanol concentration in the broth was maintained with a ratio of 10, which was much lower than 4.4 g/l with the ratio of 0.5. Besides, the Ve/Vb ratio of 10 demonstrated a 2.7-fold higher total butanol concentration (28 g/l) than that of 11 g/l obtained witha Ve/Vb ratio of 0.5. These results indicated that larger Ve/Vb improved total butanol concentration by reducing butanol toxicity in broth.

Keywords: *butanol fermentation, extraction, oleyl alcohol, tributyrin, high cell density*

1. Introduction

Butanol had the desirable properties as an alternative for premixed combustion engine fuel. It fulfilled the requirement for the spark timing and octane number as well as minimized particle emission and low carbon monoxide [1-3]. As an intermediate chemical and solvent, butanol had been used for the commercial production of various materials [4-6]. Today, butanol is majorly produced from petrochemicals [7], and biobutanol is also producible via acetone-butanol-ethanol (ABE) fermentation. The global butanol demand as a chemical feedstock has been growing increasingly [8].

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Integrated separation and fermentation enhanced butanol production by maintaining a low concentration of butanol in the media [9]. Liquid-liquid extraction provided remarkable advantages for butanol fermentation, compared with other separation methods. The equipment for the extraction unit is simple and easy to install, the recovery of the extractant is easy, butanol is extracted selectively, and requires low consumption of energy [9-13]. To select the suitable extractant, the butanol distribution coefficient, biocompatibility with butanol producer, and the ease of separation from butanol and the fermentation broth were considered [13].

The total butanol concentration may be increased if the amount of carbon source is increased. However, excessively high substrate concentration would inhibit the metabolism of *Clostridia* [9]. It is shown, by the long lag phase, before the strain can produce organic acid and solvent. As in the batch mode, fermentation substrate concentration would decrease after being consumed by the cells; it is difficult to obtain higher butanol production since the substrate is limited. Glucose concentration above 50 g/l inhibited the substrate consumption [14].

During fed-batch culture, medium or substrate is fed temporarily or continuously into the reactor, but the product is only harvested at the final of the fermentation. The feeding solutionis fed up to the maximum growth of the cell or more simply, until the maximum capacity of thereactor. To maximize the cell formation rate in a constant cell mass yield, the substrate concentration should be maintained at the value that maximizes the specific growth rate [15].

The objective of this study was to develop extractive acetone-butanol-ethanol fermentation with high cell density to enhance substrate consumption in fed-batch fermentation. The work to observe large Ve/Vb with high cell density has not been reported. To confirm what problem prevented the total butanol from increasing, the kinetics of fermentationwere studied by taking a sample at several points. Then, to increase the amount of carbon source but maintaining the substrate concentration at the normal level, fed-batch fermentation was studied.

2. Materials and Methods

2.1 Materials

C. saccharoperbutylacetonicum N1-4 ATCC 13564 was stored in the form of sand stock. Five spoons of this sand stock were inoculated in 9 ml of PG (Potato Glucose) medium in the test tube containing (g/l) grated fresh potato 150, glucose 10, $(NH_4)_2SO_4$ 0.5, and $CaCO_3$

3. *C. saccharoperbutylacetonicum* N1-4 was heat-shocked in 100°C water for 1 minute then incubated at 30°C for 24 h. All suspension was incubated in the anaerobic environment using anaeropack. This suspension was kept at 4°C as the working stock.

2.2 Inoculation

One ml of the spore suspension is refreshed in 9 ml PG medium (10% inoculation) in the test tube. *C. saccharoperbutylacetonicum* N1-4 was heat shocked in 100°C water for 1 minute then incubated at 30°C for 24 h. All suspensions were incubated in the anaerobic environment using anaeropack.

This refreshed culture was then inoculated in the preculture using 90 ml of TYA (Tryptone – Yeast – Acetate) (10% inoculation) containing glucose 20 g/l in a 200 ml flask. The medium was sparged with nitrogen gas for 10 minutes and then incubated for 15 h.

The first preculture was then continued with the second preculture to obtain high cell mass. The second preculture used 1000 ml of TYA and incubated at the optimum temperature for 15 h. Cells from the second preculture were concentrated using centrifugation at 6000 rpm for 25 minutes.

2.3 Fermentation Kinetics of High Cell Density Batch Extractive Fermentation

The concentrated cell was then inoculated in the main culture with 10% inoculation volume to set the initial cell density to 10 g/l dry cell weight. High cell density batch fermentation was conducted using TYA containing 50 g/l glucose with 40 ml working volume in a serum bottle. Oleyl alcohol was used as an extractant with Ve/Vb was 0.1 (4 ml), 0.5 (20 ml), 1(40 ml), and 10 (400 ml). Main fermentation was conducted until 96 h and was sampled for the initial several sampling times for composition in the broth and extractant phase.

2.4 Fed-Batch Fermentation with High Cell Density Extractive Fermentation

The concentrated cell was then inoculated in the main culture with 10% inoculation volume to set the initial cell density to 10 g/l dry cell weight. High cell density fed-batch fermentation was conducted using TYA containing 50 g/l glucose with 60 ml working volume in a jar fermentor. Oleyl alcohol was used as an extractant with Ve/Vb 0.5 (30 ml) and 10 (600 ml). The mixture was agitated at 120 – 130 rpm. Main fermentation was conducted until 96 h

and was sampled for several sampling time compositions in the broth and extractant phases. When the glucose concentration in the broth was less than 20 g/l, 240 g/l glucose solution was fed intothe fermentor.

2.5 Analysis

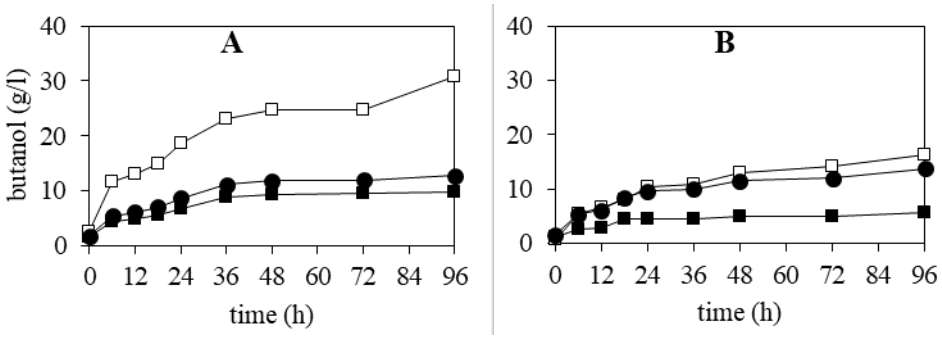
Glucose concentration as substrate in the aqueous phase was measured using High-Pressure Liquid Chromatography with an SH1011 column and a refractive index detector. Sulfuricacid, 0.05 M, was used as the mobile phase at a flow rate of 1.0 mL/min. The HPLC analysis was performed at a column temperature of 50 ºC.

Solvent concentration was analyzed using Flame Ignition Detector Gas Chromatography. Acetone, ethanol, butanol, acetate, and butyrate compounds can be measured. To measure concentration in the aqueous phase, water was used as the solvent for the samples. Tomeasure concentration in the extractant phase, methanol was used as the solvent for the samples. Helium was used as the mobile phase and the analysis was performed at a column temperatureof 50 – 170 ºC.

3. Results and Discussions

3.1 Fermentation Kinetics of High Cell Density Extractive Fermentation

C. saccharoperbutylacetonicum N1-4 strain was further studied due to its operability near ambient temperature [15]. This strain was cultured in high cell density to increase the biomass population from the initial condition [16]. A large extractant to the broth volume ratio was used to study its effect on cell growth and biobutanol fermentation.



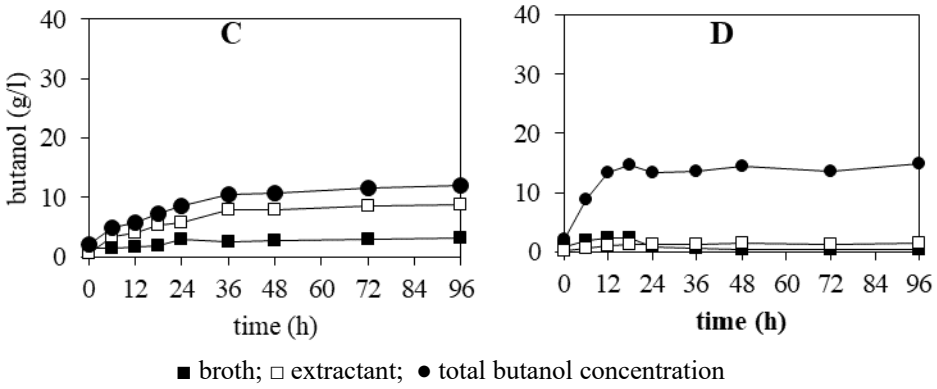


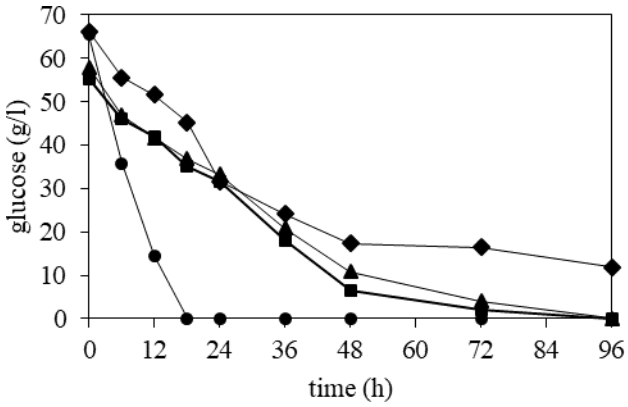
Figure 1. Time course of butanol production by *C. Saccharoperbutylacetonicum* N1-4 with high cell density in Ve/Vb ratio (A) 0.1; (B) 0.5; (C) 1.0; (D) 10.0

Figure 1 shows the fermentation kinetics of the batch extractive fermentation with highcell density. By using the high cell density fermentation, productivity was high in the early time,between 0 – 24 hours (as shown by the steeper gradient). Compared with previous studies, batch extractive fermentation was conducted using biodiesel [15], oleyl alcohol, and dodecanol [17];they resulted in a maximum dry cell weight of 1.9 g/l, 2.1 g/l, and 1.43 g/l respectively. In this study, the cell density was set at 10 g/l at the initial time and kept stable during fermentation, which provided faster substrate consumption and butanol production. However, the final total butanol produced was not significantly different by using various extractant/broth ratios. Especially for the Ve/Vb value of 10, the butanol concentration was kept stable from 18 h up to 96 h.

Table 1. Fermentation performance of *C. saccharoperbutylacetonicum* N1-4 with high celldensity and various Ve/Vb ratio

Ve/Vb	total butanol concentration (g/l)	yield (C-mol/C-mol)		productivity (g/l/h)
		butanol	solvent	max at 0-6 h
0.1	12.7	0.442	0.598	0.621
0.5	13.7	0.742	0.992	0.643
1.0	12.0	0.699	0.921	0.471
10.0	14.8	0.459	0.560	1.138

In Table 1, the highest broth-based total butanol production was obtained using a Ve/Vbratio of 10.0 of 14.8 g/l. Although it did not affect the yield, the productivity of butanol using high cell density fermentation was high, and the highest one was obtained by Ve/Vb ratio of 10.0 of 1.138 g/l/h. It was almost twice of butanol productivity using other ratios. Extraction with ahigh volume of extractant was sufficient to enhance the productivity of the strain by maintaininglow toxicity of butanol in the media [9].



using Ve/Vb ♦ 0.1; ■ 0.5; ▲ 1.0; ● 10.0

Figure 2. Glucose consumption along high cell density batch extractive fermentation usingVe/Vb

The cause of the unenhanced butanol production is related to Figure 2. This figure shows that by using the Ve/Vb ratio of 0.1, the glucose substrate was not consumed totally andremained at the end of fermentation. The toxic concentration of 15 g/l has been approached and caused the strain to enter the death phase [18]. By using the ratios of 0.5 and 1.0, the glucose consumption was similar and consumed after 96 h. By using the Ve/Vb ratio of 10.0, the glucosewas consumed totally, and no glucose remained after 18 h. High cell density was a well-knownmethod to improve glucose consumption [16, 19] but in this study, large Ve/Vb successfully fastened the glucose consumption time from 96 h by using Ve/Vb of 1.0 to only 18 h by Ve/Vb of 10.

The shortage of glucose indicated that butanol production could be increased if only the substrate was fed to the broth. Higher Ve/Vb ratio is essential to optimize the yield of butanol based on the volume of broth. To produce the same amount of butanol, a more significant amount of recyclable extractant is cost-saving rather than using the more expendable nutrient medium.

3.2 Fed-Batch Fermentation with High Cell Density Extractive Fermentation

The result of the previous experiment, which showed rapid consumption of glucose,was attempted to be solved by conducting fed-batch fermentation. Two values of Ve/Vb of 0.5and 10.0 were selected to investigate its effect. Glucose was fed twice at 6 h and 24 h.

Figure 3 shows that broth-based total butanol concentration using Ve/Vb of 10 was around twice the fold of 27.85 g/l compared with Ve/Vb 0.5 of 11.40 g/l. Compared with the similar extractive fed-batch fermentation with the Ve/Vb of 1.0 using oleyl alcohol as extractant, the total broth-based butanol production was 19.5 g/l [20], and using Ve/Vb of 4; the total butanol

production was 12. 12.3 g/l [21]. The butanol concentration in the broth was still low at 0.9 g/l, which was still far below the inhibition limit. As the concentration in the broth was low, so wasthe butanol concentration in the extractant, only 2.7 g/l. As expected from ABE fermentation withhigh cell density, besides the substrate, nutrient was also consumed rapidly and was necessary to be fed to keep the strain activity to produce butanol. A high cell density method was commonly implemented for continuous fermentation [16, 19, 22]. Figure 4 shows that the product concentration in the broth using Ve/Vb of 0.5 was still high, around 5 g/l for butanol and acetone, which may lead to a reducing effect on the strain activity. On the other hand, using the Ve/Vb ratio of 10.0, the product concentration in the broth was lower than 1 g/l. Unexpectedly, the acetone was accumulated in the broth at around 5 g/l. Acetone was also observed to cause toxicity effects to the *Clostridial* strain [23]. Further extraction of acetone during fermentation using an extractant with a high distribution coefficient for acetone such as tributyrin was sufficient to enhance butanol production [9, 21].

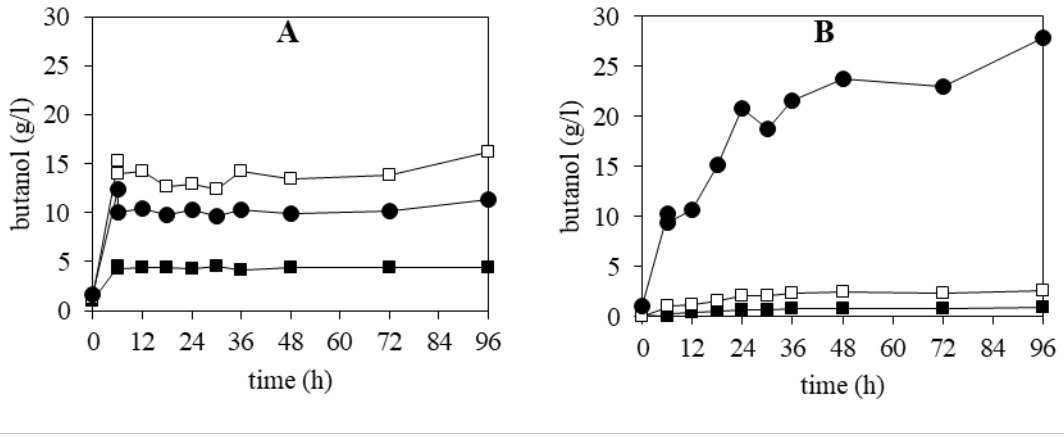
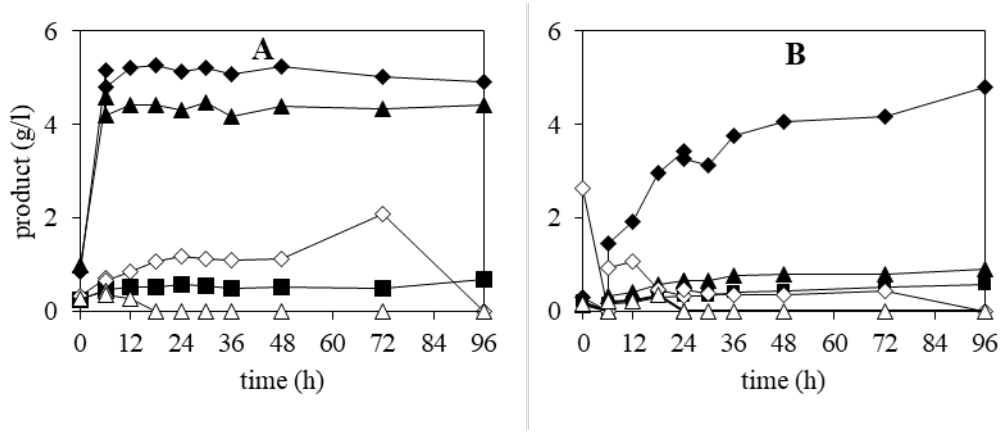


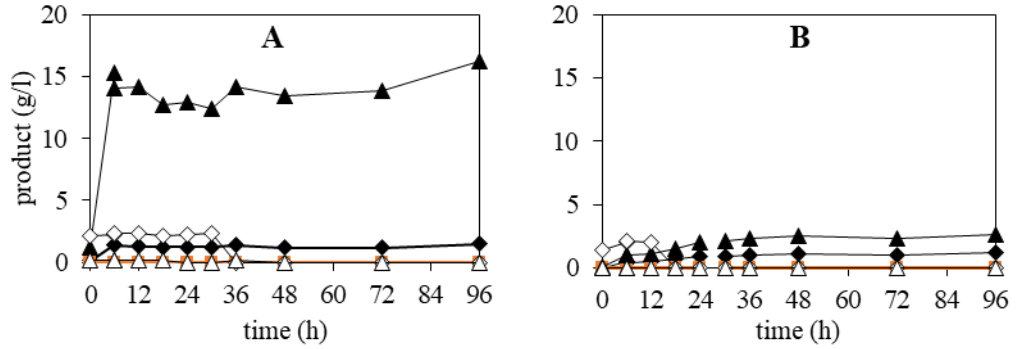
Figure 3. Butanol production using Ve/Vb ratio A) 0.5 and B) 10.0 in fed-batch fermentationusing high cell density



♦ acetone; ■ ethanol; ▲ butanol; ◊ acetate; Δ butyrate

Figure 4. Product concentration in broth using Ve/Vb ratio of A) 0.5 and B) 10.0

Product concentration in the extractant was shown in Figure 5, both for the Ve/Vb ratio of 0.5 and 10.0. The concentration of the product was still low. As the amount of product was similar, using a smaller Ve/Vb ratio, the butanol concentration in the extractant was higher. However, using a larger Ve/Vb ratio, the extraction capacity is expected to be more optimized.



♦ acetone; ■ ethanol; ▲ butanol; ◊ acetate; Δ butyrate

Figure 5. Product concentration in extractant using Ve/Vb ratio of A) 0.5 and B) 10.0

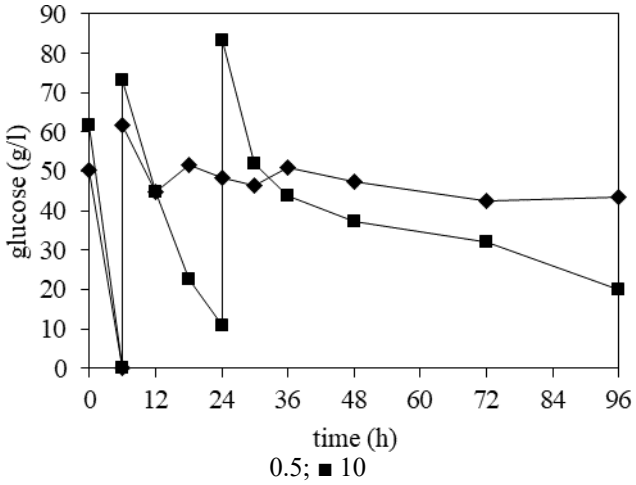


Figure 6. Glucose concentration in broth with high cell density fermentation in Ve/Vb

Even though in Figure 4B, the butanol concentration was still deficient in the broth and extractant, sugar consumption (Figure 6) and butanol production could not increase. Figure 6 shows that initial glucose was consumed very quickly in the first 6 hours. After glucose feeding at 6 h, glucose consumption was stopped at 12 h using Ve/Vb of 0.5 and was continuedup to 96 h using Ve/Vb of 10. Second, glucose feeding was added only to Ve/Vb of 10. The consumed glucose was 4.22 g and 12.18 g, respectively, for Ve/Vb of 0.5 and 10 (Table 2). Applying fed-batch fermentation, large Ve/Vb effectively enhanced the glucose consumption compared to the batch fermentation. The yield of butanol and solvent was increased by 34% from 0.309 to 0.419 C-mol/C-mol and from 0.421 to 0.614 C-mol/C-mol, respectively.

Table 2. Fermentation result

Ve/Vb	total butanol concentration (g/l)	Consumed glucose (g)	yield (C-mol/C-mol)	
			butanol	solvent
0.5	11.40	4.22	0.309	0.431
10.0	27.85	12.18	0.419	0.614

For the cost-saving purification process, it is necessary to obtain the butanol concentration in the extractant as high as possible. Probably, the Ve/Vb ratio of 10 was too large for the fed-batch process. The Ve/Vb ratio needs to be optimized more precisely. High acetone concentration, which was not selectively extracted, may also affect the butanol production.

4. Conclusion

ABE extractive fermentation using high cell density in large extractant volume has been

successfully developed. Larger Ve/Vb improved total butanol concentration and provided higher butanol yield by reducing butanol toxicity. Using high initial cell density, the lag phase during early fermentation was avoided and the higher glucose concentration was consumed. These benefitsare potential for butanol production with high productivity. This study requires further investigation to improve the feasibility of massive production.

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