Simultaneous Saccharification and Fermentation for Biobutanol Production from Corn Starch *via* ABE Fermentation

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The preparation of bio-butanol from corn starch requires saccharification and fermentation processes. In view of the fact that the pH value at the later stage of fermentation is applicable to the enzymatic hydrolysis of glucoamylase, the effects of simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) were compared in this paper. 11.2 g/L butanol and 21.5 g/L total solvent could be obtained by the SSF process, while the yield was 9.74 g/L butanol and 17.2 g/L total solvent in the SHF process. The SSF process required a shorter overall process time (120 h) than the SHF process (144 h) and resulted in a large increase of 38.9% in butanol productivity (2.25 g/Ld for SSF compared to 1.62 g/Ld for SHF). These results show that the application of SSF can reduce the fermentation overall time, simplify the fermentation process, and reduce equipment investment and operating costs.

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Keywords: Corn starch; Clostridium acetobutylicum; Simultaneous saccharification and fermentation; Butanol

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INTRODUCTION

Butanol is an important chemical widely used in the plastic industry and in chemical synthesis. As a promising and renewable biofuel, butanol production has gained attention as its production and recovery methods have become more efficient (Veza *et al*. 2021). Butanol is a potential fuel extender or even a complete gasoline replacement (Amiri 2020; Yu *et al*. 2021). With many favorable characteristics, including 25% more energy content than ethanol, higher octane value, higher flash point, lower vapor pressure, and less corrosiveness, butanol can mix with gasoline at a high ratio and is suitable for the existing fuel technology and distribution systems (da Silva and dos Santos 2017). Although butanol has great potential and advantages, the low production efficiency and high cost limits its industrial production (Sarangi and Nanda 2018). The production of butanol using biological methods cannot achieve yields beyond 13 to 14 g/L because of the toxicity of butanol to *Clostridia* cells (He *et al*. 2018; Guo *et al*. 2019).

At present, butanol is prepared mainly by chemical synthesis, but fermentation-derived butanol has been investigated. Compared with chemical synthesis, biological fermentation method has many advantages, such as abundant and economic raw materials, milder production condition, no use of heavy metal catalyst, and environmental friendly processes. Although there are many challenges to overcome, butanol production of from

feedstock will become more competitive using advanced genome editing technology to alter metabolic and regulatory pathways and developing efficient *in situ* product recovery methods to improve recovery efficiency (Arsov *et al*. 2021; Goswami *et al*. 2023).

Lignocellulosic biomass is an abundant and renewable global raw material that has been explored for biobutanol production (Guo *et al*. 2022; Riaz *et al*. 2022). Although lignocellulosic biomass resources are inexpensive and abundant, lignocellulosic biomass requires extra processes which are pretreatment, hydrolysis, and detoxification for sugar production, comparing to other feedstocks such as commercial glucose, sugarcane, and starch biomass; in addition the use of multiple processing steps has contributed to extra cost of the whole conversion (Ibrahim *et al.* 2017; Veza *et al.* 2021).

Starch-based biomass as carbon source is the most mature fermentation process (da Silva *et al*. 2022). Thang investigated acetone-butanol-ethanol (ABE) fermentation of cassava starch using *Clostridium saccharoperbutylacetonicum* N1-4; they found that batch fermentation of cassava starch resulted in 21.0 g/L of total solvent (ABE) as compared with 24.2 g/L of total solvent when using glucose (Thang *et al*. 2010). Researchers used mixed fermentation medium composed of starch wastewater and cassava as carbon source to produce butanol, and the result indicated that production cost can reduced by 30 to 40% (Luo *et al*. 2018). In all of these works, reducing sugars were obtained through liquefaction and saccharification subjected to the amylase and glucoamylase, and then butanol fermentation was carried out using the reducing sugars as carbon source. Although saccharification and fermentation are conducted separately under the optimal conditions, glucose from starch hydrolysis inhibits enzyme activity and reduces the efficiency of enzymatic hydrolysis. A high glucose concentration inhibits the early stages of strain growth (Jones and Woods 1986).

The most common bacterial strains used for ABE fermentation are *Clostridium acetobutylicum* and *Clostridium beijerinckii* (Qureshi *et al*. 2006). In this study, *C. acetobutylicum* ATCC 824 was used to produce butanol. Because of the generation of acetic acid and butyric acid, the fermentation solution drops to pH 4.0 to 4.5, which is consistent with the optimal pH of glucoamylase hydrolysis. This study combined the saccharification of starch and butanol fermentation (simultaneous saccharification and fermentation (SSF)). While adding glucoamylase, the actively growing cell was inoculated into fermentation media, and the variation of pH of the fermentation solution can accelerate saccharification of starch, leading to butanol production.

The SSF from corn starches were carried out simultaneously in the same reaction system, reducing equipment investment. In addition, glucose was used by bacteria once it was produced, which could eliminate glucose inhibition of glucoamylase activity. A high solvent production was achieved, which simplified the fermentation process to improve the economy of butanol production.

EXPERIMENTAL

Microorganisms

C. acetobutylicum ATCC 824 was maintained as spore suspensions in 25% (v/v) sterile glycerol at -80 °C. *C. acetobutylicum* ATCC 824 spores were heat-shocked for 10 min at 80 °C, followed by cooling in ice-cold water for 2 min and inoculated into fresh RCM medium, then anaerobically incubated for 16 to 20 h at 37 °C in screw-capped bottle before they transferred into solvent production medium.

Substrates and Nutrient Sources

Corn starch was purchased from Dongmei starch products factory, Fushun, Liaoning. Defoamer THIX-298 was purchased from THINGK FINECHEM. Liquozyme Supra was purchased from Novozymes, the enzyme activity is 90KNU/g. Glucoamylase SuHong GA475 was purchased from Novozymes, the enzyme activity was 500 AGU/mL. The Reinforced Clostridial Medium(RCM) medium contained (g/L): glucose, 5; peptone, 10; beef extract, 10; yeast extract, 3; soluble starch, 1; NaCl, 5; CH_3COONa, 3; L-cysteine, 0.5; agar, 0.5. The mixture was autoclaved at 115 °C for 30 min followed by cooling to room temperature.

Liquefaction of Corn Starch

Corn starch (6%) was liquefied using Liquozyme Supra (90 KNU/G) at pH 6.0 and 90 °C for 3 h with an agitation speed of 100 rpm.

Saccharification of Corn Starch

The liquefied corn starch was saccharified using SuHong GA475 (500AGU/mL) at the following conditions (pH 4.5 58 °C, pH 6.5 37 °C, pH 4.5, 37 °C) for 72 h with agitation speed of 100 rpm. The hydrolysate obtained was used as carbon source.

Simultaneous Saccharification and Fermentation

SSF treatments were conducted in batch mode in a 5L bioreactor. A total of 3L liquefied corn starch used as carbon source; other nutrient sources included (per L): 1 g yeast extract, 50 g KH_2PO_4, 50 g K_2HPO_4, 220 g CH_3COONH_4, 0.1 *p*-aminobenzoic acid, 0.1 g thiamin, 0.001 g biotin, 20 g $MgSO_4 \cdot 7H_2O$, 1 g $MnSO_4 \cdot H_2O$, 1 g $FeSO_4 \cdot 7H_2O$, and 1 g NaCl (Qureshi and Blaschek 1999). The bioreactor containing medium was sterilized at 115 °C for 30 min. Actively growing cells of *C. acetobutylicum* ATCC 824 in RCM medium and glucoamylase were added into the bioreactor after cooling to room temperature under the oxygen-free nitrogen atmosphere, and then incubated at 37 °C for 120 h. Samples were collected at various intervals. During fermentation, the pH was not controlled; defoamer with a concentration of 0.2%v/v was added as needed.

Single Fermentation

Single fermentation was conducted in a 5L bioreactor, 3 L of saccharified liquefied corn starch was used as carbon source. Other nutrient sources were the same as the medium mentioned in the SSF process.

Analytical Procedures

Glucose and butanol were measured using the Agilent 7260 High-Performance Liquid Chromatography (HPLC) equipped with refractive index detector using a BioRad Aminex HPX-87H column (7.8 × 300 mm, Bio-Rad, Richmond, CA, USA). For elution, 5 mM H_2SO_4 was used as an isocratic eluent at a flow rate of 0.7 mL/min. The temperatures of detector and column were 45 °C and 65 °C, respectively.

Acetone and ethanol were determined using a gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and DB-FFAP capillary column (30 m × 0.32 mm × 0.25 μm, Agilent Technologies). The oven temperature was programmed from 100 °C to 250 °C at a rate of 16 °C/min. Both injector and detector temperatures were set at 250 °C. The carrier gas was nitrogen, and the flow rate was 30 mL/min.

All samples were centrifuged at 10000 rpm for 5 min; the supernatant was filtered with a 0.22 μm needle filter before detection.

RESULTS AND DISCUSSION

Liquefaction and Saccharification of Corn Starch

During liquefaction, corn starch is converted to soluble, shorter-chain-length dextrins (Crabb and Mitchinson 1997; Tester *et al*. 2006). Little glucose was obtained as a carbon source for the initial growth of *C. acetobutylicum* ATCC 824. As shown in Fig. 1, the increase of amylase concentration from 0.06% to 0.6% enhanced the glucose production within 3 h. The concentration of glucose in liquefied starch increased nonlinearly from 3.01 to 7.47 g/L. As carbon source, 3.01 g/L glucose was sufficient for the initial growth of *C. acetobutylicum* ATCC 824. Considering the economy in amylase addition, 0.06% was chosen as the optimum addition amount.

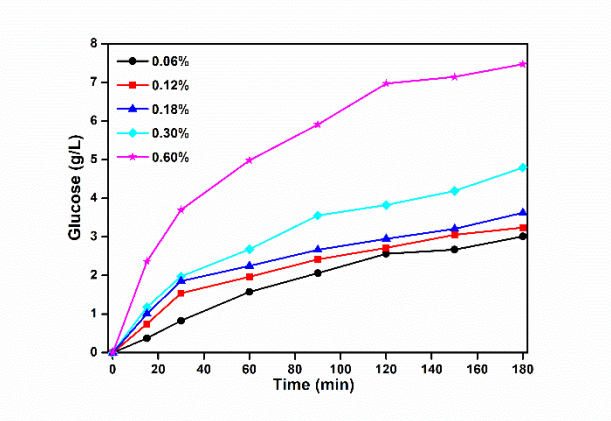

Fig. 1. Effect of amylase on glucose production in liquefaction. The liquefaction was operated at 95 °C pH 6.0, Amylase concentration is 0.06%, 0.12%, 0.18%, 0.30% and 6%, respectively.

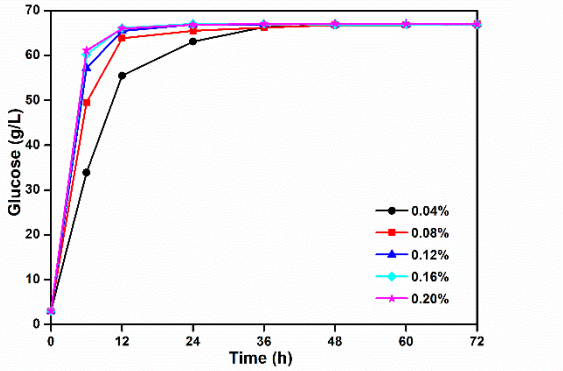

Fig. 2. Effect of different quantities of glucoamylase on glucose production. The liquefaction was operated at 58 °C at pH 4.5, glucoamylase concentration is 0.04%, 0.08%, 0.12%, 0.16% and 0.20%, respectively.

As shown in Fig. 2, the amount of glucose reached 66.8 g/L after 48 h of hydrolysis under the optimal conditions for enzyme (58 °C, pH 4.5), with the minimum enzyme dosage (0.04% corn starch). This indicates that the amount of amylase used in liquefaction stage met the requirements of the subsequent saccharification. When increasing the amount of enzyme, it takes less time to reach the maximum glucose amount, but there is no increase in glucose production.

Although the optimum temperature for usage of glucoamylase is 58 °C, the temperature of *C. acetobutylicum* ATCC 824 fermentation was 37 °C, in order to ensure the feasibility of SSF. Thus, this study investigated the performance of glucoamylase under the condition of 37 °C. In *C. acetobutylicum* ATCC 824 fermentation process, the pH in the initial stage was about 6.3. The pH decreased rapidly with the accumulation of acetic acid and butyric acid along with the fermentation progress; pH remained at around 4.5 in the end as shown in Fig. 4b. Therefore, this study investigated the performance of glucoamylase at pH 4.5 and 6.3 under the condition of 37 °C, as shown in Fig. 3.

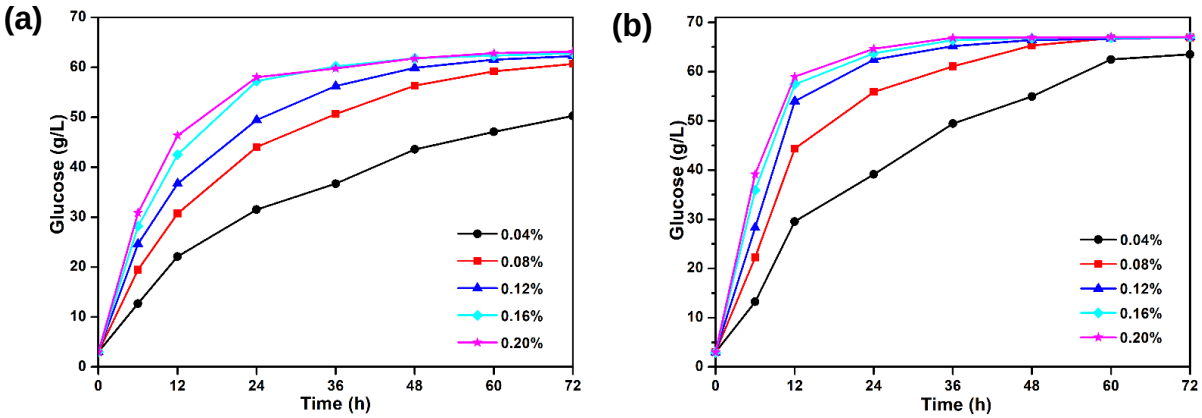

Fig. 3. Effect of different quantities of glucoamylase on glucose production under the condition of 37°C, pH 6.3 (a) and pH 4.5 (b); glucoamylase concentration was 0.04%, 0.08%, 0.12%, 0.16% and 0.20%, respectively.

As shown in Fig. 3a, 63.16 g/L glucose was produced after 72 h enzymatic hydrolysis at the highest enzyme dosage of 0.2% and pH 6.3. However, under the condition of pH 4.5, 64.6 g/L of glucose was produced after only 24 h at the enzyme dosage of 0.2%, 67.0 g/L of glucose was produced after 72 h as shown in Fig 3b. The above results show that the saccharification rate increased with the progress of SSF. Therefore, SSF is feasible in theory.

At pH 4.5, the yield of glucose was 63.5 g/L after 72 h of enzymatic hydrolysis of corn starch at the enzyme dosage of 0.04%, the yields of glucose were all more than 66.9 g/L when enzyme dosage is greater than 0.08%. Therefore, an enzyme dosage of 0.08% was chosen as the studied condition for subsequent simultaneous saccharification and fermentation of corn starch.

Batch Fermentation

Glucose was obtained as carbon source by double-enzyme method and prepared as fermentation medium; the concentration of corn starch was 6 wt% and the glucoamylase dosage was 0.08%. *C. acetobutylicum* ATCC 824 was cultured at 37 °C, with the inoculation of 5%. As shown in Fig. 4a, the product butanol increased with the extension of fermentation time. At 66 h, the butanol concentration was 9.74 g/L with a productivity of 1.86 g/Ld, and there was no significant increase afterwards. The maximum concentration of ABE was 17.224 g/L.

As shown in Fig. 4b, the pH of the fermentation liquid continued to decrease from the initial 6.3 to 4.5, and there was no significant change afterwards. This is fortunate, because the optimal pH of starch saccharification is 4.5, which is consistent with the pH of late fermentation. Therefore, SSF can be adopted, which can reduce glucose feedback inhibition.

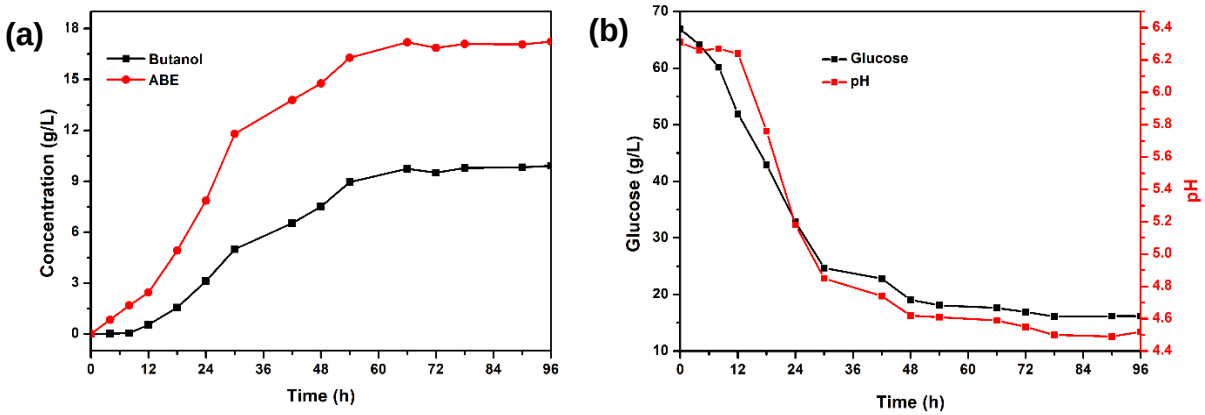

Fig. 4. Batch fermentation of enzymatic hydrolysates under the condition of 37°C, the incubation of 5%, (a) production of butanol and ABE, (b) changes of glucose and pH value

Simultaneous Saccharification and Fermentation of Corn Starch

During *C. acetobutylicum* ATCC 824 fermentation, the initial pH of the system was about 6.3, and after 48 h, the pH changed to around 4.5. The above experiments confirmed the feasibility of saccharification under fermentation conditions. Therefore, SSF was conducted at 37 °C with the incubation of 5%, saccharification enzyme dosage 0.08%, as shown in Fig. 5.

As shown in Fig. 5a, under the condition of 5% inoculation, the pH of the system was at around 6.3 in the initial fermentation stage. This pH was not good for the saccharification of corn starch. As fermentation progressed, acetic acid and butyric acid were produced, pH decreased rapidly, the saccharification rate of corn starch increased quickly, and the glucose required for the growth of strain was less than that produced by saccharification. The maximum glucose accumulation reached 37.8 g/L after 18 h of fermentation. As the fermentation progressed, glucose was continuously consumed until fermentation stopped. Finally, this culture resulted in 11.2 g/L butanol and 21.5 g/L of total ABE concentration (Fig. 5b). The productivity (2.25 g/Ld) was attained at 120 h. Both butanol and total ABE yield increased compared to batch fermentation. A small amount of glucose remained after fermentation for 120 h, which may be due to the toxicity of ABE, especially butanol to bacteria (Ezeji *et al*. 2003).

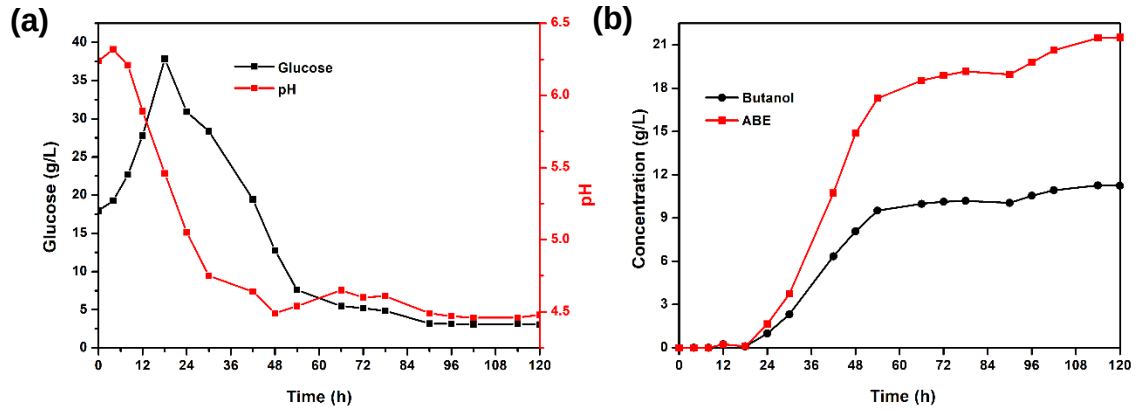

Fig. 5. SSF of corn starch at 37°C, saccharification enzyme dosage of 0.08%, the incubation of 5%, (a) changes of glucose and pH value, (b) production of butanol and ABE

In comparison to SSF, where a butanol productivity of 2.25g/Ld was obtained, SHF was poor, with a butanol productivity of 1.62g/Ld. Thus, butanol yield increased by 38.9% when using SSF.

CONCLUSIONS

The SSF of corn starch was developed by utilizing corn starch glucoamylase and the *C. acetobutylicum* ATCC 824 strain. The rate of corn starch saccharification was increased by changing the pH of the culture system. Under the conditions of 6% corn starch and 5% inoculation, the yield of butanol and total solvent in SSF was 11.2 g/L and 21.5 g/L respectively. Compared with SHF, the productivity of butanol increased by 38.9%.

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