Effect of Buffering System on Acetone-Butanol-Ethanol Fermentation by *Clostridium acetobutylicum* ATCC 824 using Pretreated Oil Palm Empty Fruit Bunch

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Change of pH has been identified as the most significant parameter in modulating the transition between the conversions of acids into solvents in acetone-butanol-ethanol (ABE) fermentation by *Clostridia*. Thus, ABE fermentation at various phosphate buffer concentrations and initial pH values was conducted using pure glucose and sugars derived from pretreated oil palm empty fruit bunch (OPEFB). A higher solvent concentration (2.93 g/L) was obtained in the fermentation using 20 g/L of glucose with buffer compared with one without buffer that produced 1.34 g/L of solvents. Approximately 8.77 and 9.15 g/L of solvents were produced from fermentation using 40 g/L of glucose with and without buffer, respectively. In the latter conditions, at an initial pH of 5.5, 8.77 g/L of solvents was obtained, which was the highest concentration compared to other initial pH values. Increasing the buffer concentration to 0.2 M at an initial pH of 6.0 resulted in acid accumulation of 16.83 g/L but reduced the solvent production to 1.36 g/L. In addition, ABE fermentation using 20 g/L of sugars from pretreated OPEFB produced 2.25 g/L of solvents with a yield of 0.13 g/g, which was comparable with fermentation using 20 g/L of glucose conducted in a buffering system.

Keywords: Acetone-butanol-ethanol (ABE) fermentation; Clostridium acetobutylicum ATCC 824; Oil palm empty fruit bunch; Buffer; Biobutanol

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INTRODUCTION

Biological production of acetone-butanol-ethanol (ABE) is currently in demand for the extraction of butanol as a biofuel. Butanol ($C_4H_{10}O$) acquisition poses the next significant challenge to meet the growth in demand for environmentally responsible and renewable fuels for use in transportation. Butanol, or butyl alcohol, is an alcohol that can be produced by microorganisms through ABE fermentation (Jones and Woods 1986; Lee *et al.* 2008; Jin *et al.* 2011). Compared to other alcoholic fuels such as ethanol and methanol, butanol has a higher heating value. Being a four-carbon alcohol, butanol contains 25% more energy than ethanol, thus reducing fuel consumption and improving mileage. Butanol has a lower volatility rate, less ignition problems, and higher viscosity than other alcohols. Because of these factors, butanol can be used in existing engine systems without any modifications (Dürre 2007; Jin *et al.* 2011). Butanol can also be distributed through the current pipeline system, as it is less corrosive when compared to ethanol and methanol (Dürre 2007). These properties of butanol are noted as better than those of other alcoholic fuels and are almost similar to those of gasoline, signifying it is a potentially great renewable energy source if its production costs can be reduced (Lee *et al.* 2008).

Acetone-butanol-ethanol fermentation by *Clostridia* has been characterised as heterotrophic fermentation that produces multiple types of by-products (Jones and Woods 1986; Sukumaran *et al.* 2011). Acetone-butanol-ethanol fermentation produces solvents (acetone, butanol, and ethanol), acids (acetic acid and butyric acid), and gasses (carbon dioxide and hydrogen) in a complex metabolic pathway. The pathway involves two main stages, acidogenesis and solventogenesis, as indicated by the stages of acid and solvent production, respectively (Lee *et al.* 2008). The change of stage from acidogenesis to solventogenesis is clearly indicated by a change of pH in the fermentation system. It is believed that acids produced in the system are required to reduce the pH to below 5 to allow the phase transition from acidogenesis to solventogenesis. However, acid accumulation may inhibit cell growth, reducing solvent production (Maddox *et al.* 2000; Ibrahim *et al.* 2012).

Increasing the buffering capacity of the medium has been suggested as a simple way to increase cell growth, substrate intake, and solvent production (Bryant and Blaschek 1988). Although there is evidence that acids inhibited the cell growth, an accumulation of certain amounts of acids is required to produce solvents. The butyric acid produced is reassimilated by the cell and converted to butanol (Hartmanis and Gatenbeck 1984), and it must be in the protonated form to permeate the cell membrane (Kell *et al.* 1981). The butyrate produced in the system was found to influence the internal pH environment of the cell, leading to solvent production. In ABE batch fermentation without pH control, the "acid crash" phenomenon naturally occurs. This happens when excess acid production takes place without switching to the solventogenic phase (Maddox *et al.* 2000).

Thus, many studies have been conducted to find a suitable pH value for solvent production and to control the pH at the same time so that suitable amounts of acids are produced for the solventogenic phase (Huang *et al.* 2004; Li *et al.* 2011; Guo *et al.* 2012). The use of buffer was believed to be the simplest way to control pH conditions in batch fermentation while simultaneously providing the chance for cells to switch from the acidogenic to the solventogenic phase (Lee *et al.* 2008). In addition, the initial pH value might be one of the factors for the phase transition from acidogenesis to solventogenesis. Zhu and Yang (2004) reported that variations of initial pH values affected the switch of the metabolic pathway from butyrate to acetate and lactate production by *Clostridium tyrobutyricum*. Thus, this present study was conducted with the aim of investigating the effects of a buffering system in ABE fermentation by *Clostridium acetobutylicum* ATCC 824 using glucose and sugars from pretreated oil palm empty fruit bunches (OPEFB). A study on the effects of phosphate buffers at various initial pH values and buffer concentrations on ABE fermentation using a glucose-based medium was also performed. Phosphate buffer was chosen in reference to a study by Bryant and Blaschek (1988) because this buffer was found to be a suitable buffer for ABE fermentation. A subsequent ABE fermentation using sugar derived from pretreated OPEFB was conducted at the appropriate tested buffer conditions.

It is important to enhance the ABE production when lignocellulosic biomass is used. In Malaysia, OPEFB is one of the most abundant forms of biomass generated from the palm oil industry, and utilisation of this lignocellulosic biomass generates added value, as has been stated in Malaysian National Biomass Strategy 2020 (Agensi Inovasi Malaysia 2013). OPFB has been tested as a carbon source for many fermentation processes including for renewable energy (Sumathi *et al.* 2008). Some recent studies have been done on utilization of OPEFB for ABE fermentation (Ibrahim *et al*. 2012; Sklavounos *et al*. 2013; Ibrahim *et al*. 2015). However, one of the biggest challenges of utilizing lignocellulosic biomass including OPEFB is low sugar concentration to stimulate the cell for solvent production. The highest sugar concentration from saccharification of OPEFB was around 30 g/L, as has been found in our previous study (Ibrahim *et al*. 2013). Therefore, maintaining a favorable pH condition has been proposed, and such a strategy was considered in the present work.

EXPERIMENTAL

Materials

Inoculum preparation

Clostridium acetobutylicum ATCC 824 was obtained from the American Type Culture Collection (ATCC, USA). The stock culture was inoculated anaerobically in reinforced Clostridial medium (RCM) (Merck, Denmark) and incubated for 24 h at 37 °C in a static Memmert incubator (Memmert, Germany). The optical density (OD) of the culture used in this experiment was fixed at ±1.0 (at Abs 620 nm). The cells were ensured in the log phase prior to the fermentation process.

Substrate preparation

Pressed and shredded OPEFB was obtained from the Dengkil Palm Oil Mill, Ulu Langat, Selangor, Malaysia. The OPEFB was soaked in commercial dish detergent (brand Sunlight) for 24 h before being washed with tap water to remove debris and oil. The washed OPEFB was dried in an oven at 60 °C for 24 h. The pretreatment of OPEFB was conducted based on a report by Umikalsom *et al.* (1998) by soaking 100 g of washed OPEFB in 2 L of 2% NaOH for 4 h and autoclaving at 121 °C for 5 min to remove the lignin that coats the internal structure of the cellulose and hemicellulose. Then, the pretreated OPEFB was washed with tap water until no alkali was detected. The presence of alkali was determined using a pH meter. The pretreated and washed OPEFB was oven-dried at 60 °C for 24 h and kept in a sealed plastic bag prior to the saccharification process.

Methods

Preparation of crude cellulase cocktail

Crude cellulase cocktail was produced by *Trichoderma asperellum* UPM1 and *Aspergillus fumigatus* UPM2 as mentioned by Abd-Aziz *et al.* (2012). The crude cellulase cocktail produced was spray dried using a spray dryer (LabPlant, UK) based on the method used by Selivanov (2005). The spray dried crude cellulase cocktail was dissolved in purified water at (0.1 g/mL), which contained 750 U/mL of CMCase, 110 U/mL of FPase, and 120 U/mL of β-glucosidase. The dissolved cellulase was diluted in 0.1 M of phosphate buffer to obtain approximately 5.0 U/mL of β-glucosidase activity to be used in saccharification of pretreated OPEFB.

Saccharification of pretreated OPEFB

The saccharification of the pretreated OPEFB was conducted in a 250-mL Erlenmeyer flask. A total of 5% (w/v) of pretreated OPEFB was put into the Erlenmeyer flask, and 100 mL of 0.1 M phosphate buffer at pH 5.5, which contained the crude cellulase cocktail prepared above was added. The saccharification process was incubated at 50 °C, 200 rpm for 72 h in a shaker incubator (Labwit, China) to convert the pretreated OPEFB into sugars following the methods described by Ibrahim *et al.* (2012). The sugars produced were recovered by centrifuging at 4000 rpm (2594 rcf) for 10 min using a centrifuge (2–6, Sigma-Aldrich, Germany) to separate the solid and liquid materials. The liquids that contained sugars were kept at -20 °C prior to medium preparation.

Medium preparation

The P2 medium described by Monot *et al.* (1982) with modification was prepared by dissolving 6 g/L of yeast extract (Bacto™) with 20 and 40 g/L of glucose, and with 20 g/L of sugars obtained from pretreated OPEFB. Both types of medium (glucose and sugars from

pretreated OPEFB) were dissolved in distilled water (for medium preparation without buffer), and in phosphate buffer (for medium preparation with buffer). Approximately 84 mL of medium was transferred into a 125-mL serum bottle and sparged with nitrogen gas for 15 min to remove oxygen. The medium was autoclaved at 115 °C for 15 min and left at room temperature for cooling. Then, the prepared medium was added with 2 mL each of filtered sterile solutions consisting of vitamins, buffer, and mineral solutions, as described by Linggang *et al.* 2013 and Razak *et al.* 2013. All the transfer processes were conducted anaerobically using syringes.

ABE fermentation

The ABE fermentation process was started by the anaerobic transfer of 10 mL of inoculum into 90 mL of prepared P2 medium. The inoculated medium was incubated at 37 °C and agitated at 120 rpm using a shaker incubator (Labwit, China) for 72 h. Approximately 2 mL of samples was drawn aseptically using a syringe at determined incubation times and subjected to product analysis.

Sampling and analysis

The samples were aseptically drawn from the fermentation bottle using a 1-mL syringe and transferred into a 1.5-mL Eppendorf tube. The protocols were conducted after removing the gas produced from the fermentation using a sterilized needle. The samples were centrifuged at 10,000 rpm for 5 min using a microcentrifuge (5415 D, Eppendorf) to separate the cells and the liquid. The liquid was analysed for pH determination, reducing sugars concentration, acetone, butanol, ethanol, acetic acid, and butyric acid concentration, while the pellet was used to determine the cell concentration. The reducing sugars concentration was analysed using the dinitrosalicylic acid (DNS) method described by Miller (1959), and the composition was analysed using high-performance liquid chromatography (HPLC) following the method described by Linggang *et al.* (2013). The concentrations of the solvents and acids were analysed using gas chromatography (GC-17A, Shimadzu, Japan) following the method outlined by Ibrahim *et al.* (2012). The cell concentration was determined based on the optical density (OD) measured at 620 nm using a spectrophotometer (GENESYS 20, Thermo Scientific, USA) calibrated with dry cell weight (DCW) as the standard. The pH was measured using a pH meter (Mettler Toledo, USA).

Solvent and acid yields expressed in g/g were calculated by dividing the total solvent or total acid produced (in g/L) by the total sugar consumption (in g/L). The biomass yield (in g/g) was determined as cell concentration (in g/L) divided by total sugar consumption (in g/L). The total sugar consumption is the amount of sugars supplied to the fermentation minus the amount of sugars present after the fermentation. The results presented are the average of at least three determinations with standard deviation.

RESULTS AND DISCUSSION

ABE Fermentation using Glucose–Based Medium without Buffer

The profile of ABE fermentation by *C. acetobutylicum* ATCC 824 in a glucose-based medium (20 and 40 g/L) without buffer at an initial pH of 5.5 with a yeast extract concentration of 6.0 g/L was determined. The glucose concentration of 20 g/L was used to mimic the ABE fermentation using 20 g/L of sugars from pretreated OPEFB. It should be noted that the maximum sugar concentration that could be obtained from enzymatic saccharification of pretreated OPEFB by cellulase was around 30 g/L, with a hydrolysis

percentage of approximately 70%. Meanwhile, a 40 g/L glucose concentration was used to check the possibility that the solvent production could be enhanced when a higher glucose concentration was used in the buffering system.

In all the ABE fermentations conducted in this experiment, the switch from the acidogenic phase (acid production) into the solventogenic phase (solvent production) was observed, while the remaining glucose after the fermentation was identified as an indicator for inhibition phenomena. The acid production began at the log phase of cell growth (within the first 24 h), while the solvent production occurred after 24 h of fermentation time, referred to as the "switch" of phase from acidogenesis into solventogenesis. In the fermentation using 40 g/L of glucose without buffer (Fig. 1), the glucose was initially converted into acetic acid before it was switched into solvent production with a total solvent concentration of 9.15 g/L. Glucose was almost fully utilised, indicating that the cells did not experience any inhibitory effect. This experiment showed that the acetic acid was predominantly produced within 24 h with the maximum acid concentration equivalent to 4.52 g/L. This acid production rate equivalent to 0.19 g/L/h was considered low in comparison with the findings of Sun and Liu (2012), who employed the same genus of *Clostridia*.

In contrast, the ABE fermentation using 20 g/L of glucose without buffer (Fig. 2) experienced an "acid crash" phenomenon. Although the solventogenic phase was observed, the glucose uptake ceased after 48 h and remained unchanged even after 72 h with residual glucose of 3.73 g/L, resulting in low solvent production (1.34 g/L). It can be observed that, unlike fermentation in 40 g/L of glucose, fermentation using 20 g/L produced both acetic and butyric acids within 24 h of fermentation time. A higher total acid concentration (6.77 g/L) was detected during the first 24 h, with an acid production rate equal to 0.28 g/L/h, 2 times higher than the acid produced in 40 g/L of fermentation, suggesting a high acid concentration was the main reason for the cell inhibition phenomenon. The pH value and the butyric acid concentration have been accepted as the major factors influencing the transition of the acidogenic to the solventogenic phase (Geng and Park 1994). However, the exact conditions remain unknown and are difficult to determine (Liu and Yang 2006).

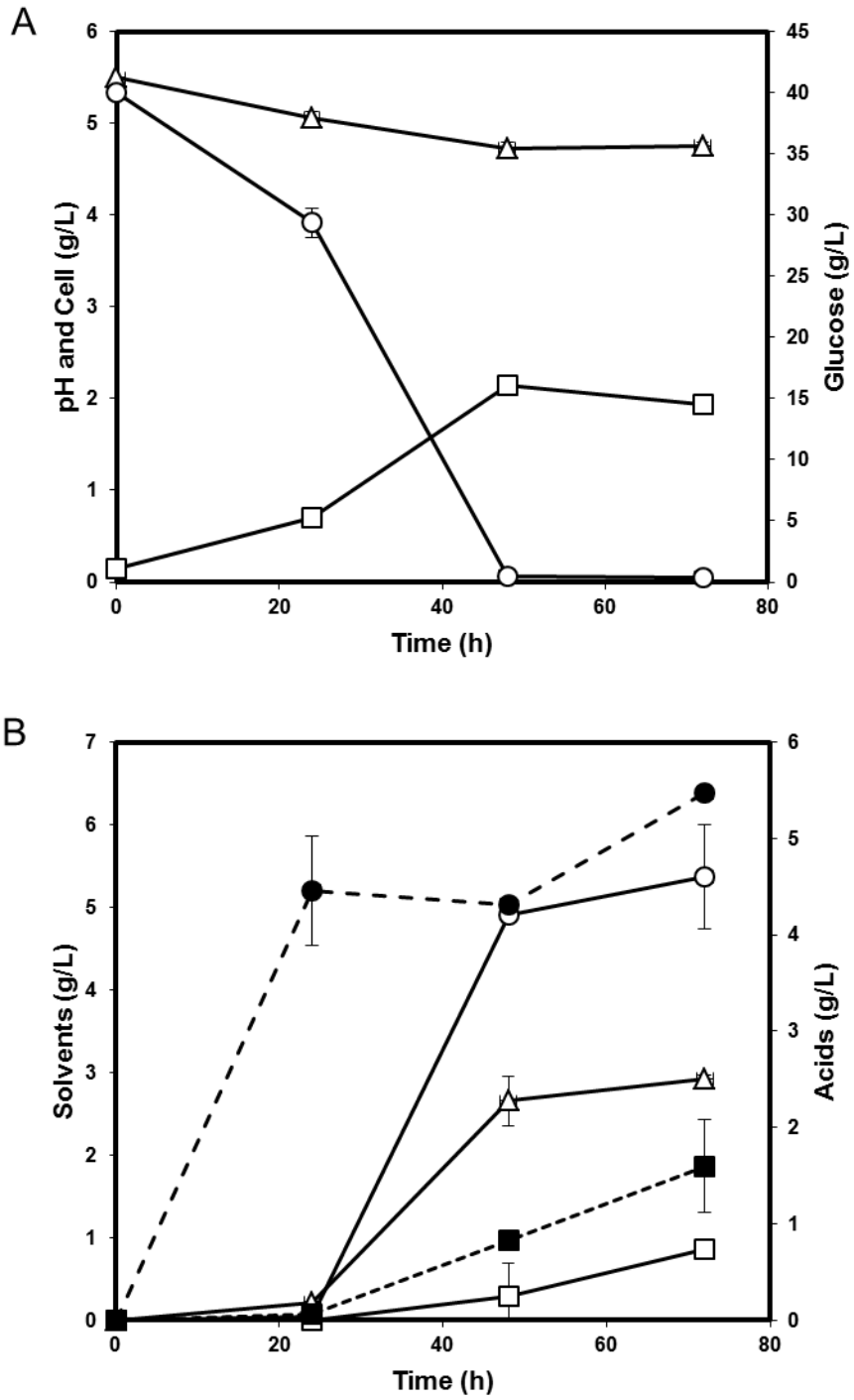

Fig. 1. ABE fermentation by *C. acetobutylicum* ATCC 824 using 40 g/L of glucose-based medium. Fermentation conducted at initial pH 5.5 without buffer. (A) Symbols represent: □: cell growth, ○: glucose consumption, ∆: pH. (B) Symbols represent: ○: butanol, □: ethanol, ∆: acetone, ●: acetic acid, ■: butyric acid

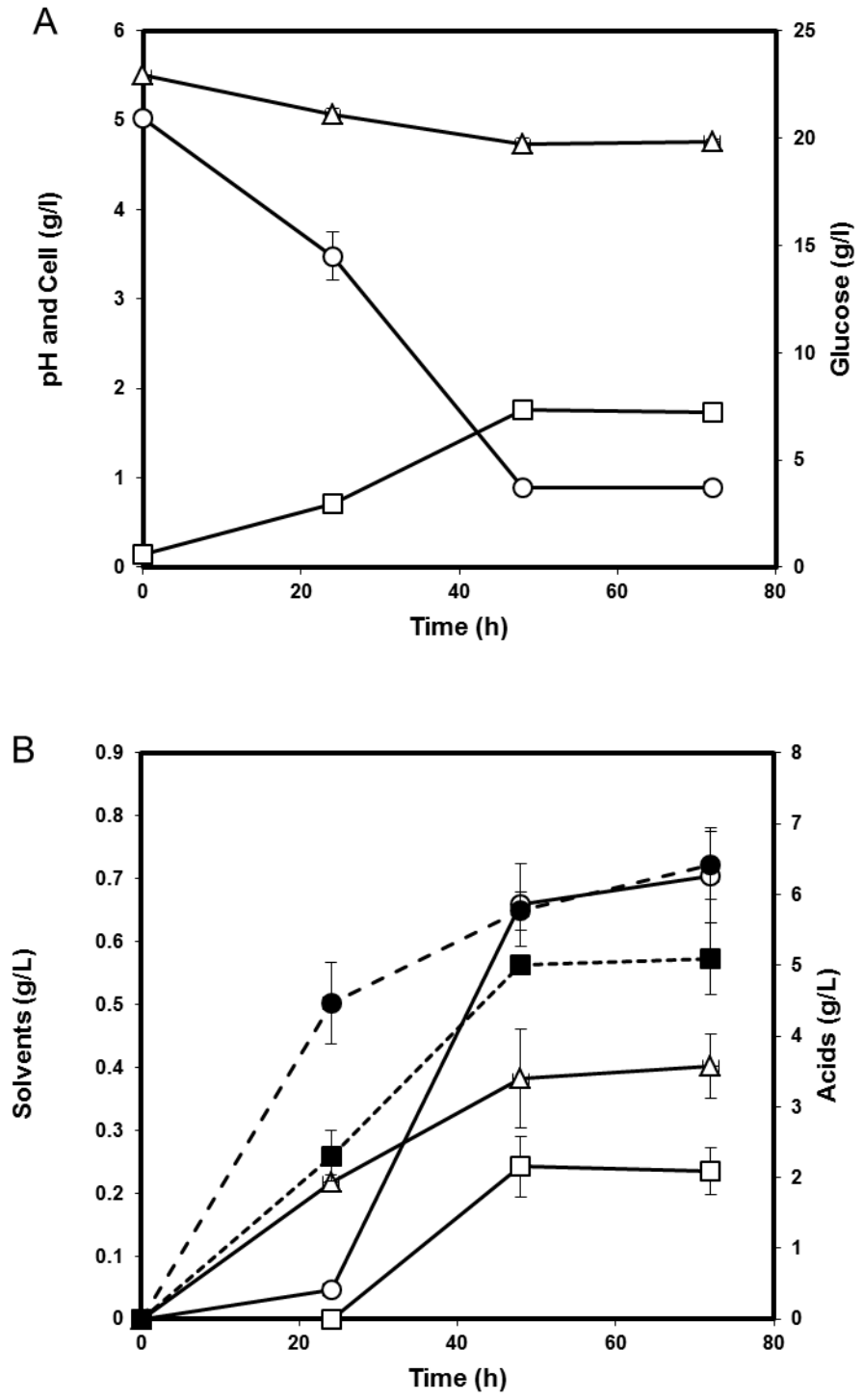

Fig. 2. ABE fermentation by *C. acetobutylicum* ATCC 824 using 20 g/L of glucose-based medium. Fermentation conducted at initial pH 5.5 without buffer. (A) Symbols represent: □: cell growth, ○: glucose consumption, ∆: pH. (B) Symbols represent: ○: butanol, □: ethanol, ∆: acetone, ●: acetic acid, ■: butyric acid

Glucose concentration might cause the cell to generate enough acids to be subsequently converted into solvents. During the solventogenic phase, the cells require acids in the protonated form to be reassimilated with glucose for solvent formation (Kell *et al.* 1981; Rogers and Gottschalk 1993). In fermentation using 20 g/L of glucose, the cell

produced more acids than solvents. This is due to the low glucose concentration, which was not enough to produce solvent that occurred after the cells reached the stationary phase. After the acid formation, the remaining amount of glucose was too low for the cells to complete the solventogenic phase, while acids were still being produced in the system. The recommencement of solvent production should occur before the "acid crash" phenomenon (usually after 60 h of fermentation time), but in this experiment, with limited glucose, the solventogenic phase ceased. This result suggested that ABE fermentation by *C. acetobutylicum* ATCC 824 tended to produce acids at a low glucose level, while solventogenesis was most likely favoured at higher glucose concentration.

Effects of Buffer on ABE Fermentation

Many researchers have observed acid accumulation during ABE fermentation. Accumulation of acid may reduce the pH to below 4.5, which can inhibit the cell growth, hence the cell cannot switch from acidogenesis to solventogenesis. Therefore, the use of buffer has been suggested as one of the methods to control the pH value, in order to allow the cell to undergo the solventogenic phase (Lee *et al.* 2008). This study investigated ABE fermentation using 20 and 40 g/L of glucose-based medium with buffer to be compared with fermentation without buffer. The comparison of ABE fermentation after 72 h of fermentation time using 20 and 40 g/L of glucose prepared in a medium with and without buffer is shown in Table 1. In this experiment, fermentation using 20 g/L of glucose with buffer produced about 30% lower amounts of acids as compared to 20 g/L fermentation without buffer, whereas about 54% higher of solvent production was observed in fermentation with buffer as compared to without buffer. The cells experienced less acid inhibition, and the shift from acidogenesis into solventogenesis was apparently better, suggesting that the presence of buffer improved the solvent production.

In fermentation using 40 g/L of glucose with buffer, the fermentation products after 72 h were almost identical to those acquired in fermentation without buffer. Both fermentations, with and without buffer, produced total solvents of 8.77 and 9.15 g/L with total acids of 4.95 and 7.41 g/L, respectively. All the glucose was utilised, indicating that the cells were not inhibited by the "acid crash" phenomenon. Thus, utilisation of buffer at this glucose concentration had no significant effect. Although there are some studies on ABE fermentation using various concentrations of glucose or sugars from various sources, as presented in Table 2, the effect of utilising low sugar concentrations on ABE fermentation with and without buffer in comparison to higher sugar concentration is not conclusive yet. It is important to study ABE fermentation at low glucose level because the maximum amount of sugars that could be produced from the saccharification of pretreated OPEFB was about 30 g/L without undergoing any concentration procedures. Results of this study will be useful for a simultaneous enzymatic saccharification and ABE fermentation, the demand for which has increased in recent years.

Table 1. ABE Fermentation by *C. acetobutylicum* ATCC 824 using Glucose-Based Medium With and Without Buffer (average ± standard deviation)

Parameters	20 g/L glucose		40 g/L glucose	
	With buffer[a]	Without buffer	With buffer[a]	Without buffer
Final pH	5.03±0.05	4.58±0.03	5.14±0.02	4.76±0.03
Glucose consumption (g/L)	18.91±0.22	17.23±0.67	39.82±0.19	39.75±0.47
Cell growth				
Cell conc. (g/L)	1.93±0.06	1.74±0.06	1.89±0.00	2.12±0.03
Biomass yield (g/g)	0.10	0.10	0.05	0.05
Solvents production				
Acetone conc. (g/L)	0.42±0.09	0.40±0.05	2.84±0.37	2.92±0.15
Butanol conc. (g/L)	2.11±0.05	0.70±0.07	5.33±0.08	5.37±0.64
Ethanol conc. (g/L)	0.40±0.16	0.24±0.04	0.60±0.42	0.86±0.10
Total solvents conc. (g/L)	2.93±0.30	1.34±0.16	8.77±0.87	9.15±0.89
Acetone yield (g/g)	0.02	0.02	0.07	0.07
Butanol yield (g/g)	0.11	0.04	0.13	0.14
Ethanol yield (g/g)	0.02	0.01	0.02	0.02
Total solvents yield (g/g)	0.15	0.08	0.22	0.23
Acids production				
Acetic acid conc. (g/L)	0.99±0.39	6.41±0.48	4.02±0.22	5.47±0.01
Butyric acid conc. (g/L)	3.18±0.27	5.45±0.51	0.93±0.01	1.94±0.48
Total acids conc. (g/L)	8.17±0.66	11.86±0.99	4.95±0.23	7.41±0.49
Acetic acid yield (g/g)	0.05	0.37	0.10	0.14
Butyric acid yield (g/g)	0.17	0.32	0.02	0.05
Total acids yield (g/g)	0.43	0.69	0.12	0.19

[a]Fermentations with buffer were conducted using 0.1 M phosphate buffer; All fermentations were conducted at an initial pH of 5.5; Data obtained after 72 h of fermentation time.

Table 2. ABE Fermentation by *C. acetobutylicum* using Glucose at Various Fermentation Conditions

Strain	Fermentation condition			Solvents (g/L)	ABE yield (g/g)	References
	Initial pH	[Glucose] (g/L)	Temp. (°C)			
C. acetobutylicum ATCC 824	Maintained at 4.5 using pH-control system	60	37	15.0	0.3	Li *et al.* (2011)
C. acetobutylicum XY16	6.5 with no pH-control	60	37	18.1	0.3	Guo *et al.* (2012)
	6.5 and maintained at pH 4.9 using pH-control system	60	37	19.2	0.3	
C. acetobutylicum ATCC 824	6.5 using 65 mM phosphate buffer	40	37	na	–	Bryant and Blaschek (1988)
C. acetobutylicum P262	6.0 with no pH-control	30	35	8.2	0.3	Madihah *et al.* (2001)
C. acetobutylicum DSM 1731 recombinant	6.5 and maintained at pH above 5.0 using pH-control system	80	37	18.5	0.2	Wang and Chen (2011)
C. acetobutylicum ATCC 824	5.5 using 1.0 M of phosphate buffer	40	37	8.8	0.2	This study
	5.5 with no pH-control	40	37	9.15	0.2	

na – not analysed or not mentioned in the report

Changes in pH throughout the fermentation with and without buffer were also observed. It was found that the buffer was able to maintain a pH above 5.0, even after 72 h of fermentation time. The initial pH value in the fermentation without buffer dropped to pH 4.58 and pH 4.76 for both fermentations using 20 g/L and 40 g/L of glucose, respectively. Although few studies have shown a requirement for the ABE fermentation to decrease the pH value below 5.0 for solvent production (Jones and Woods 1986; Lee *et al.* 2008), this study, however, found that the solventogenic phase was still initiated at pH above 5.0 and gave a comparable total solvent yield. The initiation of the solventogenic phase is influenced by the amount of acids in the broth, most probably the amount of undissociated acids instead of depending on the pH value measured during the transition process. In ABE fermentation at pH 4.8 studied by Fond *et al.* (1985), 50% of the total acids that permeated the cell were in the undissociated form. The onset of solventegenesis, however, occurred at different concentrations of acids (measured in the culture broth) because of the different concentrations of acid inside and outside the cells (Bryant and Blaschek 1988; Maddox *et al.* 2000). The intracellular acid concentration is most likely different from the concentration measured in the culture medium and, because of that, the solventogenesis occurred at various pH values.

Effects of initial pH values

The initial pH value is an important parameter that influences ABE fermentation (Zhu and Yang 2004), whereby the amount of acids produced becomes a criterion for the switch from acid into solvent production. It has been reported that the solventogenic phase does not usually occur when fermentation is conducted at an initial pH approaching neutral (Jones and Woods 1986; Maddox *et al.* 2000). However, some studies have still reported the solventogenic phase occurred in fermentation conducted at this initial pH value (mostly at pH 6.5) (Madihah *et al.* 2001; Qureshi *et al.* 2008; Sun and Lui 2012). A comparison of ABE fermentation at various pH conditions using various species of *Clostridia* and glucose concentration is summarised in Table 2. Various pH conditions have been applied for ABE fermentation, resulting in various ABE production levels. It is difficult to determine the best initial pH condition for ABE because other factors (*e.g.*, controlled and uncontrolled pH, species of microorganism employed, sugar concentration, and inhibition compounds) also affect the formation of solvents.

In the present study, acid yield increased from below 0.2 g/g of acid yield at pH lower than 6.0 to above 3.0 g/g of acid yield at pH higher than 6.5 (Table 3). A higher amount of acids was produced at higher initial pH, which then inhibited the cell metabolic pathways, as indicated by a low glucose uptake. The remaining glucose at initial pH 6.5 and 7.0 was above 14 g/L. Acids of 9.55 g/L and 7.61 g/L produced at initial pH values of 6.5 and 7.0, respectively, might be toxic to the cells. In nature, this acid production should be detoxified by the solvents produced during the solventogenic process (Rogers and Gottschalk 1993). Unfortunately, because of the high initial pH value, the cell was unable to shift to the solventogenic phase and kept producing acids until the level of acids produced became toxic to the cell. This phenomenon occurred when the combination of undissociated acetic and butyric acid above a critical threshold value affected metabolic activity and stunted the glucose uptake and solvent production. The exact value of the threshold of acids is difficult to quantify, but it is in the range of 57 to 60 mM (Evans *et al.* 1998), which is equivalent to about 4.44 g/L. Ibrahim *et al.* (2012) reported that acid inhibition occurred when acid concentration was between 5 and

13 g/L using *Clostridium butyricum* EB6. This value may vary depending on the bacterial strain or species employed for the ABE fermentation. However, the relatively high concentration of acids is toxic to the cells. The optimum pH condition reported for solvent production by *C. acetobutylicum* ATCC 824 was between 4.8 and 6.2 (Razak *et al*. 2012; Linggang *et al*. 2013; Jiang *et al*. 2014; Kudahettige-Nilsson *et al.* 2015)

Table 3. ABE Fermentation by *C. acetobutylicum* ATCC 824 at Various Initial pH Values with Buffer (average ± standard deviation)

Parameters	Initial pH				
	5.0	5.5	6.0	6.5	7.0
Final pH	5.23±0.01	5.14±0.02	4.87±0.00	4.68±0.09	4.79±0.10
Sugars consumption (g/L)	37.47±0.3	39.82±0.2	37.58±1.0	26.06±0.1	25.59±2.0
Solvents production					
Acetone conc. (g/L)	2.67±0.84	2.84±0.37	1.29±1.25	1.68±0.13	2.09±2.32
Butanol conc. (g/L)	5.27±0.05	5.33±0.08	4.06±0.36	0.85±0.18	0.28±0.35
Ethanol conc. (g/L)	0.30±0.42	0.60±0.42	0.15±0.01	0.19±0.27	0.06±0.08
Total solvents conc. (g/L)	8.24±1.31	8.77±0.87	5.50±1.62	2.72±0.58	2.43±2.75
Acetone yield (g/g)	0.07	0.07	0.03	0.06	0.08
Butanol yield (g/g)	0.14	0.13	0.11	0.03	0.01
Ethanol yield (g/g)	0.01	0.02	0.00	0.01	0.00
Total solvents yield (g/g)	0.22	0.22	0.15	0.10	0.09
Acids production					
Acetic acid conc. (g/L)	4.00±0.55	4.02±0.22	4.77±0.37	4.73±0.31	4.51±0.51
Butyric acid conc. (g/L)	1.15±0.39	0.93±0.01	1.90±0.15	4.82±0.74	3.10±2.97
Total acids conc. (g/L)	5.15±0.94	4.95±0.23	6.67±0.52	9.55±1.05	7.61±3.48
Acetic acid yield (g/g)	0.11	0.10	0.13	0.18	0.18
Butyric acid yield (g/g)	0.03	0.02	0.05	0.18	0.12
Total acids yield (g/g)	0.14	0.12	0.18	0.37	0.30

All fermentations were conducted using 40 g/L of glucose-based medium in 0.1 M phosphate buffer.
Data obtained after 72 h of fermentation.

Effects of buffer concentrations

Increasing the buffer capacity has been suggested as the simplest way to control the pH changes in ABE fermentation to subsequently improve the glucose uptake, reducing acid inhibition, and promoting the solventogenic phase (Lee *et al.* 2008). A higher acid concentration was observed in ABE fermentation conducted at initial pH values of 6.0, 6.5, and 7.0, as previously mentioned. It should be noted that, in that particular experiment, the pH dropped to below 5.0 compared to ABE fermentation at initial pH 5.0 and 5.5, which showed that the buffer capacity at 0.1 M concentration did not work. For that reason, further experiments using higher phosphate buffer concentrations (0.2, 0.4, and 0.8 M) were used to investigate the capability of these buffer concentrations to control the pH value and to reduce the inhibitory effects of acids during fermentation. The shift from acidogenesis to solventogenesis may have been induced by

the accumulation of butyric acid in the form of butyrate or undissociated butyric acid (Lin and Blaschek 1983; Terracciano and Kashket 1986). However, as mentioned before, the amount must not be above the threshold value because it may then inhibit cell metabolism.

As shown in Table 4, at buffer concentration of 0.2 M for both initial pH values of 6.0 and 6.5, the butyric acid produced was approximately 8.41 and 5.90 g/L, respectively. The total acids were above 13 g/L for both fermentations, which inhibited the ability of cells to continue their pathways. At higher buffer concentrations (0.4 and 0.8 M), lower amounts of butyric acid were obtained, but the formation of acetic acid remained high. In the metabolic pathway of *C. acetobutylicum*, acetate is required for the formation of acetone and ethanol, while butyrate for the formation of butanol (Lee *et al.* 2008; Shinto *et al.* 2008). Thus, at higher acetic acid concentration, more ethanol (pH 6.0) and acetone (pH 6.5) were produced, but very little and/or no production of butanol was observed due to lower butyric acid production. These results suggested that, by increasing the buffer capacity or by increasing the buffer concentration, the cells have the tendency to produce ethanol and acetone instead of butanol. In addition, the cells were not able to maintain their metabolism at higher buffer concentrations.

Table 4. ABE Fermentation by *C. acetobutylicum* ATCC 824 at Various Phosphate Buffer Concentrations (average ± standard deviation)

Phosphate buffer		**Final pH**	**ABE production (g/L)**			**Total ABE (g/L)**	**Acids production (g/L)**		**Total acids (g/L)**
Initial pH	Buffer conc. (M)		Acetone	Butanol	Ethanol		Acetic acid	Butyric acid	
6.0	0.2	5.01	0.53±0.01	0.64±0.02	0.19±0.00	1.36±0.03	8.42±0.10	8.41±0.47	16.83±0.57
	0.4	4.86	0.05±0.07	nd	4.37±0.02	4.42±0.09	8.09±0.48	1.76±0.95	9.85±1.43
	0.8	5.06	0.03±0.04	nd	4.20±0.08	4.23±0.12	6.50±0.20	0.61±0.17	7.11±0.37
6.5	0.2	5.16	4.88±1.28	0.51±0.03	nd	5.39±1.31	7.11±1.71	5.90±0.84	13.01±2.55
	0.4	6.49	2.10±1.32	nd	nd	2.10±1.32	4.51±0.52	3.10±0.97	7.61±1.49
	0.8	6.58	nd	nd	nd	nd	5.18±0.34	0.39±0.21	5.57±0.55

All fermentations were conducted using 40 g/L glucose-based medium.
Data obtained after 72 h of fermentation.
nd – not detected

ABE Fermentation using Sugars from Pretreated OPEFB

The ABE production was also conducted using sugars from pretreated OPEFB. The enzymatic hydrolysis of pretreated OPEFB by crude cellulase cocktail (Abd-Aziz *et al.* 2012) produced approximately 30 g/L of reducing sugars, which are contained hexose and pentose. The sugars consisted of 41% glucose, 26% xylose, 9% arabinose, and the remaining are galactose and mannose, similar sugars composition to that reported by Ibrahim *et al.* (2012). The sugars were diluted using distilled water to 20 g/L so that it was comparable to the ABE fermentation using 20 g/L of glucose-based medium.

In the present experiment, it was shown that the ABE fermentation using 20 g/L of sugars from pretreated OPEFB produced 0.37 g/L of acetone, 1.69 g/L of butanol, and 0.19 g/L of ethanol with a total solvent production of 2.25 g/L after 72 h of fermentation (Fig. 3). This solvent production was 23% lower compared with the solvents produced in a glucose-based medium with buffer, which had a total solvent concentration equal to 2.93 g/L (Table 1). The sugar consumption for these two carbon sources was slightly different, where 18.91 g/L of glucose was consumed compared to 17.11 g/L of sugars from pretreated OPEFB with ABE yields of 0.15 and 0.13 g/g for glucose and OPEFB sugars, respectively. A study by Liu and Yang (2006) found that when all types of sugars (glucose, xylose, and arabinose) from wheat bran hydrolysate were supplemented to *C. acetobutylicum*, almost all of the glucose was consumed, but approximately 50% of the xylose and 26% of the arabinose remained in the system. Fond *et al.* (1986) found that glucose was a preferable sugar for ABE fermentation, but the presence of xylose in the mixture promoted acid reassimilation. Ezeji *et al.* (2007) reported fermentation using various types of sugars (glucose, xylose, arabinose, mannose, galactose, and cellobiose), where the amount of solvents obtained from ABE fermentation using glucose was similar to the ABE fermentation using xylose by employing *C. beijerinckii* as the inoculum.

CONCLUSIONS

1. Acetone-butanol-ethanol fermentation by *C. acetobutylicum* ATCC 824 using 20 g/L of glucose-based medium produced a higher amount of solvents in the presence of 0.1 M phosphate buffer at initial pH 5.5 as compared to other pH values and buffer concentrations.
2. The phosphate buffer had insignificant effects on solvent production at higher glucose concentrations (40 g/L).
3. At higher initial pH values, the cell was inhibited by the increased amount of acids in the protonated form, which subsequently reduced the cell ability to produce solvents.
4. Higher phosphate buffer concentrations (more than 0.2 M) at higher initial pH values (6.0 and 6.5) were not favourable for the cell to produce butanol, but enhanced the formation of other solvents, *i.e.*, acetone and ethanol.

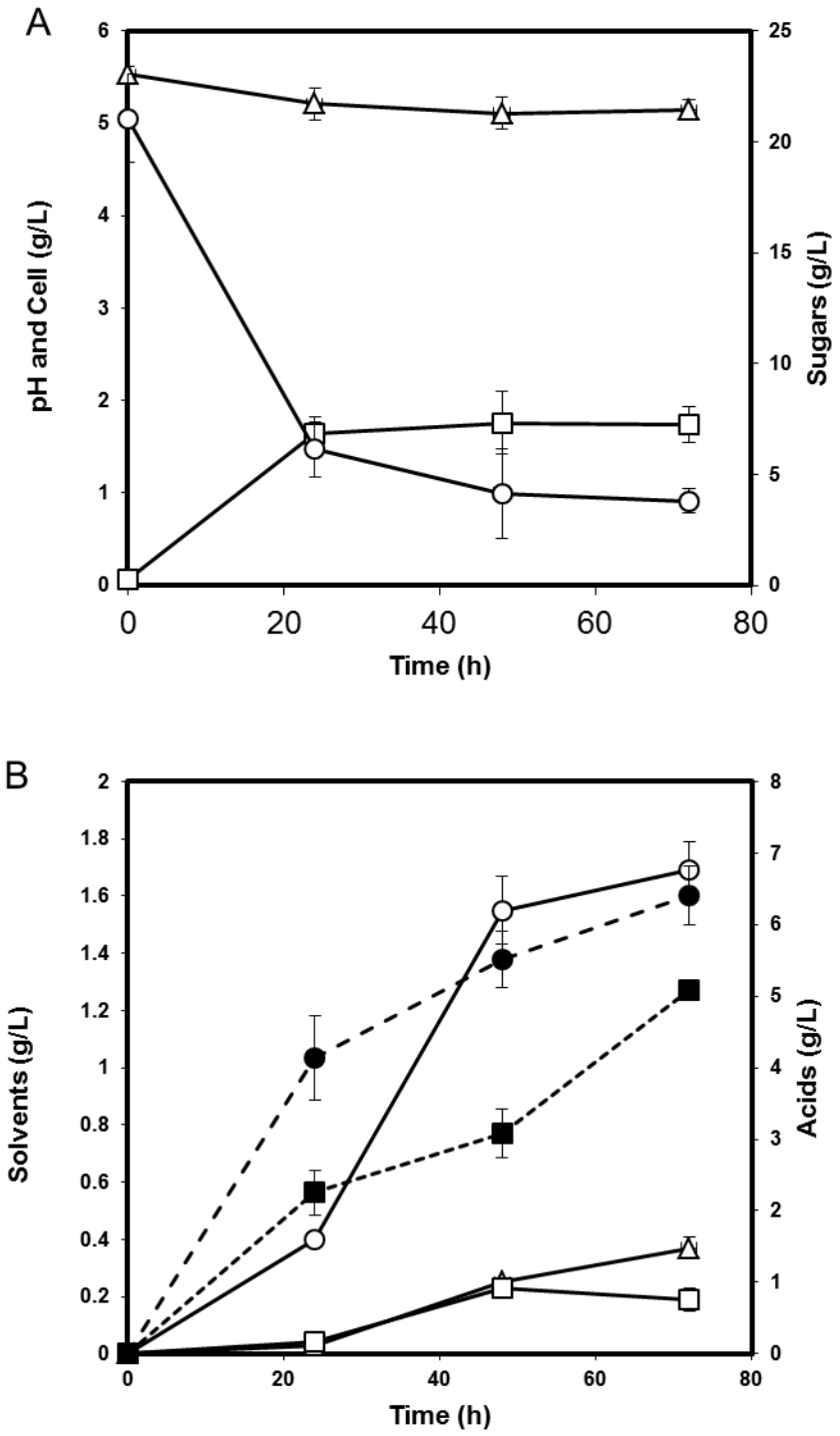

Fig. 3. ABE fermentation by *C. acetobutylicum* ATCC 824 using 20 g/L of sugars from pretreated OPEFB. Fermentation conducted at initial pH 5.5 in 0.1 M phosphate buffer. (A) Symbols represent: □: cell growth, ○: sugars consumption, ∆: pH. (B) Symbols represent: ○: butanol, □: ethanol, ∆: acetone, ●: acetic acid, ■: butyric acid.

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