Enhancement of Bioethanol Production Using a Blend of Furfural Production Residue and Tea-seed Cake

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The price of raw material, energy demand in the pretreatment step, and enzyme usage rate are the major cost factors in the process of converting biomass to bioethanol. Unwashed furfural residues (FRs) possess great potential for application in bioethanol production. Surfactant addition is an effective method to enhance the fermentation rate. In this study, unwashed FRs were used directly as raw materials to produce bioethanol. Tea-seed cake (TSC), tea seed residues that contain protein and saponin, was added in the simultaneous saccharification and fermentation (SSF) process. The effect of TSC dosage on SSF was compared. TSC was added at the dosage of 10 g/L, which resulted in a final ethanol yield of 87.2%. However, a high concentration of TSC could induce cytotoxicity in yeast. The surface tension (approximately 33.92 mM/m) at SSF using TSC-medium was much lower than that of other fermentation systems (about 64.67 mM/m). Further contact angle testing showed that TSC-medium (21.7°) had better wetting capacity than FRs (45.6°). This study provided a proposed process strategy that SSF with the addition of TSC could be a minimum consumption of chemicals and enzymes for future cellulosic ethanol production process.

Keywords: Furfural residues; Tea-seed cake; Bioethanol; Simultaneous saccharification and fermentation

INTRODUCTION

The increase in the depletion of petroleum fuels and the resulting accumulation of atmospheric greenhouse gases have raised interest in the development of sustainable technologies for biofuel production. Bioethanol is one of the most promising renewable liquid biofuels that has environmental and economic benefits (Zhang et al. 2007). However, its high processing cost is the major barrier to cellulosic ethanol production at commercial scales (Lynd et al. 2008). The price of raw material, energy demand in the pretreatment step, and the dosage of enzyme are the major cost factors in the process of converting biomass to bioethanol (Lynd et al. 2002). Therefore, it is important to eliminate these barriers for future cellulosic ethanol production.

Various types of agricultural crops such as corn, sugarcane, and wheat are the most common feedstock for bioethanol production. However, these crops used as the raw materials for fuel production raise serious food security concerns, and the price of these substrates is a major cost component in the ethanol production process (Tyner 2013). Agricultural or industrial residue is considered to be an inexpensive and highly available...
substrate for bioethanol production, which has no influence on food prices and can reduce the production costs (Valentine et al. 2012). Furfural residues (FRs), which are generated from furfural production, are one of the most promising industrial residues that could be used as a substrate for ethanol production. Annually, about 23 million tons of FRs are produced in China (Bu et al. 2011). During commercial furfural production, corn cobs are heated at approximately 170 to 185 °C under acidic conditions (5 to 8% dilute sulfuric acid) to hydrolyze all the hemicelluloses into xylose and partial cellulose to glucose (Yu et al. 2013). Thus, there exists some glucose in the wet FRs. However, a traditional washing process removes not only the inhibitors such as furfural and inorganic acid but also free glucose in the wet FRs. Moreover, the high lignin content of FRs may cause non-productive adsorption to cellulase, which is not favorable for the hydrolysis stage in the SSF process (Kumar et al. 2009, 2012). To ultimately improve SSF of FRs, various pretreatment technologies, such as green liquor and hydrogen peroxide pretreatment, acidic bisulfite pretreatment, and green liquor combined with ethanol pretreatment, have been applied to eliminate the negative effect of lignin that results in the non-productive absorption of enzymes (Yu et al. 2013, 2014; Zheng et al. 2015). However, these pretreatments required high energy and chemicals input, representing a considerable bottleneck in bioethanol production from FR (Keller et al. 2003).

Surfactant addition has a positive effect on enzymatic hydrolysis and fermentation for bioethanol production (Eriksson et al. 2002; Eckard et al. 2013; Zhang et al. 2009). Compared with pretreatments, the improvement of bioethanol production with surfactants is easily operated. Adding surfactants requires few facilities and causes little corrosion to the equipment. However, most surfactants are chemosynthetic, such that they have poor biodegradability and a negative effect on the environment (Huang et al. 2015). Natural surfactants, such as glycolipids, lipopeptides, saponin, phospholipids and microbial cells, are eco-friendly and have excellent surface activity, which make them a desirable replacement for traditional chemical surfactants (Huang et al. 2015; Menon et al. 2010).

Camellia oleifera Abel is widely distributed in the south of China, and its seed is commonly used for the production of cooking oil (Zhu et al. 2011). Tea-seed cake (TSC, the byproducts of cooking oil production) contains 10% to 17% of tea saponin, which is a non-ionic surfactant with natural, non-toxic, environmental characteristics. Some research has been conducted regarding the effects of tea saponin on the lignocellulose hydrolysis (Feng et al. 2013). Nevertheless, directly applying TSC in the promotion of bioethanol production has not been reported. In this study, unwashed FRs remained from furfural production were directly used as raw materials for bioethanol production. Different dosages of TSC in the promotion of overall ethanol yield were investigated, and the inhibitory effects of byproducts were examined. The surface tensions of the supernatants and contact angles of the solids from different fermentation system before and after SSF were also compared.

**EXPERIMENTAL**

**Materials**

The FRs were the residues from the furfural production and kindly provided by Chunlei Furfural Corporation (Hebei, China). The initial pH range of unwashed FRs was 2 to 3. The sugars in the unwashed FRs mainly consisted of 4.8% glucose and 0.57% xylose. The acetic acid content in the unwashed FRs was 3.17% (Xing et al. 2015). The
average contents of glucan, lignin, and ash were 30.3%, 47.1%, and 5.2%, respectively. The moisture content of the raw material based on wet basis was 49.9%. The wet unwashed FRs were screened with 20 meshes after being ground and then stored at -4 °C until use in subsequent experiments.

Tea-seed cake was purchased from the market. TSC was ground to obtain a 40-mesh fraction after being dried at 50 °C for 12 h. The major contents of tea saponin, proteins, and lipid were 10%, 8.8% and 7.3%, respectively. The other components of TSC were 37.6% total sugars, 12.6% glucan, 10.2% xylan, 0.2% glucose and 2.8% ash.

Methods
Microorganisms and enzyme preparation

The microorganism used glucose for producing ethanol was Saccharomyces cerevisiae in the form of dry yeast (Angel Yeast Company Ltd., China). Dry yeast was added to the culture after activating in 2% glucose solution at 36 °C for 15 min and then at 34 °C for 1 h. All reagents used were of analytical grade.

Cellulase (Celluclast 1.5 L) and cellobiase (Novozyme 188) were purchased from Novozymes (Shanghai, China). The filter paper activity of cellulase and cellobiase, measured using the International Union of Pure and Applied Chemistry (IUPAC) method, was 132 FPU/mL and 175 CBU/mL, respectively (Ghose et al. 1987). The working temperature was 50 °C and pH was 4.8.

Simultaneous saccharification and fermentation experiments

The SSF experiments were conducted at 5% substrate solids (w/v) on a shaker (Certomat-R, B-Braun, Germany) at 38 °C and 130 rpm under non-sterile conditions. In the SSF test, a 100 mL Erlenmeyer flask was used at a working volume of 60 mL. In the anaerobic cultivations, each flask was equipped with a loop trap containing sterile glycerol. The amounts of cellulase (Celluclast 1.5 L) and cellobiase (Novozyme 188) used in SSF were 15 FPU/g-cellulose and 17 CBU/g-cellulose, respectively (Zheng et al. 2015). The initial yeast cell mass was 4 g/L. The pH of the whole slurry was adjusted to 5.5 with 20% (w/v) sodium hydroxide. Three different types of SSF media were used: organic medium (1.0 g/L yeast extract, 0.5 g/L (NH4)2HPO4, 0.5 g/L MgSO4·7H2O), mineral-salt medium (0.5 g/L (NH4)2HPO4, 0.5 g/L MgSO4·7H2O), and TSC-medium (10 g/L, 20 g/L, or 30 g/L of TSC, 0.5 g/L (NH4)2HPO4, 0.5 g/L MgSO4·7H2O, respectively). Based on the different TSC dosage (10 g/L, 20 g/L, 30 g/L), the media were labeled as TSC10, TSC20, and TSC30, which corresponded to their dosage. Another group of tests without nutrition addition was set as the blank. In the experiments, FRs, water, sodium hydroxide, and fermentation medium were sterilized separately (121 °C for 20 min) and then mixed in a conical flask under sterile conditions. The enzymes and yeast were added directly to the flask. SSF was terminated after 120 h. Samples (about 2 mL) taken periodically from SSF and centrifuged at 14,000 × g for 5 min. Before analysis, the supernatant was filtered with a 0.22 μm filter to remove impurities. Ethanol and byproducts in the supernatant were determined by high-performance liquid chromatography (HPLC). Each data point was the average of two replicates. The ethanol yield was calculated by the following formula,

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\text{ethanol yield} \ (\%) = \frac{\text{ethanol at 120 h (g/L)}}{\text{initial cellulose (g/L)}} \times 100\%
\] (1)
where the initial cellulose was the sum of the cellulose content of TSC and FRs used in the SSF. The number 1.11 is the conversion factor of cellulose to glucose, and 0.51 is the conversion factor for glucose to ethanol. Thus the cellulose conversion rate was calculated assuming that 1 g of cellulose present in the liquid theoretically gave 0.568 (1.11×0.51) g of ethanol.

**Analytical methods**

The composition of unwashed FRs and TSC was analyzed according to the National Renewable Energy Laboratory (NREL) standard analytical procedure (Sluiter et al. 2008). Ethanol and by-products in the supernatant were determined by HPLC (Waters 2695e, USA) using an HPX-87H column (Bio-Rad, USA) at 65 °C and a refractive index detector at 35 °C (Tang et al. 2013). The eluent, 5 mM H$_2$SO$_4$, was flowed at 0.6 mL/min. The data were recorded as averages of replicate measurements.

**Surface tensions and contact angles**

The surface tensions of the supernatants before and after SSF were assayed according to Jian’s method on an automatic tension meter (model JK99B, Zhongchen Digital Technology Equipment Co., Ltd, Shanghai, China) at 28 °C (Jian et al. 2011). The surface tension of the distilled water was 72 ± 0.5 mN/m.

Before contact angle analysis, samples from the SSF system at 0 h and 120 h were dried at 50 °C for 24 h. The dry samples were pressed into disks of 13 mm diameter under 20 tons pressure to achieve a flat and relatively smooth surface. The sessile drop method was used for measuring water contact angle on sample surface using a contact angle goniometer OCA15Pro instrument (Data-Physics, Germany) at room temperature. A total of 10 µL of water was dropped carefully through the 0.5 mm OD needle tip onto the surface of the sample. The camera of the instrument was perfectly adjusted to take the drop image formed on the surface, and the equipment software measured the contact angle data. The average contact angle value was determined by measuring five samples.

**RESULTS AND DISCUSSION**

**Effect of TSC Dosage on Ethanol Production**

The concentration profiles of free glucose during SSF are shown in Fig. 1.

![Fig. 1. Concentration of free glucose (dashed line) and ethanol yield (solid line) during SSF](image-url)
At the beginning of SSF, the concentrations of free glucose in the specimens were different, and the results were also parallel to the concentrations of TSC that contained glucose. However, the total free glucose stayed below 0.2 g/L after 4 h in all cases, which indicated that the rate-limiting step in the overall kinetics of SSF was enzymatic hydrolysis.

The influence of fermentation media on ethanol yield was also evaluated, as shown in Fig. 1. To ensure the accuracy of ethanol yield, free glucose in the initial FRs and TSC was excluded. The blank test approached its maximum ethanol yield of 73.0% at 120 h. The maximum ethanol yield at 120 h (85.4%) was reached with the TSC10 medium, which was 12.4% higher than that in the blank test. The ethanol production was improved after the addition of fermentation media which contained TSC, except for in TSC20 and TSC30. This was due to the fact that the tea saponin of the TSC served as an accelerant to promote the adsorption of cellulolytic enzymes on the substrate and mediate the release of adsorbed enzymes (Feng et al. 2013). Although many studies have reported that pretreatment methods could improve SSF of FRs, the ethanol yield in our study was a little higher than that from FRs after different pretreatments (Bu et al. 2014; Wang et al. 2013). In addition, the requirements of yeast available nitrogen in *Saccharomyces cerevisiae* were increased by the higher sugar concentrations in fermentation broth (Childs et al. 2015). The protein of TSC likely acted as a kind of nitrogen source that is crucial to meet the nutrient requirements of yeast during fermentation (Bell and Henschke 2005). However, when TSC loading was above 20 g/L, the SSF was interrupted. These results could be explained by the negative effect of tea saponin on the activity of *Saccharomyces cerevisiae*. Some researchers have found that a high concentration of tea saponin induces cytotoxicity in microorganisms and bacteria (Zhang et al. 2014; Zhou et al. 2014). Therefore, SSF with the addition of TSC at 10 g/L could improve enzymatic hydrolysis and the overall ethanol yield.

**Effect of TSC Dosage on Byproducts during SSF Process**

Pure culture conditions in the ethanol production are generally not practical (Skinner and Leathers 2004). As a result, the emergence of bacterial contaminants in ethanol fermentation is often unavoidable (Fig. 2).

![Fig. 2. Comparison of different media on the concentration of byproducts during SSF. (a) Lactic acid; (b) acetic acid.](image-url)
Except for the desired product, a variety of byproducts will be produced during ethanol fermentation, such as lactic acid and acetic acid. Variations of byproducts in the presence of different media during the SSF process are shown in Fig. 2.

Lactic acid is one of the common byproducts in an ethanol fermentation broth. It is produced by contaminating lactic acid bacteria as a result of carbohydrate metabolism. The lactic acid is a potential inhibitor of yeast growth and metabolism when produced at concentrations of 0.2 to 0.8% w/v (Graves et al. 2006). As shown in Fig. 2(a), the yield of lactic acid was augmented along with the increase in the dosage of TSC. Compared with the blank test (3.75 g/L), the highest lactic acid content of 8.88 g/L was obtained when using TSC30-medium. This was likely due to the fact that the SSF using TSC-medium that contained sufficient nutrition was easily consumed by lactic acid bacteria in the decline phase of the yeast. And subsequently the growth of lactic acid bacteria was facilitated by the yeast (Lemaresquier 1987). Furthermore, no lactic acid was detected in the initial 24 h of SSF, and there was a sharp increase in the output of lactic acid for all the tests in the following time. These results implied that a majority of the glucose was consumed for yeast growth during the initial stage of SSF (Tang et al. 2011).

Acetic acid is a minor byproduct by Saccharomyces cerevisiae during fermentation, but toxic concentrations may be produced primarily by contaminating lactic acid bacteria and/or by acetic acid bacteria (Thomas et al. 2002). The optimal pH range for growth of Saccharomyces cerevisiae was from 5.0 to 5.5, and the cell growth ceased due to low pH in the fermentations. As indicated in Fig. 2b, the acetic acid concentration in all tests was relatively high (1.5 g/L) in the initial stage of SSF, which was due to acetic acid in the unwashed FRs (Xing et al. 2015). The acetic acid concentration increased slightly. This suggested that there was a balance between the formation of ethanol and acetic acid during the later stage. The concentrations of acetic acid were relatively low, which had no significant effect on the pH. Although acetic acid, which had a concentration range of 100 mM to 170 mM, was more inhibitory to ethanol production by yeast than lactic acid, low concentrations of acetic acid stimulated the ethanol production rate by the yeast (Taherzadeh et al. 1997; Palmqvist et al. 1999; Pampulha and Loureiro-Dias 2000; Thomas et al. 2002; Garay-Arroyo et al. 2004).

Surface Tension of SSF Liquid and Contact Angle of SSF Solid

The surface tension and contact angle were measured to further investigate the beneficial function of tea saponin in SSF. One of the typical characteristics of tea saponin is that it decreases the surface tension in a solution. Another remarkable effect of tea saponin is its maintenance of stable surface tension. However, some cellulolytic enzymes degrade tea saponin (Feng et al. 2013). The change of surface tension during the SSF process was assayed to test the stability of this non-ionic surfactant. As shown in Fig. 3a, the surface tensions of supernatants were approximately 33.92 (TSC10), 32.79 (TSC20), and 32.65 (TSC30) mN/m, respectively (SSF system with a TSC-medium at 0 h). The surface tension at SSF using TSC-medium was much lower than in the other fermentation system. The surface tension increased when the fermentation time was prolonged. This could have been due to the hydrolyzation of tea saponin and the subsequent formation of byproducts.
Contact angle measurement is an essential test for wettability alteration, which plays a very important role in the SSF process. As shown in Fig. 3b, the initial contact angle of unwashed FRs was 45.6°. Small changes in the initial contact angles of mineral-salt medium (44.2°) and organic medium (42.5°) were observed. The initial contact angles of the fermentation system with the addition of TSC dropped rapidly to 37.3° (TSC10), 27.1° (TSC20), and 21.7° (TSC30), respectively. This finding suggested that the tea saponin altered the wettability of FRs through hydrogen bonds and hydrophobic interaction (Hou et al. 2015). Furthermore, the contact angle was related to the surface tension between the liquid and the solid surface. Also, the lower surface tension would then consequently lead to a lower contact angle (Fig. 3a) (Cao et al. 2002). However, the

Fig. 3. Surface tension of the supernatants and contact angles of the solids from different fermentation system before and after SSF
final contact angles were typically larger than those in the initial stage. The results showed that the degree of degradation of tea saponin had a negative effect on the wetting capacity. And with the hydrolysis of cellulose, lignin content would increase. The lignin accumulation also played an important role in the increased contact angle.

CONCLUSIONS

1. The protein of tea-seed cake (TSC) can be used as a nitrogen source for yeast growth, and tea saponin can act as an accelerator for simultaneous saccharification and fermentation (SSF). Unwashed furfural residues (FRs) can be easily digested at relatively low TSC loading (10 g/L), and this reaction system could be promising for commercial ethanol production.

2. The maximum ethanol yield of 87.2% was reached with TSC10 medium at 96 h, which was 14.2% superior to that of the blank test (73.0%).

3. The yield of lactic acid was augmented along with the increase in the dosage of TSC. The SSF using TSC30 resulted in the highest concentration of lactic acid (approximately 8.88 g/L) after 96 h.

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