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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 in order to prevent the lag phase in the prime condition. *Clostridium saccharoperbutylacetonicum* N1-4 ATCC 13564 was employed to ferment glucose in extractive fermentation with oleyl alcohol as 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 2.7-fold higher total butanol concentration (28 g/l) than that 11 g/l obtained with a 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 particles 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], biobutanol is also producable via acetone-butanol-ethanol (ABE) fermentation. The global butanol demand as a chemical feedstock has been growing increasingly [8[.

Integrated separation and fermentation enhanced butanol production by maintaining 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 extraction unit is simple and easy to be installed, the recovery of the extractant is easy, butanol is extracted selectively, and requires low consumption of energy [9–13]. To select the suitable extractant, butanol distribution coefficient, bio-compatibility with butanol producer, and the ease of separation from butanol and the fermentation broth were consider [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

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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 on the final of the fermentation. Feeding solution is fed up to the maximum growth of the cell or more simply, until the maximum capacity of the reactor. 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. In order to confirm what problem prevented the total butanol from increasing, the kinetics of fermentation was studied by taking a sample in 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 were heat-shocked in 100^{o}C water for 1 minute then incubated at 30^{o}C for 24 h. All suspension was incubated in the anaerobic environment using anaeropack. This suspension was kept at 4^{o}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 were heat shocked in 100^{o}C water for 1 minutes then incubated at 30^{o}C for 24 h. All suspension 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 200 ml flask. The medium was sparged with nitrogen gas for 10 minutes, then incubated for 15 h.

The first preculture was then continued with the second preculture to obtain high cell mass. Second preculture used 1000 ml of TYA and incubated in the optimum temperature for 15 h. Cells from the second preculture was concentrated using centrifugation with 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 serum bottle. Oleyl alcohol was used as 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 time 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 jar fermentor. Oleyl alcohol was used as extractant with Ve/Vb was 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 composition in the broth and extractant phase. When the glucose concentration in the broth was less than 20 g/l, 240 g/l glucose solution was fed into the fermentor.

2.5. Analysis

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

Solvent concentration was analyzed using Flame Ignition Detector Gas Chromatography. Acetone, ethanol, butanol, acetate, and butyrate compound can be measured. To measure concentration in aqueous phase, water was used as the solvent for the samples. To measure concentration in 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 temperature of 50 – 170 °C.

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 in order 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.

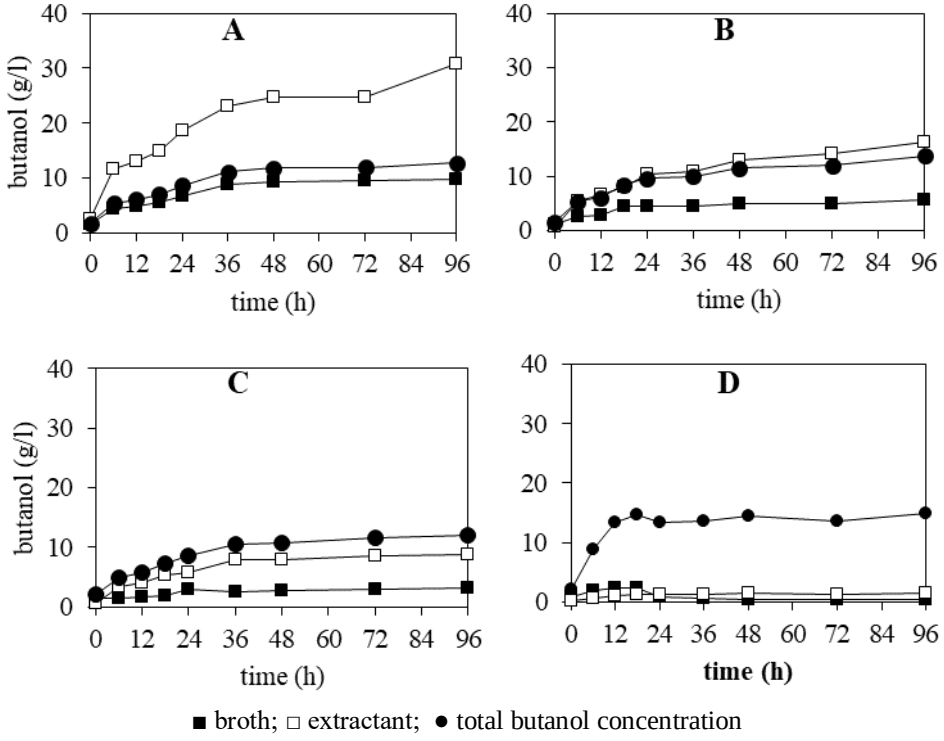


Figure 1. Time course of butanol production by *C. Saccharoperbutylacetonicum* N1-4 with high cell density in Ve/Vb ratio (A) 0.1; (B) 0.5; (C) 1.0; (D) 10.0

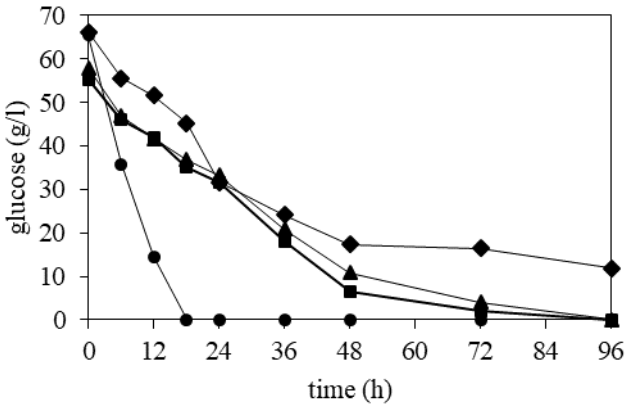
Figure 1 shows the fermentation kinetics of the batch extractive fermentation with high cell density. By using the high cell density fermentation, productivity was high in the early time, between 0 – 24 hours (as shown by the steeper gradient). Compared with previous studies, batch extractive fermentation 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 ratio. Especially for the Ve/Vb value of 10, the butanol concentration was kept stable from the 18 h up to 96 h.

Table 1. Fermentation performance of *C. saccharoperbutylacetonicum* N1-4 with high cell density 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/Vb ratio of 10 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 of 1.138 g/l/h. It was almost twice of butanol productivity using other ratios. Extraction with a high volume of extractant was sufficient to enhance the productivity of the strain by maintaining low 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 using Ve/Vb

The cause of the unenhanced butanol production was related to Figure 2. This figure shows that by using the Ve/Vb ratio of 0.1, the glucose substrate was not consumed totally and remained at the end of fermentation. The toxic concentration 15 g/l has been approached and caused the strain to enter the death phase [18]. By using the ratio 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 glucose was consumed totally, and no glucose remained after 18 h. High cell density was a well-known method to improve the glucose consumption [16, 19], but in this study, large Ve/Vb successfully fasten 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 is more fed to the broth. Higher Ve/Vb ratio is essential to optimize the yield of butanol based on the volume of broth. In order 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 the glucose, was attempted to be solved by conducting fed-batch fermentation. Two values of Ve/Vb of 0.5 and 10 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 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 19.5 g/l [20] and using Ve/Vb of 4; the total butanol production was 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 was the butanol concentration in extractant, only 2.7 g/l. As expected from ABE fermentation with high cell density, beside the substrate, nutrient was also consumed rapidly and was necessary to be fed in order 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 the 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 in around 5 g/l. Acetone was also observed to cause toxicity effect to the *Clostridial* strain [23]. Further extraction of acetone during fermentation using extractant with a high distribution coefficient for acetone such as tributyrin was sufficient to enhance butanol production [9, 21].

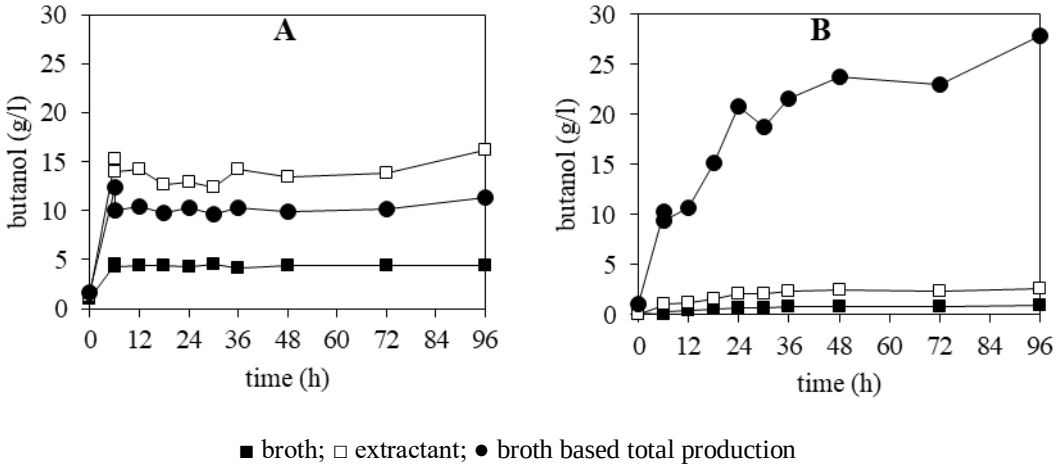
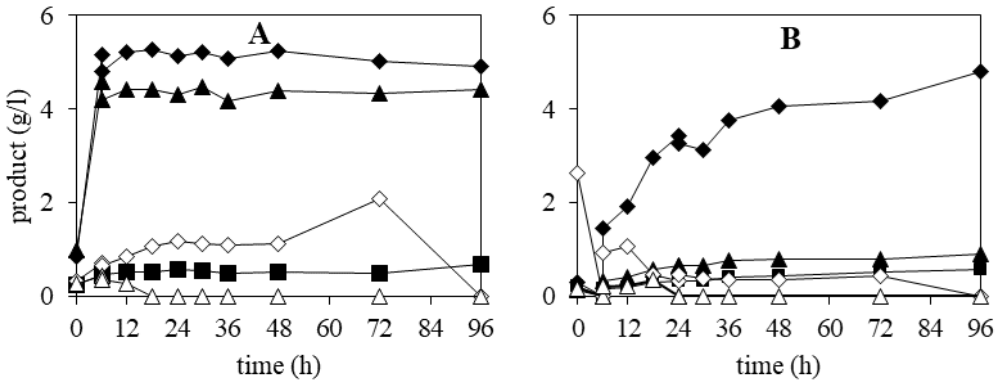


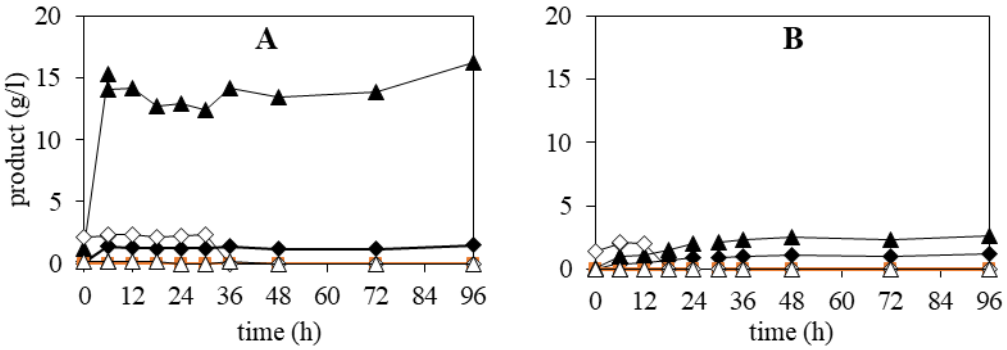
Figure 3. Butanol production using Ve/Vb ratio A) 0.5 and B) 10.0 in fed batch fermentation using high cell density



♦ 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, 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

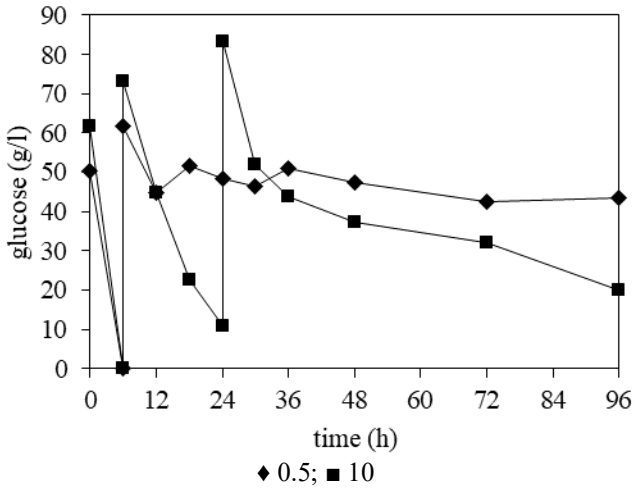


Figure 6. Glucose concentration in broth with high cell density fermentation in Ve/Vb

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 showed 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 continued up 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 provide higher butanol yield by reducing butanol toxicity. Using high initial cell density, lag phase during early fermentation was avoided and the higher glucose concentration was consumed. These benefits are potential for butanol production with high productivity. This study requires further investigation in order to improve the feasibility for massive production.

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