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Enhancement of butanol production in *Clostridium acetobutylicum* SE25 through accelerating phase shift by different phases pH regulation from cassava flour

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Abstract

A prominent delay with 12 h was encountered in the phase shift from acidogenesis to solventogenesis in butanol production when the substrate — glucose was replaced by cassava flour. To solve this problem, different phase of pH regulation strategies were performed to shorten this delay time. With this effort, the phase shift occurred smoothly and the fermentation time was shortened. Under the optimal conditions, 16.24 g/L butanol and 72 h fermentation time were achieved, which were 25.3 % higher and 14.3 % shorter than those in the case of without pH regulation. Additionally, the effect of $CaCO_3$ on "acid crash" and butanol production was also investigated. It was found that organic acids reassimilation would be of benefit to enhance butanol production. These results indicated that the simple but effective approach for acceleration of phase shift is a promising technique for shortening the fermentation time and improvement of butanol production.

Keywords: *Clostridium acetobutylicum*, ABE fermentation, pH regulation strategy, Phase shift, Cassava flour

1. Introduction

Due to the increasing concerns over the depletion of oil resources and the environmental issues, more attentions have been recently devoted to develop the renewable and green energy sources (Raganati et al., 2015b). The biofuels such as ethanol and butanol obtained from renewable resources are the excellent representatives. Among these representatives, butanol is considered the suitable second generation biofuel as it has many advantages such as higher energy content, higher octane number, less corrosive and lower solubility in water. More importantly, butanol is compatible with the current transportation pipeline and the automobile engine design. Therefore, the production of butanol through fermentation process has regained increasing attention in recent years (Jiang, W. et al., 2014, Malaviya et al., 2012).

Biobutanol can be produced through anaerobic fermentation using *Clostridium* sp. from starch-based or sugar-based feedstocks such as corn and molasses with ethanol and acetone as the major byproducts, and thus this fermentation process is designated as acetone-butanol-ethanol (ABE) fermentation (Wu et al., 2013). Some solventogenic *Clostridium* strains such as *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Clostridium saccharoperacetobutylicum* and *Clostridium saccharobutylicum* can be used as butanol producing strains during ABE fermentation. On the bases of the utilization of carbon sources, these four species can be divided roughly into two classes such as sugar and starch species. Furthermore, it was believed *C. beijerinckii* and *C. acetobutylicum* are the two major butanol producing strains in ABE fermentation industry (Keis et al., 2001, Patakova et al., 2013). Presently, the development of ABE fermentation at industrial scale has been hampered by its high cost of substrates, low final product concentrations and high product recovery costs (Kong et al., 2015). On the

other hand, economic analyses of ABE fermentation have indicated that if the final butanol concentration could be increased from 12 g/L to 19 g/L, the separation costs for butanol recovery from ABE fermentation broth would be cut in half (Ting et al., 2012). Therefore, the improvement of microbial strains, processes and substrates for its cost-competitive production is an issue of priority research (Becerra et al., 2015). Several methods such as metabolic engineering modification of producing strains, advanced fermentation techniques and downstream processing have already been used to overcome the above hampers (Fatehi. 2013, Li et al., 2014b).

ABE fermentation process could be divided into two distinct phases, i.e., acidogenic phase and solventogenic phase. Acidogenic phase occurs at logarithmic phase of cell growth and acetic and butyric acids accumulated during acidogenesis. Solventogenic phase appears in late logarithmic phase to early stationary phase of growth. These acids are re-assimilated to produce acetone, butanol, and ethanol by *clostridia* species during solventogenesis (Ellis et al., 2012). Furthermore, according to (Cheng et al., 2012), the butanol anabolism can be triggered if the pH value is below 5 and the butyric acid is over 2 g/L. However, the delay of phase shift often occurs during ABE fermentation processes. Some studies inferred that the reason accounting for this phenomenon is the "acid crash", which would often occur when excess acids (acetic and butyric acids) accumulation but without any re-assimilation. The environment that is essential for phase shift is deteriorated when an "acid crash" happens (Bankar et al., 2013, Li et al., 2012). Therefore, the development of an economic and a simple bioprocess is also desirable to avoid the phenomenon of "acid crash".

The fermentation substrate is believed as another important factor that determines the cost of butanol production (Raganati et al., 2015b). Various raw materials or renewable

agricultural wastes, such as corn (Qureshi and Blaschek. 2001) and sago starch (Al-Shorgani et al., 2012), have already been used as feedstocks for butanol production. However, a serious challenge to food security will present. Under this circumstance, the utilization of waste agricultural residues as the feedstocks for ABE fermentation has attracted more attentions. However, microorganisms were unable to grow well on such substrates unless they were subjected to a complex pretreatment such as inhibitor detoxification and dilute acid or enzymatic hydrolysis (Thirmal and Dahman. 2012). Among the available starch substances for ABE fermentation, cassava is very attractive because of its high production, low price, and particularly non-competitive with foods for cultivated land (Li et al., 2014a).

In this study, ABE fermentation by *Clostridium acetobutylicum* SE25 was carried out in a 15 L static and anaerobic fermentor. In order to solve the problem of the delay of phase shift, several pH regulation strategies (including different pH conditions, two-step pH regulation and different phases of pH regulation) were carried out. In addition, the effect of $CaCO_3$ on "acid crash" and butanol production was also investigated. The outcomes from this research indicate that the simple but effective pH regulation strategy is a promising method for more practical butanol fermentation processes.

2. Materials and methods

2.1 Microorganism and medium

C. *acetobutylicum* SE25 was maintained in 20 % (v/v) glycerol stocks at -80 ºC. The cell suspension was heat shocked for 100 S at 80 ºC followed by cooling down to room temperature on ice, and then transferred to a fresh modified tryptone-yeast extract-acetate medium (TYA medium) at 37 ºC for 16-18 h. Subsequently, 10-15 mL of

activated cultures were inoculated into 100 mL of development P2 medium, which was prepared in 250 mL screw capped bottle.

The development P2 medium consisted of the following ingredients: 60 g/L glucose, 3.0 g/L yeast extract, and 1 mL each of filter-sterilized stock solutions (minerals: 20 g/L $MgSO_4 \cdot H_2O$, 1 g/L $MnSO_4$, 1 g/L NaCl, 1 g/L $FeSO_4$; buffer: 50 g/L K_2HPO_4, 50 g/L KH_2PO_4, 220 g/L CH_3COONH_4; and vitamin: 0.1 g/L thiamin, 0.1 g/L para-aminobenzoic, 0.001 g/L biotin) was added to 1 L of P2 medium. Buffer, carbon sources and nitrogen sources were sterilized separately at 121 ºC for 15 min. Minerals and vitamins were filter-sterilized using sterile 0.2 μm membrane filters (Li et al., 2013).

The modified TYA medium was used as seed medium and it comprised of the following components: 40 g/L glucose, 2.0 g/L yeast extract, 6.0 g/L tryptone, 3.0 g/L CH_3COONH_4, 0.01 g/L $FeSO_4$, 0.2 g/L $MgSO_4 \cdot H_2O$, 0.75 g/L K_2HPO_4 and 0.75 g/L KH_2PO_4. The medium was autoclaved at 121 ºC for 15 min (Loyarkat et al., 2013).

2.2 Cassava flour pretreatment and hydrolysis

Cassava roots were obtained from a cassava-processing factory in Jiangsu, China and then were processed into cassava flour as described by Li et al., (2014a). Adequate amounts of cassava flour were added into distilled water to obtain a suspension of 80 g/L, which was prepared for subsequent enzymatic hydrolysis. Enzymatic treatment comprised of two steps: liquefaction and saccharification. Prior to liquefaction, $CaCl_2$ (1 g/L) was supplemented into cassava flour suspensions to obtained a concentration of 40 mg-Ca^{2+}/L and the pH was adjusted to 6.5 using 2 M NaOH or 2 M HCl. For liquefaction, a minuscule amount of α-amylase (8 U/g-cassava) was added into the

cassava flour suspensions. The enzymatic reaction was carried out at 100 ºC for 45 min, followed by cooling down to 60 ºC in a water bath and a decrease in pH to 4.5 using 2 M HCl. Subsequently, saccharification was performed by adding β-glucoamylase (120 U/g-cassava) at 60 ºC for 4 h. The enzyme was inactivated by heating at 80 ºC for 5 min after saccharification. The liquefied cassava flour was used as the medium for ABE production (Yang et al., 2015). α-amylase (20,000 U/mL) and β-glucoamylase (100,000 U/mL) were purchased form Boli Biological Products Co. Ltd, Taizhou, China.

2.3 Batch fermentation

ABE fermentation process was conducted in a 15-L bioreactor (LiFlus GX, Biotron Corp, China) with an initial working volume of 9 L containing the hydrolyzed cassava flour, sterilized in an autoclave (121 ºC for 15 min). Anaerobiosis was achieved by sparging oxygen-free high purity nitrogen gas into medium before and after inoculation. The pH of fermentation broth was adjusted by 2 M HCl or 2 M NaOH. The fermentation process was carried out at 37 ºC until the cell ceased secreting butanol and the operating pressure of the fermentor was controlled in a range of 0.02-0.04 MPa throughout the whole fermentation process. Before collecting samples from fermentation broth, agitation was first done for a short time (5 min, 200 rpm). And 10 mL samples were then regularly collected for the analysis of organic acids (acetic and butyric), total sugar and ABE.

2.4 Analytical methods

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Spectronic 200, Thermo scientific). The concentrations of glucose

and starch were determined using the method described by Thang et al., (2010).

Solvents and organic acids concentration were quantified by internal standard method using a gas chromatography (GC) system (GC-2010, Shimadzu Scientific Instruments, Japan) equipped with a flame ionization detector (FID) , along with capillary column (PED-20M, 30 m, 0.32 mmID,0.4 μm df). The column temperature was maintained at 210 ºC, the injector temperature at 280 ºC and the detector temperature at 240 ºC. The carrier gas and internal standard were nitrogen gas, isobutanol, isobutyric acid and phosphoric acid (for acidification), respectively. All samples were clarified by centrifugation at 10,000 rpm for 5 min and 0.4 μL of sample was injected. Solvent productivity was estimated as the solvent concentration achieved (g/L) divided by the fermentation time (h). The solvent yield was defined as the amount (expressed in g/g) of solvent produced from 1 g of total sugar (Guo et al., 2013).

3. Results and discussion

3.1 Comparison of glucose-based and cassava-based ABE fermentation performance

As an abundantly available and inexpensive feedstock, cassava is very suitably applied in ABE fermentation. In this study, C. *acetobutylicum* SE25 was cultivated on cassava flour and P2 fermentation medium to compare their fermentation performances. Furthermore, cassava flour (80 g/L) was used as the sole substrate for butanol production. As can be seen from Figs. 1b and d, when P2 medium was used as fermentation medium, the pH of the fermentation broth rebounded promptly after declining to their bottom levels. In contrast, the pH of the fermentation broth dropped down to bottom levels and then maintained at these levels for a long period (about 12 h) when using cassava substrate. In fact, this period represented the time delay in phase

shift from acidogenesis to solventogenesis, and which was about 12 h and 6 h for cassava flour and P2 fermentation medium, respectively. Furthermore, the lowest pH was 4.35 ± 0.19 and 4.45 ± 0.21, respectively, when using glucose and cassava flour as substrate. Moreover, it was found the final total acid concentration was 2.27 ± 0.11g/L and 2.44 ± 0.12 g/L, with glucose and cassava flour as substrate, respectively.

As shown in Figs.1 a and c, when cassava flour was used as fermentation medium, the highest concentration of total solvent and butanol reached 19.15 ± 0.93 g/L and 12.96 ± 0.66 g/L, respectively. The productivities of total solvent and butanol were 0.23 g/(L·h) and 0.15 g/(L·h), respectively. For glucose-based ABE fermentation, 18.13 ± 0.84 g/L of total solvent (containing 3.72 ± 0.14 g/L acetone, 1.37 ± 0.07 g/L ethanol and 13.04 ± 0.66 g/L butanol) was obtained at the end of the fermentation. The productivities of butanol and total solvent were 0.22 g/(L·h) and 0.30 g/(L·h), respectively, which were about 46.7 % and 30.4 % higher than those of the cassava flour-based ABE fermentation. On the other hand, in China, the prices of glucose and cassava were 580-725 $ and 153-177 $ per 1,000 Kg, respectively (www.taobo.com). Furthermore, in order to obtain 1,000 Kg butanol by ABE fermentation, based on the preceding results, the quantities of consumption glucose and cassava flour were about4,600 Kg and 6,170 Kg, respectively. Consequently, the feedstock cost for ABE fermentation were about 2,668-3,335 $ per 1,000 Kg and 944-1,092 $ per 1,000 Kg, respectively. Therefore, the low cost of cassava determines that cassava-derived ABE fermentation is more competitive than glucose-derived. Additionally, about 4,560,000 tons of cassava is produced in 2012 and cassava is a nonfood crop in China (Jiang et al., 2014). Thus, cassava is a potential resource for the production of butanol if the problem of the phase shift delay could be successfully resolved and the fermentation time could

be simultaneously shortened.

3.2 Effect of different regulated pH values on butanol production

In previous experiments, it was found that the phase shift could not successfully complete in the process of phase shift period if the pH value of the fermentation broth maintained at a low level for a long time, and then affected the production of butnaol. To deal with the problem of the phase shift delay, the pH values were regulated at five levels: 4.5, 5.0, 5.5, 6.0 and 6.5. However, a small amount of insoluble solid could not be utilized if the method of direct fermentation of gelatinized cassava starch to acetone, butanol, and ethanol was used. Therefore, The OD value was very difficultly determined. Thus, cassava flour was hydrolysised by the method of enzymatic hydrolysis, and then was filtered by the method of suction filtration. The filtrate was used in this experiment. In addition, in our previous study, corn steep liquor was believed as a better nitrogen source for ABE fermentation from cassava flour (Li et al., 2014b). Therefore, corn steep liquor (1.5 g/L) was supplemented into the fermentation medium. The different pH of fermentation broth was obtained by 2 M HCl or 2 M NaOH.

The results, reported in table 1 and Fig. 2, clearly demonstrate that glucose conversion, fermentation time and solvent production are strongly pH dependent. When the pH values were increased from 5.5 to 6.5, the final concentrations of residual sugar were 8.41 g/L, 6.45 g/L and 7.38 g/L, respectively, which were obviously lower than those in other groups (27.56 g/L and 16.63 g/L). However, the production of butanol in these conditions were 7.50 ± 0.37 g/L, 6.45 ± 0.33 g/L and 5.94 ± 0.28 g/L, respectively, which were all lower than that of pH 5.0 (8.49 ± 0.42 g/L). Therefore, it was suggested that the higher pH conditions would be beneficial to cell growth, and the fermentation

time was then shortened. However, the concentration of total organic acid also increased at the same time, but the cells cannot be timely re-assimilated. On the other hand, when the pH value was maintained as 4.5, the phase shift could not also successfully complete. The concentration of residual sugar (27.56 g/L) was the highest under this condition.

Although the problem of the phase shift delay cannot be successfully solved in this experiment. However, an inspirational phenomenon was observed, which the higher pH value conditions would be beneficial to cell growth, but in contrast more solvents were obtained if the pH was keep at a lower level. Therefore, when the different phases of pH regulation strategy was used, which not only successfully solved the problem of the phase shift delay, but also shortened the fermentation time, and then improved the production of solvent.

3.3 Accelerating phase shift and improving the ABE fermentation performance by the different phases of pH regulation

The importance of pH in ABE fermentation has been known for a long time (Marchal et al., 1985). Nishio et al., (1983) also reported that the optimum pH was different between the period of the cell growth and the secretion of ABE by the producing strains. Therefore, in order to solve the problem of the phase shift delay and improve the ABE fermentation performance, the different pH regulation strategies were performed in the process of ABE fermentation.

3.3.1 Two-step pH regulation strategy

In a first approach, to promote the rapid growth of cells, the pH was kept constant at 6.0 during the phase of acid production (acidogenesis). After the OD_{600} value reached

2.0, the pH was controlled to 4.5 to accelerate phase shift. As shown in Fig. 3, when the pH was kept constant at 6.0 during the phase of acidogenesis, the cell could quickly enter the exponential phase and the OD_{600} value reached 2.0 at 18 h. The concentration of residual sugar was 36.31 ± 2.21 g/L at 18 h, which was almost close to the value (35.19 ± 1.68 g/L) at 24 h without pH control (Fig.1c). Therefore, the results from this case indicated that this condition could promote good growth of microorganisms. However, if the pH was controlled at 4.5 after the OD_{600} value reached 2.0, the consumption rate of sugar rapidly decreased. The final concentration of residual sugar and butanol were 27.01 ± 1.35 g/L and 4.18 ± 0.21 g/L, respectively, which only decreased by 25.6 % and slightly increased compared with the values at 18 h. The concentration of total organic acids have always increased until 70 h, which indicated that the organic acids was constantly secreted by cells, then the phase shift could not occur smoothly because the cells could not re-assimilate organic acids for ABE production. Therefore, the purpose of accelerating phase shift has not achieved by the method of two-step pH regulation.

3.3.2 Different phases of pH regulation strategy

From the above results, when the pH was rapidly dropped by the two-step pH regulation, the phase shift not only could not occur smoothly, but also accelerate the cell death, and the fermentation performance was then finished ahead of the normal time. However, the higher pH values conditions were unfavourable for ABE fermentation (Table 1). Therefore, different phases of pH regulation strategies were performed in this case. Specifically, two ways were used, in a first approach, the initial pH was set at 6.0, when the OD_{600} reached 1.6, the artificial pH regulation was relieved. The results

obtained in this condition are presented in Fig. 4(a). For the second approach, the initial pH was also adjusted at 6.0, however, the without pH regulation was performed after the OD_{600} reached 1.4. At the same time, 3.0 g/L of CaCO3 was added into the fermentation medium. The second approach of pH regulation which gave preferable results is illustrated in Fig. 4(b).

As can be seen in Fig. 4, when the first approach was used, the cell could rapidly enter the logarithmic growth phase. However, after the artificial pH regulation was relieved, the pH of the fermentation broth obviously decreased from 6.0 to 5.35 ± 0.24 (Fig. 4a), which illustrated that a lot of organic acids (acetic and butyric acids) were produced. The fermentation entered the acidogenic phase. Furthermore, the lowest pH was 4.57 ± 0.22 after 30 h of fermentation. Subsequently, the pH gently increased and remained at 5.11 ± 0.21 until the end of fermentation. These results indicate that the cell can normally grow. The final production of butanol was 10.87 ± 0.53 g/L, which had greatly enhanced compared with the methods of different pH regulation strategy. At the same time, the concentration of residual sugar had also greatly decreased. However, the final total production of organic acids (acetic and butyric acids) were 3.33 ± 0.20 g/L, which was 56.3 % higher than the value in the previous studies (Li et al., 2014b). The cell growth, sugar consumption and total solvent production would be probably inhibited if higher total organic acids (acetic and butyric acids) accumulation in the fermentation broth (Madihah et al., 2001). Therefore, the results indicated that the phase shift could not occurred smoothly by this method.

When the second approach was performed, the fermentation process was consistent with the first method before the first 12 h. When the spontaneous pH regulation was functioned and 3.0 g/L of $CaCO_3$ was added into the fermentation broth (Fig. 4b). The

descent speed of the pH of the fermentation was slightly lower than that of the first approach. However, the lowest pH was 4.68 ± 0.23 after 30 h of fermentation, which was a little higher than that of the first method. Furthermore, the final total concentration of organic acids (acetic and butyric acids) were1.88 ± 0.08g/L, which was 77.1 % lower compared with the first method. Therefore, the capability of reassimilation of organic acids of this method was higher than the first one. Li et al., (2012) have reported that partial organic acids were re-assimilated and ABE formation was observed during the solventogenic phase. At the same time, the phase shift occurred smoothly in this condition, which enhanced total solvent and butanol production. On the other hand, the final concentration of residual sugar was 0.88 ± 0.04 g/L, which was the lowest value in these pH regulation methods. The concentrations of butanol, organic acids and residual sugar were hardly changed after 72 h of fermentation. Therefore, the results from this condition indicated that the fermentation time was 72 h, which was 14.3 % shorter than that of the process without pH regulation (Fig. 1c). In addition, the final butanol production and yield was 16.24± 0.70 g/L and 0.26 g/g, respectively, which were 25.3 % and 18.2 % higher than those of the process without pH regulation (Fig. 1c).

$CaCO_3$ exerted an observably positive effect on acetone and butanol production, but gave almost no effect on the production of ethanol. This effect could be attributed to the buffering effect of carbonate (Kanouni et al., 1998, Raganati et al., 2015a). Moreover, due to the presence of bivalent ions (Ca^{2+}), $CaCO_3$ was found to improve the stability of membrane proteins (Richmond et al., 2011) and butanol production (Han et al., 2013, Isar and Rangaswamy. 2012). Due to the higher organic acids accumulation in tapioca and potato starch fermentations, butanol production, cell growth and sugar utilization

could be probably inhibited. In contrast, lower organic acids accumulation in the fermentation of corn and sago starches correlates with increased solvent production and cell growth during solventogenic phase (Madihah et al., 2001). In this study, it was found that the phase shift smoothly occurred and the fermentation time was shortened when the second approach was used. The lowest total organic acids concentration was achieved when the production of butanol was at the highest value. It could be concluded that organic acids reassimilation was benefit to ABE fermentation. Therefore, according to the above results, the simple but effective accelerating phase shift method is a promising technique for improvement of butanol production.

3.4 Efficiency of accelerating phase shift strategy assay

The delayed phase shift was triggered when cassava substrate was used. The consecutively low level of pH in the fermentation broth could be the reason for the delay of phase shift, which meant an "acid crash" happened. Maddox et al., (2000) reported that the environment that is necessary to shift from acidogenic phase to solventogenic phase was deteriorated when an "acid crash" happened, so that the excessively produced organic acids could not be normally converted into solvents. Some methods for accelerating phase shift have currently achieved, but the final acids concentration was still high and the fermentation time was not be shortened (Li et al., 2014b, Li et al., 2012). In the present study, in order to accelerate phase shift, a novel approach was proposed. As a result, the phase shift occurred smoothly and the butanol production was increased compared with the without accelerating phase shift strategy. Table 2 shows butanol production in comparison with other accelerating phase shift strategies. Among them, the highest butanol production (16.24 g/L) was achieved when

the different phases of pH regulation strategy was performed. Therefore, when compared to other methods, although the type and concentration of substrates were various, the method of the different phases of pH regulation with $CaCO_3$ addition was superior to the others in respect to butanol production.

In order to facilitate commercial ABE fermentation, reducing production cost and enhancing the butanol production are promising ways. In present study, cassava flour, as a perspective substrate, was used for ABE fermentation. Furthermore, a novel way was developed to deal with the problem of phase shift delay, followed by enhancing butanol production in *C. acetobutylicum* using different phases of pH regulation strategies. The results demonstrated that this simple but effective method is available and valuable. Thus, it is believed that this novel approach could also provide a valuable reference for other researches in biotechnology.

4. Conclusions

The phase shift delay and a little longer fermentation time were encountered when cassava was used. To solve those problems, a different phase of pH regulation strategy with $CaCO_3$ addition was performed. In summary, The phase shift occurred smoothly and the fermentation time was shortened when this method was used, and then 16.24 g/L butanol and 72 h fermentation time were obtained, which were 25.3 % higher and 14.3 % shorter than those of none pH regulation. Therefore, the report demonstrated that this method is a simple but effective approach for accelerating phase shift which could enhance the butanol production.

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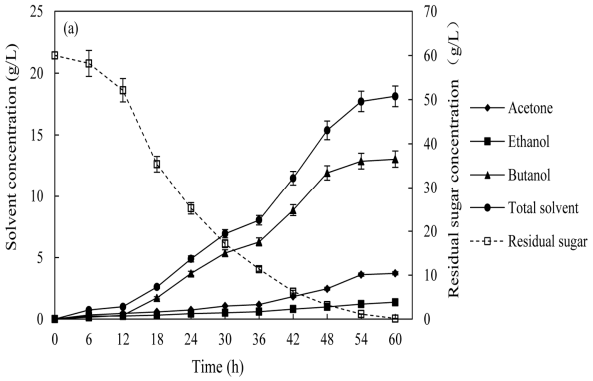
Figure captions

Fig. 1 Performance comparisons of ABE fermentation using glucose (a and b) and cassava flour (c and d)

Fig. 2 Butanol production during ABE fermentation under different pH conditions

Fig. 3 ABE fermentation with the two-step pH regulation

Fig. 4 ABE fermentation with different phases of pH regulation



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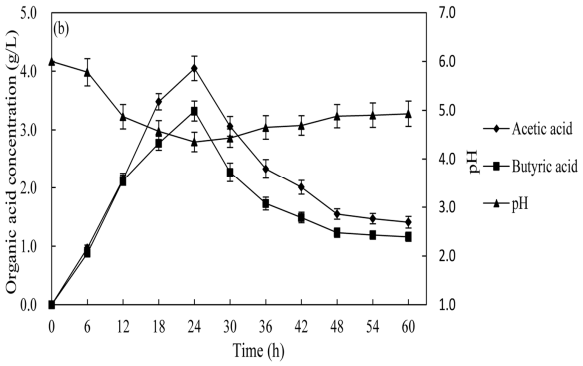

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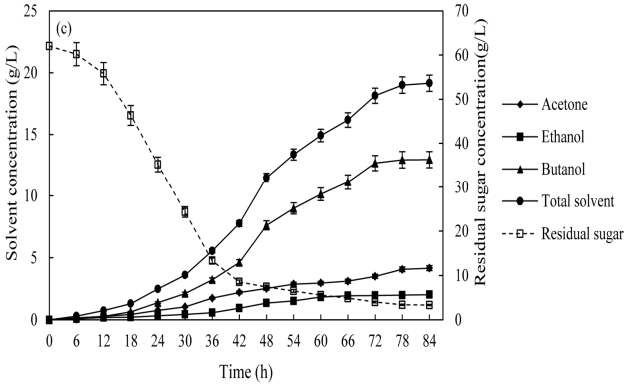

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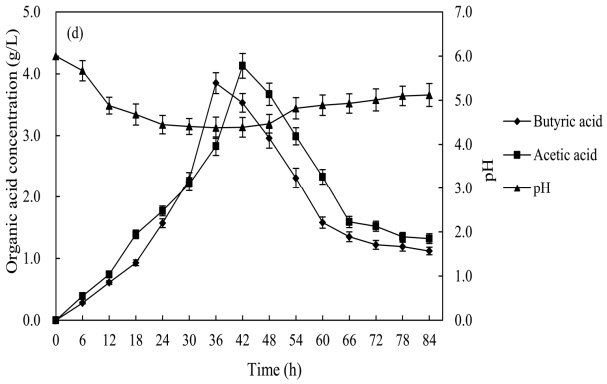

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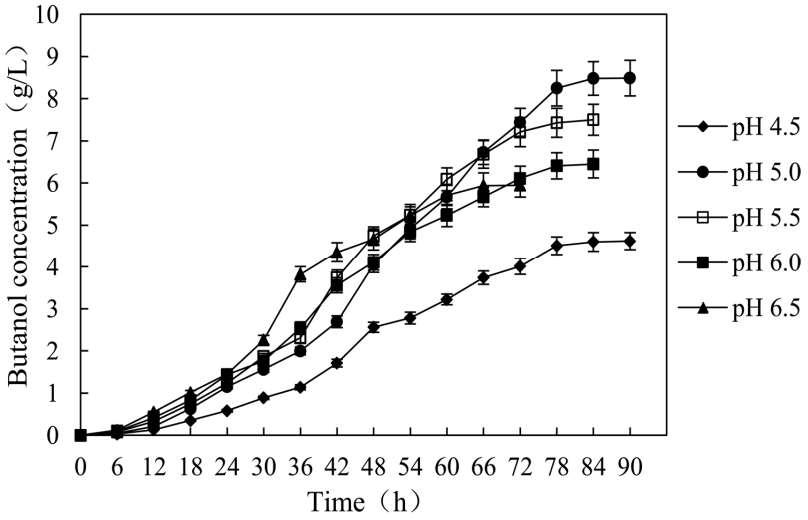

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4 Fig. 2

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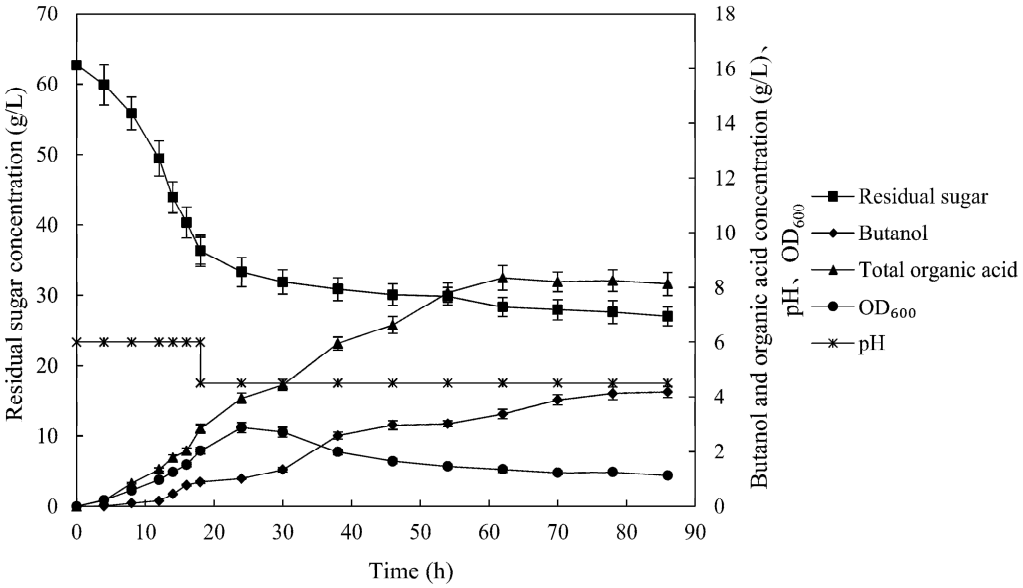
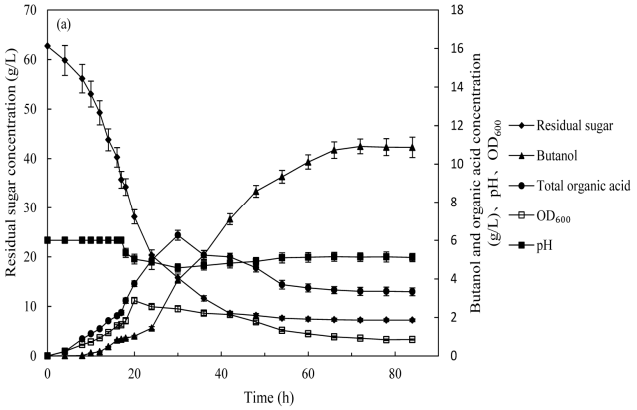


Fig. 3



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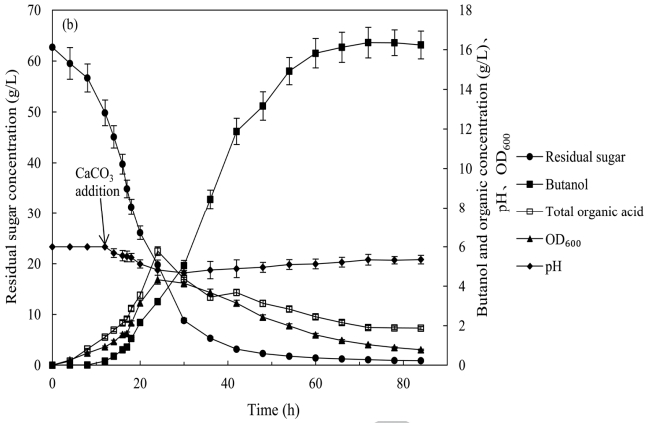

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5 Fig. 4

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Table 1

Results of the ABE fermentation under different pH conditions from cassava folur[a]

Parameter/performance	4.5	5.0	5.5	6.0	6.5
Initial sugar concentration (g/L)	62.7	62.7	62.7	62.7	62.7
Residual sugar concentration (g/L)	27.56	16.63	8.41	6.45	7.38
Butanol concentration (g/L)	4.65 ± 0.21[b]	8.49 ± 0.42	7.50 ± 0.37	6.45 ± 0.33	5.94 ± 0.28
Ethanol concentration (g/L)	0.43 ± 0.02	1.06 ± 0.05	1.15 ± 0.06	0.97 ± 0.04	0.93 ± 0.02
Acetone concentration (g/L)	1.91 ± 0.10	2.94 ± 0.13	2.18 ± 0.11	2.67 ± 0.13	2.50 ± 0.13
Total solvent concentration (g/L)	6.99 ± 0.35	12.49 ± 0.61	10.83 ±0.49	10.09 ± 0.46	9.37 ± 0.48
Total acid concentration (g/L)	9.13 ± 0.48	4.41 ± 0.21	5.98 ± 0.26	7.46 ± 0.38	8.38 ± 0.40
Fermentation time (h)[c]	90	90	84	84	72
Butanol productivity g/(L·h)	0.05	0.09	0.09	0.08	0.08

[a]80 g/L cassava flour was used as the fermentation medium,

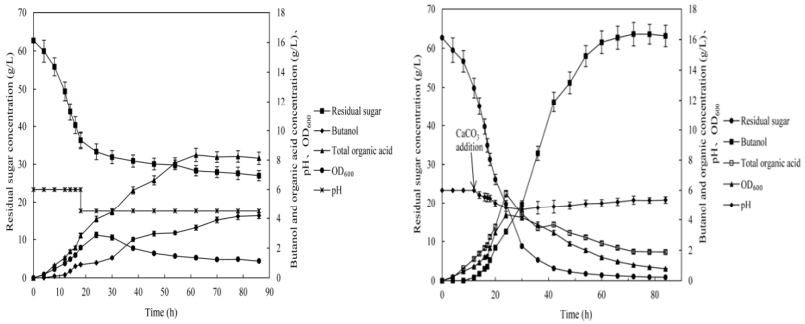
[b]Values are means of three replicates with ± SD

[c]Fermentation time was determined based on the cell cease secreting butanol

Table 2

Comparison of butanol production using different accelerating phase shift strategies in batch fermentation

Strain	Substate	Butanol production (g/L)	Strategies	References
C. *acetobutylicum* L7	Glucose (70 g/L)	12.6	Addition of $ZnSO_4 \cdot 7H_2O$	Wu et al. (2013)
C. *acetobutylicum* SE36	Cassava flour (80 g/L)	14.92	Addition of corn steep liquor	Li et al. (2014)
C. *acetobutylicum* ATCC824	Cassava meal (100 g/L)	13.60	Addition of yeast extract	Li et al. (2012)
C. *acetobutylicum* SE25	Cassava flour (80 g/L)	4.18	Two-step pH regulation	This study
C. *acetobutylicum* SE25	Cassava flour (80 g/L)	16.24	Different phases of pH regulation	This study



Different phase of pH regulation strategies with $CaCO_3$ addition were performed to deal with the problem of phase shift delay when cassava flour was used as the substrate for ABE fermentation. With this effort, the phase shift occurred smoothly and the fermentation time was shortened. Furthermore, the effect of $CaCO_3$ on "acid crash" and butanol production was also investigated. It was found that organic acids reassimilation would be of beneficial to enhance the butanol yield.

Graphical abstract

We consider the highlights of this paper as follows:

1. A severe delay of phase shift was observed in ABE fermentation from cassava flour.

2. Phase shift was smoothly triggered due to different pH regulation strategy performance.

3. Direct fermentation of cassava flour to produce butanol was successfully achieved by SE25.

4. The correlation between phase shift and produced butanol was investigated.

5. The higher and lower pH would respectively be beneficial to cell growth and solvent production.