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Combination of biological pretreatment with liquid hot water pretreatment to enhance enzymatic hydrolysis of *Populus tomentosa*

Wei Wang [a], Tongqi Yuan [b], Kun Wang [b], Baokai Cui [a], Yucheng Dai [a,*]

[a] Institute of Microbiology, Beijing Forestry University, Beijing 100083, China
[b] Institute of Biomass Chemistry and Technology, Beijing Forestry University, Beijing 100083, China

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ABSTRACT

A novel stepwise pretreatment of combination of fungal treatment with liquid hot water (LHW) treatment was conducted to enhance the enzymatic hydrolysis of *Populus tomentosa*. The results showed that lignin and cellulose increased with the elevating temperature, while significant amount of hemicellulose was degraded during the LHW pretreatment. A highest hemicellulose removal of 92.33% was observed by combination of *Lenzites betulina* C5617 with LHW treatment at 200 °C, which was almost 2 times higher than that of sole LHW treatment at the same level. Saccharification of poplar co-treated with *L. betulina* C5617 and LHW at 200 °C resulted in a 2.66-fold increase of glucose yield than that of sole LHW treatment, and an increase (2.25-fold) of glucose yield was obtained by the combination of *Trametes ochracea* C6888 with LHW. The combination pretreatment performed well at accelerating the enzymatic hydrolysis of poplar wood.

1. Introduction

Populus tomentosa is a wide distributed tree species in China, as a result of its widespread availability, sustainable production and low starting value, it has the potential to serve as the feedstock for production of fuel ethanol.

Conversion of lignocellulosics to ethanol employs three major steps including (1) pretreatment to breakdown the lignin and open the crystalline structure of cellulose, (2) hydrolysis with a combination of enzymes to reduce cellulose to glucose and (3) microbial fermentation of glucose to ethanol (Sun and Cheng, 2002). However, a major barrier in the commercialization of a lignocelluloses based ethanol process is pretreatment, which constitutes one-third of the total production costs (NREL, 2000). Typical physical and chemical pretreatments, such as microwave, ionizing radiation, steam explosion, dilute acid, alkali, and oxidation or varied combination, require special instrument and consume a lot of energy (Mosier et al., 2005b). Moreover, they often generate some inhibitors to subsequent enzymatic hydrolysis and microbial fermentation apart from producing acidic or alkaline waste water, which needs pre-disposal treatment to ensure environmental safety (Keller et al., 2003).

Microbial pretreatment, as an environmental friendly and low cost pretreatment approach for enhancing enzymatic saccharification and fermentation of lignocellulosic biomass to ethanol, is attracting increasing attention in recent years (Keller et al., 2003; Taniguchi et al., 2005; Hwang et al., 2008; Zhang et al., 2007; Shi et al., 2008, 2009). So far, lots of studies on pretreatments with various white rot fungi have been reported. Four white rot fungi were applied to pretreatment of white pine and tulip tree for enzymatic hydrolysis, and found that 450 mg glucose/g pretreated wood was obtained by *Trametes versicolor* in 30 days (Hwang et al., 2008). Yu et al. (2009a) studied the effect of biological pretreatment with the selective white rot fungus *Echinodontium taxodii* on enzymatic hydrolysis of Chinese willow and China-fir, the results showed that the hydrolysis ratios increased 4.7-fold for hardwood and 6.3-fold for softwood after pretreatment for 120 days. Pretreatment with *Ceriporiopsis subvermispora* for enzymatic hydrolysis of corn stover was investigated, and overall glucose yields of 66.61% were achieved with 35-days microbial pretreatment (Wan and Li, 2010). These reports showed a great potential with biological pretreatment in conversion of lignocellulosic biomass to ethanol. However, relative low efficiency, considerable loss of carbohydrates and long residence periods are the three major disadvantages for the fungal pretreatment (Yu et al., 2009b). New strategies should be adopted to overcome these feeble sides.

Recently, liquid water under elevated temperature and pressure, namely hot compressed water or hydrothermal processing, has received renewed attention. Liquid hot water (LHW) pretreatment has been shown to be effective in pretreatment of lignocellulosic biomass by partially hydrolyzing the hemicelluloses and disrupting the lignin and cellulose structures, thus increasing the surface area (Mosier et al., 2005b). The advantages of LHW

* Corresponding author. Tel./fax: +86 10 6233 6309.
E-mail addresses: wish101@yahoo.cn (W. Wang), yuchengd@yahoo.com (Y. Dai).

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pretreatment contained: limited corrosion problems, no sludge generation, low capital and operational costs and negligible loss of cellulose under normal operating conditions (Liu, 2010).

In order to enhance the efficiency of the fungal pretreatment and lower the severity requirements of the LHW pretreatment, a two-step pretreatment was employed. The wood flour of *P. tomentosa* was pretreated with white rot fungi *Lenzites betulina* and *Trametes ochracea*, respectively, and then dealt with LHW. The component changes of pretreated wood and sugar yield from enzymatic hydrolysis were both determined to evaluate the effects of combination pretreatments on *P. tomentosa*.

2. Methods

2.1. Microorganism and inoculums preparation

The two white rot fungi, *L. betulina* C5617 and *T. ochracea* C6888, were isolated from Liaoning and Hebei provinces in china, respectively. The organisms were preserved on 2% (w/v) malt-extract agar (MEA) plates at 4 °C in laboratory. The two fungi were activated in 100 mL basic medium (g/L: glucose 20, yeast extract 5, KH_2PO_4 1, $MgSO_4$ 0.5, VB_1 0.01), and cultured on a rotary shaker at 28 °C with a speed of 150 rpm. Mycelial pellets were harvested after 5 days, added 100 mL distilled water and then mixed with a laboratory blender for 30 s at 5000 rpm. This suspension would act as inoculums.

2.2. Raw materials

Fresh poplar (*P. tomentosa*) from countryside of Beijing was chopped into small pieces and air-dried. The samples were ground, and the particles below 0.9 mm were prepared for the subsequent pretreatment with white rot fungi and LHW, respectively.

2.3. Biological pretreatment of Poplar wood

The biological pretreatment was carried out in a 250 mL Erlenmeyer flask with 5 g of air-dried poplar wood and 12.5 mL of distilled water. The samples were sterilized in the autoclave for 20 min at 121 °C and inoculated with 5 mL inoculums. The cultures were incubated statically at 28 °C for 4 weeks. The non-inoculated samples were served as the control. All experiments were performed in triplicate.

2.4. LHW pretreatment of poplar wood

The experiments were conducted in batch tube reactors fabricated from 316 stainless steel tubes, with a length of 4.5 inches, an outside diameter of 1.0 inch, wall thickness of 0.065 inch, and a total volume of 50 mL. The reactor was filled with 2.5 g raw or bio-pretreated poplar wood and 25 mL distilled water to achieve a 10% w/v of dry matter mixture. After the slurry was loaded, the 316 stainless steel caps were fitted onto each end of the tubes. Then a drying oven was used for heat-up of the tubes. When the oven reached the targeted temperature (140, 160, 180, and 200 °C), the residence time began to record. After 30 min, the reaction was ended by quenching the tube in room-temperature water, which caused the temperature of the internal tube to drop below 100 °C in less than 5 min.

Wet material was vacuum filtered to obtain water-insoluble residues. The residues after filtration were extensively washed to neutralize with distilled water, and then dried at 35 °C for 24 h for further analysis (Lu and Zhou, 2011).

2.5. Enzymatic hydrolysis

Commercial cellulase preparation (Celluclast 1.5 L), produced by *Tricoderma reesei* ATCC 26921, was purchased from Sigma. A typical hydrolysis mixture consisted of 0.2 g of pretreated sample, 10 mL of 50 mM sodium acetate buffer (pH 4.8) supplemented with 40 μL antibiotics tetracycline and 20 μL cycloheximide, and 35 FPU/g substrate of cellulase. The mixture was incubated at 50 °C in a rotary shaker at 150 rpm for 96 h. Samples were taken from the reaction mixture at certain interval and centrifuged for 10 min at 10000 rpm, stored at −20 °C for further assay. Experiments were all performed in duplicate.

2.6. Analytical methods

The chemical composition of raw material and pretreated residues was determined according to NREL LAP "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter et al., 2008) using HPAEC. The HPAEC system (Dionex ISC 3000, USA) was equipped with an amperometric detector, AS50 autosampler, a carbopacTM PA-20 column (4 × 250 mm, Dionex), and a guard PA-20 column (3 × 30 mm, Dionex). Cellulose contents were calculated based glucose using anhydro corrections of 0.9, hemicellulose contents were calculated based the sum of xylose, galactose and arabinose, using 0.88 as anhydro corrections for xylose and arabinose, and 0.9 for galactose.

The glucose in the supernatant after enzymatic hydrolysis was also analyzed by HPAEC. The glucose yield was calculated as follows:

$$\text{Glucose yields}(\%) = \frac{\text{amount of glucose in enzyme hydrolysate} \times 0.9}{\text{amount of cellulouse in pretreated sample}} \times 100$$

3. Results and discussion

3.1. Effects of pretreatments on chemical components

After 4 weeks of biological pretreatment with *L. betulina* C5617 and *T. ochracea* C6888, the bio-pretreated samples and raw material were further treated by LHW at 140, 160, 180 and 200 °C for 30 min, respectively. The composition of different chemical components in residues after various pretreatments might be seen in Fig. 1.

As one of the main components of plant cell wall, lignin limits the enzymatic hydrolysis of lignocellulosic biomass by cross-linking with cellulose and hemicelluloses (Fan et al., 1987). To expose the highly ordered crystalline structure of cellulose and facilitate substrate access by hydrolytic enzymes, reducing the lignin content of the biomass is expected (Sun and Cheng, 2002). Recently, white rot fungi were thought to be the most promising organisms that can efficiently metabolize lignin in a variety of lignocellulosic materials (Hatakka, 1983; Sawada et al., 1995; Keller et al., 2003). In this work, significant decrease of acid insoluble lignin (AIL) only happened in the step of fungal pretreatment, *L. betulina* C5617 and *T. ochracea* C6888 decayed 12.7% and 11.89% of AIL, respectively. When the bio-treated samples and raw materials were subject to LHW, however, an increase of AIL was observed with the increasing temperature, this is contradictory to previous works concerning LHW pretreatment with flowing hot water through cellulosic biomass in a small tubular flowthrough reactor, which performed better at degrading lignin than that of batch reactor, and high flow rates enhanced lignin removal, especially at elevated temperatures (Liu and Wyman, 2003, 2004a, 2004b). The accumulation of lignin may result from the condensation and precipitation of the lignin