

## The Effect of Initial Butyric Acid Addition on ABE Fermentation by *C. acetobutylicum* NCIMB 619

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**Abstract.** The addition of organic acids to the growth medium has been shown to stimulate solvent production and protect against the degeneration of ABE-producing clostridia. The objective of this study is to demonstrate the effect of introduction of butyric acid in the fermentation media on the solvent production by *C. acetobutylicum* NCIMB 619. In this study, batch cultures were carried out in *Reinforced Clostridial Media* (RCM) containing 0.0015, 2.0 and 5.0 g/l of butyric acid, simultaneously with a control media (without addition of butyric acid) at 37°C for 72 hours. Samples were periodically withdrawn for analysis purposes. It was found that the presence of butyric acid during the early stage of the fermentation process favor the solvent yield to glucose utilization and productivity up to 139%. By adding butyric acid, the concentration of acetone, butanol and ethanol generated was improved significantly. In the case of 5.0 g/l butyric acid addition, acetone, butanol and ethanol production was increased approximately 92, 8 and 2 folds, respectively compared to control culture. The result obtained in this study showed that the addition of butyric acid has successfully enhanced the cell growth and subsequently promoted solvent production by *C. acetobutylicum* NCIMB 619.

**Keywords:** Acetone-butanol-ethanol, organic acid, clostridia.

### 1. Introduction

The production of butanol by acetone-butanol-ethanol (ABE) fermentation used to be one of the largest bioprocesses until the 1950s, but later it was replaced by the less expensive petroleum-based chemical synthesis. In recent years, interest in bio-based butanol has been revived primarily due to concerns with fossil fuel depletion, and microbial production of butanol is considered to be a potential source of liquid fuels.

The metabolic pathway of solvent-producing *Clostridium* consists of two distinct phases, acidogenesis and solventogenesis [1]. In general, during acidogenesis, cell growth is exponential and organic acids are produced together with hydrogen gas, which results in pH reduction. Solventogenesis followed subsequently where cell growth enters the stationary phase and the organic acids are reutilized to produce acetone, butanol and ethanol. This reutilization of organic acids increases the pH value of the broth. It was reported that the addition of organic acids in the broth triggered the metabolic transition from the acidogenic phase to solventogenic phase [1-4]. Therefore in the present study, butyric acid was introduced early in the fermentation media to demonstrate its contribution in solvent production.

### 2. Approach and Methods

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## 2.1. ABE Fermentation

*C. acetobutylicum* NCIMB 619 obtained from British Culture Collection NCIMB LTD., Scotland, UK was used in this study. Laboratory stocks of *C. acetobutylicum* were routinely maintained and kept as a single colony as spore suspensions in liquid Reinforced Clostridial Media (RCM) at 4°C. The bacterial reactivation were performed by transferring stock culture into fresh deoxygenized RCM liquid medium and incubated at 37°C for 30 hours. Followed by inoculum production by inoculating 10% (v/v) of reactivated culture in another volume of fresh deoxygenized RCM liquid medium and grew in the similar temperature for 10 hours. All procedures were carried out anaerobically and inoculum with optical density of 1.2 to 1.4 at 680 nm was used in the fermentation. Different volumes of pure butyric acid were added into the media prior to fermentation process which took place subsequently in four 250mL flasks (including control culture) at a temperature of 37°C for 72 hours without stirring. Periodically, samples were withdrawn for analysis purposes.

## 2.2. Analytical Method

The pH reading was determined by pH meter (Mettler Toledo, USA) while the cell concentration was estimated by optical density with a UV-spectrophotometer at 680 nm. The concentration of acetone, butanol, and ethanol was evaluated by Agilent Technologies gas chromatograph model 6890N, equipped with flame ionization detector (FID) and capillary column Equity<sup>TM</sup>-1 (Supelco) with dimension of 30m x 0.32m x 1.0µm. The oven temperature was programmed at 40°C (8 min) and later increased to 130°C (4°C min<sup>-1</sup>) for 5 min. The detector and injector were set at 250°C with the carrier gas (helium) flow rate of 1.5ml min<sup>-1</sup>. The reducing sugar concentration in the sample was measured using DNS method.

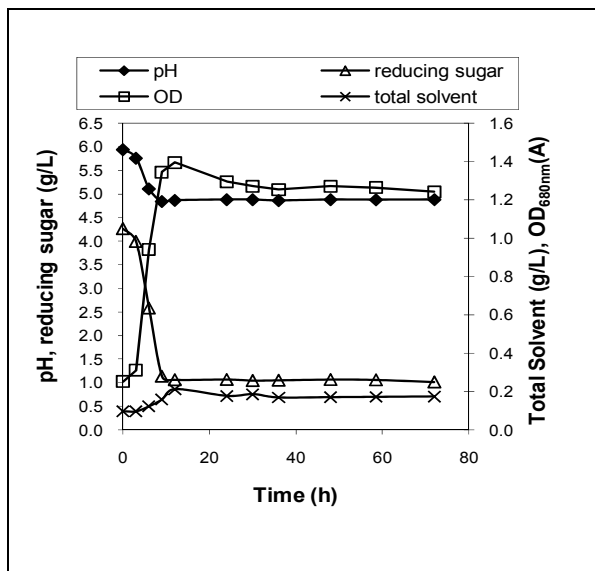
## 3. Results and Discussion

To investigate the effect of addition of butyric acid, batch cultures were carried out in RCM medium containing 0, 0.0015, 2.0 and 5.0 g/l butyric acid. Figure 1 illustrated the effect of initial addition of butyric acid on pH of the broth, cell optical density and reducing sugar concentration. The culture without acid addition demonstrated a common pattern of bacterial growth with 3 hours of lag phase, followed by the exponential cell growth and peaked at 12 hours of fermentation time before it reduced gradually to the end of fermentation. The addition of butyric acid, as low as 0.0015 g/l enhanced the bacterial growth as the OD reading rose directly upon incubation, with higher value compared to that in the control media (Figure 1b). This was probably contributed by the changes of initial pH value towards 5.0 (more acidic) which favors the bacterial growth [1-4]. Moreover, the exponential growth was extended up to 24 hours with the addition of 2.0 g/l butyric acid. This trend supported the findings of previous studies that the early presence of organic acid offered a protection against the degeneration of ABE-producing clostridia [2, 5-8]. However, as the concentration increased to 5.0 g/l, the growth rate was decreased (indicated by reduced OD reading) and this suggested that admittance of butyric acid higher than 2.0 g/l inhibited the exponential growth phase and simultaneously promoted the solventogenesis where the cell growth was stationary. A similar trend was observed in a previous study [4].

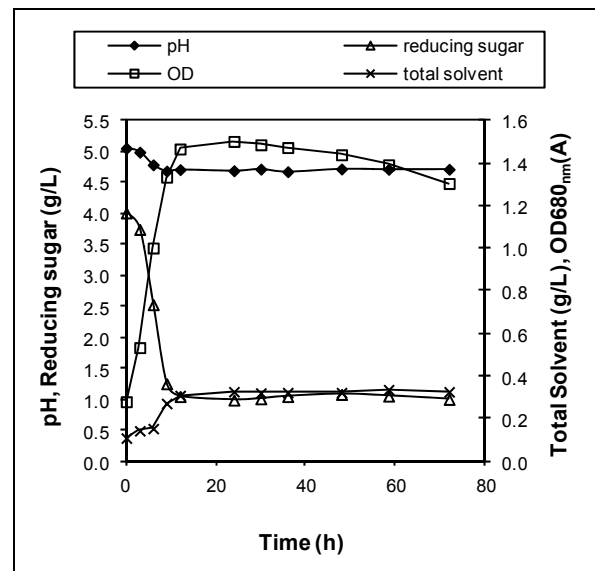
The amount of solvents (ABE) produced in the medium containing 5.0 g/L was greater than those produced in the medium containing other butyric acid concentrations (Table 1). Furthermore, with additional butyric acids, the glucose utilization rate was lower and results in higher solvent yield.

Butyric acid in broth, particularly undissociated butyric acid, has been shown to trigger solvent production by *C. acetobutylicum* [3-4, 9]. Furthermore, the increase in yield and production of solvents were also reported upon the addition of butyric acid and acetic acid to cultures of *C. acetobutylicum*, *C. beijerinckii* and *C. saccharoperbutylacetonicum* N1-4 [2, 4-8]

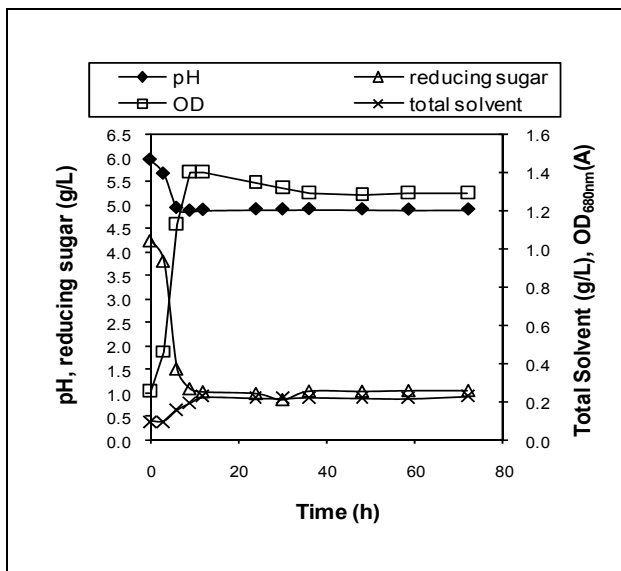
Besides that, previous studies have shown that pH of the fermentation media has a significant influence on ABE fermentation where it determine the organic acid or solvent production [2-4, 10-11]. It is reported that organic acid production is enhanced at higher pH while solvents are mainly produced at lower pH. The addition of butyric acid helped to maintain the media at low pH value which induced a metabolic shift from acidogenesis to solventogenesis and thus, enhanced the total solvent production.



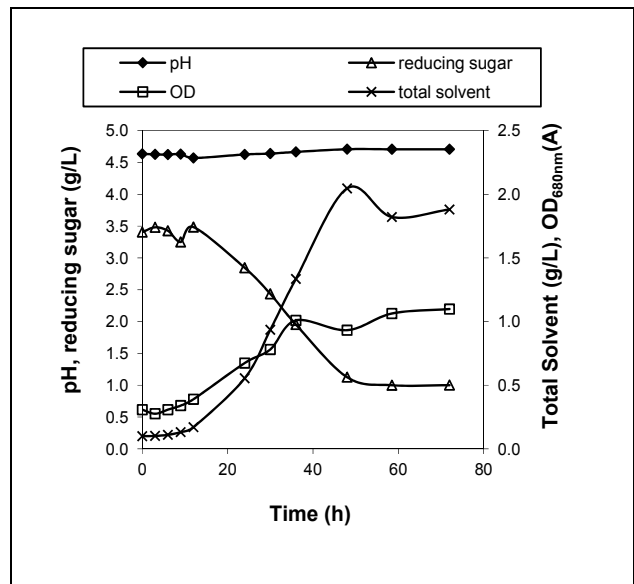
(a)



(c)



(b)



(d)

Fig. 1: Growth profiles of *C.acetobutylicum* NCIMB 619 over 72 hours fermentation with butyric acid initial addition at several concentrations: (a) 0 g/l; (b) 0.0015 g/l; (c) 2.0 g/l, (d) 5.0 g/l.

Table 1: Effect of butyric acid in ABE fermentation

Butyric acid (g/l)	Production <sup>a</sup>			Solvent yield <sup>b</sup> (g/g)	Productivity <sup>c</sup> (g/l/h)
	Acetone(g/l)	Butanol (g/l)	Ethanol (g/l)		
0	0.008	0.149	0.067	0.066	0.018
0.0015	0.011	0.156	0.094	0.071	0.003
2.0	0.04	0.237	0.056	0.113	0.006
5.0	0.739	1.184	0.122	0.898	0.043

<sup>a</sup> Maximum solvent production

<sup>b</sup> Total solvent production per glucose utilization

<sup>c</sup> Total solvent production over fermentation time

From the plots of Figure 1, it was apparent that solvent production was ceased at fermentation time of 12 hours for the medium containing 0, 0.0015 and 2.0 g/l of butyric acid. However, for 5.0 g/l acid addition, the solvent production continued up to 48 hours with higher solvent concentration. This outcome could be

explained by relating the importance of sufficient glucose concentration for solvent production. After 12 hours of fermentation, the remaining glucose concentration in the broth was approximately 1.0 g/l. At this point, the solvent production was ceased due to glucose insufficiency, except those with 5.0 g/l acid addition. With the presence of adequate sugar concentration, organic acids were continuously transformed into solvents. Following 48 hours of fermentation, although high butyric acid still presented in the broth (1.1 g/l), solvent production was inhibited as reducing sugar concentration in the broth has reached the limiting point.

Previous researchers reported that butyric acid was reutilized and converted to butanol via three metabolic enzymes, which are acetoacetyl-CoA: acetate/butyrate: CoA transferase, NADH-dependent butyraldehyde dehydrogenase and NADH-dependent butanol dehydrogenase [1, 7]. Since these dehydrogenases require reducing power, such as NADH obtained by glycolysis from glucose, no butanol production by feeding of only butyric acid may result from insufficient NADH due to the absence of glucose. Consequently, energy sources like glucose are necessary for butyric acid utilization and butanol production.

#### 4. Conclusion

The result obtained in this study showed that the initial addition of butyric acid promoted solvents production by *C. acetobutylicum* NCIMB 619, under sufficient sugar concentration. Upon butyric acid addition, more organic acids were presented in the broth and subsequently converted into solvents. Apart from that, with the introduction of butyric acid, the initial pH was adjusted to a lower value which favors solvent production. Highest solvent concentration of 2.1 g/l was obtained with the addition of maximum concentration of butyric acid.

#### 5. Acknowledgement

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