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Original Research Paper

Enhancement of specific butanol yield from glucose with butyrate as co-substrate by *Clostridium beijerinckii* NCIMB 8052

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ABSTRACT

***Clostridium beijerinckii* NCIMB 8052 with solventogenesis genes located in the chromosome has been identified as a promising strain for butanol production by acetone-butanol-ethanol (ABE) fermentation, but its specific butanol yield is not satisfactory. To enhance butanol yield from glucose with butyric acid as co-substrate, effect of concentration of glucose, butyric acid and inoculum were investigated by batch cultures of the strain, while the metabolic characteristics was discussed. It was found that the free butyric acid could be detoxified by enriching inoculum of the strain. Batch feeding of butyric acid could improve the availability of glucose and cell growth, but was less help to improve butanol yield. With 5 g/L butyric acid as the co-substrate of 30 g/L glucose and 0.59 g-dry cell/L inoculums in the culture, a butanol production of about 9.91 g/L was obtained. The specific butanol yield of 0.52 g/g by glucose consumed was the highest so far. The additional butyric acid could not only induce solventogenesis rapidly but also change the fermentation process, resulting in an accelerating formation of butanol and an enhancement of specific butanol yield.**

Key words: *Clostridium beijerinckii*, acetone-butanol-ethanol fermentation, co-substrate, butyric acid, specific butanol yield.

INTRODUCTION

In the past decades, an increasing attention has been devoted to the conversion of biomass to biofuels (bioethanol, biobutanol and biodiesel) as energy substitutes, due to the increasing global energy demand and the limited supply of nonrenewable fossil fuel (Nigam and Singh, 2011). Butanol is higher in heat energy but lower in hygroscopicity, making it fit better with the current fuel distribution infrastructure (Weber *et al.,* 2010). Thus, biobutanol has not only been considered as a promising substitute to automobile fuel in transportation industry, but also attracted an increasing interest in both academia and industrial sectors (Wang *et al.,* 2014). However, biobutanol application has been hampered by the high production cost resulted in the low butanol productivity of fermentation processes (Napoli *et al.,* 2010). More and more researches have focused on the enhancement of butanol yield in the fermentation processes (Formanek *et al.,* 1997; Lee *et al.,* 2008; Al-Shorgani *et al.,* 2012a; Wang *et al.,* 2013; Li *et al.,* 2014).

Fermentative butanol production from sugars is known as acetone-butanol-ethanol (ABE) fermentation (Jones and Woods, 1986; Yang *et al.,* 2013) and has been successfully implemented with the genus *Clostridium*, particularly *C. acetobutylicum* (Lin and Blaschek, 1983; Dürre, 2008). However, the plasmid in where the solventogenesis genes located is easily lost within the fermentation process and would result in organic acid accumulation without switch to solvent (Cornillot *et al.,* 1997). *C. beijerinckii* NCIMB 8052 has been identified as an alternative strain because of its solventogenesis genes located in the chromosome and the stability in solvent production (Wilkinson *et al.,* 1995; Ezeji *et al.,* 2004).

The ABE fermentation by clostridia can be divided into two stages, that is, acidogenesis phase and solventogenesis phase in sequence (Jones and Woods, 1986). The first stage is characterized by the production of acetic and butyric acids along with rapid cell growth. During the second stage, solvents, including acetone, butanol and ethanol, are formed by reutilization of the organic acids produced in the first stage, resulting in a pH increase and cell growth cease. Butyric acid above 1.5 g/L has been reported as a triggering substance to solventogenesis in clostridia (Terracciano and Kashket, 1986; Awang *et al.,* 1988; Richter *et al.,* 2012), and pH of about 5 required by the transformation from acidogenesis to solventogenesis was considered as an indirect effect (Bahl *et al.,* 1982; Monot *et al.,* 1984a). Butanol production by ABE fermentation from glucose and butyric acid as co-substrate has been extensively investigated and 2-3 g/L butyric acid was suggested to enhance glucose utilization and butanol productivity (Al-Shorgani *et al.,* 2012b; Oshiro *et al.,* 2010; Tashiro *et al.,* 2004; Tashiro *et al.,* 2007; Wang *et al.,* 2013). It is significant to optimize the doses of the glucose and butyric acid to enhance butanol yield.

Though some researchers have focused on the fermentative butanol production by *C. beijerinckii* NCIMB 8052, the specific butanol yield from substrates is not satisfactory (Holt *et al.,* 1984; Lee *et al.,* 2008). To enhance the specific butanol yield by strain NCIMB 8052, fermentation of glucose and butyric acid as co-substrate were carried out by batch cultures in the present research. After optimum concentration of glucose, butyric acid and inoculum were identified, effect of batch feeding of butyric acid on glucose conversion and butanol production was further evaluated.

MATERIALS AND METHODS

Microorganism and inoculum preparation

C. beijerinckii NCIMB 8052 was obtained from the China General Microbiological Culture Collection Center (CGMCC). The strain was anaerobically incubated in peptone-yeast glucose (PYG) medium at 37°C for 2 days and then stored as a suspension of spores at 4°C. One liter of the PYG medium contained polypeptone 5.0 g, tryptone 5.0 g, yeast extract 10.0 g, $CaCl_2$ 0.008 g, $MgSO_4 \cdot 7H_2O$ 0.016 g, K_2HPO_4 0.04 g, KH_2PO_4 0.04 g, $NaHCO_3$ 0.4 g, NaCl 0.08 g and glucose 10 g.

For inoculum preparation, the stored spore suspension was transferred to fresh P2 medium and then incubated at 120 rpm and 37°C for 18-22 hours. Prior to the inoculation, the stored culture was left at room temperature (about 22°C) for 30 min to pre-activate the cells. To diminish the effect of fermentation products in the fermented broth and that were attracted to the surface of cells, cells harvested by centrifugation (3000 rpm, 5 min) were washed once with 0.9% NaCl solution and then suspended in 0.5 mL of NaCl solution. The suspended cells were used as the inoculum. The P2 medium contained per 1 L distilled water: glucose 10.0 g, yeast extract 3.0 g, K_2HPO_4 0.5 g, KH_2PO_4 0.5 g, *p*-Amino benzoic acid 0.001 g, thiamine 0.001 g, biotin 0.0001 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $MnSO_4 \cdot H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, NaCl 0.01 g.

ABE Fermentation

Batch cultures were carried out in Hungate tubes (18 × 150 mm). Each of the tube was 18 mL and loaded with 10 mL P2 medium containing a certain concentration of glucose, butyric acid and inoculum. Butyric acid was added as sodium salt. All cultures were incubated at 37°C and 120 rpm for 5 days with an initial pH 5.0 adjusted by 0.1 mol/L HCl and NaOH solution. Each test was performed in triplicate.

To obtain a certain glucose concentration in P2 medium, a designated volume of glucose concentrate solution was added into the mediums. The real concentration in the mediums was detected at the beginning of incubation. Glucose concentrate solutions with concentration of 100, 200 and 300 g/L were prepared, separately. All of the concentrate were sterilized in an autoclave at 121°C for 20 min and then cooled down to room temperature for use.

Analytical Methods

The concentration of glucose was determined following the method reported by Ghose (Ghose, 1987). Cell concentration was estimated by the optical density method using a predetermined correlation between optical density at 600 nm (OD_{600}) and dry cell weight. OD_{600} was measured using a spectrophotometer (UV-2450; SHIMADZU, Japan). The concentration of acetic acid, butyric acid, and solvents in culture supernatant were determined by a gas chromatograph (SP-6800A, Shandong Lunan Instrument Factory, China) equipped with a flame ionization detector and a 30 m capillary column (Stabilwax-DA, i.d.0.32 mm, 11054, Restek). The oven temperature was increased from 50°C to 170°C at the rate of 20°C/min by a temperature-programming. The injector and detector temperatures were both set at

Table 1. Effect of glucose concentration in ABE fermentation.

Initial Glucose (g/L)	Residual (g/L)		Production (g/L)		Consumption (g/L)	
	Glucose	Butyric acid	Cell density	Butanol	Butyric acid	Glucose
0.00±0.00	0.00±0.00	3.29±0.22	0.16±0.01^{A}	0.16±0.07^{A}	0.00±0.26^{A}	0.00±0.00^{A}
9.96±0.60	0.6±0.02	0.53±0.16	1.44±0.07^{B}	4.89±0.50^{B}	2.47±0.25^{BC}	9.36±0.60^{B}
19.67±0.83	1.96±1.15	0.40±0.05	2.18±0.01^{E}	7.17±0.61^{C}	2.77±0.27^{C}	17.71±0.80^{C}
29.64±1.77	7.13±0.92	0.70±0.03	2.01±0.11^{DE}	8.87±0.22^{D}	2.37±0.46^{BC}	22.51±2.60^{D}
38.99±0.84	14.93±2.45	0.65±0.23	1.89±0.20^{CD}	9.21±0.59^{D}	2.33±0.14^{BC}	24.05±3.18^{D}
49.71±0.91	24.35±0.24	0.65±0.21	1.67±0.23^{BC}	8.64±0.57^{D}	2.34±0.06^{BC}	25.35±1.09^{D}
60.16±1.34	37.48±4.03	0.81±0.15	1.59±0.11^{B}	9.36±0.78^{D}	2.21±0.21^{BC}	22.68±3.62^{D}

In the same column, with same superscript are not significantly different at P=0.05 level.

210°C. Nitrogen was used as the carrier gas. OD_{600}, pH, substrate and product concentrations were measured once every 12 or 24 h.

Statistical analysis

Multiple one-way analysis of variance and Duncan's multiple-range test were conducted to investigate the effect of glucose concentration on substrate utilization, butanol production and biomass. All analyses were performed using SPSS 19.0 (IBM Corporation, Armonk, NY, USA). All the results were expressed as mean ± SD unless it was expressly stated.

Calculations

Butanol yield from glucose consumed ($Y_{butanol}$, g/g) were estimated using equation (1),

$$Y_{butanol}=P/S \quad (1)$$

where P is butanol production (g/L) and S is the consumed glucose (g/L).

Specific yield of butanol from substrates ($Y_{butanol/carbon}$, mol/mol) in terms of the moles of carbon were estimated by equation (2),

$$Y_{butanol/carbon} = (C_{butanol}\times4)/(C_{butyrate}\times4 + C_{glucose}\times6) \quad (2)$$

where $C_{butanol}$ is butanol produced (mmol/L), $C_{butyrate}$ and $C_{glucose}$ are consumption of butyric acid (mmol/L) and glucose (mmol/L), respectively.

RESULTS

Effect of glucose concentration on butanol production

Effect of glucose concentration on butanol production by *C. beijerinckii* NCIMB 8052 were investigated in batch cultures. The batch fermentations were performed in P2 medium with a initial butyric acid of 3 g/L and an inoculum of 0.10 g-dry cell/L. Six glucose concentration of 10, 20, 30, 40, 50, and 60 g/L were evaluated, respectively, along with a control that with no glucose in the broth. As illustrated in Table 1, after fermentation for 5 days, only 0.16 g/L butanol was observed in the control cultures. Butanol production was remarkably enhanced upon the supply of glucose. When glucose in the medium increased from 10 to 40 g/L, butanol produced was improved from 4.89 to 9.21 g/L. However, with an initial glucose concentration of about 50 or 60 g/L in the fermentation systems, the butanol production was not sufficient preponderance over that with 30 or 40 g/L glucose in the medium. The statistical analysis (p=0.05) indicated that no significant difference had been revealed in the production of butanol, and the consumption of glucose and butyric acid when glucose ranged from 30-60 g/L in the cultures.

Though the obtained biomass in the fermentation system with an initial glucose concentration of about 20 g/L was the highest among the seven fermentation systems, it was not significantly different from that with an initial glucose about 30 g/L. But the butanol production with 30 g/L glucose was observable higher than that with 20 g/L glucose. The results suggested that the optimum glucose concentration was 30 g/L, with 3 g/L butyric acid as co-substrate, for *C. beijerinckii* NCIMB 8052 to form butanol. It was found that the residual glucose increased remarkably along with the increase of initial glucose concentration in the fermentation systems. There was about 7.1 g/L glucose that still remained in the fermentation system even with the initial glucose of about 30 g/L. Thus, it became an important issue to improve glucose conversion to enhance butanol yield.

It was surprised to find that any ethanol exactly existed in all fermentation processes with glucose or/and butyric acid as carbon source. Normally, *Clostridium* will produce acetone, butanol and ethanol together in ABE

Table 2. Effect of butyric acid concentration in ABE fermentation.

Initial butyric acid (g/L)	Residual (g/L)		Cell density (g/L)	Butanol (g/L)	Consumption (g/L)	
	Glucose	Butyric acid			Butyrate	Glucose
0.04±0.04	16.95±1.43	0.14±0.05	1.73±0.02^{A}	2.07±0.03^{A}	0.00±0.06^{A}	11.68±1.58^{A}
3.03±0.34	12.00±0.78	0.71±0.23	1.41±0.06^{B}	8.63±0.64^{B}	2.32±0.57^{B}	17.58±0.37^{B}
4.75±0.13	24.37±0.59	4.62±0.22	0.4±0.10^{C}	0.43±0.14^{C}	0.13±0.35^{C}	4.08±0.07^{C}
8.02±0.31	28.65±1.13	7.82±0.58	0.1±0.02^{D}	0.00±0.00^{C}	0.20±0.50^{D}	0.33±1.69^{D}
9.80±0.25	29.45±1.35	9.65±0.22	0.1±0.01^{D}	0.00±0.00^{C}	0.15±0.03^{D}	0.72±1.19^{D}

In the same column, with same superscript are not significantly different at P=0.05 level.

fermentation with different ratio depending the type of strains (Lee *et al.,* 2008; Wang *et al.,* 2013). Therefore, the issue of no ethanol produced by *C*. *beijerinckii* NCIMB 8052 in the present research is very sensitive and should be carefully handled. Another *C*. *beijerinckii* NCIMB 8052 has been purchased from the more authoritative organization American Type Culture Collection, and a comparability study will be carried out to reveal why the *C*. *beijerinckii* NCIMB 8052 obtained from CGMCC does not produce ethanol and how it works.

Effect of butyric acid on butanol production

A series concentration of butyric acid was employed to evaluate the butanol production with a constant glucose of 30 g/L and an inoculum of 0.10 g-dry cell/L in the broths. It was found that an increased butyric acid concentration to a certain extent could enhance butanol formation in the ABE fermentation, but an excessive feed would suppress the activity of the strain, resulting in a low butanol production. As shown in Table 2, cell growth and butanol formation of the strain were both seriously suppressed with a butyric acid concentration of 5 g/L in the broth, yet no observable metabolic activity could be found with a butyric acid concentration above 8 g/L.

As shown in Figure 1A, a butanol as low as 2.07 g/L was obtained with only 30 g/L glucose in the broth. When 3 g/L butyric acid as co-substrate of the glucose was added into the broth, the butanol production was remarkably improved to 8.92 g/L (Figure 2A). Cell growth in the cultures, both with and without co-substrate butyric acid, reached almost the same maximum cell density of about 1.8 g-dry cell/L after fermenting for 36 h, and then came into their stationary phases synchronously (Figure 1B and Figure 2B). pH in the cultures with no supply of butyric acid was slightly decreased from the initial 5.0 to 4.2 (Figure 1B). On the contrary, a pH of about 5.0 was maintained throughout the fermentation process in the cultures that were supplied with butyric acid (Figure 2B), which resulted in the buffer effect of sodium butyrate (Lee *et al.,* 2008).

Cultures with glucose as the only carbon source (Figure 1A) experienced an accumulation of acetic and butyric acids in the first 12 h, counted for 0.25 and 0.14 g/L, respectively (data of the acetic acid was not shown in the figures due to its low concentration). But no observable changes in the concentrations were further found since the 36 h. The low concentration of volatile fatty acids indicated that the acidogenesis had been inhibited with the pH less than 5.0 (Monot *et al.,* 1984). It has been reported that acidogenesis phase would convert to solventogenesis phase at pH of about 5.0 (Terracciano and Kashket, 1986). As illustrated in Figure 1A, the fermentation shifted from acidogenesis to solventogenesis within the time from the 12th-24th hour, corresponding to the pro-phase of the exponential growth phase (Figure 1B). Concentrations of acetone and butanol increased rapidly during the exponential phase, and then continued to increase slightly until a maximum of 1.39 g/L and 2.07 g/L, respectively, at the 72nd hour. Though there was about 18.85 g/L glucose left in the broth, the fermentation process stopped from then on.

With 3 g/L butyric acid as co-substrate of glucose, production of solvents could be observed within the first 12 h and the supplied butyric acid was assimilated at a rate corresponding to the solvent accumulation through the exponential phase (12th-36th hours) (Figure 2A). The butanol producing rate within the exponential phase was calculated to be 0.33 g/L/h with a consumption rate of butyric acid and glucose of 0.11 and 0.52 g/L/h, respectively. Butanol formation since the 36th hour was comparatively gradual until a maximum of 8.71 g/L was reached at the 60th hour with a residual glucose of 12.45 g/L and butyric acid of 0.79 g/L in the broth.

Effect of inoculum concentration on butanol production

The results, as shown in Figure 1 and Figure 2, indicated that the supply of butyric acid had caused the rapid occurrence of solventogenesis and the butanol production was enhanced as a result. And Table 1 and Figure 2 illustrated that the optimum glucose concentration was 30 g/L with 3 g/L butyric acid as co-substrate for *C. beijerinckii* NCIMB 8052 to form butanol. However, an excessive supply of butyric acid suppressed

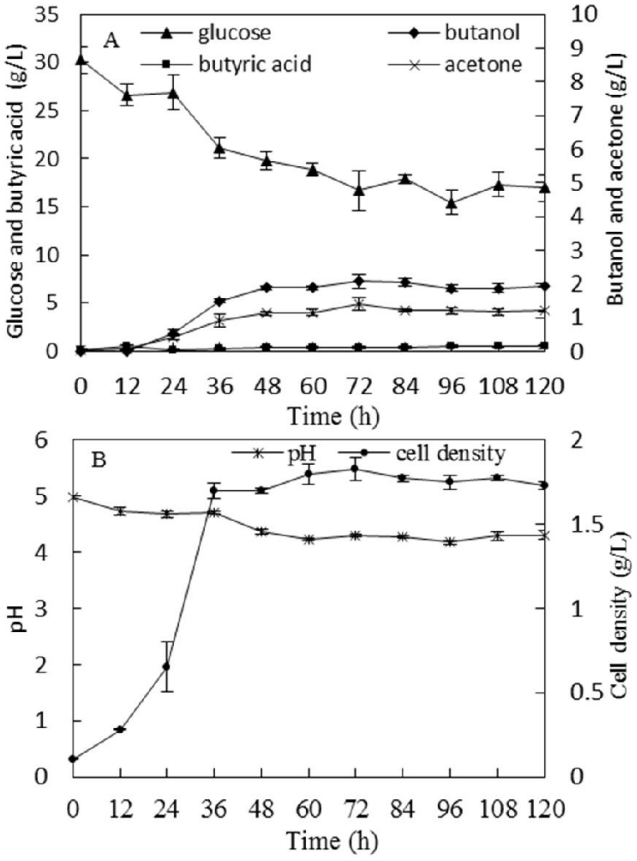

Figure 1. Glucose and products (A), pH and biomass (B) in the batch cultures of C. beijerinckii NCIMB 8052 without the addition of butyric acid.

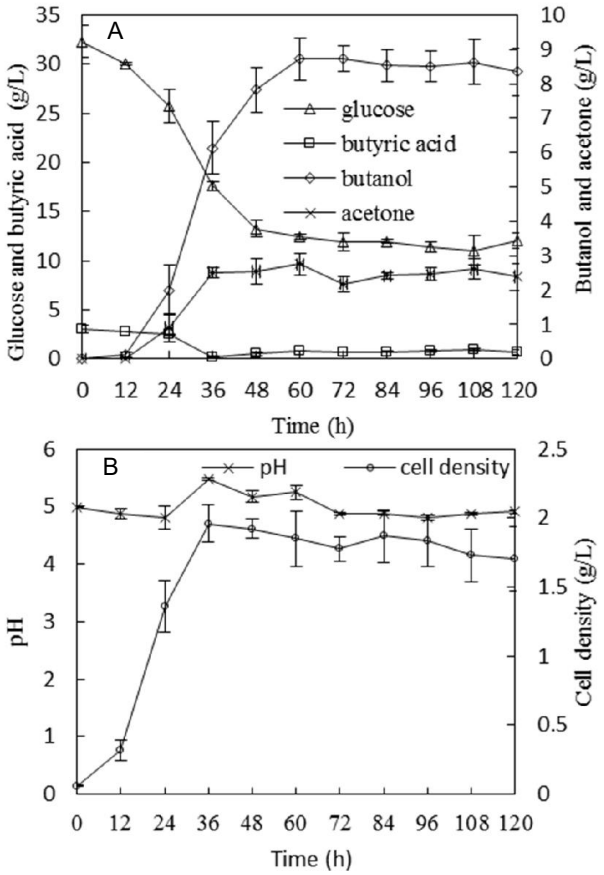

Figure 2. Substrates and products (A), pH and biomass (B) in the batch cultures of C. beijerinckii NCIMB 8052 with the addition of 3 g/L butyric acid.

the activity of *C. beijerinckii* NCIMB 8052, resulting in a lower butanol production (Table 2). To facilitate the assimilation of butyric acid for the further enhancement of butanol production, effect of inoculum concentration on butanol production was evaluated with P2 medium containing 30 g/L glucose and 5 g/L butyric acid at pH 5.0.

As illustrated in Table 3, the butanol production in the cultures with the same 5 g/L butyric acid had been enhanced by the increased inoculums. After fermenting for 120 h, the cell density and butanol concentration in the fermentation systems with an inoculum of 0.11 g-dry cell/L was as low as 0.32 g-dry cell/L and 0.48 g/L, respectively. The cell growth and butanol production were remarkably enhanced to 1.31 g-dry cell/L and 9.91 g/L, respectively, as the inoculum was increased to 0.59 g-dry cell/L. Though a slightly higher butanol production of 10.16 g/L came up to the cultures with an inoculum concentration of 0.75 g-dry cell/L, no significant difference in butanol production, as well as the cell growth and consumption of glucose and butyric acid, was found when compared to that of the 9.91 g/L. Obviously, inoculum concentration of 0.59 g-dry cell/L of *C. beijerinckii* NCIMB 8052 for butanol production from 30 g/L glucose with a co-substrate of 5 g/L butyric acid was more feasible.

Effect of feeding method of butyric acid on butanol production

As illustrated in Table 1 and Table 2, there always was some glucose left in the cultures after fermentation, which should be used to deoxidize butyric acid to produce more butanol in the batch cultures. To improve the glucose utilization in ABE fermentation, effect of batch feeding of butyric acid on glucose consumption, butanol production and cell growth was evaluated by feed-batch culturing. The ABE fermentation was started up at an initial butyric acid and glucose concentration of 5 and 30 g/L, respectively, with 0.59 g-dry cell/L inoculums in the culture (pH5.5). The butyric acid in the broth was intermittently enriched to 3 g/L whenever the concentration decreased to 1.5 g/L or less. The pH all through the fermentation process ranged from 5.0-5.8

Table 3. Butanol production with inoculum concentration using mixture of 30 g/L glucose and 5 g/L butyric acid as substrate.

Inoculum concentration (g/L)	Residual (g/L)		Production (g/L)		Consumption (g/L)	
	Glucose	Butyric acid	Cell density	Butanol	Butyrate	Glucose
0.11±0.03	24.67±0.86	4.61±0.32	0.32±0.09^{A}	0.48±0.15^{A}	0.14±0.45^{A}	4.06±1.69^{A}
0.36±0.05	12.28±2.04	1.49±0.33	0.69±0.12^{B}	5.87±0.40^{B}	3.24±0.39^{B}	17.57±0.97^{B}
0.59±0.09	10.04±0.16	0.48±0.03	1.31±0.26^{C}	9.91±0.71^{C}	4.42±0.37^{C}	19.03±1.91^{BC}
0.75±0.04	7.97±1.81	0.44±0.03	1.30±0.14^{C}	10.16±0.37^{C}	4.64±0.11^{C}	21.66±0.86^{C}
0.85±0.05	8.92±1.63	0.38±0.02	1.47±0.08^{C}	9.22±0.84^{C}	4.79±0.04^{C}	20.72±1.98^{C}

In the same column, with same superscript are not significantly different at P=0.05 level.

naturally.

Figure 3 showed that glucose consumption and cell growth were tremendously enhanced by the supplementary of butyric acid. Since the second supplement at the 48th hour, the total glucose and butyric acid consumption were improved to 28.86 and 5.94 g/L, respectively, observably higher than that with no supplement of butyric acid. Although a cell density of 2.19 g-dry cell/L was obtained at last, the final butanol concentration of 9.84 g/L in broth was very similar to that without any supplemental butyric acid. The results demonstrated that the batch feeding of butyric acid could improve the availability of glucose, but had little effect on butanol formation.

DISCUSSION

Butanol yield by *C. Beijerinckii* NCIMB 8052

For butanol production by ABE fermentation, most researches have focused on *C. acetobutylicum*, *C. saccharoperbutylacetonicum* and C. *beijerinckii* over the past decade (Al-Shorgani *et al.,* 2012a; Formanek *et al.,* 1997; Lee *et al.,* 2008; Li *et al.,* 2014; Tashiro *et al.,* 2004; Wang *et al.,* 2013). Though butyric acid as co-substrate of glucose has extensively been investigated to enhance butanol production in batch cultures, the specific butanol yield by substrate is still not satisfactory due to the incomplete conversion of glucose (Al-Shorgani *et al.,* 2012b; Holt *et al.,* 1984; Monot *et al.,* 1984a; Oshiro *et al.,* 2010; Tashiro *et al.,* 2004; Tashiro *et al.,* 2007; Wang *et al.,* 2013). To enhance butanol yield, effect of concentration of glucose, butyric acid and inoculums were investigated by batch cultures of *C. beijerinckii* NCIMB 8052. Table 3 showed that, with 5 g/L butyric acid as the co-substrate of the 30 g/L glucose and 0.59 g-dry cell/L inoculums in the culture, a butanol production of about 9.91 g/L was obtained.

As illustrated in Table 4, the butanol production of C. *beijerinckii* BA101 and *C. saccharoperbutylacetonicum* N1-4 was as high as 18.60 and 17.00 g/L, respectively (Formanek *et al.,* 1997; Tashiro *et al.,* 2004). But the specific butanol yields of glucose consumed and that of substrates (glucose together with butyric acid) were only 0.32 g/g and 0.53 mol/mol (Formanek *et al.,* 1997), and 0.35 g/g and 0.48 mol/mol (Tashiro *et al.,* 2004), respectively. For *C. beijerinckii* NCIMB 8052, in the present research, the specific butanol yield of glucose consumed and that of substrates reached 0.52 g/g and 0.65 mol/mol, respectively, much higher than the reported ones.

C. beijerinckii NCIMB 8052 has been recognized as a potential strain for butanol production because of its solventogenesis genes in the chromosome and the ability to utilize a broad spectrum of carbohydrates (Cornillot et al., 1997; Lin and Blaschek, 1983). A butanol production of 11.2 g/L had been reported in ABE fermentation of *C. beijerinckii* NCIMB 8052 (Lee *et al.,* 2008; Wang *et al.,* 2013), obviously higher than that of 9.91 g/L in the present research. However, its specific butanol yield of glucose consumed and that of substrates reached 0.45 g/g and 0.62 mol/mol, respectively, less than that obtained in the present research. This result suggested that specific butanol yields of the strains with high butanol production such as *C. beijerinckii* BA101 and *C. saccharoperbutylacetonicum* N1-4 can further be improved by optimizing concentration of butyric acid and inoculums in the batch cultures. Furthermore, strain improvement by gene engineering techniques has suggested an approach to enhance the butanol production of *C. beijerinckii* NCIMB 8052. For example, *C. beijerinckii* BA101 is a mutant strain of *C. beijerinckii* NCIMB 8052, and its butanol produced in batch cultures could reach a surprising concentration as high as 18.6 g/L (Formanek et al., 1997). This result demonstrated that more productive new strains can be developed by mutagenesis and genetic engineering.

Removal of toxicity of butyric acid by inoculum concentration

As known, acetic and butyric acids would be produced in the acidogenesis phase of ABE fermentation and the

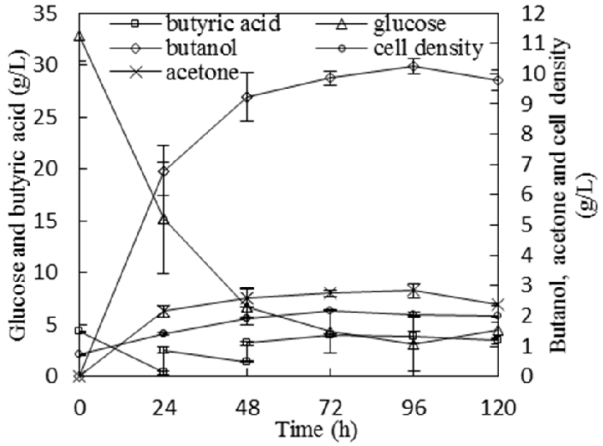

Figure 3. Glucose consumption, butanol production, cell growth and pH in the cultures batch-fed with butyrate.

Table 4. Strains with high butanol production and their butanol yield from glucose and butyric acid as co-substrate in batch cultures.

Strain	Butanol produced (g/L)	Glucose consumed (g/L)	Butyric acid consumed (g/L)	$Y_{butanol}$ [a] (g/g)	$Y_{butanol/carbon}$ [b] (mol/mol)	References
C. *beijerinckii* NCIMB 8052	11.20	24.88	3.96	0.45	0.62	Lee *et al.,* 2008
C. *beijerinckii* NCIMB 8052	10.20	24.87	4.40	0.41	0.56	Wang *et al.*, 2013
C. *beijerinckii* BA101	18.60	57.30	0.00	0.32	0.53	Formanek *et al.,* 1997
C. acetobutylicum ATCC824	13.00	55.00	2.53	0.24	0.36	Li *et al.,* 2014;
C. saccharoperbutylacetonicum N1-4	17.00	49.18	5.00	0.35	0.48	Tashiro *et al.,* 2004
C. saccharoperbutylacetonicum N1-4	9.59	29.97	5.00	0.32	0.42	Al-Shorgani *et al.,* 2012a
C. beijerinckii NCIMB 8052	9.91	19.03	4.42	0.52	0.65	Present study

a, butanol yield from glucose consumed; b, butanol yield from glucose with butyric acid as co-substrate.

free butyric acid by its nature is toxic to the bacteria (Herrero, 1983; Herrero *et al.,* 1985; Jones and Woods, 1986). And the shift of acetic and butyric acids to solvents can act as a detoxification mechanism to avoid the accumulation of organic acids to a toxic level (Bahl *et al.,* 1982; Costa, 1981; George and Chen, 1983; Hartmanis *et al.,* 1984). It has been reported that cell growth would be inhibited by 50% with a total butyric acid of 6-13 g/L or a free butyric acid ranged from 0.25-0.5 g/L (Herrero *et al.,* 1985; Monot *et al.,* 1984a; Monot *et al.,* 1984b). Concentration of free butyric acid is mostly related to the pH in a fermentation system. The percentage of free form in the total butyric acid would increase from 6% to 66% when pH decreases from 6.0 to 4.5 (Häggström, 1985). To trigger solventogenesis, as shown in Figure 1 and Figure 2, the pH in the batch cultures of *C. beijerinckii* NCIMB 8052 was maintained at about 5.0. With pH 5.0, there was about 40% of the total butyric acid existed in free form. This suggested that the supplied butyric acid as co-substrate for butanol production should be less than 2 g/L in total.

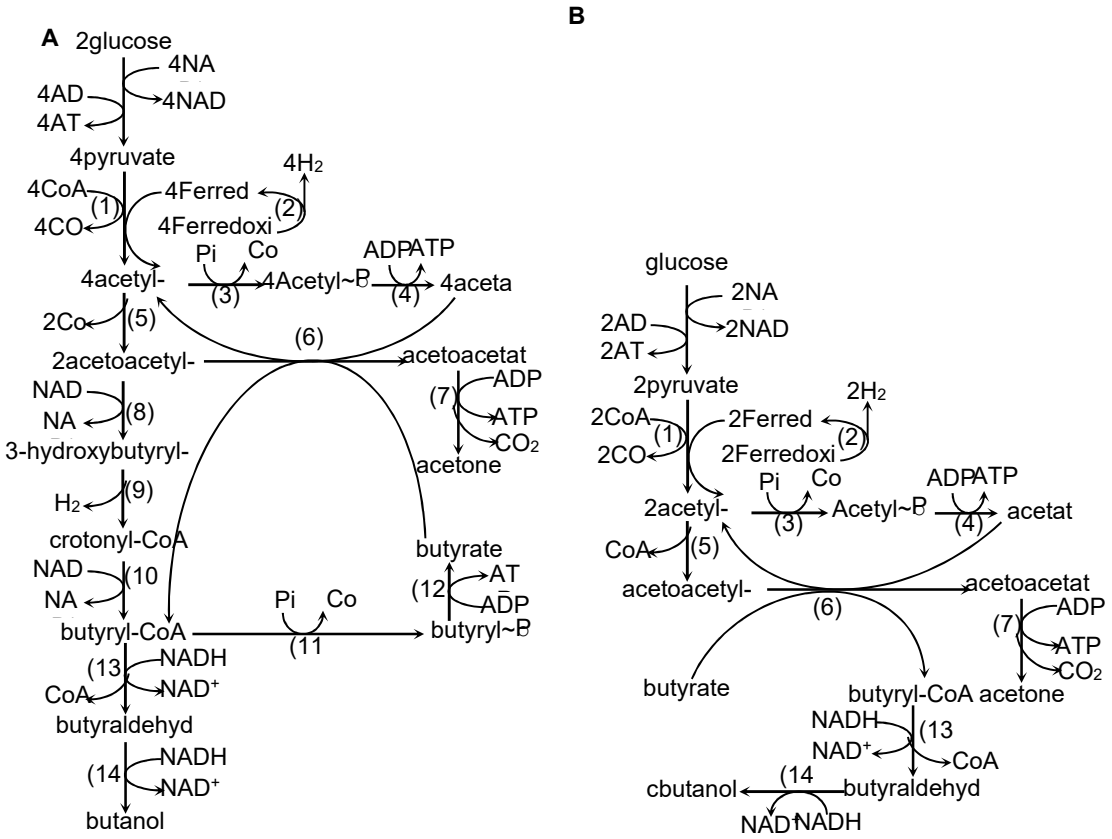

Figure 4. Metabolic pathways to solvents in C.beijerinckii NCIMB 8052. (A) Metabolic pathways to solvents by glucose fermentation. (B) Metabolic pathways of external butyrate to solvents with glucose as co-substrate. Enzymes are indicated by numbers as follows: (1) pyruvate-ferredoxinoxidoreductase; (2) hydrogenase; (3) phosphate acetyltransferase; (4) acetate kinase; (5) acetyl-CoA acetyltransferase; (6) CoA-transferase; (7) acetoacetate decarboxylase; (8) 3-hydroxybutyryl-CoA dehydrogenase; (9) crotonase; (10) butyryl-CoA dehydrogenase; (11) phosphate butyltransferase; (12) butyrate kinase; (13) butyraldehyde dehydrogenase; (14) butanol dehydrogenase.

Table 3 showed that, with 30 g/L glucose and 5 g/L butyric acid in the broth, inoculated with 0.11 g-dry cell/L, the growth and metabolic activity of *C. beijerinckii* NCIMB 8052 were badly inhibited at pH about 5.0. When the inoculum concentration was enriched to 0.59 g-dry cell/L, cell growth and butanol production were remarkably improved to 1.31 g-dry cell/L and 9.91 g/L, respectively. When the inoculum volume was further enhanced, increment in cell growth and butanol production were no longer observed. The result suggested that the toxicity of free butyric acid could be removed to a certain extent by enrichment of inoculum. The inoculum concentration ranged from 0.59-0.75 g-dry cell/L in batch cultures was found to be favorable for butanol production by *C. beijerinckii* NCIMB 8052.

Metabolism of the strain in mixture of glucose and butyric acid

As shown in Figure 1, with glucose as the only carbon source in the cultures, butyric acid formation was observed in the first 12 h without any butanol produced. Thereafter, butanol formation occurred and was accumulated in the fermentation system until the stationary phase came up since the 48th hour. The result indicated that acidogenesis phase and solventogenesis phase had occurred in sequence within the ABE fermentation process (Jones and Woods, 1986; Napoli *et al.,* 2010), which was in accordance with the two-phase ABE fermentation pathways in C. *beijerinckii* NCIMB 8052 (Figure 4A). Because the actual fermentation process is rather complex and very delicate to be efficiently controlled (Chauvatcharin *et al.,* 1998), the butanol yield from glucose is relatively low in conventional ABE fermentation. With no additional supply of butyric acid in the broth, only 2.07 g/L of butanol was produced by C. *beijerinckii* NCIMB 8052 in the present research (Figure 1A).

On the other hand, as the triggering substance to solventogenesis in clostridia at a controlled pH of about

5.0, butyric acid has been extensively investigated as co-substrate of glucose to enhance butanol yield (Awang *et al.,* 1988; Bahl *et al.,* 1982; Richter *et al.,* 2012; Yang *et al.,* 2013). It was found that the extrinsically supplied butyric acid could not only enhance the butanol yield, but also change the fermentation process. As illustrated in Figure 2, the solventogenesis could promptly occur in the fermentation systems with butyric acid as the co-substrate of glucose, and no significant acidogenesis phase had be identified from the ABE fermentation process with a maximum butanol of about 8.71 g/L observed at the 60th hour. It was worthwhile to note that the consumption of butyric acid was slow within the first 24 h, but became faster in the next 12 h along with a rapid production of butanol. The result indicated that the fermentation pathways might have transformed.

The slight decrease of butyric acid concentration in fermentation systems with glucose and its co-substrate butyric acid coexisted in the medium (Figure 2) indicated that conventional ABE fermentation pathways (Figure 4A) dominated the fermentation process within the first 24 h, while conversion of the additional butyric acid to butanol was weak. Within the time from the 24th to the 36th hours, consumption of the butyric acid sped up, indicating more of the additional butyric acid was converted to butanol (Figure 2). Within this phase, the acetoacetyl-CoA from glycolysis was no longer converted to 3-hydroxybutyryl-CoA and further to butyryl-CoA and butanol, but used to react with butyric acid to form butyryl-CoA and acetone (Figure 4B). The formed butyryl-CoA was further reduced to butanol with the NADH supplied by glycolysis (Shinto *et al.,* 2007; Tashiro *et al.,* 2007). Thus, accumulation of butanol and acetone could be observed both together in this phase. The shortened metabolic path had not only sped up the butanol formation but also enhanced the specific butanol yield by glucose and butyric acid as co-substrates.

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