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Original article

Impact of pH and butyric acid on butanol production during batch fermentation using a new local isolate of *Clostridium acetobutylicum* YM1

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ABSTRACT

The effect of pH and butyric acid supplementation on the production of butanol by a new local isolate of *Clostridium acetobutylicum* YM1 during batch culture fermentation was investigated. The results showed that pH had a significant effect on bacterial growth and butanol yield and productivity. The optimal initial pH that maximized butanol production was pH 6.0 ± 0.2. Controlled pH was found to be unsuitable for butanol production in strain YM1, while the uncontrolled pH condition with an initial pH of 6.0 ± 0.2 was suitable for bacterial growth, butanol yield and productivity. The maximum butanol concentration of 13.5 ± 1.42 g/L was obtained from cultures grown under the uncontrolled pH condition, resulting in a butanol yield ($Y_{P/S}$) and productivity of 0.27 g/g and 0.188 g/L h, respectively. Supplementation of the pH-controlled cultures with 4.0 g/L butyric acid did not improve butanol production; however, supplementation of the uncontrolled pH cultures resulted in high butanol concentrations, yield and productivity (16.50 ± 0.8 g/L, 0.345 g/g and 0.163 g/L h, respectively). pH influenced the activity of NADH-dependent butanol dehydrogenase, with the highest activity obtained under the uncontrolled pH condition. This study revealed that pH is a very important factor in butanol fermentation by *C. acetobutylicum* YM1.

1. Introduction

Butanol is a highly efficient biofuel that has earned renewed attention recently due to its potential as a renewable and environmentally friendly biofuel substitute for gasoline. Butanol is produced by acetone-butanol-ethanol (ABE) fermentation by anaerobic solvent-producing *Clostridium* strains (Al-Shorgani et al., 2012a; Chandrasekaran and Bahkali, 2013). Butanol as a fuel offers many superior advantages to ethanol, including its high energy content, less corrosive nature, low freezing point, high octane number, high hydrophobicity and the fact that it can be blended with gasoline or used directly in current engines without any modifications (Al-Shorgani et al., 2012b,d; Dürre, 2007; Qureshi et al., 2014).

However, the production of butanol via the ABE fermentation process by *Clostridium* suffers from some set-backs, including butanol toxicity due to product accumulation, low yield and productivity, and the high cost of the substrate and product recovery. Although significant efforts have been made and newer methods have been proposed in increasing numbers to curb these limitations in the fermentation process, more research is required to address these issues (Al-Shorgani et al., 2012c).

Proposed approaches, including screening for new strains of solvent-producing microbes capable of producing higher concentrations of butanol with higher tolerance against high concentrations of solvent, using renewable and readily available wastes, and optimization of the fermentation process could contribute to solving the issues related to the low productivity of butanol production.

Typically, ABE production by *Clostridium* species is achieved during biphasic fermentation. The first phase is called the acidogenic phase. During this phase, the major products are acetate,

Abbreviations: ABE, acetone-butanol-ethanol; NADH-BDH, NADH-dependent butanol dehydrogenase.

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butyrate, hydrogen and carbon dioxide. This acidogenic phase is usually associated with the exponential growth phase of cell division. The second phase is the solventogenic phase, in which the produced acids are reconsumed by the cells and used to produce acetone, butanol and ethanol (or isopropanol instead of acetone in some strains of *Clostridium beijerinckii*) (Andersch et al., 1983; S.Y. Lee et al., 2008; Servinsky et al., 2014). However, the shift from the acidic phase to the solvent formation phase is not well understood. Undissociated butyric acid and changes in pH have been reported to be involved in triggering the shift from acid production to solvent production in solvent-producing *Clostridium* strains (Gottwald and Gottschalk, 1985; Monot et al., 1984). Although studies have investigated the mechanism behind ABE fermentation, the enzymatic regulation of the shift mechanism from the acidogenic phase to the solventogenic phase are not understood. Moreover, the role of pH-induced gene regulation on butanol fermentation needs further investigation to optimize the continuous fermentation of butanol using *Clostridium acetobutylicum* on an industrial scale (Haus et al., 2011).

In this study, *C. acetobutylicum* YM1 was used to produce butanol in a batch fermentation process under different pH strategies with and without the addition of butyric acid. The objective was to investigate the influence of pH and butyric acid on butanol production by strain YM1. This strain is one of the solvent-producing *Clostridia* strains that was recently isolated from Malaysian agricultural soil and has been used for the production of biohydrogen and butanol (Abdeshahian et al., 2014; Al-Shorgani et al., 2013, 2015).

2. Materials and methods

2.1. Microorganism

The newly isolated strain of *C. acetobutylicum* YM1 was used in this study (GenBank accession No. KC969670). This microorganism was isolated from a local agricultural soil in Malaysia. The isolate was maintained as a stock in glycerol and stored at −30 °C. Prior to the experiments, the isolate were activated in tryptone yeast-extract medium (TYA) supplemented with 20 g/L glucose.

2.2. Medium and fermentation conditions

The effect of pH on butanol fermentation in a batch culture of *C. acetobutylicum* YM1 was studied in a 5 L bioreactor (INFORS HT, Switzerland) with a working medium volume of 3 L. TYA medium supplemented with glucose as a carbon source at a concentration of 50 g/L was used in this study. The TYA consisted of tryptone (6 g/L), yeast extract (2 g/L), $MgSO_4{\cdot}7H_2O$ (0.3 g/L), ammonium acetate (3 g/L), KH_2PO_4 (0.5 g/L) and $FeSO_4.7H_2O$ (0.01 g/L). Butyric acid was added in some experiments.

The medium was sterilized by autoclaving at 121 °C and 15 psi for 15 min prior to use. Nitrogen gas was used to create anaerobic conditions by flushing the medium for 4 min before inoculation. The fermentation conditions for the experiments were as follows: temperature 30 °C, inoculum size of 10% and bioreactor agitation at 200 rpm throughout the fermentation period. The pH was automatically adjusted to the desired pH value by feeding with either NaOH (5 M) or HCl (5 M). The investigation into the effect of the initial pH on butanol production was conducted in 100 mL serum bottles with a working volume of 50 mL using TYA medium supplemented with 30 g/L glucose.

2.3. Analytical methods

Samples of fermentation broth were collected at appropriate time points for the analysis of solvents, acids, cell growth and glucose. The samples were centrifuged at 7000 rpm prior to analysis for butanol, acids and glucose. Butanol and butyric acid were analyzed using a gas chromatograph (7890A GC-System, Agilent Technologies, USA) equipped with a flame ionization detector (FID). A 30 m capillary Supelco column (Equity 1^{TM} column) was used, and the detection and injection temperatures were set as 240 °C and 260 °C, respectively. Helium was used as the carrier gas with a flow rate of 1.5 mL/min.

Cell growth was measured using a UV–Vis spectrophotometer (Shimadzu, UV mini-1240, Japan) at 600 nm. Glucose was measured using a glucose kit [glucose oxidase kit; GOD, (E.C. 1.1.34), Roche Ltd., Switzerland] following the manufacturer's instructions. The intracellular protein of the cells was measured according to the protocol of Bradford (Bradford, 1976).

2.4. Butanol dehydrogenase activity

For determining the activity of NADH-dependent butanol dehydrogenase (NADH-BDH), *C. acetobutylicum* YM1 samples were collected and cell free extracts were prepared using the method described by Salleh et al. (2008). NADH-BDH was measured in the forward (butanol formation) direction at 30 °C. To measure the NADH-BDH activity, 50 mM of butyraldehyde and 0.5 mM of NADH in Tris-HCl buffer (50 mM, pH 6) was used. The volume of reaction mixture was 1 mL and it contains 6% (v/v) of cell lysate or enzyme solution except the blank control. The reaction was initiated by the addition of 60 µL enzyme solution, and the absorbance of the solution at 340 nm was recorded for 500 s using a UV–Vis spectrophotometer (Shimadzu-UV 1800, Japan). One unit (U) of activity is equivalent to amount of enzyme required for oxidization of 1 µmol of NADH/min. The specific activity of NADH-BDH was calculated as the enzyme activity (U) per mg protein.

3. Results and discussion

3.1. Effect of initial pH on butanol production

The initial pH of the fermentation medium in butanol production is an important factor that significantly affects the fermentation process. The effect of different initial pH values of 5.0, 5.5, 6.0, 6.2, 6.5 and 7.0 (adjusted by addition of either 3 M HCl or 3 M NaOH) were investigated in TYA medium supplemented with 30 g/L glucose. Fig. 1 shows the production of butanol in batch fermentation of *C. acetobutylicum* YM1. The results showed that an initial pH of 6.0 and 6.2 represented the optimal pH values for butanol production; these initial pH values resulted in the production of 6.22 and 6.28 g/L of butanol, respectively. Lower butanol

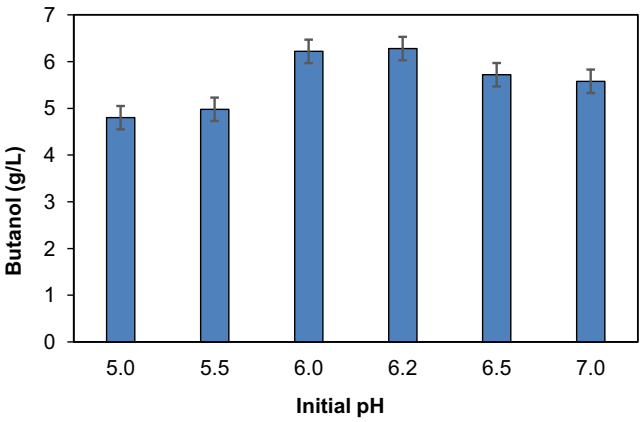

Fig. 1. Effect of initial pH on butanol production by *C. acetobutylicum* YM1.

production was obtained in cultures at other pH values. *Clostridium* is an anaerobic microorganism; as such, it seems to be unable to maintain the internal pH value (intracellular pH) at a more or less constant level. Based on these results, it is clear that the initial pH noticeably affected butanol production. This result could be attributed to a direct effect on enzyme activities and metabolic pathways (Lay, 2001). The optimal pH for butanol fermentation using different carbohydrates is usually between pH 5.0 and pH 6.5 (Jones and Woods, 1986). Accordingly, we found that *C. acetobutylicum* YM1 preferred an initial pH of 6.2 for butanol production using glucose as the sole carbon source. It was previously proposed that the optimum initial pH of ABE fermentation is in the range of 5.5–6.0 (Malaviya et al., 2012). Our results are in agreement with those of Li et al. (2014) who studied butanol production by *Clostridium* sp. strain BOH3 at various pH values ranging from pH 4.0 to 8.0 during batch fermentation and concluded that an initial pH value of 6.0 resulted in the highest butanol production.

3.2. Effect of uncontrolled pH

The optimal initial pH for *C. acetobutylicum* YM1 was found to be in the range of 6.0–6.2. To estimate the effect of uncontrolled pH on solventogenesis initiation, cell growth and final butanol production, strain YM1 was cultivated in a 5 L bioreactor. The fermentation medium was adjusted initially to pH 6.0. Then, the culture after inoculation was allowed to run without pH control. Fig. 2 shows the fermentation profile of growth, pH, butanol and butyric acid formation.

During the first 22 h, the pH decreased during the log phase (exponential growth phase) to 4.43 concomitant with increasing cell growth. The decrease in pH was associated with an increase in butyric acid production. After 22 h, the pH remained constant for 15 h, then re-increased until it reached pH 4.9 at 72 h. A decrease in the butyric acid concentration was observed during the period of constant pH, indicating a re-assimilation of butyric acid by the cells as a precursor of butanol production.

Notably, when butanol formation was initiated at 13 h, the glucose uptake began to slow and the butyric acid concentration decreased. Moreover, the drop in pH stopped and remained constant at 4.46 ± 0.2 for approximately15 h. The slow utilization of glucose was associated with the shift to solvent and butyric acid utilization, which may be attributed to the use of butyric acid as a co-substrate. Similar pH behavior in uncontrolled ABE fermentation process has been previously reported for solvent-producing *Clostridium acetobutylicum* (Husemann and Papoutsakis, 1990; Yang et al., 2013).

Butanol production (solventogenic phase) was initiated at 13 h when the cell growth reached the stationary growth phase. Once butanol production was initiated, the butyric acid concentration began to decrease in an inverse manner. The final butanol production was recorded as 13.5 ± 1.42 g/L at the fermentation time of 72 h. A butanol yield and productivity of 0.27 g/g and 0.188 g/L h, respectively, were recorded at the same time point.

3.3. Effect of controlled pH

To evaluate the influence of pH control on solventogenesis initiation, cell growth, butyric acid production and final butanol production, *C. acetobutylicum* YM1 was cultivated in a 5 L bioreactor and the pH was controlled at different levels (pH 6.0, 5.5, and 4.8) by feeding the culture with NaOH (5 M) or HCl (5 M). Additionally, in a separate group, the pH was controlled during the fermentation phase at pH 6.0. In the first instance, the pH was maintained at pH 6.0 during the first 12 h of the cultivation, then was left uncontrolled for the remainder of the fermentation process. In the second group, the pH was uncontrolled for the first 12 h (initial pH 6.0), and then controlled at pH 6.0 thereafter by automatic feeding with NaOH (5 M) or HCl (5 M).

As described above, we found that an initial pH of 6.0 was optimal for butanol production for the YM1 strain. Hence, we first controlled the pH of the cultures at 6.0 throughout the fermentation process. The details obtained for butanol production, cell growth, and butyric acid and glucose consumption are presented in Fig. 3.

When the pH was maintained at 6.0, cell growth reached its maximum at 14 h with an OD_{600} of 2.479. Moreover, the stationary phase was very short, and after 17 h cell growth started to decline. Maintaining the pH at 6.0 inhibited growth and butanol production compared to the uncontrolled pH condition, and butyric acid utilization ceased once cell growth began to decrease. Butanol production reached 2.2 g/L at 24 h and slowly rose to a maximum of 2.53 g/L at 52 h. Butyric acid production increased during the growth phase and reached 2.2 g/L at 19 h. Butyric acid was utilized once butanol production was initiated; correspondingly, its concentration dropped to 1.76 g/L at 23 h. Subsequently, butyric acid increased to a final concentration of 2.53 g/L. Glucose was con-

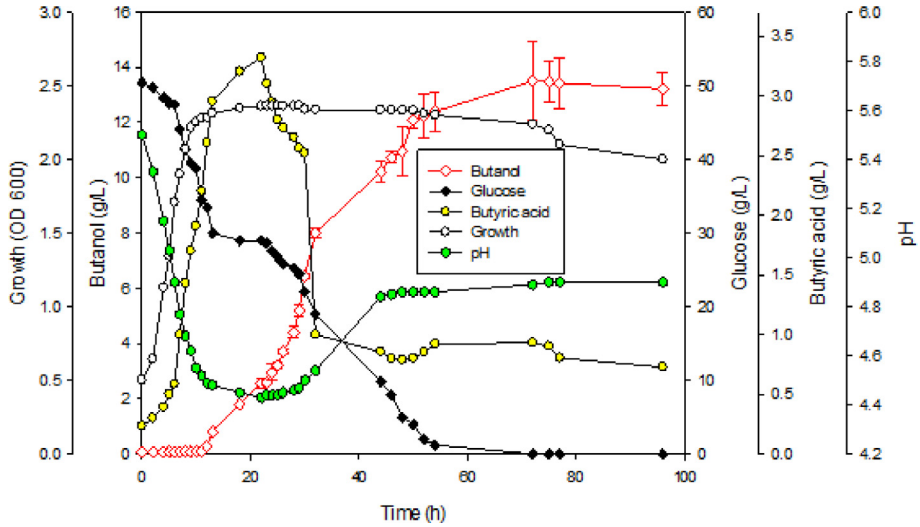

Fig. 2. Effect of uncontrolled pH (initial pH 6.0) on cell growth, pH, glucose utilization and the production of butanol and butyric acid.

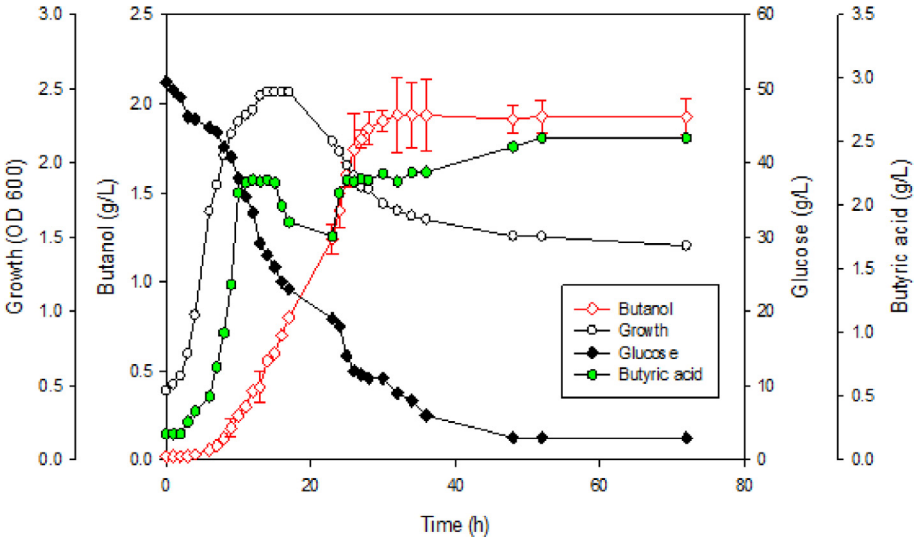

Fig. 3. Effect of controlled pH 6.0 on cell growth, pH, glucose utilization and the production of butanol and butyric acid.

sumed with an uptake rate of 0.67 g/L h, leading to a final glucose concentration of 2.94 g/L.

Butanol production was significantly decreased in the controlled pH cultures at pH values of 6.0, 5.5 and 4.8 compared to the uncontrolled pH culture. However, cell growth of all of the cultures was favored during the exponential growth phase. The biomass of cultures grown under pH control at pH 6.0 after 12 h of cultivation time (controlled pH at 6.0 in the second phase) showed a decline in growth and a shortened stationary growth phase (Figs. 3 and 7).

In this study, we found that butanol production commenced when the culture pH dropped to 4.8 ± 0.2 in the uncontrolled pH culture; hence, we decided to apply a controlled pH of 4.8. The biomass growth in the cultures with controlled pH at 5.5 and 4.8 exhibited a longer stationary growth phase compared to the culture with controlled pH at 6.0 (Figs. 4 and 5). Moreover, the butanol production was higher in the uncontrolled pH culture compared to the cultures with controlled pH at 6.0 (Table 1). The results from the controlled pH experiments at 5.5 and 4.8 revealed a significant decrease in butanol production, while butyric acid production was comparable or higher than the uncontrolled pH culture condition. The pH controlled experiment at pH 4.8 produced the highest yield of butyric acid (6.45 g/L). Thus, controlling the pH of the fermentation medium at pH 6.0, 5.5 and 4.8 noticeably inhibited butanol formation but not glucose utilization, growth during the exponential phase or butyric acid production.

At controlled pH 4.8, a high concentration of butyric acid (6.45 g/L) was produced during the acidogenic phase, with 5.05 g/L obtained at the end of the fermentation process. Thus, little butanol production (5.20 g/L) was recorded. Although an accumulation of acids during ABE fermentation could cause an acidic crash during butanol production, no inhibition of *C. acetobutylicum* YM1 growth was observed (Fig. 5). It has been reported that controlling ABE fermentation under low pH conditions can cause an inhibitory effect on bacterial activity (Maddox et al., 2000). However, the low final pH in the uncontrolled cultures was normal and did not show any inhibitory effects on ABE fermentation.

While studying the effect of pH control on one of the fermentation stages of ABE fermentation (acidogenic phase or solventogenic phase), we found that controlling the first phase (first 12 h) at pH 6.0 led to the production of a higher butanol concentration. Additionally, the growth biomass was more pronounced and the stationary phase was longer compared to cultures controlled at pH 6.0 throughout fermentation (Fig. 6). Butyric acid levels were

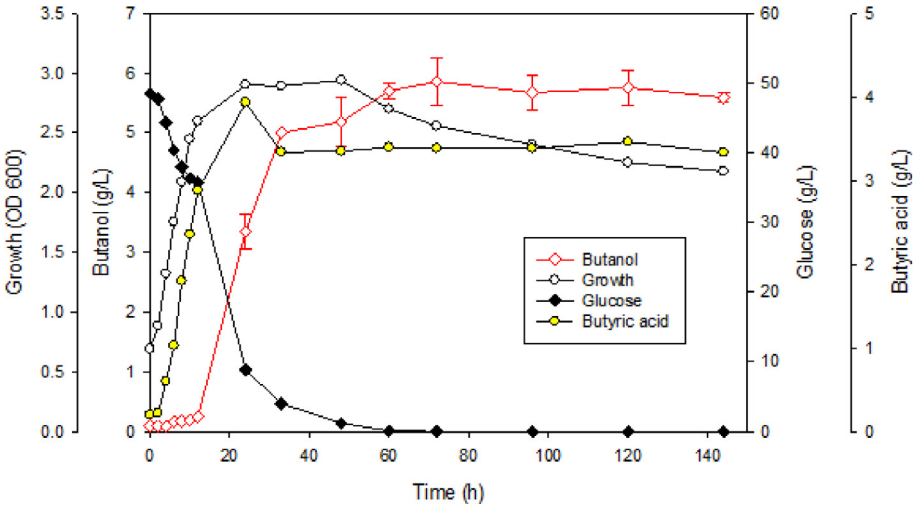

Fig. 4. Effect of controlled pH at 5.5 on cell growth, pH, glucose utilization and the production of butanol and butyric acid.

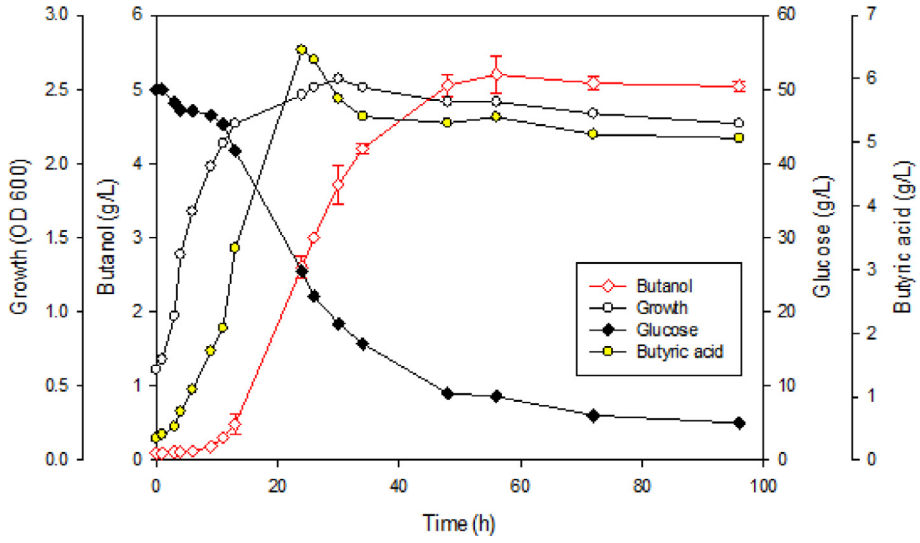

Fig. 5. Effect of controlled pH at 4.8 on cell growth, pH, glucose utilization and the production of butanol and butyric acid.

Table 1
Influence of different pH values on the butanol fermentation process by *C. acetobutylicum* YM1.

	Uncontrolled pH (initial pH 6.0)		Controlled pH			Controlled pH at 6.0 with addition of 4 g/L butyric acid		Controlled pH for 12 h at pH 6.0	Controlled pH after 12 h at pH 6.0
	–	With 4 g/L butyric acid	6.0	5.5	4.8	Addition time 0 h	Addition time 14 h		
Maximum cell growth (OD_{600})	2.370	2.248	2.479	2.940	2.570	2.586	2.446	2.401	2.559
Maximum butanol production (g/L)	13.5 ± 1.42	16.5 ± 0.80	1.937 ± 0.21	5.86 ± 0.4	5.20 ± 0.64	2.252 ± 0.31	1.896 ± 0.54	4.389 ± 0.17	1.655 ± 0.65
Fermentation time (h)	72	101	32	72	56	48	96	96	52
Final pH	4.9	5.55	–	–	–	–	–	5.7	–
Maximum butyric acid	3.32	5.74[a]	2.20	3.93	6.45	4.47[a]	6.177[a]	3.577	3.08
Butanol productivity (g/L h)	0.188	0.163	0.061	0.081	0.093	0.047	0.020	0.046	0.032
Butanol yield $Y_{P/S}$ (g/g)	0.27	0.345	0.047	0.117	0.123	0.048	0.041	0.091	0.033

[a] Supplemented culture with 4 g/L butyric acid.

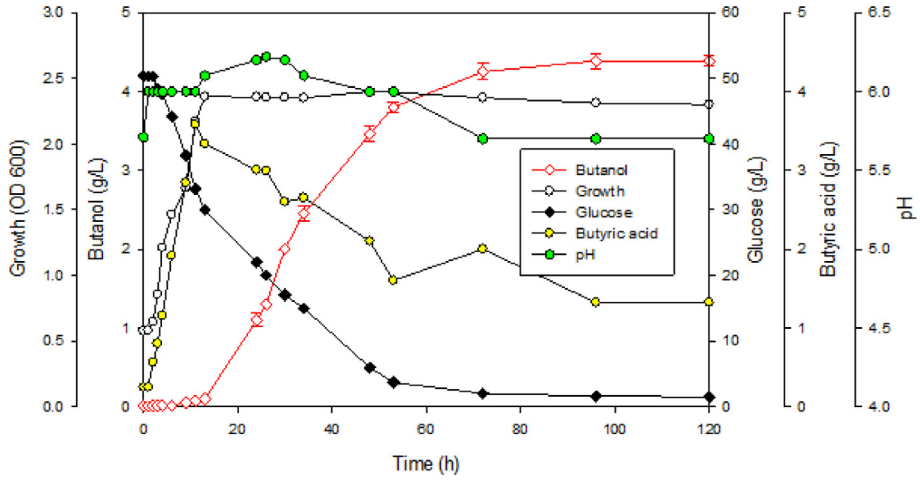

Fig. 6. Profile of cell growth, pH, butyric acid, glucose consumption and butanol production under two-stage pH control: the pH was controlled at 6.0 for 12 h, and then the fermentation was run without control.

also decreased when the pH was increased to 6.2 as a result of butyric acid uptake after the induction of butanol formation.

Fig. 7 shows the fermentation profile of pH controlled cultures after 12 h at pH 6.0. The production of butanol was lower than was observed in the culture with controlled pH during the first 12 h at pH 6.0. However, the cell growth profile was similar to the profile recorded in the pH controlled culture at pH 6.0. Continuous feeding with chemicals to maintain the pH at 6.0 seems to

contribute to the decrease in the exponential growth phase and consequent decrease in butanol production in *C. acetobutylicum* YM1 cultures.

The low production of butanol may be a result of the accumulation of salts from the release of intracellular undissociated acids into the extracellular environment. Undissociated acids are secreted from cells through the cellular membrane into the medium, resulting in a low external pH and a correspondingly higher internal pH with a subsequent proton release inside the cells (Jones and Woods, 1989).

In our study, we applied a two-stage pH strategy. First, the pH was controlled at 6.0 for 12 h, and then the culture was allowed to proceed without pH control (Fig. 6). The second strategy utilized a starting pH of 6.0 without no pH control for 12 h, followed by a controlled pH at 6.0 throughout the remainder of the fermentation process (Fig. 7). Although the butyric acid concentration in the medium was observably high, this approach did not improve the production of butanol.

Recently, Li et al. (2014) found that a two-stage pH-shift strategy was optimal for a high specific growth rate and specific butanol production rate by *Clostridium* sp. BOH3. In this strategy, pH 6.0 was controlled for the first 6 h of cultivation, then the pH was allowed to drop until it reached 5.0. Then, the pH was again controlled at 5.0 until the end of the fermentation process (Li et al., 2014). In contrast, the results of our study revealed that cultivation of *C. acetobutylicum* YM1 under controlled pH conditions was not suitable for butanol production.

Amador-Noguez et al. (2011) reported similar observations when the culture of *C. acetobutylicum* was grown under pH control at 6.0. In this study, growth during the stationary phase was short and no acid re-utilization or obvious solventogenic phase was observed (Amador-Noguez et al., 2011).

In contrast, Geng and Park (1993) reported that controlled culture of *C. acetobutylicum* B18 at pH 6.0 was optimal for butanol production, glucose consumption, cell growth, and butyric acid uptake. Further increases in the pH beyond 6.5 (controlled pH conditions) resulted in a swift switch to the acid production mode (Geng and Park, 1993).

Therefore, the suitable pH for solvent production seems to depend on the particular strain and fermentation conditions used (Jones and Woods, 1986). The optimal pH for our strain *C. acetobutylicum* YM1 for maximum butanol production involved the application of an initial pH of 6.0 ± 0.2 without any control throughout the fermentation process.

3.4. Effect of butyric addition without control pH

Biomass growth and pH patterns during growth and butanol fermentation by *C. acetobutylicum* YM1 were similar under uncontrolled pH conditions with and without butyric acid supplementation (Figs. 2 and 8). The pH drop during the exponential growth phase was associated with an increase in the production of butyric acid. When butanol production was initiated, the pH decline ceased and the observed pH values increased over time. Cell growth in the stationary phase remained constant for a much longer time than was observed under a controlled pH of 6.0. The bacteria produced butyric acid in the acidogenic phase even following an initial supplementation of the medium with butyric acid; the maximum butyric acid level reached 5.74 g/L at 18 h before declining due to its consumption associated with butanol formation (Fig. 8).

3.5. Effect of butyric acid addition with controlled pH at 6.0

Supplementation of the fermentation medium with butyrate, acetate, lactate and propionate has been shown to improve final ABE production without affecting the onset of the solventogenic phase (Al-Shorgani et al., 2012a; Fond et al., 1985; Husemann and Papoutsakis, 1990; Yoshida et al., 2012). To evaluate the effect of butyric acid supplementation on fermentation under a controlled pH of 6.0, butyric acid (4 g/L) was added initially to the fermentation medium (before the inoculation); in another experiment, butyric acid was added after 14 h of fermentation. Both cultures were run under controlled pH conditions at pH 6.0.

In this study, the addition of butyric acid to the fermentation medium under non-optimal pH conditions did not improve the production of butanol. Compared to the culture with uncontrolled pH, the production of butanol in the butyric acid-supplemented culture at a controlled pH of 6.0 was very low. The addition of butyric acid initially or after 14 h in cultures with controlled pH at 6.0 produced 2.25 g/L and 1.896 g/L butanol, respectively. In contrast, butanol production was significantly higher (16.50 ± 0.8 g/L) under uncontrolled pH conditions (Table 1). These results indicate that pH plays a very important role in butanol production. As shown in Figs. 9 and 10, the majority of the added butyric acid was not consumed; growth was accelerated during the log phase but experienced a very early decline after the bacteria reached the stationary phase. However, no significant butanol was produced under controlled pH conditions at pH 6.0 with the presence of butyric acid in the medium, thus ultimately leading to a final concentration of un-utilized butyric acid. Although the bacterial growth was significantly affected by the controlled pH condition (pH 6.0) during the stationary phase, no effect was observed in the first phase (exponential growth phase).

Table 1 summarizes the production of butanol, butyric acid, the yield and productivity of butanol and the growth of strain YM1 under the different pH conditions applied in this study. The results obtained showed that the optimal pH for strain *C. acetobutylicum* YM1 used in this study was an initial pH of 6.0 with no further pH control. The yield and productivity of butanol produced from TYA medium under the uncontrolled fermentation process were 0.27 g/g and 0.188 g/L h, respectively. The addition of 4 g/L of butyric acid led to an enhancement of the butanol concentration from 13.5 ± 1.42 g/L to 16.50 ± 0.8 g/L. The yield (0.345 g/g) and productivity of butanol (0.163 g/L.h) when the medium was supplemented with butyric acid were also markedly improved.

Hüsemann and Papoutsakis (1988) reported a linear correlation between butanol and the undissociated butyric acid concentration at the initiation of solvent. The threshold concentration of undissociated acids that initiate solvent production is 19 mM (acids produced by the microbe itself); concentrations beyond this threshold decrease H_2 production and the onset of solvent formation (Van Ginkel and Logan, 2005). The amount of undissociated acids that can induce the shift towards solvent production may differ among solvent-producing *Clostridium* strains.

Acetate and butyrate supplementation of the medium used to culture *Clostridium beijerinckii* NCIMB 8052 was found to improve solvent production and affect the ratio of produced butanol/acetone. This effect may result from metabolic changes in ABE production. Additionally, it was observed that supplementation of the medium with butyrate prohibited the culture from degeneration phenomena due to continuous subculturing. The addition of 36 mM butyrate to the culture of *Clostridium beijerinckii* significantly enhanced butanol production, achieving 11.2 g/L butanol with a butanol/acetone ratio of 3:1, while only 0.45 g/L butanol was produced without butyrate supplementation (S.M. Lee et al., 2008).

During the growth of *Clostridium acetobutylicum* on a phosphate-limited synthetic medium, the bacteria were unable to maintain a constant intracellular pH (pH inside the cells), and acid (acetic and butyric) production was allowed during the acidogenic phase as well as ABE production during the solventogenic

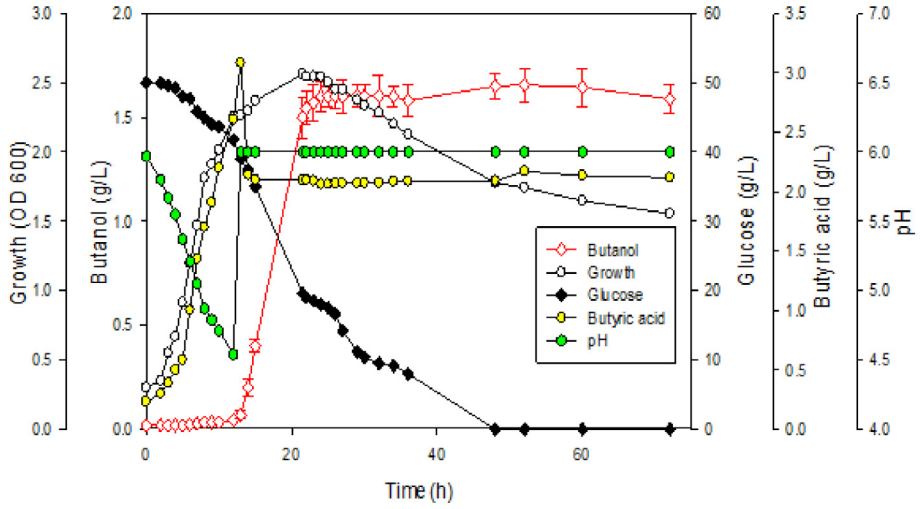

Fig. 7. Profile of cell growth, pH, butyric acid, glucose consumption and butanol production under two-stage pH control: the culture was started with an initial pH of 6.0 and run without control the pH. After 12 h it was controlled at 6.0 for the remainder of the fermentation time.

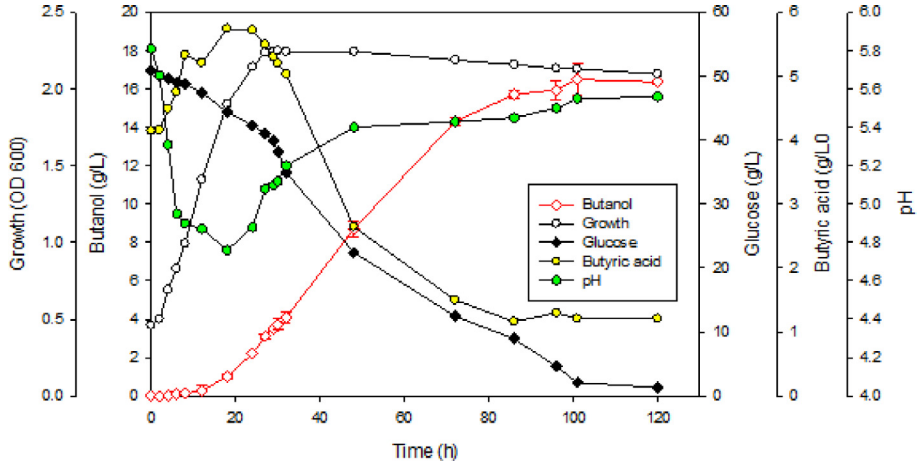

Fig. 8. Profile of cell growth, butyric acid, glucose consumption and butanol production in batch culture of YM1 under uncontrolled pH condition and a 4 g/L butyric acid supplementation.

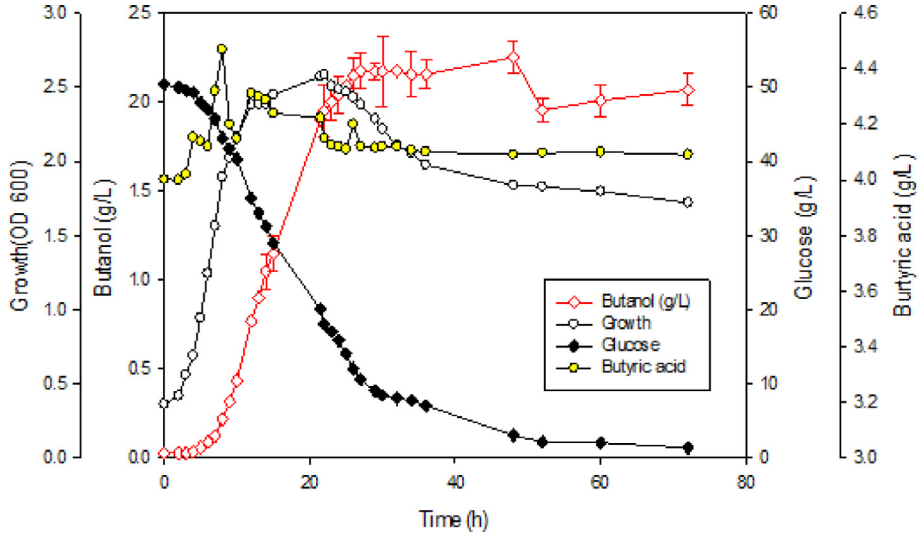

Fig. 9. Profile of cell growth, butyric acid, glucose consumption and butanol production in a batch culture of YM1 under controlled pH at 6.0 with initial supplementation with 4 g/L butyric acid.

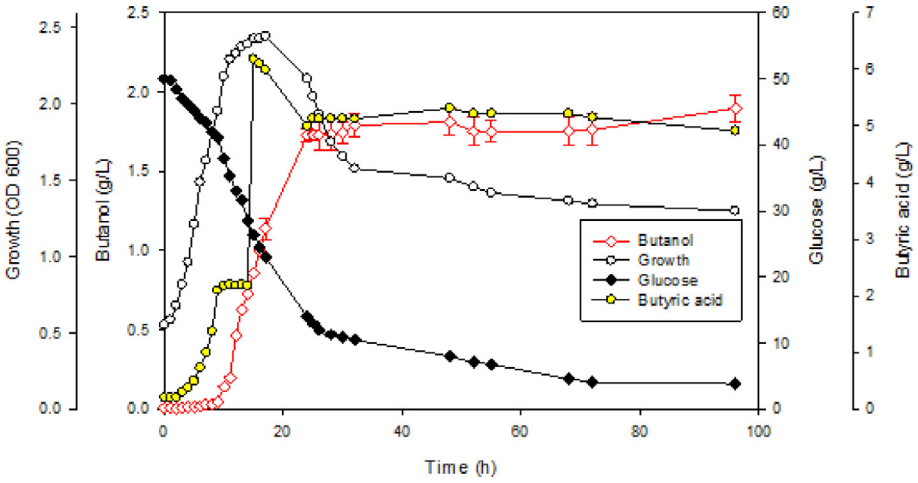

Fig. 10. Profile of cell growth, butyric acid, glucose consumption and butanol production in a batch culture of YM1 under controlled pH conditions (pH 6.0) with supplementation of 4 g/L butyric acid after 14 h of fermentation.

phase. When the external pH values ranged between 5.9 and 4.3, the intracellular pH was maintained at a constant ΔpH of 0.9–1.3. Similarly, a continuous culture of *Clostridium acetobutylicum* under solvent- producing conditions presented with a similar ΔpH (Gottwald and Gottsehalk, 1985).

During ABE fermentation, cultures that are grown in poorly buffered media without pH control do not shift to solvent formation; therefore, only acids are produced. Moreover, the cells die because sporulation does not occur under these conditions (Jones and Woods, 1986).

The solvent concentrations produced by *C. beijerinckii* VPI 13436 under controlled pH conditions at pH 6.8 were comparable to the solvent concentrations in uncontrolled cultures in which the pH was allowed to drop to below 5.0 (George and Chen, 1983).

Although suitable pH conditions in the medium may contribute to the initiation of solvent formation, a decrease in pH is required to allow the shift to solvent formation. However, pH is not the only triggering factor (Jones and Woods, 1986; Long et al., 1984).

Yang et al. (2013) found that butanol formation not only correlated with cell growth but also correlated with the pH of the fermentation process and the concentration of undissociated butyric acid (Yang et al., 2013).

It was also reported that when the internal pH fell below 5.5, the culture was unable to shift to solvent production; the solvent production process requires enzyme synthesis or at least enzyme activation (Andersch et al., 1983; Gottwald and Gottschalk, 1985). At low internal pH levels, the shift from acid formation to solvent production may not be possible. Therefore, the cell energy status required to perform the shift must be similar to conditions corresponding to an internal pH above 5.5.

It has been suggested that the main triggering factor that induces the culture shift under certain conditions is the concentration of undissociated butyric acid (Monot et al., 1983). The effect of undissociated acids in this process is based on the changing concentrations of butyrate and acetate. However, the effect is not related to the undissociated or dissociated forms of the acids themselves because the uncharged acids are biochemically inert compounds (Gottwald and Gottschalk, 1985). Thus, Gottwald and Gottschalk (1985) proposed raising the concentrations of internal butyrate; this approach raised the butyryl phosphate and butyryl coenzyme A concentrations. Usually, low phosphate and coenzyme A concentrations are the basis of the stimulating mechanism for solvent formation. Under these conditions, butyryl coenzyme A or butyryl phosphate and acetoacetyl coenzyme A are readily available substrates for butanol and acetone production. Subsequently, the shift from acid to solvent production occurs as follows.

First, butyrate is synthesized, the cell is maintained at a ΔpH, and the accumulation of internal butyrate leads to an increase in the concentration of butyryl coenzyme A (phosphate) and subsequent decrease in the concentration of coenzyme A (phosphate). These conditions provide the signal to the cell for the shift towards solvent production. When the signal is provided under appropriate internal pH conditions that are suitable for the protein synthesis machinery, the enzymes that are required for solvent production are synthesized or activated.

3.6. Effect of pH on NADH-dependent butanol dehydrogenase activity

In this study, we investigated the activity of the NADH-dependent butanol dehydrogenase enzyme (NADH-BDH) during fermentation at three different pH values. These pH conditions included non-controlled pH fermentation with an initial pH of 6.0, controlled pH at 6.0 for 12 h, and controlled fermentation at pH 6.0 for the remainder of the process. Fig. 11(a)–(c) shows the growth profile of *C. acetobutylicum* YM1, butanol production and NADH-BDH activity for the three studied pH conditions in a 5 L bioreactor. The profile of the specific NADH-BDH activities at three pH values is presented in Fig. 11d. The highest specific activity of NADH-BDH and the highest butanol production were observed when the pH was not controlled. Under all of the pH conditions studied, the maximum specific activities of NADH-BDH were achieved at 24 h in the non-controlled pH cultures and at 48 h in the cultures with controlled pH for 12 h or controlled pH during the fermentation process (Fig. 11).

The results obtained in this study show that NADH-BDH activity is proportional to the growth of the *C. acetobutylicum* YM1 strain; this finding is in agreement with a recent study by Jiang et al. (2014) that reported that the total enzyme activity correlated with cell growth during the solventogenic phase (Jiang et al., 2014). Lower NADH-BDH activities and lower butanol production were observed in pH-controlled fermentation media at pH 6.0 compared to the non-pH-controlled culture (Fig. 11c). As indicated in Fig 11c, maintaining the fermentation at pH 6.0 led to a short period of bacterial growth, a condition that permitted only a very short period of solvent production as the cells exited the exponential growth phase.

The NADH-BDH enzyme has a higher activity level in cultures without pH control. Subsequently, butanol production was higher

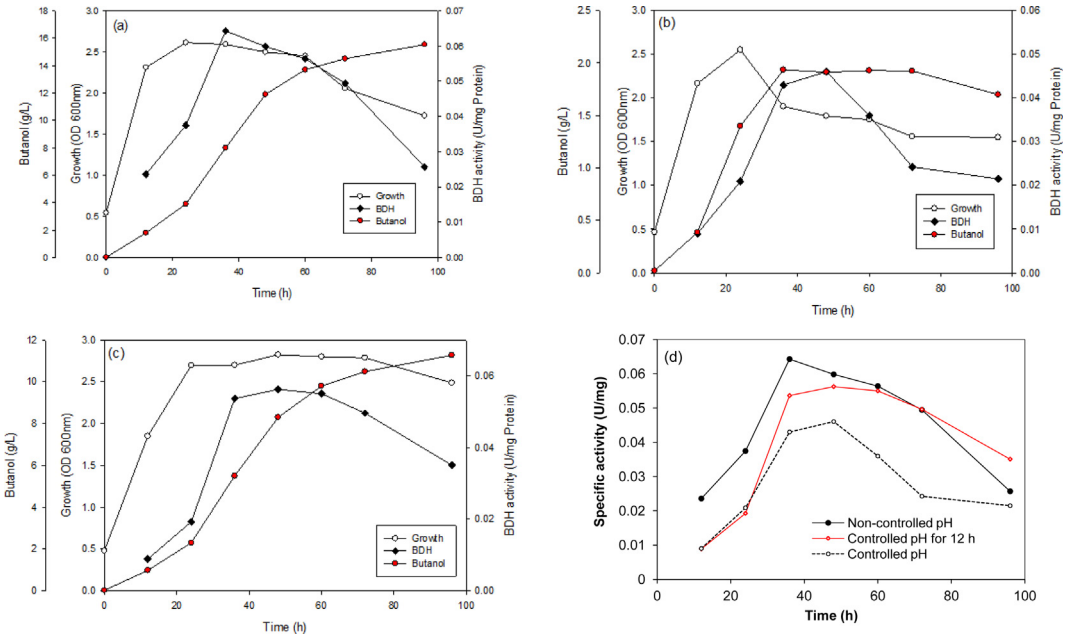

Fig. 11. Profile of growth, butanol production and NADH-dependent butanol dehydrogenase activity in butanol fermentation by *C. acetobutylicum* YM1 (a) in non-controlled pH culture, (b) controlled pH fermentation at 6.0, (c) fermentation controlled at pH 6.0 for 12 h and then allowed to proceed without control and (d) comparison of the specific activity of NADH-BDH under three different pH strategies.

in the non-controlled pH culture and lower when the pH was maintained at 6.0 throughout the fermentation process.

Based on these results, we conclude that the metabolites of *C. acetobutylicum* YM1 are significantly affected by the pH. Moreover, NADH-BDH activity directly reflects butanol formation in that high activity indicates high butanol production. The results show that pH control plays a very important role in butanol fermentation by solvent-producing *Clostridium* strains.

4. Conclusions

This study revealed that pH is a very important factor in butanol fermentation by *C. acetobutylicum* YM1. The suitable pH for butanol production in batch fermentation cultures of *C. acetobutylicum* YM1 is an initial pH of 6.0, followed by a lack of control throughout fermentation. Controlling the pH did not support butanol production by *C. acetobutylicum* YM1. Moreover the controlled pH conditions led to a shortened stationary phase characterized by declining growth and ultimately decreased butanol production. The presence of butyric acid in the medium was not necessary to initiate the solventogenic phase and enhance the production of butanol at inappropriate pH conditions. When the pH was controlled, a short solventogenic phase (stationary phase) was observed regardless of the presence of butyric acid. This effect may be due to an acid crash of fermentation under controlled pH conditions.

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