

Utilization of Steam-Exploded Corn Straw to Produce Biofuel Butanol *via* Fermentation with a Newly Selected Strain of *Clostridium acetobutylicum*

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The feasibility of utilizing corn straws to produce butanol *via* fermentation with *Clostridium acetobutylicum* was evaluated. The supernatant of enzymatically hydrolyzed supernatant of steam-exploded corn straws was used as the raw material. A bacterial strain was selected from *Clostridium acetobutylicum* zzu-02 and *Clostridium beijerinckii* zzu-01, which was capable of fermenting the enzymatically hydrolyzed supernatant of steam-exploded corn straw to produce butanol with high yield. The optimal fermentation conditions for the selected strain with enzymatically hydrolyzed supernatant of steam-exploded corn straw were also investigated and they were determined as follows: sugar concentrations in enzymatically hydrolyzed solution of steam exploded corn straws, 57.5 g/L; initial pH, 6.3; the amount of added CaCO₃, 5g/L; the bacterial inoculation concentration to enzymatically hydrolyzed solution, 6%; fermentation temperature, 37 °C, the amounts of the added nutritional elements, *i.e.* yeast extract, CH₃COONH₄, KH₂PO₄, and C₆H₆N₂O, 0.8, 6.0, 0.5, and 0.25 g/L, respectively. Under these conditions, the butanol yield reached 9.88 g/L. Based on the butanol metabolism pathways, supplementation of a small amount of C₆H₆N₂O was found to effectively increase the yield of butanol production.

Keywords: Steam-exploded corn straw; Biofuel; Enzymatically hydrolyzed supernatant; Butanol; *Clostridium acetobutylicum*; *Clostridium beijerinckii*; Fermentation

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INTRODUCTION

Butanol is not only an important organic solvent and a raw material for chemical industry (Guo *et al.* 2012), but it also is a new type of promising biofuel (Pyrgakis *et al.* 2016; Al-Shorgani *et al.* 2018; Cimino *et al.* 2018; Diaz and Tost 2018; Oh *et al.* 2018). Traditionally, acetone-butanol fermentation is a large-scale fermentation process using corn starch as the raw material (Luo *et al.* 2017; Faraji *et al.* 2018). With the gradual exhaustion of fossil fuel resources, and the potential shortage of food and oil as well as the emergence of problems caused by unfavorable environmental factors such as the increasing greenhouse effects, utilization of agricultural wastes as the raw materials to produce chemical raw materials and the energy-generating substances has attracted more and more attention (Zheng *et al.* 2017; Michaels *et al.* 2018).

Currently, utilization of the fermentation of biomass such as lignocellulose *etc.* to generate butanol is becoming a hot research topic (Ezeji *et al.* 2007; Qureshi *et al.* 2010; Farmanbordar *et al.* 2018; Maiti *et al.* 2018; Perez-Bibbins *et al.* 2018). A number of factors such as the types of fermentation bacteria, raw materials, and the ways of pretreatment of raw materials, as well as the fermentation conditions all have significant impacts on the yield of solvent production. Dilute acid treatment of the ethanol extracted biodegradable fraction of municipal solid waste at 140 °C for 60 min resulted in a liquor containing 23 g/L glucose and 41 g/L soluble starch, which was fermented to the highest ABE (acetone-butanol-ethanol) concentration of 17 g/L with productivity of 0.24 g/L/h (Farmanbordar *et al.* 2018). Parameter optimization (time, pH, and substrate concentration) for acid-catalysed brewery industry liquid waste hydrolysate utilization using central composite model technique produced 307.9 g/kg glucose. Also, 10.62 g/L of ABE was produced by subsequent clostridial fermentation of the substrate (Maiti *et al.* 2018). The butanol concentration of the fermentation with hydrolytic solution of 'sugarcorn' by *Clostridium beijerinckii* was 8.3 g/L in 257 h (Chen *et al.* 2017).

The steam-explosion method is a way by which the biomass raw materials are at first steamed by the treatment with high temperature and high pressure for a certain period of time, and that is followed by a sudden decompression. By this way, the chemical decomposition of components of the raw materials, mechanical fracture, and structural recombination can be achieved (Boren *et al.* 2018). This study aimed to test the feasibility of utilizing corn straw to produce butanol *via* bacterial fermentation. By using the enzymatically hydrolyzed supernatant of steam-exploded corn straw (Zhang *et al.* 2017) as the raw material for fermentation, we selected a bacterial strain capable of generating butanol with higher yield and optimized its fermentation conditions. We also investigated the effects of supplement of a small amount of C₆H₆N₂O on the yield of butanol production. This study could provide the experimental basis for increasing the yield of butanol production through proper utilization of the agricultural wastes.

EXPERIMENTAL

Materials

The strain of *Clostridium acetobutylicum* zzu-02 was cultured and optimized by The Biochemical Industry Laboratory of College of Chemical Industry and Energy Resources, Zhengzhou University (Henan, China). The bacterial spores were cultured with 5% (w/w) corn mash in ratio of corn starch: deionized water of 1:20 and stored at 4 °C. *Clostridium beijerinckii* zzu-01 was conserved by The Biochemical Industry Laboratory of College of Chemical Industry and Energy Resources, Zhengzhou University. The bacterial spores were cultured in culture medium and stored at 4 °C.

Corn straw samples were collected from the experimental field (50 m×80 m) located at the Suburb of Nanyang City at Henan Province, China. Water steam was channeled to a sealed explosion tank containing naturally air-dried, 1 to 3 mm pieces of corn straw. The pressure within the tank was rapidly increased to 1.5 MPa and then maintained for 5 min. The pressure was suddenly decompressed. The steam-exploded corn straw made in this way were saved for subsequent use. The steam exploded corn straw contained 32.3% cellulose and 16.0% hemicellulose.

Methods

Formulation of culture mediums

Culture Medium for Clostridium acetobutylicum zzu-02: the commercially available corn starch obtained from the local market was directly autoclaved under 0.1 MPa at 121 °C for 15 min and dissolved in deionized water to make 5% (w/w) of corn mash with pH 6.3.

Culture Medium for Clostridium beijerinckii zzu-01: this medium contained 500 mL of tryptone pancreatic digest of casein, 500 mL of beef infusion broth, 5.0 g sodium chloride, 5.0 g glucose, 1 g agarose, 0.5 g sodium thioglycollate, and 10 g meat scraps and with pH 7.2 to 7.4.

Fermentation Medium: This medium containing 0.8 g yeast extract, 0.5 g KH_2PO_4 , 6 g $\text{CH}_3\text{COONH}_4$, 0.25 g $\text{C}_6\text{H}_6\text{N}_2\text{O}$, per liter was brought up to 1L with enzymatically hydrolyzed supernatant of steam exploded corn straws at pH 6.3.

Preparation of seed liquids

Clostridium acetobutylicum zzu-02 conserved in the freezer was taken out and inoculated into corn mash culture medium in a proportion of 6% (v/v), *i.e.* 6 mL of *Clostridium acetobutylicum zzu-02* spore solution was inoculated into 94 mL of corn mash culture medium, and cultured anaerobically at a constant temperature of 37 °C for 25 h. *Clostridium beijerinckii zzu-01* conserved in the freezer was taken out and inoculated into seed culture medium in proportion of 6% (same as above), and cultured anaerobically at a constant temperature of 37 °C for 28 h.

Screening bacterial strain

Clostridium beijerinckii zzu-01 and *Clostridium acetobutylicum zzu-02* were inoculated, respectively, into the screening medium. At the end of fermentation, the butanol yields produced by two strains were checked and compared. The strain that generated higher yield of butanol was selected as the bacterial strain for this study.

Fermentation conditions

The fermentation conditions were: Sugar concentrations in enzymatically hydrolyzed solution of steam exploded corn straws, 57.5 g/L; initial pH, 6.3; the amount of added CaCO_3 , 5g/L; the bacterial inoculation concentration to enzymatically hydrolyzed solution, 6%; fermentation temperature, 37 °C, the amounts of the added nutritional elements, *i.e.* yeast extract, $\text{CH}_3\text{COONH}_4$, KH_2PO_4 , and $\text{C}_6\text{H}_6\text{N}_2\text{O}$, 0.8, 6.0, 0.5, and 0.25 g/L, respectively.

Optimization of butanol fermentation conditions

Because the major components in the enzymatically hydrolyzed supernatant of steam-exploded corn straw are mainly sugars, supplementation of certain amounts of nutrients for bacterial growth can enable the bacterial cells to grow and ferment normally. In this study, in addition to testing bacterial strains, effects of initial pH value, the amounts of added CaCO_3 and the sugar concentrations in the enzymatically hydrolyzed supernatant on the butanol yield were also examined. Moreover, the effects of nutritional elements including $\text{CH}_3\text{COONH}_4$ (A), yeast extract (B), KH_2PO_4 (C) and $\text{C}_6\text{H}_6\text{N}_2\text{O}$ (D) were examined using an orthogonal experiment, which was designed by using $L_9(3^4)$ orthogonal experimental table (Table 1). Each orthogonal experiment was repeated three times.

Table 1. Orthogonal Factor Level Table

Level	Factors			
	A/(g/L)	B/(g/L)	C/(g.L ⁻¹)	D/(g/L)
1	5.5	0.6	0.5	0.15
2	6.0	0.7	0.6	0.25
3	6.5	0.8	0.7	0.30

Fermentation of enzymatically hydrolyzed supernatant of steam-exploded corn straw

Three-hundred milliliters of fermentation culture medium was transferred to a 500 mL-conical flask. The prepared bacterial seed solution was inoculated into the fermentation culture medium in a proportion of 6% and incubated at a constant temperature of 37 °C for 70 h.

Analysis*Measurement of the reducing sugar contents*

The reducing sugar contents were measured by using 3,5-dinitrosalicylic acid (DNS) method as described previously (Ghose 1987).

Measurement of butanol content

Butanol content was measured by using gas chromatography with a GC7980 gas chromatograph (Shanghai SiMo Analytical Instruments LLC, Shanghai, China). Capillary column was used, and the temperature at sample injection port was 230 °C, column temperature was 70 °C, and the running time was 6 min. The program was set without increasing temperature and without splitting injection. The injection volume was 1 µL, the flow rate of H₂ gas was 30 mL/min, the air flow rate was 300 mL/min, and the internal standard used was isobutanol.

Calculation of conversion rates

The conversion rates of butanol and total solvent were calculated using the following equations:

$$\text{Conversion rate of butanol (\%)} = \frac{\text{Concentration of butanol}}{\text{Concentration of total sugars} - \text{Concentration of sugar residue}} \times 100\%$$

$$\text{Conversion rate of total solvent (\%)} = \frac{\text{Concentration of total sugars} - \text{Concentration of sugar residues}}{\text{Concentration of total sugars}} \times 100\%$$

For both equations, the concentration units for butanol, total solvent and sugar residues were all expressed as g/L.

RESULTS AND DISCUSSION

Temperature is the important influencing factor for fermentation. The suitable fermented temperature of *Clostridium acetobutylicum* is 37 °C according to several studies

(Xue *et al.* 2016; Dong *et al.* 2018; Luo *et al.* 2018). Suitable growth temperature for *Clostridium acetobutylicum* zzu-02 was also 37 °C in each experiment. Thus, corn stover enzymolysis liquid was fermented with *Clostridium acetobutylicum* zzu-02 at 37 °C.

Screening Bacterial Strains

The bacterial strains were screened mainly based on the butanol yield produced by the tested strains. Analyses of the butanol yield produced by two strains after fermentation revealed that the butanol yield produced from the enzymatically hydrolyzed supernatant of steam-exploded corn straws containing 52 g/L reducing sugars by fermentation with *Clostridium acetobutylicum* zzu-02 and *Clostridium beijerinckii* zzu-01 was 6.925 and 5.238 g/L, respectively. The butanol yield produced by fermentation with *Clostridium acetobutylicum* zzu-02 was significantly higher than that produced by fermentation with *Clostridium beijerinckii*-zzu-01. The difference in butanol yield between two strains may be because higher concentrations of carbon sources may cause significant inhibition of the growth of *Clostridium beijerinckii* (Lee *et al.* 2008). Thus, *Clostridium acetobutylicum* zzu-02 was selected as the experimental strain for this study.

Effects of Sugar Concentrations in Enzymatic Hydrolysis Solution on Butanol Yield

In order to enhance the butanol production intensity, the sugar concentration is required to be increased as high as possible in such circulation that no product inhibition occurs and the butanol yield is not reduced. An amount 15% (w/w) of steam exploded corn straws in a ratio of corn straw/deionized water of 3/20 was enzymatically hydrolyzed. The hydrolyzed solution was centrifuged at 1500 rpm for 10 min. The supernatant was saved and its sugar concentration was measured to be 57.5 g/L, which was relatively low. After fermentation, the concentration of sugar residues was low, indicating that the sugars in the fermented solution had almost been used up.

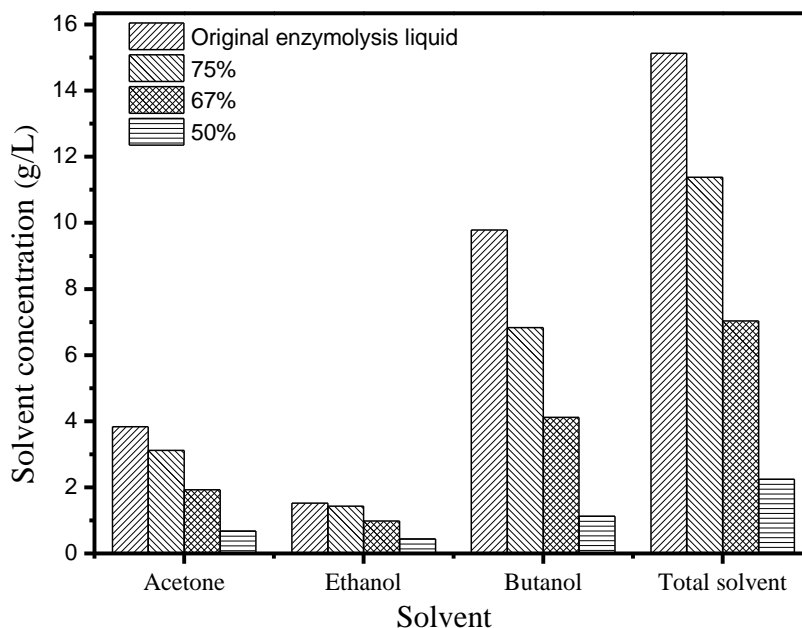


Fig. 1. Solvent concentrations in various concentrated supernatants after fermentation

In order to increase the sugar concentration, an attempt was made to concentrate the enzymatically hydrolyzed supernatant. The supernatant solution was concentrated to 75 (mass fraction, the same as below), 67, and 50% of the original supernatant, respectively. After concentration, the sugar concentrations reached 76.7, 86.2, and 115 g/L, respectively (Table 2). The concentrated supernatants with different sugar contents were fermented, and the changes in the butanol yield and total solvent yield were monitored and compared.

There were relatively large differences in butanol concentrations and total solvent concentrations in various concentrated supernatants after enzymatic hydrolysis (Fig. 1). The butanol concentration and total solvent concentration in the enzymatically hydrolyzed supernatants of the original supernatant were the highest. The higher the concentration degree was, the lower were the butanol concentration and total solvent concentration.

Table 2. Concentrations of Reducing Sugars in Various Concentrated Supernatants before and after Fermentation

Degree of concentration	Sugar concentration before fermentation (g/L)	Sugar concentration after fermentation (g/L)
50%	115	97
67%	86.3	53.8
75%	76.7	30.5
0 (Original enzymatic hydrolysis solution)	57.5	1.3

There were also differences in reducing sugar contents in various concentrated supernatants before and after fermentation (Table 2). The higher the content of reducing sugar content was in the enzymatic hydrolysis solution of the original supernatant, the lower were the amounts of sugars that were consumed during fermentation.

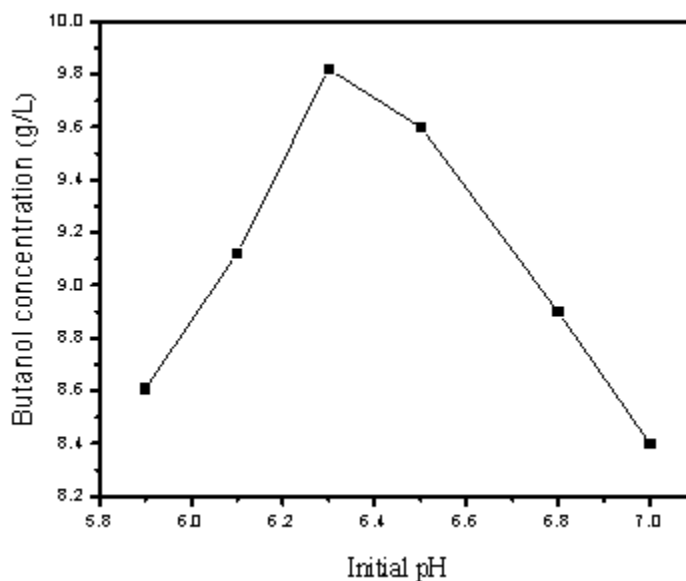


Fig. 2. Effects of initial pH values on butanol yield

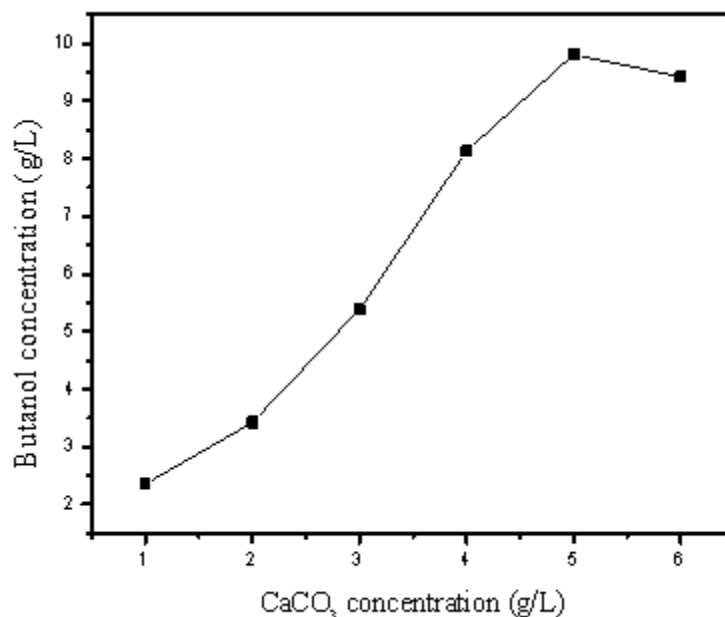


Fig. 3. Effects of the concentrations of the added CaCO₃ on butanol yield

Because of the presence of substances that inhibit the bacterial growth in the enzymatically hydrolyzed solution of steam-exploded corn straw, with the increase in the sugar contents of concentrated solutions, the sugar content was increased and the concentrations of the inhibitory substances were also increased. Thus, when the enzymatically hydrolyzed supernatant was concentrated to 50% of the original supernatant, the butanol concentration was 1.13 g/L, which was very low. Thus, in order to increase the production intensity, appropriate pretreatment of corn straw is needed first before enzymatic hydrolysis. The inhibitory substances generated during enzymatic hydrolysis of steam-exploded corn straws were found to be mainly formic acid (4.05g/L) and furfural (3.86 g/L) *etc.* Pretreatment of the corn straws by washing with water before enzymatic hydrolysis can reduce the contents of formic acid and furfural and thus, can increase the butanol yield.

Effects of Initial pH Value, Amount of Added CaCO₃ and Bacterial Inoculum Concentration on Butanol Yield

Another key factor that affects the butanol yield is the initial pH value. If the initial pH value is too low, it is not favorable for bacterial growth, whereas if the initial pH value is too high, it is not favorable for solvent production. After being detoxified, the pH value of the enzymatically hydrolyzed supernatant of steam-exploded corn straw was around 5.95 ± 0.05 . The pH values were adjusted with anhydrous ammonia. The effects of the changes in pH values on the butane yield were examined to determine the optimal initial pH value at which the butanol yield produced by fermentation of steam -exploded corn straw was the highest one. The effects of the initial pH values within the range of 5.9 to 7.0 on fermentation of steam-exploded corn straws were examined. It was found that within this initial pH range, there were not big differences in butanol yield and that when the initial pH value was 6.3, the butanol yield was slightly higher (Fig. 2).

When butanol is produced by pilot fermentation, during the fermentation process, the butanol yield reaches the maximal value only when the pH value was maintained in the

range of 4.5 to 5.0. CaCO_3 is an important buffering substance for butanol fermentation with steam-exploded corn straws. When corn straw was utilized to produce butanol, if CaCO_3 was not added, the pH value would be reduced to 3.78 during fermentation, and this resulted in the fermentation solution that was too acidic, which was not favorable for the growth of *Clostridium acetobutylicum* and thus, affected the butanol yield. The effects of changes in CaCO_3 concentrations at the range of 1 to 6 g/L in the fermentation solution on the butanol yield were investigated. The results showed that when the concentration of the added CaCO_3 reached 5g/L, the butanol yield was the highest (Fig. 3). When its concentration was too high or too low, CaCO_3 reduced the butanol yield.

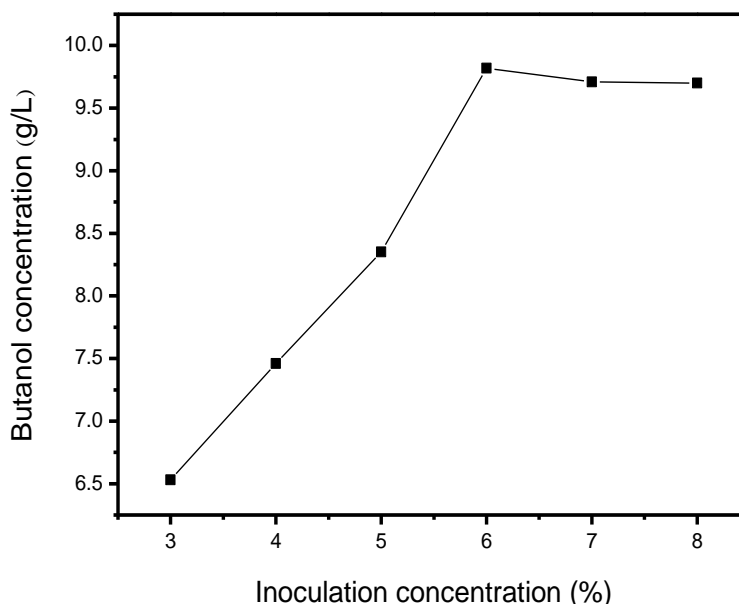


Fig. 4. Effects of inoculation concentrations on butanol yield

The bacterial inoculation concentrations also caused effects on butanol yield to a certain extent. The butanol yield was increased with the increase in inoculation concentrations. When the proportion of the inoculation concentration reached 6%, butanol yield was not further increased but was kept stable (Fig. 4). Thus, the proportion of 6% was selected as the inoculation concentration for fermentation in this study.

Effects of Supplement of Nutritional Elements on the Butanol Yield

The results of orthogonal experimentation showed that the influence degrees of different nutritional elements, *i.e.* $\text{CH}_3\text{COONH}_4$ (A), yeast extract (B), KH_2PO_4 (C), and $\text{C}_6\text{H}_6\text{N}_2\text{O}$ (D), which were supplemented to the enzymatically hydrolyzed supernatant on butanol yield were in the order (from high to low) of $A > C > B > D$. Among the nutritional elements, the effect of $\text{CH}_3\text{COONH}_4$ on butane yield was the highest one (Table 3). Analysis on the results of orthogonal experiment revealed that the combination of $A_2B_3C_1D_2$ was the optimal one. Under these conditions, the butanol concentration in enzymatically hydrolyzed supernatant was 9.726 g/L (Fig. 5).

Table 3. Results of L9 (3⁴) Orthogonal Experiment

Entry	A	B	C	D	Butanol concentration (g/L)
1	1	1	1	1	7.637
2	1	2	2	2	7.412
3	1	3	3	3	7.532
4	2	1	2	3	8.635
5	2	2	3	1	8.423
6	2	3	1	2	9.726
7	3	1	3	2	7.638
8	3	2	1	3	7.547
9	3	3	2	1	7.432
k_1	7.527	8.065	8.303	7.831	
k_2	8.928	7.794	7.826	8.259	
k_3	7.539	8.230	7.864	7.905	
R	1.401	0.436	0.477	0.428	

When *Clostridium beijerinckii* was used as the bacterial strain and yeast extract was used as the bacterial nutrient, the yield of solvent production from the hydrolysis solution of wheat straw reached 0.6 g/(L·h) (Qureshi *et al.* 2007). Yeast extract is also suitable to be used as the nutrient for the fermentation of steam-exploded corn straw with of *Clostridium acetobutylicum*. Supplementation of CH₃COONH₄ provided the nitrogen source for the growth of *Clostridium acetobutylicum* in the enzymatically hydrolyzed supernatant of steam-exploded corn straws. CH₃COOH was also an important chemical for ABE fermentation, which was closely related to the effective expression of enzymes during the processes of acid production and solvent production (Gu *et al.* 2009). Thus, supplementing of the proper amount of CH₃COONH₄ was favorable for fermentation and could increase the butanol yield and the utilization rate of sugars. Among the trace nutritional elements, K⁺ is essential for cellular growth. When K⁺ concentration was zero, fermentation did not produce solvents but produced only acids. With the increase in K⁺ concentrations, the yield of solvent production was increased. However, when K⁺ concentration reached certain level, the yield of solvent production was not further increased but was kept constant (Yi *et al.* 2018). Thus, supplement of the appropriate amount of KH₂PO₄ not only provided a phosphate source but also provided K⁺, which was beneficial for increasing the yield of solvent production.

C₆H₆N₂O is an important component for the formation of co-enzymes NAD and NADP. Both NAD and NADP are the co-enzymes of a number of dehydrogenases. The nicotinamide moiety of two co-enzymes has the functions of reversible hydrogenization and dehydrogenization, which plays an important role in transferring electrons and protons in the redox reactions during many biological oxidation processes (Lee *et al.* 2018). Within the butanol metabolism pathway, from the steps of acetyl-CoA to butanol, four dehydrogenases, *i.e.* 3-hydroxybutyrate dehydrogenase, isobutyryl coenzyme-A dehydrogenase, butyraldehyde dehydrogenase, and butanol dehydrogenase, are involved in these oxidation-reduction reactions. Thus, supplement of a small amount of C₆H₆N₂O can accelerate butanol pathway and thus, is favorable for butanol production.

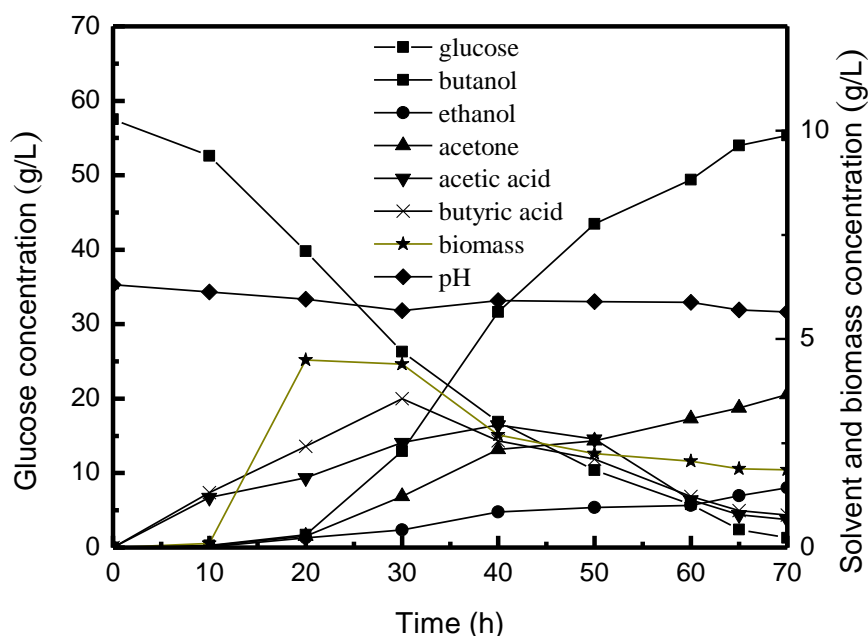


Fig. 5. Butanol from steam-exploded corn stover

Verification of the Experimental Results under the Optimized Conditions

In order to verify the reliability of the optimized fermentation conditions, the yield of solvent production with steam-exploded corn straws was compared to those with several other sugar sources including corn starch, enzymatically hydrolyzed solution of bran, and glucose solution.

It was found that the butanol yields produced by *Clostridium acetobutylicum* fermentation with steam-exploded corn straws, enzymatically hydrolyzed solution of bran and glucose solution were all around 10g/L. The yield produced with steam-exploded corn straws was slightly lower than that with corn starch but slightly higher than that with enzymatically hydrolyzed solution of bran (Table 4). These results indicate that the optimized conditions are suitable for butanol production by *Clostridium acetobutylicum* fermentation with steam-exploded corn straws as the raw materials.

Table 4. The Yields of Solvent Production by *Clostridium Acetobutylicum* Fermentation with Different Sources of Raw Materials

Entry	Carbon sources	Acetone (g/L)	Ethanol (g/L)	Butanol (g/L)	Total Solvents (g/L)	Conversion of butanol (%)	Conversion of total solvents (%)
1	Corn straws	4.14	1.80	9.88	15.82	17.58	28.15
2	Bran	4.42	1.68	9.79	15.89	17.5	28.5
3	Corn starch	4.53	1.81	10.23	16.61	19.3	31.3
4	Glucose	3.47	1.41	8.05	12.93	15.4	24.7

CONCLUSIONS

1. *Clostridium acetobutylicum* zzu-02 was selected, which was capable of fermenting steam-exploded corn straws and producing higher butanol yield.
2. The optimal conditions for fermentation by this strain with steam exploded corn straws were determined, and the butanol yield reached 9.88 g/L under these conditions.
3. According to the butanol metabolism pathway, supplementation of a small amount of C₆H₆N₂O can effectively increase the butanol yield

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