# Ferrous Ion and Medium Composition Effects on Acidogenic Phase in Biobutanol Production from Molasses

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**Abstract**. *Clostridium acetobutylicum* B530 has ability to convert sugar into biobutanol through two phases, i.e. acidogenic and solventogenic. This fermentation process is often hampered by high raw material cost and low product yield. In order to suppress the production cost, the molasses, a byproduct of sugar cane process production, was used as carbon source in this research. Molasses has nitrogen content in a small amount, thus could be negating the beef extract component, which is expected not to affect the growth of *C. acetobutylicum* B530 and also can reduce the production cost. In addition, a certain amount of $Fe^{2+}$ (ferrous ion), a precursor in the formation of the enzyme ferredoxin, was added to the fermentation medium to contribute in the synthesis of acetyl-CoA, so that the formation of acidogenic products such as butyric acid and acetic acid is affected. This study aimed to investigate the effect of ferrous ion and the medium composition in acidogenic phase. The addition of 20 ppm $FeSO_4.7H_2O$ in the fermentation medium without beef extract can increase the concentration of butyric acid by 20% at a temperature of 35°C, while acetic acid concentration decreased by 6%. According to those results, it is expected that the product selectivity of butanol will increase in solventogenic phase. In addition, the removal of beef extract in the fermentation medium does not affect the kinetics of growth of *C. acetobutylicum* B530.
Keywords: *Clostridium acetobutylicum* B530, acidogenic phase, ferrous ion, molasses

## 1. Introduction

Development of alternative energy from biomass is needed to overcome the crisis of fossil-based natural resources. One of the promising biofuel developments is the production of biobutanol from agroindustry waste. Biobutanol has excellent properties as substitute fuel for gasoline, such as less hygroscopic, lower vapor pressure, and high energy content. Moreover, blending of butanol and gasoline is possible at any concentrations, and there is no need for adjustment of vehicle and engine technologies [1,2].

Biobutanol can be produced with biological process using solventogenic bacteria, such as clostridia. *Clostridium acetobutylicum* is biological agen that is often used in the production of acetone, butanol and ethanol (ABE). These bacteria have the ability to convert fermentable sugar into acids (acidogenic phase) followed by the reactions to produce ABE solvents (solventogenic phase).

The mole ratio of the solvent production (acetone:butanol:ethanol) is approximately 3:6:1 [3]. The acidogenic phase occurs when the *C. acetobutylicum* growth is in the exponential phase, while the solventogenic phase occurs when the cell growth slowed [4]. Metabolic pathways of ABE solvents production from glucose by *C. acetobutylicum* is illustrated in **Figure 1**.

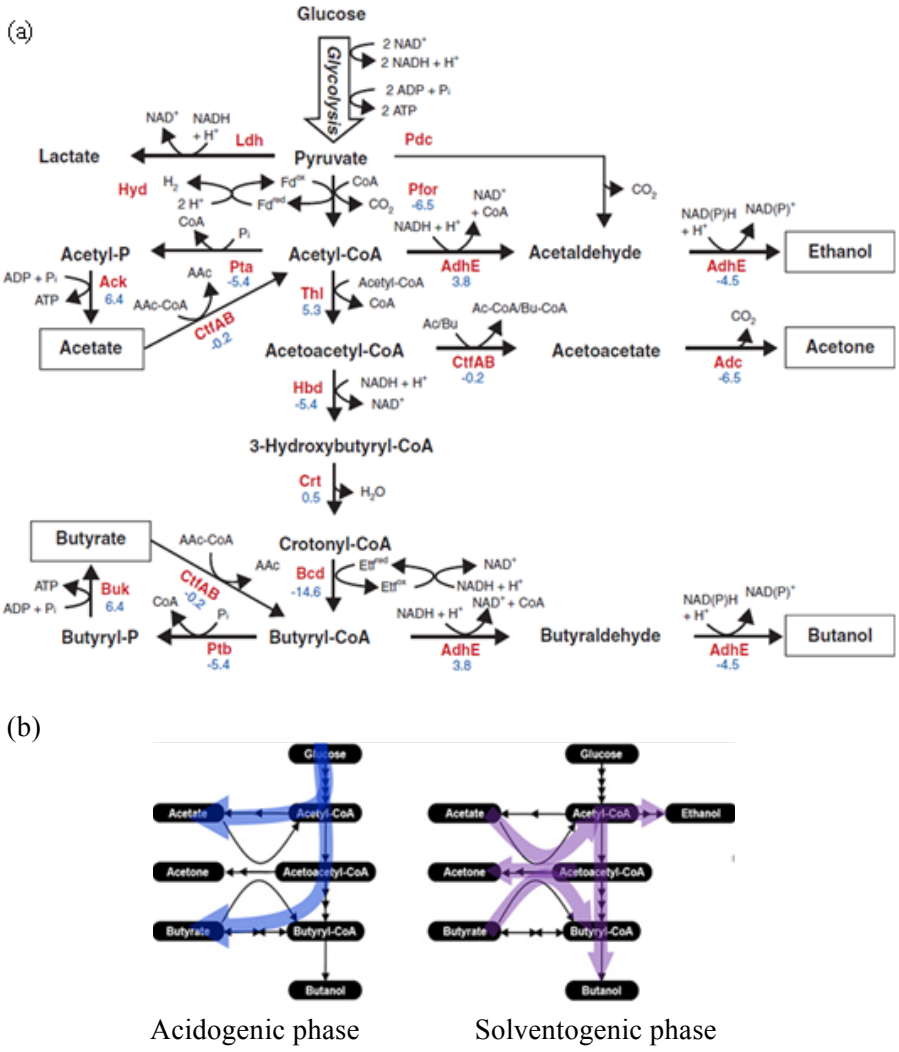


**Figure 1.** (a) Metabolism pathway of *C. acetobutylicum* to produce ABE solvents from glucose [5] and (b) acidogenic and solventogenic phases [6]

The acids produced in acidogenic phase will serve as precursor in solventogenic phase, so that the yield of each acid can affect the selectivity of the solventogenic products. The yield of ABE solvents can be affected by environmental conditions such as pH, temperature, and nutritional supplements. Previous studies reported that the addition of ferrous ion can increase the yeld of ABE solvents [7-9]. Ferrous ion is used as a cofactor for ferredoxin in the synthesis of acetyl-CoA. Acetyl-CoA plays an important role as precursor for the synthesis of all desired products, including acetogenic products. This study deals with the proper concentration of ferrous ion given in the fermentation process in order to obtain high yield of butyric acid which is expected to spur a high butanol selectivity. Fermentation stage observed in this study is limited to the exponential phase of *C. acetobutylicum* growth so it is possible to assess the effect of ferrous ion in acidogenic phase.

*C. acetobutylicum* can grow well in a reinforced clostridial medium (RMC). However, RCM has expensive constituent components, such as beef extract for nitrogen and vitamin sources, leading to not reliable process for commercial application. In addition. this study also examines the use of sugarcane molasses to replace the use of glucose and eliminate the use of beef extract in medium. Sugarcane molasses is a byproduct of the sugar industry and can be obtained at a cheap price compared to the price of glucose. Molasses has a high sugar content, i.e. 55-65%-w, so it is appropriate to be used as a carbon source. In addition, molasses also contains nitrogen and vitamins in small amounts. It is estimated that the use of molasses in the absence of beef extract in the medium does not affect the growth of *C. acetobutylicum*.

## 2. Material and Methods

### *2.1. Strain and culture maintenance*

*Clostridium acetobutylicum* B530 used in this study is a gift from ARS (Agricultural Culture Collection), USA. The culture was grown in 50 ml vial bottle under anaerobic condition at 37°C. Before fermentation, inoculum was prepared by incubation at 37°C for 24 hours. Cultures were stored at 4°C and reseeded into fresh medium every 14 days.

### *2.2. Pretreatment of sugarcane molasses*

Decalcification or addition of sulfuric acid was performed as cane molasses pretreatment in this study. Distillated water was added to 60 grams of cane molasses until the volume 1 L and then the mixture was acidified to pH 3.5 with $H_2SO_4$ 96%. Following water bath at 60°C for 2 h, the mixture was centrifuged at 3000 rpm for 2 h using ultra speed centrifuged HERMLE Z 36 HK. Mixture was filtered using Whatman filter paper no. 1 (particle retention = 11 µm; diameter = 9 cm). Before being used for fermentation, the supernatant was neutralized to pH 6.5 with NaOH 4 N.

### *2.3. Fermentation mediums*

The fermentation medium was composed of pretreated cane molasses 6%-w/v. The fermentation medium contained the following components per liter of distilled water: 10 g pepton (Bio Basic Inc., Canada), 5 g NaCl (Bio Basic Inc., Canada), 3 g yeast extract (Himedia, India), 3 g $CH_3COOH$ (Bio Basic Inc., Canada), 1 g soluble starch (Bio Basic Inc., Canada), dan 0.5 g L-cystein HCl (Bio Basic Inc., Canada). 10 g beef extract (Oxoid, England) was added to certain experiment. The final pH was adjusted to 6.8. The variations of fermentation medium in this study are tabulated in **Table 1**.

**Table 1.** Variation of fermentation medium

| | Carbon source | Beef extract | $FeSO_4.7H_2O$ (ppm) | Incubation temperature (°C) |
|---|---|---|---|---|
| **Glu** | Glucose | + | - | 37 |
| **Mol#1** | Molasses | + | - | 37 |
| **Mol#2** | Molasses | - | 10 | 37 |
| **Mol#3** | Molasses | - | 20 | 37 |
| **Mol#4** | Molasses | - | 20 | 35 |
| **Mol#5** | Molasses | - | 30 | 35 |

+ added to the medium
- not given in the medium

### *2.4. Fermentation*

Fermentation studies were conducted in 30 mL vials covered with rubber and stainless steel seal. 20 mL medium listed in **Table 1** were put in those vials and sterilized in autoclave at 121°C for 20 min. 10%-v/v of inoculum was applied into the sterilized fermentation medium. Inoculation was performed in the anaerobic chamber. Anaerobic chamber used in this research shown in **Figure 2**. Nitrogen gas

was filtered and used for purging the anaerobic chamber up to no oxygen gas inside the chamber. The fermentations were incubated for 120 hours at certain temperatures (35 or 37$^{o}$C). Samples were taken periodically to analyze the composition of fermentation products. Gasses formed during fermentation ($CO_2$ and $H_2$) were taken intermittently with sterile syringe. The observations of acidogenic stage were performed on the first 30 hours.

**Figure 2.** A simple anaerobic chamber connected with a nitrogen gas tube and equipped with a UV lamp.

*2.5. Measurement of biomass density and cell dry weight*

The optical cell density was determined at 600 nm using *spectrophotometer optima* SP-300. Cell dry weight was obtained from the following procedure. The samples of fermentation were centrifuged at 6000 rpm for 15 min. After removing the supernatant, pellets were subsequently put into oven at 60°C until the constant mass was obtained. The constant weight indicated the cell dry weight.

*2.6. Morphology observation*

Gram staining was performed to check the morphology of *C. acetobutylicum* B530. *C. acetobutylicum* B530 is a gram positive bacteria, so it will give purplish color. Morphology observation was performed using a Nikon Eclipse E200MV RS microscope with 100x focus.

*2.7. Product quantification*

The concentration of glucose, acetic acid, and butyric acid were measured using HPLC refractive index detector (RID) (Waters 2414) with Aminex HPX87H column (Biorad,CA) autosampler waters 2707. $H_2SO_4$ 0.005 M was used as mobile phase with flow rate of 0.6 ml/min. The temperature operation was 60°C.

## 3. Results and Discussion

*3.1. Effect of fermentation medium modification to the growth kinetics of C. acetobutylicum*

The curve of *C. acetobutylicum* growth is depicted in **Figure 3**. *C. acetobutylium* growth in a medium containing glucose and beef extract (Glu) were observed faster than the growth in medium containing 6%-w/v molasses (with (Mol#1) or without (Mol#2) beef extract. The doubling time of *C. acetobutylicum* in a medium containing glucose and beef extract is higher than in ones containing molasses (see **Table 2**). It is quite acceptable because the molasses has a sugar content, which is more complex than glucose. *C. acetobutylicum* growths in molasses mediums with beef extract (Mol#1) or without beef extract (Mol#2) do not show a significant difference. The specific growth rate and the doubling time of *C. acetobutylicum* in both the fermentation medium also have similar values.

Sugarcane molasses contains various carbon sources and other nutrients, such as 30%-w/v sucrose, 13%-w/v fructose, 7.2%-w/v glucose, 0.4%-w/v lipid, and vitamins. Naturally, cane molasses contain ± 3%-w/v of nitrogen, so that the fermentation without beef extract component does not affects the growth of *C. acetobutylicum* B530 [11]. The results showed that molasses can be potentially as a carbon source and may negate beef extract in a medium. Further discussion after this section will look at the use of molasses as fermentation medium of *C. acetobutylicum*.

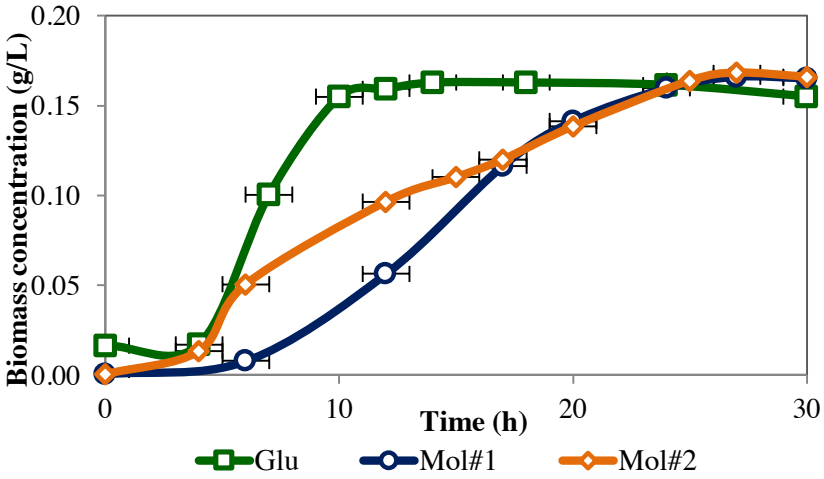


**Figure 3.** Growth profile of *C. acetobutylicum* B530 in different fermentation mediums (square for Glu, circle for Mol#1, dan diamond for Mol#2). The compositions of medium were mentioned in subsection *2.3*.

**Table 2.** The specific growth rate and doubling time of *C. acetobutylicum* B530 in different fermentation mediums

| | μ ($h^{-1}$) | τ (h) |
|---|---|---|
| **Glu** | 0.369 | 1.879 |
| **Mol#1** | 0.245 | 2.824 |
| **Mol#2** | 0.247 | 2.807 |

*3.2. pH changes during fermentation*

The decreasing of pH was observed during molasses fermentation (with or without beef extract) as presented in **Figure 4**. This decrease in pH indicates the occurrence of acids production that took place on acidogenic phase. This pH decrease occurred in the exponential phase of *C. acetobutylicum* growth. The similar profiles of pH decrease indicate that the presence or absence of beef extract in molasses fermentation does not affect significantly in pH change of the medium during fermentation [5,6]. Low pH is one of the prerequisite conditions for *Clostridium sp*. to swith phase from acidogenic phase to solventogenic phase. Previous studies explained that optimum pH condition for culture to enter solventogenic phase is 4.5 [5,12,13].

*3.3. Effect of iron ions in the production of acids in the acidogenic phase*

Molasses fermentations by *C. acetobutylicum* were conducted in several concentrations of ferrous ion (10 ppm, 20 ppm, and 30 ppm) and different temperatures (35°C and 37°C) as mentioned in **Table 1**. The fermentation done in a medium without the addition of ferrous ion (Mol#1) was used as a base case. The acids detected as acidogenic products in this study were acetic acid and butyric acid. Acetic and butyric acid obtained from the fermentation of molasses on acidogenic stage for the medium variations of Mol#2 (10 ppm $Fe^{2+}$, 37°C), Mol#3 (20 ppm $Fe^{2+}$, 37°C), Mol#4 (20 ppm $Fe^{2+}$, 35°C),

and Mol#5 (30 ppm $Fe^{2+}$, 35°C) were compared with those obtained from the fermentation in Mol#1 (0 ppm $Fe^{2+}$, 37°C). The percentage of acid productivity on different media relative to the productivity on Mol#1 is given in **Figure 5**.

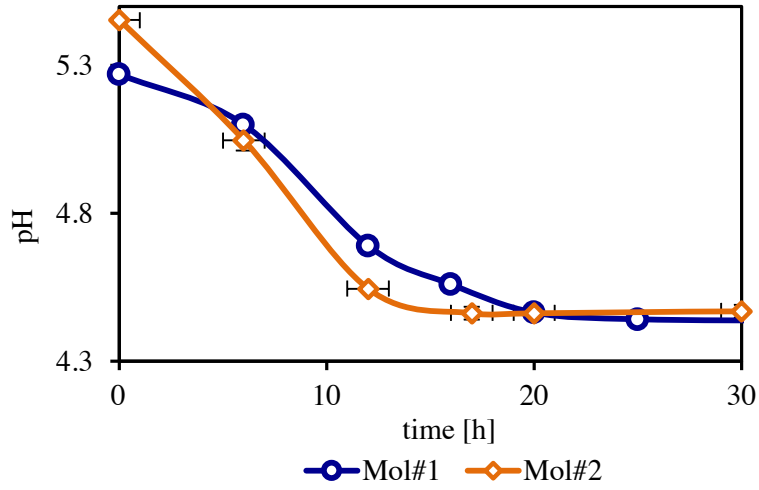


**Figure 4.** pH profile of molasses fermentation by *C. acetobutylicum* B530 in different mediums (circle for Mol#1, dan diamon for Mol#2)

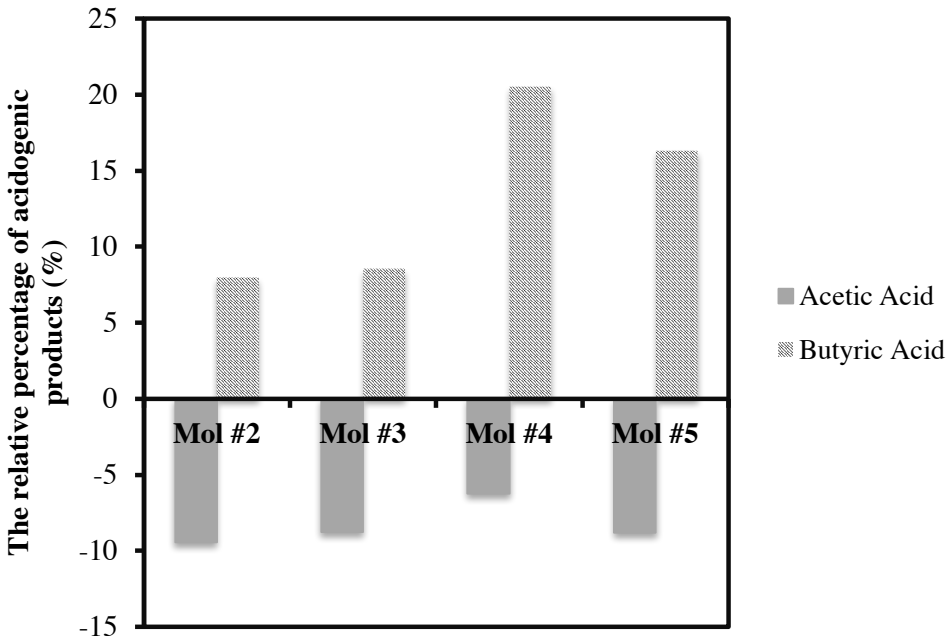


**Figure 5.** The percentages of acidogenic products from molasses fermentation using different medium (Mol#2, Mol#3, Mol#4, and Mol#5) relative to fermentation products using Mol#1 medium. The composition of medium is given in **Table 1**.

Result shows that the molasses fermentation conducted with the addition of 20 ppm of ferrous ion (at 35°C) is able to produce butyric acid by 20% higher than the base case (Mol#1), while acetic acid

produced is 6% smaller. This result is expected to improve the selectivity toward biobutanol. The composition of those acidogenic products could influence the ratio of ABE solvent production. Ferrous ion can affect the metabolism of *C. acetobutylicum*. As shown in *C. acetobutylicum* metabolism pathway, acidogenic phase begins with the formation of acetyl-CoA, which is synthesized from the oxidation of pyruvic acid by the ferredoxin. Ferredoxin is an enzyme containing iron and sulfur as essential substances involved in the oxidation, therefore the addition of $FeSO_4.7H_2O$ will increase the production of this enzyme [14]. Furthermore, lowering the incubation temperature slightly could decrease the rate of cell metabolism, so that the performance of enzymes involved in acidogenic synthesis product will increase [12]. High productivity of butyric acid will enhance biobutanol production, because butyric acid plays an important role as precursor in the synthesis of biobutanol. The increase of acetic acid concentration as acidogenic product will provide more energy for biobutanol synthesis [9].

## 4. Conclusions

Sugarcane molasses can be used as a potential carbon and also vitamin sources in the fermentation by *C. acteobutylicum*. The addition of $FeSO_4.7H_2O$ into the fermentation medium can enrich the production of butyric acid in the acidogenic phase. 20 ppm $FeSO_4.7H_2O$ that was added into the molasses fermentation medium (at $T = 35^oC$) can increase about 20% of butyric acid productivity compared to the molasses fermentation without ferrous ion (at $T = 37^oC$). The increase in the production of butyric acid could be expected to increase the selectivity toward biobutanol in ABE fermentation.

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