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Simultaneous saccharification and fermentation and a consolidated bioprocessing for Hinoki cypress and *Eucalyptus* after fibrillation by steam and subsequent wet-disk milling

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HIGHLIGHTS

- Combination pretreatment of ST and WDM for biomass utilization was evaluated.
- Mild-condition ST prevents the generation of fermentation inhibitors.
- ST can facilitate the fibrillation during WDM.
- High glucose production yield was obtained from both hardwood and softwood.
- ST–WDM improved the fermentation product yield obtained from SSF and CBP.

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ABSTRACT

An advanced pretreatment method that combines steam treatment (ST) with wet disk milling (WDM) was evaluated using two different species of woods, viz., Hinoki cypress (softwood) and *Eucalyptus* (hardwood). Bioconversion of the pretreated products was performed using enzymatic saccharification via a commercial cellulase mixture and two types of fermentation processing, i.e., yeast-based simultaneous saccharification and fermentation (SSF) and *Clostridium thermocellum*-based consolidated bioprocessing (CBP). A higher yield of glucose was obtained in the enzymatic saccharification and fermentation products from SSF and CBP with pretreatment consisting of WDM after ST, as compared to either ST or WDM alone. Maximum ethanol production via SSF and CBP were 359.3 and 79.4 mg/g-cellulose from Hinoki cypress, and 299.5 and 73.1 mg/g-cellulose from *Eucalyptus*, respectively. While the main fermentation product generated in CBP was acetate, the total products yield was 319.9 and 262.0 mg/g-cellulose from Hinoki cypress and *Eucalyptus*, respectively.

1. Introduction

Recently, second-generation biofuels produced from lignocellulosic biomass, such as agricultural by-products, forest residues, and dedicated energy crops, have garnered attention over first-generation biofuels produced from food crops such as cereals, sugar crops, and oil seeds, because of the increased prices resulting from competition of such crops with food crops (Sims et al., 2010). Lignocellulosic biomass is considered to be a sustainable energy source that has the potential to be converted to biofuels that can replace fossil fuels, but this conversion is challenging, given that it is mainly composed of three robust structural biopolymers, namely, cellulose, hemicellulose, and lignin. The robust and complex structure of lignocellulosic biomass requires a multi-step process; thus, the bioconversion of lignocellulosic biomass mainly consists of three steps: pretreatment, enzymatic hydrolysis, and fermentation (Mosier et al., 2005). This increases the production cost of biofuels, especially owing to the cost of the enzymes (Klein-Marcuschamer et al., 2012).

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Simultaneous saccharification and fermentation (SSF) combines the enzymatic hydrolysis and fermentation of sugars (Brethauer and Wyman, 2010). SSF overcomes the inhibition of cellulase by hydrolysis products such as glucose and short cellulose oligomers, because these products can be fermented immediately (Lin and Tanaka, 2006). However, the primary disadvantage of SSF is the optimum temperature for enzymatic hydrolysis (45–60 °C) exceeds compatible temperatures for yeast and many bacterial biofuels fermentations (Brethauer and Wyman, 2010; Bhalla et al., 2013). Still, SSF is an attractive strategy for increasing cellulose conversion while maximizing enzyme use since the soluble sugar levels do not reach levels that might inhibit the fermentation microorganism.

Another fermentation approach, consolidated bioprocessing (CBP), has been investigated increasingly in recent years (Olson et al., 2012). In CBP, enzyme production by microorganisms, enzymatic saccharification, and fermentation of the resulting sugars to the desired products proceeds simultaneously without the need for additional enzymes. There are two approaches to develop microorganisms for a CBP system. One is the "native strategy", in which the biofuels production capability of a cellulolytic microorganism is improved using metabolic engineering, and the other is the "recombinant strategy", in which the capability for cellulose hydrolysis is introduced into a highly capable non-cellulolytic microorganism by genetic engineering (Lynd et al., 2002; La Grange et al., 2010; Olson et al., 2012).

Clostridium thermocellum is one of the most popular anaerobic, thermophilic, cellulolytic microorganisms for CBP (McBee, 1950; Taylor et al., 2009; Argyros et al., 2011) that fits the native strategy. It generates an extracellular multi-enzyme complex, called the cellulosome, on the surface of the cell membrane which is composed of various different types of glycosyl hydrolases, such as cellulases, hemicellulases, and carbohydrate esterases (Bayer et al., 1983; Shoham et al., 1999). The cell membrane-tethered cellulosome binds to cellulose particles, and facilitates solubilization of lignocelluloses. However, *C. thermocellum* produces multiple products such as ethanol, acetic and lactic acid, and others have addressed removing the acid production by genetic modification (Argyros et al., 2011).

In both biological conversion technologies described above, pretreatment that does not lead to production of inhibitors is preferred. A large number of pretreatment methods have been developed to date (Mosier et al., 2005; Alvira et al., 2010). Among these, dilute acid pretreatment in particular has been considered as one of the most promising pretreatment approaches in terms of economic feasibility (Esteghlalian et al., 1997). However, this pretreatment is known to produce inhibitors of the biological conversion step.

On the other hand, hot compressed water treatment (HCWT) has been known to be an environmentally friendly pretreatment, because it does not require any additives such as acids, bases, organic solvents, or other chemicals (Mok and Antal, 1992; Yu et al., 2010; Nitsos et al., 2013). However, the severe HCWT conditions required to produce the required effects can also result in production of inhibitors of enzymatic hydrolysis, and microorganism growth and fermentation (Ximenes et al., 2010; Yu et al., 2010; Nitsos et al., 2013).

In our previous reports, we investigated the use of wet disk milling (WDM) fibrillation after partial removal of hemicelluloses and lignin with HCWT, or steam treatment (ST) that employs milder conditions than those generally used in HCWT. We found that ST resulted in significant improvements in enzymatic saccharification of lignocellulosic biomass and enhanced the sugar recovery yield (Lee et al., 2010; Hideno et al., 2012). In addition, WDM in combination with HCWT or ST has advantages for reducing energy consumption of milling and enzyme loading, of which the cost constitutes a significant portion of the overall cost of the bioprocess.

Given this background, we combined ST and WDM with the goal to reduce the production of inhibitors. We applied this combined pretreatment to Hinoki cypress (softwood) and *Eucalyptus* (hardwood), and compared the effects of this pretreatment approach with those of ST or WDM alone, and those of conventional acid-catalyzed HCWT using SSF and CBP processing.

2. Methods

2.1. Materials

Wood chips of Hinoki cypress (*Chamaecyparis obtusa*) and *Eucalyptus* were kindly supplied by Maniwa City (Okayama, Japan) and purchased from Oji Paper Co., Ltd., respectively. *Eucalyptus* wood chips were mixtures of several species (mainly *Eucalyptus globulus*). These wood materials were milled to a size of less than 3 mm by cutter milling and were stored under dry conditions until required for use.

Acremonium cellulase (Meiji Seika Co., Tokyo, Japan) which was derived from *Talaromyces cellulolyticus* (formerly known as *Acremonium cellulolyticus* (Fujii et al., 2014)), Cellulosin GM5 (HBI Enzymes Inc., Hyogo, Japan), and Optimash BG (Genencor International, Palo Alto, CA, USA) were used for enzymatic saccharification. For SSF, *Saccharomyces cerevisiae* D5A (ATCC 200062) was used, and enzymes Spezyme CP (Genencor-Danisco, Beloit, WI, USA) and Accellerase BG (Genencor-Danisco, Beloit, WI, USA) were used. Thermophilic bacterium, *C. thermocellum* (ATCC 27405) was used for CBP. *C. thermocellum* was a gift from Dr. Xiongjun Shao at Dartmouth College, Hanover, NH, USA. *S. cerevisiae* was provided by the National Energy Renewable Laboratory (NREL, Golden, CO, USA). Other chemicals were purchased from commercial sources.

2.2. ST and WDM

Wood powder was soaked in water (10 wt% suspension) and left overnight at room temperature (20–22 °C). ST was conducted at 150 °C for 2 h, using an autoclave (SPT-3050P, ALP Co., Ltd., Tokyo, Japan). The pressure during processing was 0.38 MPa. After ST treatment, the sample was cooled to room temperature and exposed to WDM.

WDM was carried out using a disk mill (Supermasscolloider MKCA6-2, Masuko Sangyo Co., Ltd., Saitama, Japan), as described in our previous reports (Lee et al., 2010; Hideno et al., 2012). The apparatus was equipped with two ceramic nonporous disks. The concentration of the ST-product was adjusted to 5 wt%. The clearance of the two disks was adjusted to 20–40 μm and the rotation speed was set to 1800 rpm. Milling operation cycles, in the range of 1–10, were performed; duration was recorded for each milling cycle, and each WDM time was calculated on the basis of the weight of the dried biomass placed in the disk mill. The energy consumption of each operation was calculated from the voltage, current, and recorded duration. Thus-obtained WDM samples were vacuum-filtrated to concentrate the solid content and used in enzymatic hydrolysis, SSF, and CBP.

2.3. Sulfuric acid-catalyzed HCWT

Sulfuric acid-catalyzed HCWT was conducted according to our previous report (Yee et al., 2012). In brief, the sample was soaked overnight in 0.5% H_2SO_4 at a ratio of 9 mL of acid per gram of dry sample and centrifuged at 8000 rpm, for 30 min, at 4 °C in a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instruments, Wilmington, DE, USA). The sample (2.5 g dry weight per tube)