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Analysis Effect of Butyrate Addition on Butanol Production by PVA-Immobilized *Clostridium acetobutylicum* ATCC 824 in Batch Culture Fermentation

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ABSTRACT

The cultivation of PVA-immobilized *Clostridium acetobutylicum* ATCC 824 in CRM medium including 60 g/L of glucose as a carbon source was used to produce butanol. The addition of various butyrate concentrations was analyzed to stimulate metabolic pathways toward butanol production. Anaerobic incubation conditions were maintained at a temperature of 37°C for 24-36 h. The A-B-E batch fermentation was performed at the optimum condition as follows: temperature of 37°C, the glucose concentration of 60 g/L, and pH of 4.5 (controlled). The highest butanol production of 14.94 g/L was achieved with butyrare addition of 2.5 g/L, and the highest butanol yield of 0.61 mol butanol/mol glucose was achieved at the addition of 5 g/L. The experimental result showed that butanol production and yield increase when the addition of butyrare is less than 5 g/L. Despite that, with further higher butyrare concentration, butanol production decreases accompanied by decreased glucose consumption. The lowest butanol production of 11.74 g/L was obtained at the addition of 7.5 g/L butyrare.

Keywords: Butanol, butyrate, Clostridium acetobutylicum ATCC 824, glucose, batch culture fermentation

Introduction

Nowadays, butanol and biobutanol can be alternative fuels, which is more promising than ethanol. The properties of butanol are more similar to gasoline and have several advantages such as higher energy content, less volatility, hygroscopicity, less corrosive, and better mixing with gasoline in any proportion (Lin et al., 2015). To enhance the butanol production performance and improve the feasibility and cost for industrial-scale butanol production, several methods such as increasing the internal concentration of acids, particularly the butyric acid, to stimulate the metabolic switch from acidogenesis to solventogenesis by supplementing various uncouplers (Gottwald & Gottschalk, 1985) or organic acids (Tashiro et al., 2004; Tashiro et al., 2007) or decreasing the pH (Bahl et al., 1982; Geng& Park, 1993) was investigated.

Production of butanol through the Acetone-Butanol-Ethanol (ABE) fermentation by using the solventogenic clostridia has recently attracted a considerable amount of attention due to its value as a promising product (Durre, 2007). ABE fermentation process includes two phases. The first phase is known as the acidogenic phase. During this phase, the acid formation pathways are activated in which carbohydrate substrates particularly glucose are fermented to organic acids. The products of this phase are acetate, butyrate, hydrogen, and carbon dioxide. This acidogenic phase usually occurs during the exponential growth phase of *Clostridium* species. The second

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phase is the solventogenic phase in which acids reassimilation occurs. The products obtained in this phase are mainly acetone, butanol, and ethanol (Jojima et al., 2008).

Several Clostridia microorganisms which are typically required under anaerobic conditions and rod-shaped bacteria can use to produce butanol. These species can utilize different substrates such as organic and amino acids, other organic compounds, polyalcohols, and different sugars (from simple monosaccharides including several hexoses and pentoses to complex polysaccharides) to produce butanol and other solvents. *C. beijerinckii, C. acetobutylicum, C. saccharoacetobutylicum and C. saccaroperbutylacetonicum* can achieve butanol production performance with higher butanol yield. According to a previous study of butanol production by *Clostridium acetobutylicum* YM1 in batch culture fermentation, the highest butanol concentrations, yield, and productivity of 16.50 ± 0.8 g/L, 0.345 g/g, and 0.163 g/L h, respectively was obtained under uncontrolled pH condition and butyric acid addition of 4.0 g/L (Al-Shorgani et al., 2017). Others, *Clostridium pasteurianum* CH4 in batch production of butanol can produce the highest butanol production of 12.0 g/L and yield of 0.34 mol butanol/mol glycerol (Lin et al., 2015).

This research was focused to enhance the butanol production by *C. acetobutylicum* ATCC 824 in batch culture fermentation with and without the addition of butyric acid. The result was to investigate the effect of butyric acid addition on butanol production by strain ATCC 824.

Material and Methods

Microorganism

Clostridium acetobutylicum ATCC 824 was purchased from the Bioresource Collection and Research Center (BCRC), Taiwan. Each experiment was started from a spore culture of *C. acetobutylicum*. To grow the spores of *C. acetobutylicum*, spore culture was heat shocked for 2 min at 70°C and transferred to fresh pre-culture medium (PCM) at 37°C for 24-36 h. After pre-culture, the culture was transferred to the fermentation medium (clostridium reactor medium, CRM) (Li et al., 2011) and it was inoculated with initial cell loading of 0.017 g/l (OD_{600}=0.05).

Substrate and Medium Preparation

The composition of pre-culture medium (PCM) as follows (in g/L): glucose, 20; yeast extract, 5; tryptone, 1; biotin, 0.01; p-Aminobenzoic acid, 0.01; Na_2SO_4, 0.18; K_2HPO_4 , 0.175; $Na_2MoO_4 \cdot 2H_2O$, 0.24; $CoCl_2 \cdot 6H_2O$, 0.24; $CaCl_2 \cdot 2H_2O$, 1.5; $FeCl_3$,16.20; $CuSO_4$, 0.16; $ZnSO_4 \cdot 7H_2O$, 0.52; $MnSO_4 \cdot H_2O$, 1.7; $MgSO_4 \cdot 7H_2O$, 24.6; H_2SO_4 (6M) 28 ml. The pH was adjusted to 4.8 before sterilization. The pH was not controlled in pre-culture. The butanol fermentation medium, which was known as clostridia reactor medium (CRM) had the following composition (in g/L): glucose, 60; yeast extract, 5; KH_2PO_4, 0.75; K_2HPO_4, 0.75; $(NH_4)_2SO_4$, 2; NaCl, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; L-cysteine-HCl, 0.5. Initial pH was adjusted to around 6 before sterilization.

Immobilization of Clostridium acetobutylicum with Poly-vinyl-alcohol (PVA)

For cell immobilization with poly-vinyl-alcohol (PVA) gel, 2500 ml of culture solution (at 5.0-6.0 of OD_{600}) including *Clostridium acetobutylicum* was centrifuged (10000 rpm, 5 minutes, 4°C). After centrifugation, the supernatant was removed and the pellet was re-dissolved with 20 ml of 0.9% NaCl solution. 80 ml of 11.25% (w/v) PVA solution was mixed with the bacterial culture. After the mixing process, the PVA solution was extruded in form of droplets into a stirred buffer solution, which contained NaH_2PO_4 0.4 M, Na_2HPO_4 0.1 M, and H_3BO_3 70 g/l. The formed PVA gel beads were further stirred for another 2 hours and washed using pre-cold sterilized RO water. Then, the PVA gel beads were stored in sterilized ice water for 48-72 hrs. Before the fermentation, the PVA beads must be re-cultivated twice with CRM containing glucose 20 g/l without pH controlling and CRM containing glucose 60 g/l with pH controlling at 4.5. After pre-culturing, the PVA-immobilized cells would be used for the fermentation in different conditions.

Batch Butanol Fermentation with PVA-immobilized cells of Clostridium acetobutylicum

The PVA immobilized cells were pre-cultured with original CRM with glucose of 20 g/l for 24 h and the pre-cultured PVA-immobilized cells were transferred into another fresh CRM contain 60 g/l glucose for A-B-E fermentation with various of butyric acid concentration of 2.5, 5, and 7.5 g/l, respectively. The initial culture pH was 4.5 and the culture pH was maintained at 4.5 by auto-titration.

Analytical Methods

UV/Vis spectrophotometer at an absorbance of 600 nm was used to determine cell concentration of *C. acetobutylicum* in solution. High-performance liquid chromatography (HPLC) with refractive index detection (RID) was used to determine the amount of total carbon source (glucose) consumed during the fermentation process. The concentrations were calculated by extrapolation of measured peak area against the calibration curve of standard components. The gas chromatography (GC) with a flame ionization detector (FID) was used to detect the concentration of acetone, ethanol, and butanol. The result of total butanol production divided by fermentation time was used to calculate the productivity of butanol which was expressed as g/l/h. The butanol yield was calculated as total butanol production divided by the total carbon source utilized and was expressed as mol/mol.

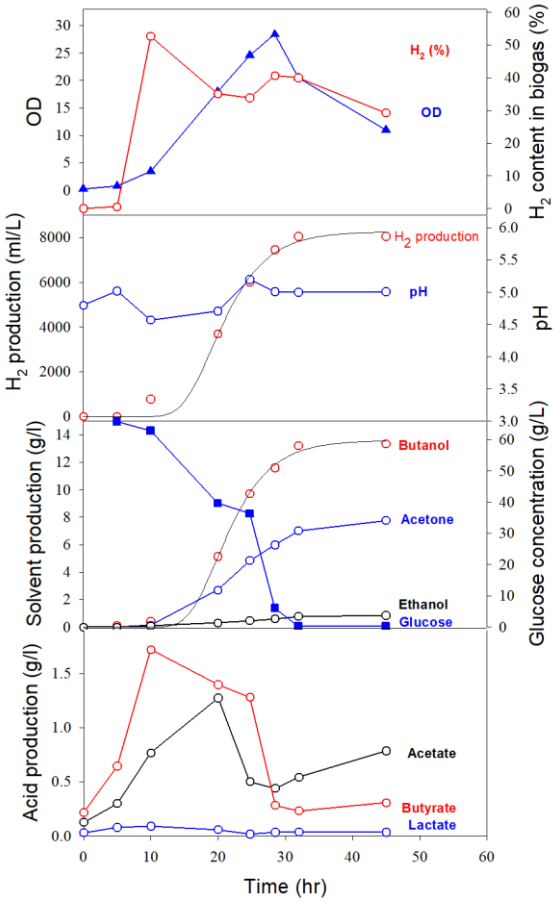
Results and Discussion

The effect of initial butyric acid concentration on butanol production performance by immobilized *C. acetobutylicum* ATCC 824 was investigated in batch culture. Table 1. shows butanol production and yield increase when butyric acid is less than 5 g/L. However, with further higher butyric acid concentration, butanol production decreases followed by decreased glucose consumption. Previous studies also indicated that the addition of butyric acid to cultures of *C. beijerincki* and *C. acetobutylicum* can increase the yield and production of butanol, although the exact mechanism is still not clear. Therefore, initiate the solventogenesis stage earlier, shorten the acidogenesis stage, activate or synthesize the enzymes for solventogenesis, and increase the carbon flow towards butanol production pathway, resulting in the higher butanol productivity, production and yield can occur by the addition of butyric acid (Ezeji et al., 2004; Ramey &Yang 2004; Tashiro et al., 2004; Qureshi et al., 2006; Tashiro et al., 2007).

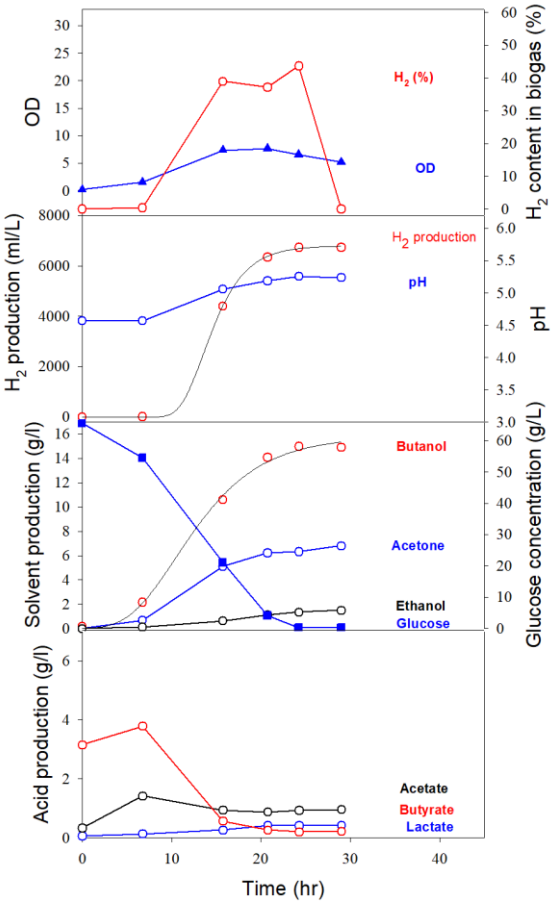
When the concentration of butyric acid increased, maximum cell density (as measured by optical density) and maximum butanol productivity will decrease. It was reported that the addition of exogenous butyric acid to suspension cells of *C. acetobutylicum* lowered the internal pH, almost eliminated the pH gradient (Bowles & Ellefson, 1985), and had an inhibitory effect on the cell growth (Tashiro et al., 2004) and it was also noticed that butyric acid has more inhibition effect than butanol (Bowles & Ellefson, 1985). According to the result, 5 g/L of the initial butyrate addition was the optimal butyrate concentration to enhance the butanol production and yield in batch culture.

C. acetobutylicum ATCC 824 can produce butanol through the two metabolic phases particularly by the addition of butyric acid concentration. In the first fermentation phase, butyric acid were produced, and the solventogenic stage was initiated when butanol rapidly increases followed by a decrease of butyrate. From Figure 1. with the addition of supplemented butyric acid on the culture, the butyrate concentration increased initially which indicated the direct utilization of the added butyrate without an increase in the concentration of butyric acid (Ventura & Jahng, 2013). It was reported that the conversion from butyric acid to butanol includes three metabolic enzymes, i.e., acetoacetyl-CoA: acetate/butyrate: CoA transferase, NADH-dependent butyraldehyde dehydrogenase, and NADH-dependent butanol dehydrogenase, and two molecules of NADH are used as a cofactor for butyraldehyde dehydrogenase and butanol dehydrogenase (Tashiro et al., 2007). Because these dehydrogenases need NADH as reducing power obtained from the glycolysis from glucose, the butyrate formation through acidogenic phase of glucose

metabolism is essential for the utilization of butyric acid and the production of butanol (Tashiro et al., 2004).



(A)



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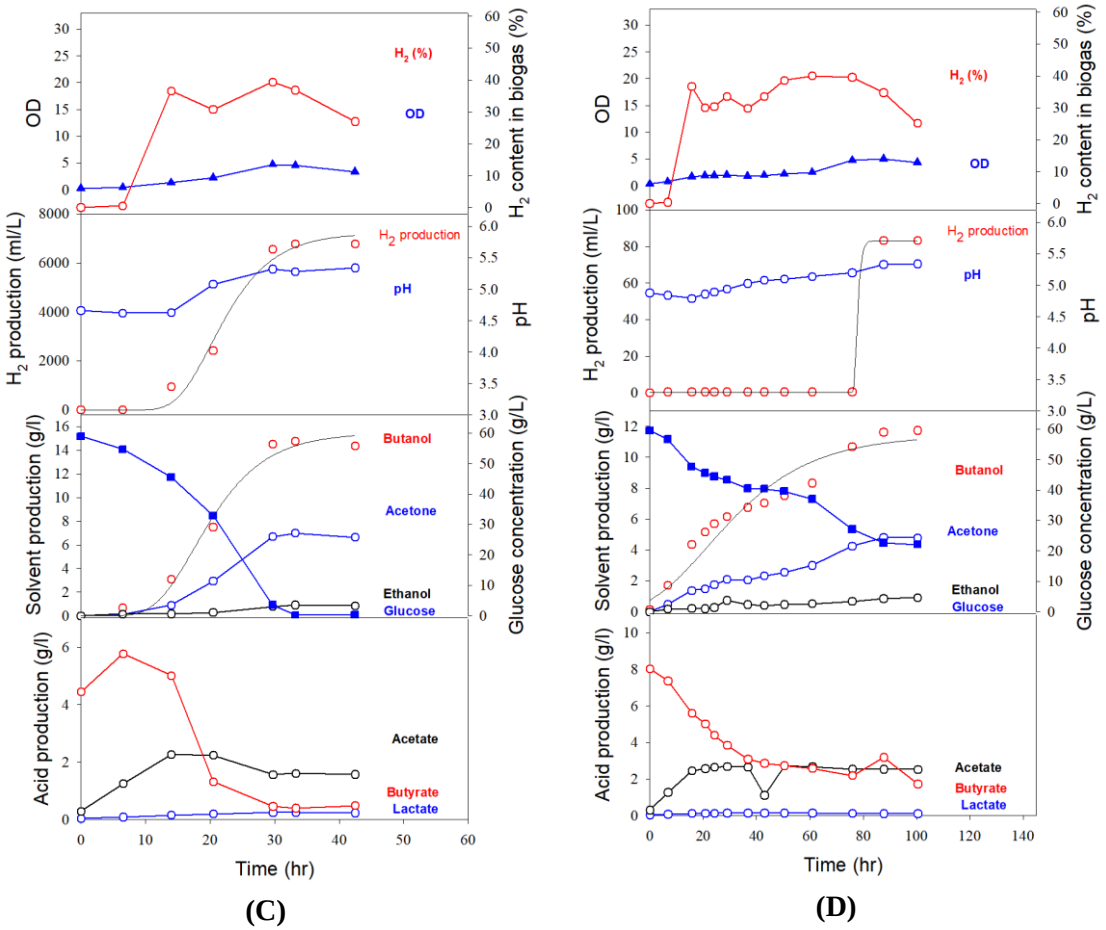

Figure 1. Butanol fermentation with the initial butyrate concentrations of 0 g/L (A), 2.5 g/L (B), 5 g/L (C) and 7.5 g/L (D)

Table 1. Comparison of butanol production with PVA-immobilized *Clostridium acetobutylicum* at different of butyrate concentration in batch culture fermentation

Butyrate concentration (g/L)	Glucose utilization	Butanol production (g/L)	Butanol productivity (g/L/h)	Butanol yield (mol butanol/mol glucose)	Maximum OD_{600}
0	100%	13.35	1.15	0.48	28.4
2.5	100%	14.94	1.10	0.56	7.68
5.0	100%	14.78	0.94	0.61	4.72
7.5	66%	11.74	0.19	0.73	5.03

Conclusion

This research was investigated the analysis effect of butyric acid addition on butanol production by *C. acetobutylicum* ATCC 824 in batch fermentation. The highest result butanol production of 14.94 g/L was attained at butyric acid addition of 2.5 g/L. Furthermore, 5 g/L of butyrate addition giving the highest butanol yield of 0.61 mol butanol/mol glucose. The butanol production and yield increase, when butyrate concentration is less than 5 g/L. On the other hand, with higher butyrate concentration, butanol production decreases followed by decreased glucose consumption. According to the result, 5 g/L of the initial butyric acid addition was the optimal

concentration and the addition of butyric acid can increase the butanol production and yield in batch fermentation.

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