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Simultaneous saccharification and co-fermentation of steam-exploded poplar wood to ethanol with *Escherichia coli* KO11

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Steam explosion pretreatment is a promising method for the preparation of lignocellulosic biomass for biofuels production. Steam explosion was performed to assess the effectiveness of pretreating poplar wood for ethanol production. Maintaining steam pressure at 4 MPa for 10 min, 66.8% of hemicellulose and 5.3% of cellulose were hydrolyzed. The scanning electron microscopy observation, FT-IR spectroscopy analysis and X-ray diffraction analysis showed that hydrolysis of hemicellulose and modification of cellulose and lignin structures improved the accessibility of cellulose to hydrolytic enzymes. Cellulase production by *Trichoderma reesei* RUT C-30 was initiated with inocula prepared by different methods. It was found that the composition of pre-culture medium and inoculum age have minimal or no impact on cellulase production. Using the steam-exploded poplar wood as feedstock with cellulase loading of 20 FPIU g^{-1} substrate, ethanol production by gene-engineered *Escherichia coli* KO11 reached 3.6 g L^{-1}, corresponding to 51.4% of the maximum theoretical yield based on glucan remaining in the input substrate. The ethanol yield was limited by the low solid content of steam-exploded residue, and inhibited by the inhibitors released in the pretreatment process. So the steam explosion process needs to be improved for lower water content and less inhibitors, and detoxification is necessary to achieve a good fermentability of pretreated poplar wood to ethanol.

Key words: Steam explosion pretreatment, Simultaneous saccharification and co-fermentation, Poplar wood, Ethanol.

INTRODUCTION

With the rising tightness of the world's energy supply, growing attention has been devoted to ethanol fuel, but the real cause was the inevitable depletion of fossil fuels. However, nearly all bioethanol fuel is produced by fermentation of sugar from food crops, corn and sugarcane [1]. On the other hand, the utilization of cellulosic materials with relatively low cost and plentiful supply is very limited. The central technological impediment to bioconversion of lignocellulosic biomass to ethanol is the general absence of low-cost and efficient technology for delignification to liberate cellulose and hemicellulose from their complex with lignin, depolymerization of cellulose and hemicellulose to monose, and fermentation of mixed hexose and pentose to ethanol [2-3].

Steam explosion pretreatment is a promising method for the preparation of lignocellulosic biomass for biofuels production. The utilization of lignocellulosic biomass is limited by the hard-textured structure existing among the three main components of the plant cell wall - cellulose, hemicellulose and lignin [4]. Steam explosion as a pretreatment method is used to overcome the physical and chemical barriers of lignocellulosic materials quickly and efficiently. Steam explosion can be carried out to pretreat a great variety of lignocellulosic feedstocks including forestal and agricultural residues [5]. One of the central economic impediments to the bioconversion of lignocelluloses was the cost of cellulolytic enzymes [6-7]. Many efforts have been made in cost reduction of cellulolytic enzyme usage for the commercialization of cellulosic ethanol [8-9]. Fermentable sugars from the hydrolysis of cellulose and hemicellulose by pretreatment with cellulolytic enzymes include hexose and pentose. However, most microbes consume the mixed sugars sequentially because of the carbon catabolite repression existing in most microbes [10]. To overcome this barrier, specific strains of *Escherichia coli*, *Saccharomyces cerevisiae*, and *Zymomonas mobilis* have been engineered for simultaneous fermentation of hexose and pentose to improve the efficacy of the overall process [11]. In this study, cellulase fermentation was carried out with *Trichoderma reesei* RUT C-30. The preparation methods of the inoculum were tested to find out the effect of pre-culture medium composition and inoculum age on cellulase production. Pretreatment of poplar wood was performed in a custom-made steam explosion device, trying to evaluate the steam explosion process for preparing poplar wood as feedstock for

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ethanol production. The steam-exploded poplar wood was converted to ethanol in a process termed SSCF (simultaneous saccharification and co-fermentation) using *T. reesei* cellulase and *E. coli* KO11 as genetically engineered ethanol-producing bacteria efficiently converting both hexose and pentose sugars to ethanol [12]. The SSCF process integrates the enzymatic hydrolysis of cellulose with the simultaneous fermentation of hexose and pentose to ethanol. Furthermore, scanning electron microscopy (SEM), FT-IR spectroscopy and X-ray diffraction analysis were carried out to evaluate the physical/chemical changes of poplar wood.

EXPERIMENTAL

Steam explosion pretreatment of poplar wood

Poplar wood was cut into chips of 5 cm × 3 cm × 0.2 cm. Steam explosion pretreatment was carried out in a custom-made device produced by Haian Huada Petroleum Instrument Corporation, Jiangsu Province, China. This device is mainly composed of two parts, an electrical heating high pressure steam generator and a steam explosion reactor with a working volume of 3 L. The maximum operating pressure and temperature are 10 MPa and 220°C, respectively. The steam explosion process involves first filling a vertical cylinder with poplar wood chips, and then inletting saturated steam. Maintaining the pressure at 4 MPa for 10 min, with a sharp release of pressure steam explosion pretreatment was accomplished, and the steam-exploded residue was collected and used as feedstock for ethanol production. The steam-exploded residue contained 34.4 g L^{-1} of poplar wood.

Preparation of inoculum for cellulase production

Trichoderma reesei RUT C-30 used as cellulase producer was obtained from the CCICC (China Center of Industrial Culture Collection). The culture was maintained on potato dextrose agar slants for 7 days at 28°C and then stored at 4°C. Conidia were harvested by washing the slant with 3 mL of sterile distilled water. The spore concentration in the conidial suspension was determined by counting with a blood cell counting chamber. 1 mL of spore suspension (10^7 spores mL^{-1}) was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of pre-culture medium and placed on an orbital shaker at 200 rpm and 28°C for 2 days.

Cellulase production

A 5 % (v/v) inoculum concentration was used to initiate the cellulase production. Cellulase fermentation was carried out in 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium at 200 rpm and 28°C for 5 days with initial pH of 4.8. The cellulase fermentation medium was composed of 12 g cellulose, 24 g bran, 10 g tryptone, 100 mL Mandels nutrient salts solution, 1 mL Mandels trace elements solution, and 50 mL 1 mol L^{-1} citrate buffer per liter [13]. The cellulase was collected from the fermentation broth by centrifugation (5000 *g*, 10 min), and concentrated by ultrafiltration.

Preparation of inoculum for ethanol fermentation

Escherichia coli KO11 was used as ethanol producer. The *Zymomonas mobilis* genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh*B) were integrated into the chromosome of *E. coli* within or near the pyruvate formate-lyase gene (*plf*), so that the gene-engineered *E. coli* KO11 can efficiently convert both hexose and pentose sugars to ethanol [12]. *E. coli* KO11 was grown on plates containing solid LB medium supplemented with 20.0 g L^{-1} glucose and 0.6 g L^{-1} chloramphenicol [12], and incubated at 30°C for 24 h. Seed cultures were grown on liquid LB medium supplemented with 20.0 g L^{-1} glucose at 30°C for 24 h with agitation of 100 rpm. Cells were harvested by centrifugation (5000 *g*, 5 min) and used as an inoculum to provide an initial concentration of 33 μg/ml dry weight (about 0.1 OD_{550}).

Simultaneous saccharification and co-fermentation (SSCF)

The SSCF runs were performed in a 3 L fermentor (BioFlo 110, New Brunswick, United States) with a working volume of 2 L at 38°C with agitation of 150 rpm and pH control at 5.5. Steam-exploded poplar wood was introduced in the fermentor with cellulase loading of 20 FPIU g^{-1} substrate. The ethanol fermentation medium was composed of 27.3 g steam-exploded poplar wood, 10 g tryptone, 1 g KH_2PO_4, 0.5 g K_2HPO_4, 3 g/l $(NH_4)_2SO_4$, 0.4 g $MgCl_2 \cdot 6H_2O$ and 0.02 g $FeCl_3 \cdot 6H_2O$ per liter. A 4 mol L^{-1} KOH solution and a 6 mol L^{-1} HCl solution were used for pH control.

Analytical methods

Cellulase activity was measured as filter paper activity according to the method recommended by Ghose [14]. One international unit of cellulase activity is the amount of enzyme that forms 1 μmol glucose per min during the hydrolysis reaction. The cellulose, hemicellulose and lignin were measured using a method described previously [15]. Ethanol was determined by using gas chromatography (Clarus 500 GC, PerkinElmer, United States) with

1-propanol as internal standard, as described earlier [16]. A scanning electron microscope (JSM-6380LV, JEOL, Japan) was used to take images of treated and untreated samples at 10 kV acceleration voltage after gold coating. The FT-IR spectra were obtained on an FT-IR spectrophotometer (FTIR-8400S, Shimadzu, Japan) using a KBr disc. Infrared transmittance between 400 cm^{-1} and 4000 cm^{-1} was measured. X-ray diffraction analysis was done by X-ray powder diffractometry (D8-Advance, Bruker, Germany) to determine the crystallinity of samples. The sample was scanned in the 2θ values from 10° to 30°, and the resultant graphs were printed out using the software OriginPro 8.0, and the crystallinity index was calculated by using the software MDI JADE 5.0. Statistical analysis was performed using the software SPSS 19.0.

RESULTS AND DISCUSSION

Cellulase production by Trichoderma reesei RUT C-30

Many methods of inoculum preparation, including different carbon sources in pre-culture medium and different ages of inocula, have been reported for cellulase production [17-21]. The preparation method could be a contributory factor in improving cellulase production. In this study, nine preparation methods were selected and compared to investigate the effect on cellulase production (Table 1). With the same inoculum concentration of 5 % (v/v), cellulase production was initiated and the cellulase yield calculated by filter paper activity measured after 5 days of culture. According to the fermentation results shown in Fig.1, the composition of pre-culture medium and inoculum age have minimal or no impact on cellulase production.

From the time course of ethanol production in Fig. 2, it is seen that these remaining fermentable sugars are difficult to be converted to ethanol. The unhydrolyzed poplar wood implies that inhibitors have formed in the steam explosion pretreatment and inhibited cellulase activity. The unutilized reducing sugars imply that the growth and activity of the ethanol-producing strain *E. coli* KO11 were inhibited. In this study, the ethanol yield was low, caused by the low concentration of pretreated poplar wood loaded in the fermentation medium. The steam-exploded residue with a water content as high as 96.5% was further diluted by loading the crude cellulase solution, which ultimately led to a substrate concentration of 27.3 g L^{-1}. Another cause is that inhibitors are released during the pretreatment process. So detoxification is necessary to achieve efficient fermentability of steam-exploded feedstock to ethanol.

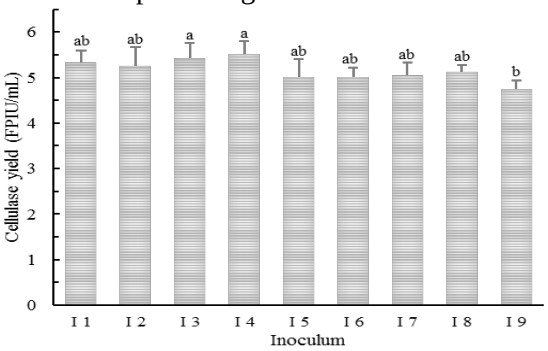

Fig. 1. Cellulase yield with different methods of inoculum preparation. Clusters followed by the same letter are not significantly different at P=0.001, according to least significant difference test.

SSCF of steam-exploded poplar wood with E. coli KO11

The SSCF of steam-exploded poplar wood was carried out with cellulase loading of 20 FPIU/g substrate (Fig. 2). The maximum ethanol yield reached 3.6 g L^{-1}, corresponding to 51.4% of the maximum theoretical yield based on glucan remaining in the input steam-exploded poplar wood. It was observed that 16.7 g L^{-1} of solid substrate and 2.7 g L^{-1} of reducing sugars remained.

Table 1. Preparation methods of inoculum of *T. reesei* RUT C-30 for cellulase production

Inoculum	Pre-culture medium composition					Culture time (days)
	Basic nutrients [a]	Glucose (g L^{-1})	Cellulose (g L^{-1})	Lactose (g L^{-1})	Tween 80 (mL L^{-1})	
I 1	√	20				2
I 2	√	20	10			2
I 3	√		7.5			4
I 4	√		10			4
I 5	√		10		0.5	4
I 6	√	30				2
I 7	√	20			0.5	2
I 8	√			30		2
I 9	Spore suspension (10^7 spores)					

[a] The basic nutrients contain 1 g L^{-1}peptone, 100 mL L^{-1} Mandels nutrient salts solution, 1 mL L^{-1} Mandels trace elements solution, and 50 mL L^{-1}1 M citrate buffer.

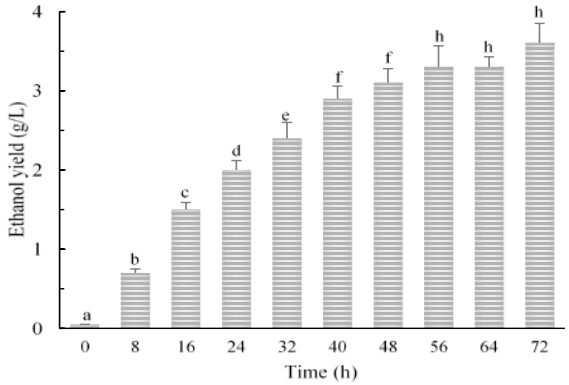

Fig. 2. Time course of ethanol production from steam-exploded poplar wood. Clusters followed by the same letter are not significantly different at P=0.001, according to least significant difference test

Compositional analysis

The compositional changes of the poplar wood during the steam explosion pretreatment and SSCF are presented in Table 2. In the steam explosion pretreatment process, 16.3% of the dry weight of poplar wood were lost, including 66.8% of hemicellulose and 5.3% of cellulose. During the SSCF process, 62.2% of cellulose and 76.2% of hemicellulose were hydrolyzed and utilized. From this analysis results, no significant changes in lignin content were observed. The high-pressure steam chemically modified lignin structure improved the accessibility of cellulose to hydrolytic enzymes [4]. Due to condensation and re-polymerization reactions, the lignin content remained almost unchanged during steam explosion pretreatment [4, 22].

Scanning electron microscopy analysis

Poplar wood samples were observed using SEM to establish what occurred to the physical structure of poplar wood after steam explosion and enzymatic hydrolysis.

Fig. 3 (b) clearly presents that unorganized and loose structure of the poplar wood fiber bundles was generated by the steam explosion compared to the untreated one shown in Fig. 3 (a). The picture of residual solids after enzymatic hydrolysis in the SSCF process (Fig. 3 (c)) suggests that steam explosion pretreatment significantly improved the biodigestibility of poplar wood. The irregular fiber bundles (Fig. 3 (b)) were hydrolyzed into small fragments (Fig. 3 (c)). It can be speculated that the improvement in biodigestibility by steam explosion pretreatment was due to the modification of the compact structure of cellulose and lignin and the increase in the accessible surface area.

Fig. 4 presents the FT-IR spectra of raw poplar wood, steam-exploded poplar wood and fermentation residue. The sharp peak at 1736 cm^{-1} in the spectrum of raw poplar represents the carboxyl groups, which are the main constituents of hemicellulose [23]. As shown in Fig. 4, this characteristic hemicellulose peak almost disappeared in the pretreatment process, which is consistent with the compositional analysis result that steam explosion pretreatment has led to the hydrolysis of the major part of the hemicellulose. The changes observed in the peak at 898 cm^{-1} are due to absorption of cellulose [24]. The decrease in intensity of this characteristic peak indicates the hydrolysis of cellulose during pretreatment and fermentation processes.

X-ray diffraction analysis

The poplar wood samples were analyzed by X-ray diffraction to characterize their crystallinity and the diffraction patterns are shown in Fig. 5. The diffraction peaks at 2θ angles of 16.5° and 22.5° correspond to crystalline cellulose [25]. These two characteristic peaks markedly weakened.

Table 2. Composition of poplar wood samples

	Dry weight loss (%)	Cellulose (%)	Hemicellulose (%)	Lignin and ash (%)	Cellulose removal (%)	Hemicellulose removal (%)
Raw poplar wood	0	45.0±3.7 A [a]	22.3±1.6 A	20.5±1.4 A	0	0
Pretreated poplar wood	16.3±0.7 A	42.6±3.9 B [b]	7.4±0.6 B	20.4±0.8 A	5.3±0.8 A	66.8±6.4 A
Pretreated poplar wood after SSCF	48.2±5.1 B	17.0±2.3 C	5.3±0.6 C	19.4±1.3 A	62.2±6.8 B	76.2±4.7 B

[a] Values are expressed as mean±SEM (n=3). Values in the same column followed by different capital letters are significantly different at P=0.05, according to least significant difference test. [b] The data in the rows are based on the oven dry untreated poplar wood. For example, 42.6 doesn't mean that the oven dry solid residue contains 42.6% cellulose, but infers that the residue (84.7% of untreated biomass) contains 50.3% cellulose.

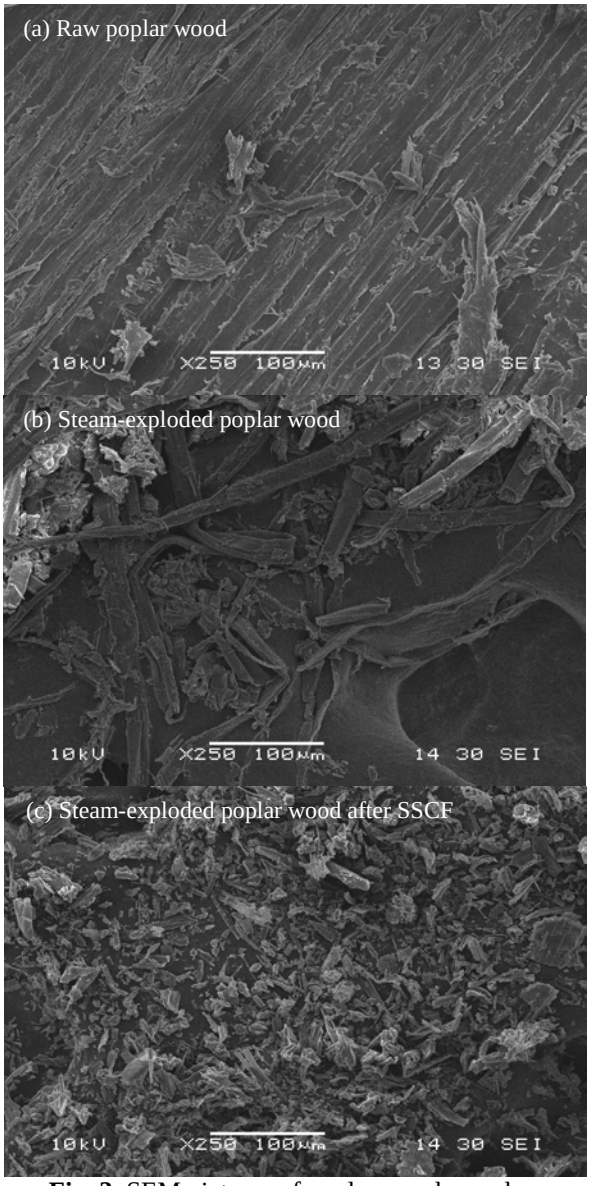

Fig. 3. SEM pictures of poplar wood samples.

SSCF of steam-exploded poplar wood with E. coli KO11

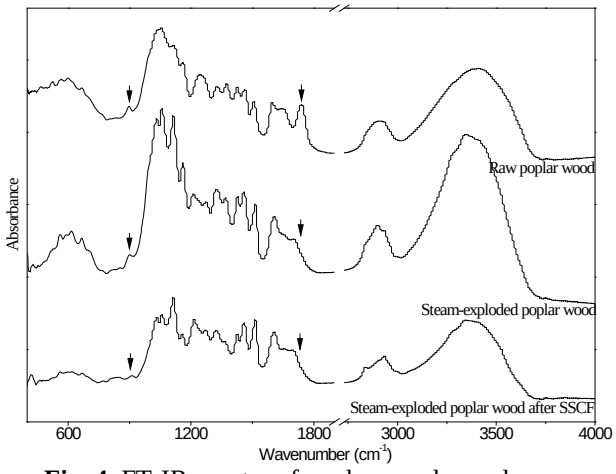

Fig. 4. FT-IR spectra of poplar wood samples.

The steam-exploded poplar wood had a decreased relative crystallinity index due to the phase change in the crystal structure from cellulose I to cellulose III [26-27]. With the degradation of cellulose by cellulase enzymes in the SSCF process, the relative crystallinity further decreased. These results are consistent with the observations from compositional analysis and FT-IR spectroscopy analysis.

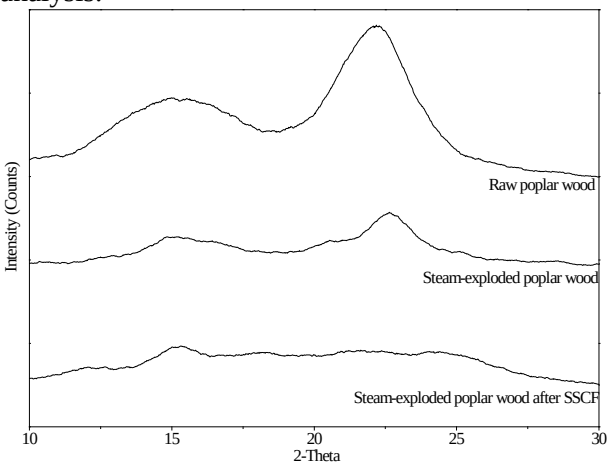

Fig. 5. X-ray diffraction intensity curves of poplar wood samples.

CONCLUSION

Steam explosion is shown to be a practicable method for quickly and efficiently pretreating poplar wood for ethanol production. By steam-explosion at 4 MPa for 10 min, 66.8% of hemicellulose and 5.3% of cellulose were hydrolyzed. Using the steam-exploded poplar wood as feedstock with cellulase loading of 20 FPIU g^{-1} substrate, ethanol yield reached 3.6 g L^{-1}, corresponding to 51.4% of the maximum theoretical yield based on glucan remaining in the input substrate. The ethanol yield was limited by the low solid content of steam-exploded residue, and inhibited by the inhibitors released in the pretreatment process. So the steam explosion process needs to be improved for lower water content and less inhibitors, and detoxification is necessary to achieve a good fermentability of pretreated poplar wood to ethanol.

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