Research Article

Biobutanol Production Using High Cell Density Fermentation in a Large Extractant Volume

Rizki Fitria Darmayanti[a,b*], Yukihiro Tashiro[b], Kenji Sakai[b],
Kenji Sanomoto[b,c], Ari Susanti[a], Bekti Palupi[a], Meta Fitri Rizkiana[a]

[a] Department of Chemical Engineering, University of Jember, Jalan Kalimantan No. 37, Tegalboto, Jember 68121, Indonesia

[b] Faculty of Agriculture, Kyushu University, 744 Motooka Nishi-ku, Fukuoka 819-0395, Japan

[c] Bio-Architecture, Kyushu University, 744 Motooka Nishi-ku, Fukuoka 819-0395, Japan

ABSTRACT. Biobutanol is well known as a suitable substitute for gasoline, which can be applied without engine modification. Butanol toxicity to the producer strain causes difficulties to grow strain of higher than 4 g/L dry cell weight and to produce butanol higher than 20 g/L. Fermentation using high initial cell density has been reported to enhance butanol productivity. In addition, oleyl alcohol has been recognized for effective extraction of butanol because of its selectivity and biocompatibility with reduced the effect of toxicity. Butanol fermentation with high cell density and large extractant volume has not been reported and is expected to improve butanol production in a minimum medium volume setting. *Clostridium saccharoperbutylacetonicum* N1-4, *C. beijerinckii* NCIMB 8052 (8052), and *C. acetobutylicum* ATCC 824 (824) were used in this study. Three kinds of media, TYA, TY, and TY-CaCO3, were used in this conventional extractive fermentation. Then, in situ extractive fermentation with Ve/Vb ratios at 0.1, 0.5, 1.0, and 10 were used. Total butanol concentration was defined as the broth-based total butanol, which is the total amount of butanol produced in broth and extractant per the volume of broth. TYA medium yielded the highest total butanol concentrations at N1-4 (12 g/L), 8052 (11 g/L), and 824 (15 g/L), and the highest partition coefficient (3.7) among the three media with similar Ve/Vb ratio at 0.5. N1-4 yielded the highest increment of total butanol production (22 g/L) in the extractive fermentation with high cell density. Low butanol concentration of 0.8 g/L in the broth was maintained using the extractant at a broth volume ratio (Ve/Vb) much lower than 4.4 g/L with a ratio of 0.5. Ve/Vb ratio of 10 which provided 2-fold higher total butanol concentration (28 g/L) than that of 11 g/L obtained using a Ve/Vb ratio of 0.5. These results indicated that a larger volume of extractant to broth improved total butanol concentration by reducing butanol toxicity and led to high medium based butanol yield in fermentation using high cell density. ©2020. CBIORE-IJRED. All rights reserved

Keywords: biobutanol, high cell density, extraction, fermentation, large extractant volume

1. Introduction

Biobutanol is a known suitable substitute for gasoline as an internal combustion engine fuel. Compared to bioethanol, biobutanol possess several attractive properties. Compared to ethanol, which has a lower flashpoint, butanol is safer to be stored under a larger range of temperatures (Ramey *et al.* 2004). Butanol is also not as corrosive as ethanol of which its hydrophilicity causes damage to the engine (Lapuerta *et al.* 2017). Gasoline fuel mixture using up to 40% butanol has been proven to provide good performance without negative impact on the current spark ignition engine models (Merola *et al.* 2012).

Some native species of *Clostridia* strain have been found to be potential microbes for ABE fermentation. *Clostridium acetobutylicum*, *C. beijerinckii*, and *C. saccharoperbutylacetonicum* were proposed to have similar metabolic pathways (Sakuragi *et al.* 2011). They convert various glucose and starch into acetic acid, lactic acid, and butyric acid via acidogenesis and then converted to acetone, ethanol, and butanol, carbon dioxide, and hydrogen gases. Compared to *C. acetobutylicum* and *C. beijerinckii*, there is only a few number of published reports on butanol production by using *C. saccharoperbutylacetonicum*, a strain which could thrive at a near ambient temperature.

C. saccharoperbutylacetonicum N1-4 (ATCC 13564) has been proven to be able to convert many kinds of substrates to butanol, such as glucose, lactic acid (Oshiro *et al.* 2010), xylose (Zheng *et al.* 2013), cellobiose (Noguchi *et al.* 2013), xylan (Al-Shorgani *et al.* 2011), cellulose (Nakayama *et al.* 2011), sago starch (Madihah *et al.* 2001), cassava (Thang *et al.* 2010), hydrolyzed agricultural waste

* Corresponding author: r.f.darmayanti@unej.ac.id

(Zverlov *et al.* 2006), untreated and hydrolyzed palm oil waste (Mun *et al.* 1995), wheat straw hydrolysate (Qureshi *et al.* 2008), and wood (Zheng *et al.* 2015).

Biobutanol is not produced yet in massive production as biofuel due to its low yield concentration i.e mostly less than 2 % (w/v) in the broth because of high toxicity of butanol to the strain (Lu *et al.* 2012). Butanol concentration of higher than 15 g/L could inhibit the growth of the strain and suppress the ability of cells to maintain internal pH, resulting in a lower intracellular ATP level and reduced glucose uptake (Bowles *et al.* 1985). These bottlenecks require a large amount of energy to recover butanol from the dilute fermentation broth, which drives up the cost of production.

Using the ordinary fermentation approach, it is relatively hard to grow the cell mass of higher than 4 g/L dry cell weight. Previous studies have reported on the use of bioreactors for high free cell density fermentation. High free cell density is feasibly applied, whether through combination with the batch in ethanol fermentation (Gron *et al.* 1996, Rosini *et al.* 1986) or xylitol fermentation (Kim *et al.* 2004). In fed-batch fermentation, high free cell density has been used in combination with adsorption separation (Yang *et al.* 1995). High cell density in continuous culture approach was applied in pH-stat ABE fermentation using butyric acid (Tashiro *et al.* 2004), cell recycling using ultrafiltration (Pierrot *et al.*1986), and ABE fermentation under phosphate limitation (Schlote *et al.* 1986). For high free cell density fermentation, desired parameters could be maintained namely, high cell mass and density, volumetric productivity, yield of desired product, product concentration in the fermentation broth, productivity stability, and reduced fermentation time. Compared to fermentors using immobilized cells, fermentors with free cells are advantageous in providing homogeneity of the media, which facilitates better diffusion in the fermentor that reaches up to 106 g/L DCW with ABE productivity of 11.0 g/L/h (Tashiro *et al.* 2004).

Liquid-liquid extraction is preferred for butanol separation from ABE fermentation broth to reduce butanol concentration in the media for a more supportive growing environment. Oleyl alcohol is known as a suitable biocompatible extractant with a high distribution coefficient of butanol (3.2) (Ranjan *et al.* 2012). By maintaining a low concentration of butanol in the broth, extraction has been proven to enhance total butanol concentration (Darmayanti *et al.* 2018). ABE fermentation using high free cell density with large extractant volume has not been reported previously. Both of these methods improved butanol productivity.

The objective of this study was to investigate ABE extractive fermentation using high cell density. Three types of wild strain and three types of media were used to select one strain with the best performance for this fermentation mode. It is expected to develop biobutanol production with high total butanol concentration by combining high cell density and extraction with large extractant volume.

2. Materials and Methods

2.1 Microorganism

C. acetobutylicum ATCC 824, *C. beijerinckii* NCIMB 8052, and *C. saccharoperbutylacetonicum* N1-4 ATCC 13564

were preserved in the form of sand stock. Five-spatula of this sand stock was inoculated into 9 mL of PG (potato glucose) medium in a test tube containing (g/L) grated fresh potato 150, glucose 10, (NH4) 2SO4 0.5, and CaCO3 3. *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 were heat-shocked in an 80 °C water bath for 10 minutes then incubated at 37 °C water for 1 min followed by incubation at 30 °C for 24 h. *C. saccharoperbutylacetonicum* N1-4 was heat-shocked at 100 °C for 24 h. All suspension was incubated in an anaerobic chamber using Anaeropack (Mitsubishi Gas Chemical, Inc., Tokyo, Japan). This suspension was kept at 4 °C as the working stock.

2.2 Inoculation

One-millilitre of the spore suspension was revived in 9 mL PG medium (10% inoculation) in a test tube. *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 were heat-shocked at 80 °C water for 10 min and then incubated at 37 °C water for 1 min followed by incubation at 30 °C for 24 h. *C. saccharoperbutylacetonicum* N1-4 was heat-shocked at 100 °C for 24 h. All suspension was incubated in anaerobic environment using Anaeropack.

The revived culture was then inoculated into the preculture medium containing 90 mL of TYA (tryptone–yeast–acetate) (10% inoculation) containing glucose at 20 g/L and other components in a 200 mL flask as shown in Table 1. The medium was sparged using nitrogen gas for 10 min, then incubated for 15 h. Bacto tryptone and yeast extract (Becton and Dickinson, Co., Maryland, MD, USA) were used.

2.3 Optimum Medium for Extractive Fermentation

Precultured strain was then inoculated into the main fermentation medium of 20 mL in a 100 mL serum bottle. Three kinds of media were examined namely, TYA, TY (tryptone–yeast), and TYC (tryptone–yeast medium pH-buffered using $CaCO_3$) containing glucose at 50 g/L. This main culture fermentation was integrated with oleyl alcohol as the extractant at $Ve/Vb = 0.5$ or volume of extractant of 10 mL.

Table 1
Composition of medium for preculture and extractive fermentation using *C. acetobutylicum* ATCC 824, *C. beijerinckii* NCIMB 8052, and *C. saccharoperbutylacetonicum* N1-4 ATCC 13564

Component	TYA	TY	TYC
Yeast extract	2 g	2 g	2 g
Tryptone	6 g	6 g	6 g
$MgSO_4.7H_2O$	0.3 g	0.3 g	0.3 g
KH_2PO_4	0.5 g	0.5 g	0.5 g
$FeSO_4.7H_2O$	10 mg	10 mg	10 mg
CH_3COONH_4	3 g	-	-
$(NH_4)_2SO_4$	-	2.57 g	2.57 g
$CaCO_3$	-	-	3 g

The main fermentation was carried out until 96 h and samples for the initial (0 h) and final (96 h) fermentation were collected for the analysis of composition in the broth and extractant phases.

2.4 Strain Selection

After the medium selection experiment, TYA was found to be the most suitable medium for extractive fermentation. Then, the strain selection was conducted using three types of wild strain and at various Ve/Vb ratios. Using the same method for reviving and preculturing, the culture was then continued using a second preculture to obtain higher cell mass. The second preculture used 1,000 mL of TYA with incubation at the optimum temperature for 15 h.

Cells from the second preculture were concentrated using centrifugation at 6,000 rpm for 25 min and then inoculated into the main culture at 10% inoculation volume at an initial cell density of 10 g/L dry cell weight. High cell density batch fermentation was conducted using TYA containing 50 g/L glucose with 8 mL working volume in a 100 mL serum bottle. Oleyl alcohol was used as extractant with Ve/Vb at 0.1 (0.8 mL), 0.5 (4 mL), 1 (8 mL), and 10 (80 mL). The main fermentation was carried out until 96 h and samples for the initial (0 h) and final (96 h) were collected for the analysis of composition in the broth and extractant phases.

2.5 Analysis

The density of the cells was analyzed using optical density of the sample at 562 nm (OD_{562}) using UV-1600 visible spectrophotometer (Bio-Spec; Shimadzu; Kyoto, Japan). One unit at OD562 corresponds to 0.246 g dry cell weight (DCW) per liter. The concentration of glucose in the aqueous phase was analyzed using high-pressure liquid chromatography (HPLC; US HPLC-1210; Jasco, Tokyo, Japan) with a SH1011 column and a refractive index detector (Shodex, Tokyo, Japan). Temperature of the HPLC column was set at 50 °C and the carrier for mobile phase was 0.05 mM H_2SO_4, flowing at 1.0 mL/min, in a 20 mL volume of injection. Acetone, butanol, ethanol, butyric acid, and acetic acid were analyzed using a gas chromatography (GC) system (6890A; Agilent Technologies, Palo Alto, CA, USA), equipped with a flame ionization detector and a 15-m capillary column (INNOWax; i.d. 0.53 mm; 19095N-121; Agilent Technologies). Methanol was used as diluent for the samples of the extractant, and water was used as diluent for the samples of the aqueous phases.

2.6 Determination of Fermentation Parameters

Parameters based on the analyzed data were calculated as follows:

$$K_d = \frac{[Product]_e}{[Product]_b} \tag{1}$$

where K_d is the distribution coefficient of products (acetone, butanol, ethanol, butyric acid, and acetic acid) between the extractant phase and the aqueous phase, $[Product]_e$ and $[Product]_b$ are the concentrations of product (g/L) in the extractant and in the broth, respectively.

$$[BuOH]_{Total} = \frac{[BuOH]_b V_b + [BuOH]_e V_e}{V_b} \tag{2}$$

where $[BuOH]_{Total}$ is the total butanol concentration (g/L-broth), butanol produced in all the phases per broth volume used. $[BuOH]_b$ is the butanol concentrations in the broth (g/L) and the cell beads (g/L), while $[BuOH]_e$ is the butanol concentration in the extractant (g/L). V_b is the total volume of the broth and the cell beads, while V_e is the volume of the extractant (L).

$$Y_{BuOH/S} = \frac{\frac{m_{BuOH} N_{BuOH}}{MW_{BuOH}}}{\frac{m_{Glc} N_{Glc}}{MW_{Glc}}} \tag{3}$$

where $Y_{BuOH/S}$ is the yield of butanol to consumed substrate (C-moles/C-moles), m_{BuOH} is the total amount of butanol produced (g) and m_{Glc} is the total glucose consumed (g). MW_{BuOH} is the molecular weight (g/mole) of butanol and MW_{Glc} is the molecular weight of glucose. N_{BuOH} is the number of carbons in butanol (i.e., 4) and N_{Glc} is the number of carbon in glucose (i.e., 6).

3. Results and Discussions

3.1 Optimum Medium for Extractive Fermentation

ABE fermentation was conducted using three types of media with different composition. They were Tryptone – yeast extract (TY), tryptone – yeast extract – calcium carbonate (TYC) and tryptone – yeast extract – acetate (TYA). The objective of this experiment was investigating the optimum medium for extractive ABE fermentation.

According to the result in Fig. 1, TYA yielded the highest production of biobutanol. As the butanol concentration in the broth increases, the butanol concentration in extractant achieved the aqueous–extractant phase equilibrium at a faster rate. The highest production of butanol achieved by using TYA medium was up to 11.2 g/L for *C. beijerinckii*, 11.6 g/L for *C. saccharoperbutylacetonicum*, and 15.0 g/L for *C. acetobutylicum*.
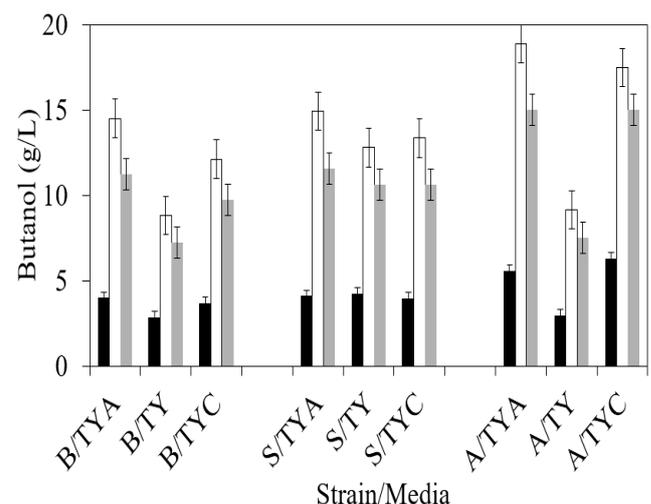
Fig. 1 Total butanol concentration using various fermentation media and Ve/Vb of 0.5 after 96 h; ■ broth, □ extractant, ▨ broth based total production; B = *C. beijerinckii* NCIMB 8052, S = *C. saccharoperbutylacetonicum* N1-4, A = *C. acetobutylicum* ATCC 824

The presence of the acetate holds a role to buffer the solution so that the acidity of the medium does not change significantly, which is necessary to maintaining the activity of the strain to produce butanol (Gao *et al.* 2016). Calcium carbonate also functions as the buffer in the medium (Han *et al.* 2013), but butanol production was lower when TYA is used. This could be attributed to the acetate content in the medium, which stimulates the solventogenesis phase, as it is the intermediate compound in the metabolic pathway between acidogenesis and solventogenesis (Gao *et al.* 2015).

TYA not only increased the performance of fermentation, but also favored the extraction with the highest distribution coefficient of 3.66 among the three kinds of media (Table 2). The electron in the acetate belonging to the oxygen atom in the nucleophilic carboxylic part attracts the hydrogen in the water, while the electrophilic hydrogen atom in the ammonium attracts the oxygen atom in the water (Nahringbauer *et al.* 1970). This phenomenon created the hydrogen bond between ammonium acetate and water, which led to the increase in the tension of aqueous phase. It forces the hydrophobic compound, in this case, butanol to be transitioned into the extractant phase.

As the effect of the transitioned butanol to the extractant selectively, the butanol concentration in the broth was reduced during fermentation. This shifted the strain to produce more butanol than other product (Li *et al.* 2014). This was proven with the highest yield of butanol obtained using TYA, while the other solvent yield remained stable using these three kinds of medium.

3.2 Extractive Fermentation Using High Cell Density in a Large Extractant Volume

Based on the result of the previous experiment, TYA medium was selected for the high cell density experiment. High cell density was applied to enhance the butanol product, which is highly possible to occur in a large volume of extractant. It was obtained by using a double preculture method.

Low butanol concentration of 0.8 g/L in broth (Fig. 2) was maintained at an extractant to broth volume ratio (*Ve/Vb*) of 10, which was much lower than 4.4 g/L with the ratio of 0.5. *Ve/Vb* ratio of 10 provided more than 2-fold higher total butanol concentration (22 g/L) compared to 11 g/L obtained with a *Ve/Vb* ratio of 0.5. Low butanol concentration is necessary to maintain its concentration below the toxicity limit for the producer strain.

Table 2
Distribution coefficient of butanol and product yield in the different fermentation medium using oleyl alcohol as extractant

Strain	Media	K_d[a]	$Y_{BuOH/S}$[b]	
		Butanol	Butanol	Acetone + ethanol
	TYA	3.66	0.510	0.111
C. beijerinckii NCIMB 8052	TY	3.13	0.420	0.094
	TYC	3.31	0.390	0.093
	TYA	3.66	0.559	0.111
C. saccharoperbutyl- acetonicum N1-4	TY	3.04	0.432	0.099
	TYC	3.40	0.433	0.105
	TYA	3.40	0.641	0.170
C. acetobutylicum ATCC 824	TY	3.10	0.519	0.146
	TYC	2.78	0.499	0.156

[a] Distribution coefficient
[b] Product yield based on substrate consumption (C-moles/C-moles)
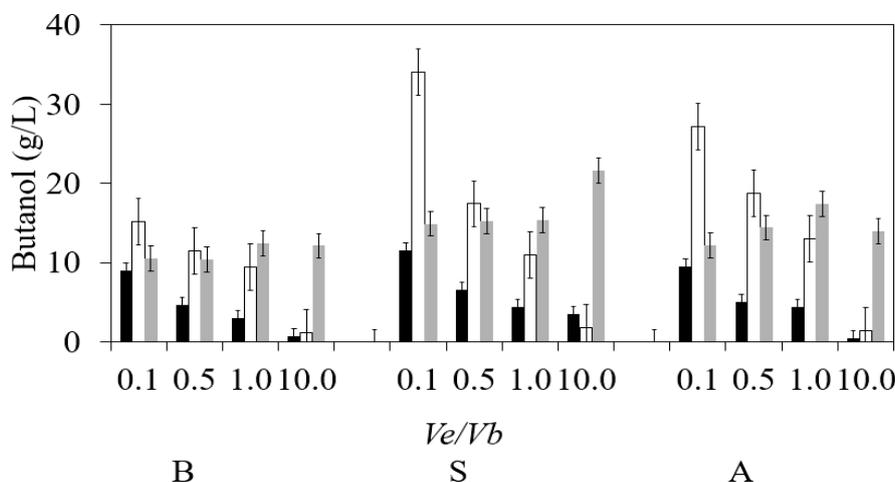
Fig. 2 Effect of *Ve/Vb* to total butanol concentration after 96 h using oleyl alcohol as extractant; ■ broth, □ extractant, ▨ total butanol concentration; B = *C. beijerinckii* NCIMB 8052, S = *C. saccharoperbutylacetonicum* N1-4, A = *C. acetobutylicum* ATCC 824

Table 3
Yield, consumed substrate, and distribution coefficient using high cell density extractive fermentation

Sample	$Y_{BuOH/S}$[a]		Consumed glucose[b]
	Butanol	Solvent	
B/0.1	0.499	0.659	37.4
B/0.5	0.362	0.535	37.3
B/1.0	0.396	0.527	44.1
B/10.0	0.334	0.498	43.2
S/0.1	0.468	0.612	50.3
S/0.5	0.458	0.608	40.8
S/1.0	0.593	0.655	34.5
S/10.0	0.682	0.924	40.2
A/0.1	0.392	0.512	56.7
A/0.5	0.781	0.947	29.2
A/1.0	0.570	0.665	55.9
A/10.0	0.421	0.513	58.4

B = *C. beijerinckii* NCIMB 8052; S = *C. saccharoperbutylacetonicum* N1-4; A = *C. acetobutylicum* ATCC 824; [a]Product yield based on substrate consumption (C-mol/C-mol); [b]Consumed glucose concentration (g/L)
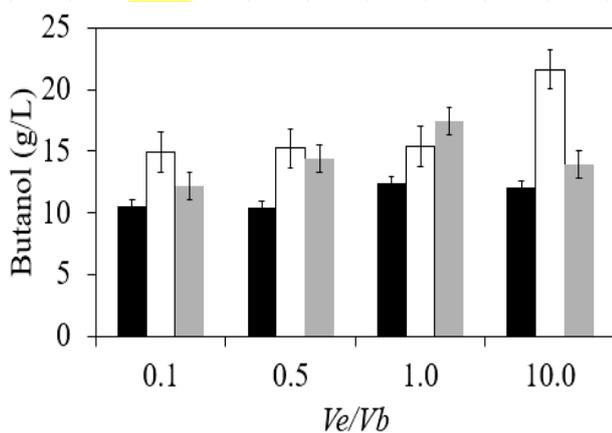
Fig. 3 Total butanol concentration using high cell density extractive fermentation with oleyl alcohol as extractant after 96 h; ■ *C. beijerinckii* NCIMB 8052, □ *C.saccharoperbutylacetonicum* N1-4, ■ *C. acetobutylicum* ATCC 824

When the high cell density culture was applied, maximum broth-based total butanol concentration increased to 12.4 g/L by using *C. beijerinckii*, 21.6 g/L by using *C. saccharoperbutylacetonicum*, and 17.4 g/L by using *C. acetobutylicum*. The result was similar to the batch extractive fermentation using oleyl alcohol using *C. acetobutylicum* ATCC 824 as conducted by Roffler *et al*. (1987) which achieved a butanol concentration of 19.6 g/L. The butanol production could be further increased if more extractant volume was added. The combination of high cell density and large extractant volume had successfully utilized all glucose substrate of 50 g/L to obtain high total broth-based butanol concentration.

Fig. 3 shows the comparison of the three wild strains to produce butanol using high cell density extractive fermentation. N1-4 yielded the highest increment of total butanol production (22 g/L) in the extractive fermentation using high cell density. This three types of strain have the similar metabolic pathway (Tashiro *et al*. 2013), but the result above shows that *C. saccharoperbutylacetonicum*

N1-4 performed fermentation with the highest total butanol production overall. In the inhibitive environment, N1-4 strain is a wild type strain with high robustness compared to other strains (Yao *et al*. 2017). This is an economically beneficial result of the fermentation process because this strain has a lower optimized incubation temperature (30 °C) compared to *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824 (37 °C). Besides, the pH of the media changes during fermentation, with an observed value between 5.3 – 6.4. This is the optimum acidity for *C. saccharoperbutylacetonicum*, meanwhile, for the other two strains, the optimum pH is above 6.5 (Keis *et al*. 2001).

For *C. beijerinckii* the total butanol concentration was not significantly different. Furthermore, a large volume of extractant led to low butanol yield. *C. saccharoperbutylacetonicum* achieved a higher yield of 0.682 (Table 3) when a large *Ve/Vb* of 10.0 was used. Hence, in this study, the highest total butanol concentration was obtained by using the 10.0 *Ve/Vb* ratio. When the extractant volume was increased up to 1.0 *Ve/Vb* ratio, the broth-based butanol total concentration was not significantly different. However, the higher *Ve/Vb* ratio resulted in a higher broth-based butanol total concentration. These results indicate that a larger volume of extractant to broth improved total butanol concentration by reducing butanol toxicity and led to high medium-based butanol yield in fermentation using high cell density.

4. Conclusion

This study established ABE fermentation with a high cell density large *Ve/Vb* ratio. TYA resulted in the highest total butanol concentration, butanol yield, and butanol distribution coefficient among the media examined. When high cell density extractive fermentation was applied, N1-4 yielded the highest total butanol concentration compared to strains 824 and 8052. *Ve/Vb* improved total butanol concentration and provided higher butanol yield

by reducing butanol toxicity. Further investigation is necessary to upscale biobutanol production using fed-batch or continuous mode because high cell density fermentation requires high substrate supply.

Acknowledgments

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