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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

Akio Kumagai [a,1], Shunsuke Kawamura [a,1], Seung-Hwan Lee [a,b], Takashi Endo [a,*], Miguel Rodriguez Jr. [c], Jonathan R. Mielenz [d]

[a] Biomass Refinery Research Center, National Institute of Advanced Industrial Science and Technology (AIST), 3-11-32 Kagamiyama, Higashi-Hiroshima, Hiroshima 737-0046, Japan
[b] Department of Forest Biomaterials Engineering, College of Forest and Environmental Sciences, Kangwon National University, 192-1 Hyoja, Chuncheon 200-701, South Korea
[c] Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6226, USA
[d] BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6226, USA

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).

* Corresponding author. Tel./fax: +81 82 420 8278.
E-mail addresses: t-endo@aist.go.jp (T. Endo), biofuels4me@gmail.com (J.R. Mielenz).
[1] First and second authors were equally contributed.

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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)

was loaded into 10 cm × 1 cm Hastelloy steel tubular pretreatment reactors (Industrial Alloys Plus, Inc., Utica, KY, USA). The reactors were pre-heated in boiling water for 2 min, and then transferred to a fluidized sand bath (Omega fluidized bath FSB1; Techne Co., Princeton, NJ, USA) at 180 °C, for 7.5 min. The reactors were cooled by quenching in an ice bath. The biomass was removed from the reactors and washed with 100 mL Milli-Q water per gram of dry sample. The samples were stored at −20 °C until required for use.

2.4. Enzymatic hydrolysis

Enzymatic saccharification was carried out using an enzyme cocktail containing 10 FPU Acremonium cellulase per gram of biomass, supplemented with 0.167 mL Cellulosin GM5 for Hinoki cypress or 0.2 mL Optimash BG for *Eucalyptus*. Cellulosin GM5 and Optimash BG were added to enhance the activities of mannanase and xylanase, respectively. These enzymes were added to 1 g of the pretreated samples, along with 30 mL of 50 mM acetate buffer (pH 5.0). The reaction was allowed to proceed at 45 °C for 72 h, with agitation using a rotary shaker. The sugar production was quantified using a high performance liquid chromatography (HPLC) system, as described below.

2.5. SSF

SSF of the pretreated control and sample was performed using *S. cerevisiae* D5A (ATCC 200062), 15 FPU per gram cellulose of Spezyme CP, and a 25% volume ratio to Spezyme CP of Accellerase BG as per manufacturer recommendations and as described in our previous method (Yee et al., 2012). The fermentation bottles were loaded with 1 g of dry wood materials and 20 mL of YPD media lacking glucose, and autoclaved at 121 °C for 20 min before being cooled. Yeast, precultured in YPD medium (Difco, Detroit, MI, USA), sterile water, and enzymes were added, and the bottles were sealed without flushing with nitrogen. SSF was conducted at 35 °C with shaking at 150 rpm, using a rotary shaker. Samples were not removed from the bottles during fermentation. Instead, weight loss was used to monitor the progress of the fermentation, according to previous reports (Mielenz et al., 2009; Yee et al., 2012). Weight loss was measured after venting to release CO_2, using a sterile needle, and was monitored during fermentation without removing samples from the bottle. At the end of the fermentation, samples were taken to analyze metabolites and glucose residue. Experiments were conducted in biological duplicates.

2.6. CBP

CBP fermentation was performed using *C. thermocellum* (ATCC 27405) according to a previous report (Yee et al., 2012). MTC media was used; its composition is described in detail elsewhere (Zhang and Lynd, 2003; Yee et al., 2012). Fermentation was conducted in 125-mL anaerobic serum bottles with a 70 mL working volume containing 0.8 g of dry wood materials. The fermentation bottles were loaded with wood materials and MTC media without some components and they were autoclaved at 121 °C for 30 min and then cooled. After autoclaving, the further sterile components of MTC medium were added. The inoculum of *C. thermocellum* was grown in 125-mL fermentation bottles sealed under nitrogen with 50 mL of the same media and a carbon source of 5.0 g/L Avicel PH-105 (FMC BioPolymer, Philadelphia, PA, USA), at 58 °C, using a rotary shaker at 125 rpm. CBP fermentation was conducted at 58 °C with orbital shaking at 125 rpm, in the anaerobic fermentation bottles. Monitoring of weight loss profiles and analysis of metabolites and glucose residue were performed as for SSF.

2.7. HPLC analysis

Monomeric sugars, produced by enzymatic saccharification, were analyzed using an HPLC system (Jasco Co., Ltd., Tokyo, Japan) equipped with a refractive index detector (RI-2031 Plus, Jasco Co., Ltd., Tokyo, Japan) and with an Aminex HPX-87P column (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 80 °C with a flow rate of 1.0 mL/min of Milli-Q water.

Metabolites generated by SSF and CBP were analyzed using HPLC (LaChrom Elite® system, Hitachi High Technologies America, Inc., Pleasanton, CA, USA) equipped with a refractive index detector (model L-2490). The products and carbohydrates were separated using an Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, USA), at a flow rate of 0.5 mL/min of 5.0 mM sulfuric acid and a column temperature of 60 °C.

2.8. Scanning electron microscope (SEM)

The morphological characteristics of the pretreated samples were observed by SEM using an S-4800 SEM (Hitachi Co., Tokyo, Japan). The samples for SEM were thoroughly washed with *tert*-butyl alcohol and freeze-dried. Before SEM observation, the sample was coated with a thin layer of osmium.

3. Results and discussion

3.1. Pretreatment

Individual pretreatment of ST and WDM, and combination treatment of ST and WDM (ST–WDM) were performed to compare the effects of pretreatments on SSF and CBP. Hydrothermal pretreatment of lignocellulosic biomass is known to allow selective removal of hemicellulose from lignocellulose (Mok and Antal, 1992; Yu et al., 2010; Nitsos et al., 2013). Since the partial removal of hemicellulose facilitates mechanical fibrillation and the fibrillation of wood improves enzymatic saccharification (Inoue et al., 2008; Lee et al., 2010; Hideno et al., 2012), WDM was carried out after ST in this study. The effect of the combined pretreatment of ST and WDM on enzymatic saccharification was investigated and compared with the effects of pretreatment using ST or WDM only. Even though the conditions applied in ST in this study was milder in terms of temperature (150 °C) and pressure than those generally used in HCWT, ST at 150 °C is still considered to allow the selective removal of hemicellulose from the lignocellulosic biomass and to limit inhibitor production (Lee et al., 2010; Hideno et al., 2012). This partial removal of hemicellulose is thought to facilitate mechanical fibrillation, facilitating enzymatic saccharification.

Table 1 summarizes the WDM time and the energy consumption of WDM, taking the energy consumption of ST into consideration. The WDM time and WDM energy consumption were calculated on the basis of the weight of the dried biomass inserted into the disk mill for each milling cycle, and the cumulative values are shown in this table. During WDM, the fibrillated product was homogeneously dispersed in water and the biomass formed a viscose paste with increasing WDM cycle number. The graphic representation of the relation between WDM cycle number and WDM time is shown in Supplementary Fig. 1. Each WDM cycle increased the total milling time so that at the same level of 5 cycles of WDM, the WDM time for samples exposed to ST was about 12.4 and 2.3 times longer for Hinoki cypress and *Eucalyptus*, respectively, compared to samples only treated by WDM. The difference in the required WDM time for the Hinoki cypress and *Eucalyptus* may be attributed to the distinctions between hardwood and softwood, such as fiber length and chemical composition (Hägglund et al., 1956; Bobleter, 1994; Assor et al., 2009). The use of ST followed

Table 1
WDM cycle number, WDM time (min/kg-biomass weight), and energy consumption (MJ/kg-biomass weight) in WDM. Samples treated using a single pretreatment are described with "-only", and samples treated with WDM in combination with ST are described with "ST–WDM", followed by a WDM cycle number.

	Sample	WDM cycle number	WDM time (min/kg-biomass weight)	Energy consumption (MJ/kg-biomass weight)
Hinoki cypress	ST-only	–	–	0.80[a]
	WDM-only	10	1.39×10^3	3.07
	ST–WDM3	3	1.17×10^3	3.48[b]
	ST–WDM4	4	1.86×10^3	4.49[b]
	ST–WDM5	5	2.61×10^3	6.16[b]
Eucalyptus	ST-only	–	–	0.80[a]
	WDM-only	7	0.62×10^3	1.47
	ST–WDM3	3	0.34×10^3	1.49[b]
	ST–WDM4	4	0.60×10^3	1.83[b]
	ST–WDM5	5	0.93×10^3	2.45[b]

[a] The energy consumption of ST-only was calculated using the weight of the dried biomass, assuming that the ST was performed with a maximum volume (10-wt% wood powder suspension) of the autoclave used in this study.
[b] The energy consumption of ST–WDM was the sum of the energy consumption of ST-only and the cumulative value of the energy consumption after each cycle of WDM treatment.

by WDM treatment yielded a higher initial viscosity for the suspended solids than those not steam treated. This is possibly due to partial removal of hemicellulose by ST which typically yields more highly fibrillated samples. Well-fibrillated sample tends to indicate high viscosity and take long WDM time.

The morphologies of the pretreated products were observed by SEM (Supplementary Fig. 2). In ST-only pretreated Hinoki cypress and *Eucalyptus*, micron-scale fibers were observed. The fiber surface appeared to be covered with an unknown compound, which may be lignin generated from breakdown of the cell wall or lamella between the fibers. Furthermore, some nanoscopic fibers were partially separated from the surface, indicating that most of these remained bound to each other via hemicellulose and lignin interaction to form the micron-scale fibers. However, structural disruption of the fibers was also clearly seen in ST-only products, which is likely because of the partial removal of hemicellulose in particular. This disrupted structure should facilitate mechanical fibrillation. On the other hand, fine, nano-scale fibrous structures, up to hundreds of nanometers, were observed in WDM-treated samples, both with or without ST, which was not seen in ST-only products. When comparing samples exposed to WDM-only or ST–WDM, the same morphological characteristics were obtained with a shorter WDM duration in samples exposed to ST–WDM. This fine structure should facilitate enzymatic saccharification, as well as SSF and CBP.

3.2. Enzymatic saccharification

Fig. 1 shows the time course for enzymatic saccharification of the pretreated products, calculated on the basis of the initial glucan content of the raw biomass. Although the raw wood powders (particle size < 3 mm) of Hinoki cypress and *Eucalyptus* comprised fine particles, their glucose production yields were found to be only 2.8 and 6.1 wt% after a 72-h hydrolysis period, respectively. In the case of Hinoki cypress, the glucose production yield of the ST-only product was almost the same as that obtained with the raw material. The glucose production yield of *Eucalyptus* exposed to ST-only was found to be less than 20 wt%. WDM-only resulted in glucose production yields less than 50 wt% in both samples. However, the enzymatic digestibility was markedly improved when employing ST–WDM. By increasing the WDM time, the glucose production yields after a 72-h enzymatic saccharification of Hinoki cypress and *Eucalyptus* reached 96.8 and 98.4 wt%, respectively. These values are almost twofold higher than those of obtained from WDM-only samples. The dependency of glucose production yield on WDM duration is summarized in Supplementary Fig. 3, in which the differences in glucose yield between samples exposed to WDM-only or ST–WDM, for the same WDM duration, can be seen. This result indicates that ST before WDM may enable to make fibrillation and enzymatic saccharification more efficient and improve the production cost of biofuels.

3.3. SSF

Fig. 2 shows the effect of SSF duration on weight loss monitored during fermentation. In the case of Hinoki cypress, weight loss of the control without biomass, raw material without any pretreatments, and samples treated with ST-only were lower than 3 wt%, and they showed virtually the same trend during SSF. In the case of *Eucalyptus*, weight losses of the above-described three samples were less than 6%, but in the order of ST-only, raw material, and control samples (highest to lowest). More particularly, there was

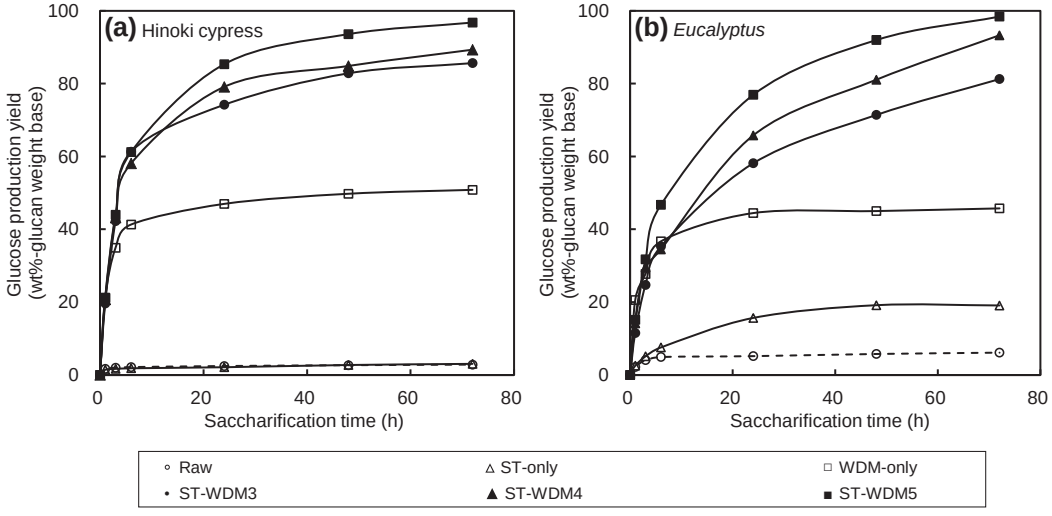

Fig. 1. Glucose production yield time-course measured by enzymatic saccharification of pretreated biomass. Enzymatically-hydrolyzed biomass included (a) Hinoki cypress and (b) *Eucalyptus* pretreated with ST-only (△), WDM-only (□), or a combination of ST and WDM with various milling cycles, described with "ST–WDM", followed by a WDM cycle number (ST–WDM3 (●), ST–WDM4 (▲), and ST–WDM5 (■)). Raw material (○) without pretreatment was used for control sample.

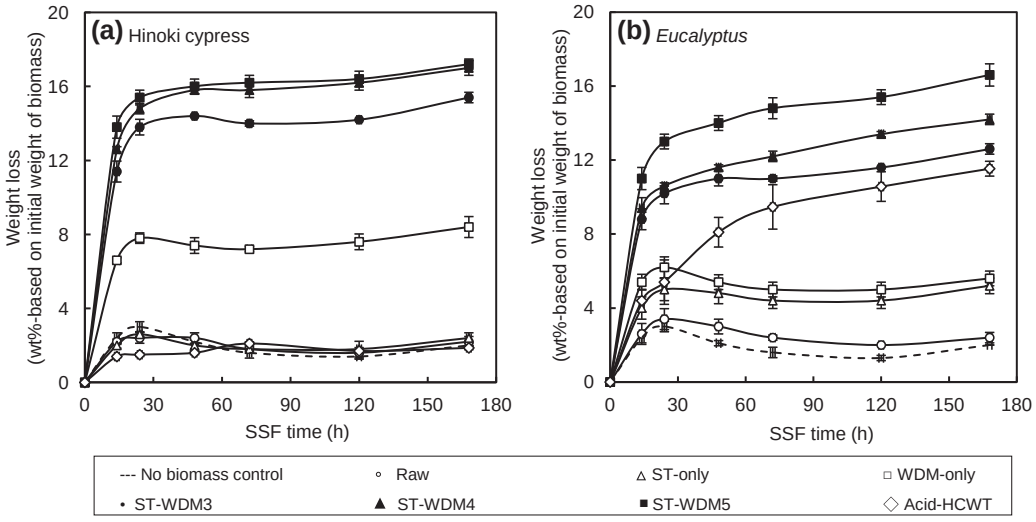

Fig. 2. Fermentation weight loss over time for yeast growing on pretreated biomass in SSF. Biomass used for SSF were (a) Hinoki cypress and (b) *Eucalyptus*, pretreated with ST-only (△), or WDM-only (□), a combination of ST and WDM with various milling cycles, described with "ST–WDM", followed by a WDM cycle number (ST–WDM3 (●), ST–WDM4 (▲), and ST–WDM5 (■)), or conventional pretreatment with sulfuric acid-catalyzed HCWT (◇). No biomass sample (dotted line) and raw material (○) without pretreatment were used for control sample.

a difference in weight loss between Hinoki cypress and *Eucalyptus* after ST-only with *Eucalyptus* yielding more weight loss. This indicates that ST was not effective for this softwood. Indeed, it is well known that hydrothermal treatment is less effective for softwoods (Ando et al., 2000; Assor et al., 2009) because of the different structure of the lignin and hemicellulose (Bobleter, 1994). For instance, hardwood lignin has a less cross-linked structure than softwood lignin does, and hardwood hemicellulose contains more acetyl groups, which can act as acid catalysts during ST (Bobleter, 1994; Assor et al., 2009). Furthermore, acid-catalyzed HCWT of *Eucalyptus* resulted in greater weight loss than in Hinoki cypress, most likely due to the same reasons as described above.

In both Hinoki cypress and *Eucalyptus*, weight loss with WDM-only treatment was higher than observed with ST-only. Furthermore, the weight loss seen with ST–WDM was markedly increased. Even compared with acid-catalyzed HCWT, ST–WDM resulted in a higher weight loss value with Hinoki cypress yielding a higher apparent weight loss rate that the *Eucalyptus*, but both substrates eventually yielding similar total weight loss. This indicates that WDM fibrillation of the disrupted cell wall structure after ST was more effective in promoting accessibility of the cellulose surface to the cellulolytic enzymes, by separating the cellulose microfibrils from one another, and thereby increasing the specific surface area (Hideno et al., 2009; Endo, 2010). Additionally, Hinoki cypress was more accessible to the enzymes than *Eucalyptus* because Hinoki cypress achieved equilibrium more quickly than *Eucalyptus* in both enzymatic saccharification and SSF (Figs. 1 and 2).

Fig. 3 shows the effect of various pretreatments on the yields of the fermentation products. The main fermentation product was ethanol by yeast. However, very little ethanol was produced from raw material in the absence of pretreatment, for both Hinoki cypress and *Eucalyptus*. Acetate and glycerol (less than 5 mg/g-cellulose) were produced even from raw materials not treated by any pretreatment including WDM and ST.

With ST-only, the yields of total fermentation products, and particularly those of ethanol were higher for *Eucalyptus* than for Hinoki cypress. On the other hand, WDM-only resulted in a higher yield of ethanol in Hinoki cypress, suggesting Hinoki cypress is more susceptible to mechanical disruption than *Eucalyptus*. As seen for weight loss, yields of all fermentation products were markedly increased when using ST–WDM. In particular, ethanol production yields for ST–WDM5 were 1.9- and 3.4-fold higher in Hinoki cypress and *Eucalyptus*, respectively, in comparison to those obtained with WDM-only. In addition, the yields of ethanol, based on the initial weight of biomass, for ST–WDM5 were 187.9 and 174.9 mg/g-initial biomass weight in Hinoki cypress and *Eucalyptus*, respectively (Supplementary Fig. 4). These results correspond well with the results for weight loss during SSF and glucose

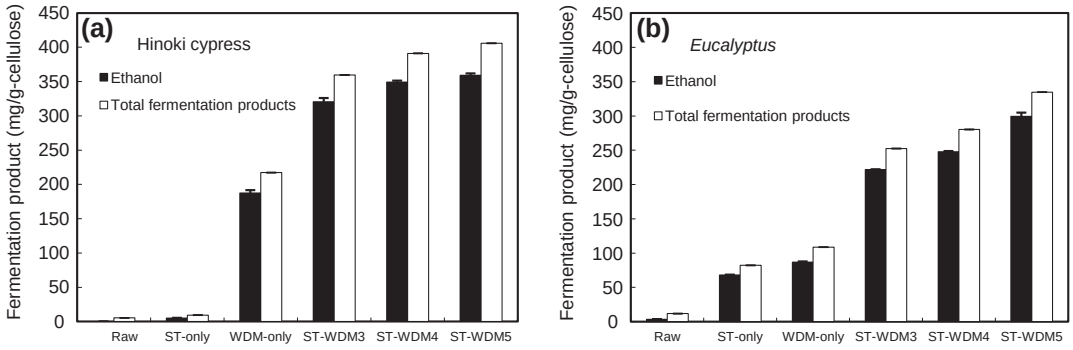

Fig. 3. Fermentation product yields produced by SSF of pretreated biomass. Biomass used for SSF were (a) Hinoki cypress and (b) *Eucalyptus* pretreated with ST-only, WDM-only, or a combination of ST and WDM with various milling cycles, described with "ST–WDM", followed by a WDM cycle number. The black bar represents the yield of ethanol, and the white bar represents the yield of total fermentation products.

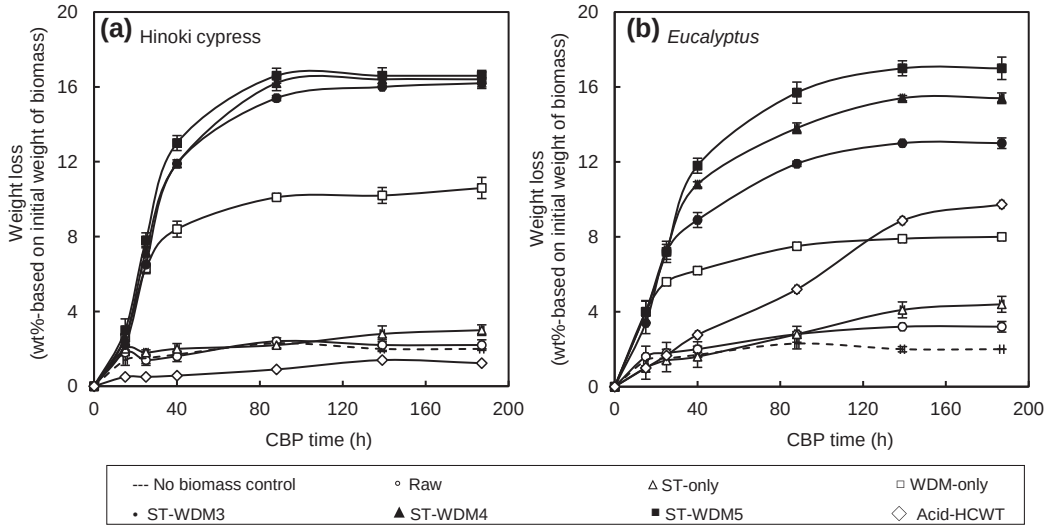

Fig. 4. Fermentation weight loss over time of *C. thermocellum* growing on pretreated biomass during CBP. Biomass used for CBP were (a) Hinoki cypress and (b) *Eucalyptus*, pretreated with ST-only (△), WDM-only (□), a combination of ST and WDM with various milling cycles, described with "ST–WDM", followed by a WDM cycle number (ST–WDM3 (●), ST–WDM4 (▲), and ST–WDM5 (■)), or conventional pretreatment with sulfuric acid-catalyzed HCWT (◇). No biomass sample (dotted line) and raw material (○) without pretreatment were used for control sample.

production yield by enzymatic saccharification, suggesting that the glucose produced by enzymatic saccharification was effectively fermented by yeast.

3.4. CBP

CBP with *C. thermocellum* was conducted using the same pretreated samples used for SSF. The fermentation was also monitored by measuring weight loss over time, and these results are summarized in Fig. 4. The weight loss profile during CBP for the different pretreatments revealed a very similar tendency as those obtained with SSF, showing that ST–WDM was the most effective pretreatment.

Fig. 5 shows the effect of various pretreatments on the yields of fermentation products obtained by CBP. *C. thermocellum* is capable of utilizing only the hexoses, but not the pentose sugars, to produce ethanol, acetate, and lactate as main fermentation products (Taylor et al., 2009). Acetate was the main product with every pretreatment, and the amount produced was more than twice that of the ethanol produced, with an undetermined portion coming from deacetylation of remaining hemicellulose. The significant production of acetate had also previously been observed with CBP, using *C. thermocellum*, of microcrystalline cellulose (Avicel) and paper pulp sludge (Chinn et al., 2007a,b). As expected, lactate production during CBP was also higher than during SSF. Similar to SSF, ST–WDM resulted in a higher yield of all fermentation products than did the other pretreatments. However, the ethanol production yield during CBP was lower than that obtained during SSF. Compared to the highest ethanol yield (359.3 and 299.5 mg/g-cellulose) obtained from Hinoki cypress and *Eucalyptus*, respectively, during SSF, yields of only 79.4 and 73.1 mg/g-cellulose were obtained during CBP, when using ST–WDM5. However, total product (ethanol, acetate plus lactate) yields of 319.9 and 262.0 mg/g-cellulose from Hinoki cypress and *Eucalyptus*, respectively, show the CBP approach can effectively convert both substrates to fermentation products, especially Hinoki cypress.

The energy consumption of ST–WDM3 for Hinoki cypress and *Eucalyptus* were comparable to those of each WDM-only process (Table 1), but much higher bioconversion yields were obtained from enzymatic saccharification, SSF, and CBP after ST–WDM3, as described above. The energy consumption (0.80 MJ/kg) for ST under the mild conditions used in this study was much lower than that required for general HCWT at 180 °C to achieve a sufficient bioconversion yield (6.6 MJ/kg) (Hideno et al., 2009). In addition, the energy consumption of ST–WDM for Hinoki cypress and *Eucalyptus* (3.48–6.16 and 1.49–2.45 MJ/kg) were significantly lower

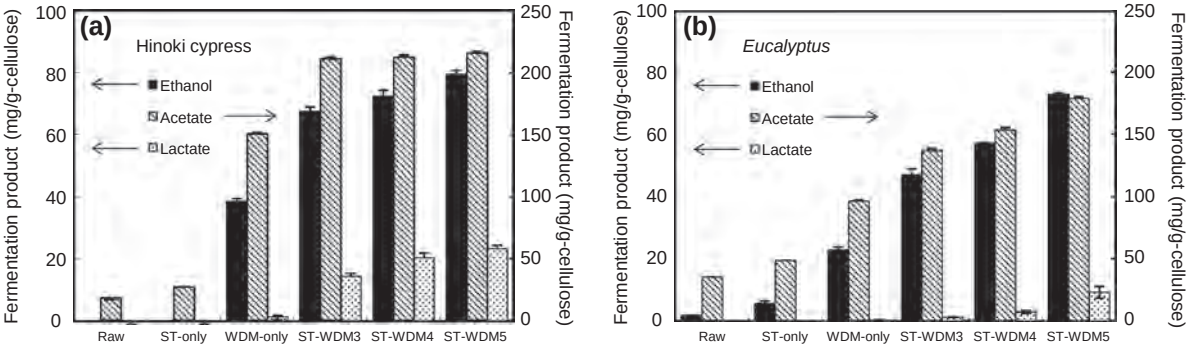

Fig. 5. Fermentation product yields for CBP generated from pretreated biomass. Biomass used for CBP were (a) Hinoki cypress and (b) *Eucalyptus* pretreated with ST-only, WDM-only, or a combination of ST and WDM with various milling cycles, described with "ST–WDM", followed by a WDM cycle number. The black bar represents the yield of ethanol, the slashed bar represents yield of acetate, and the dotted bar represents the yield of lactate.

than for other mechanical pretreatment, such as ball milling (108 MJ/kg) (Hideno et al., 2009). It has also been reported that pretreatment by WDM in combination with ST is advantageous for achieving a higher bioconversion yield with lower enzyme loading (Hideno et al., 2012). This advantage will also benefit cost reduction of the SSF process, due to the low enzyme loading required to achieve sufficient ethanol production by ST–WDM.

4. Conclusions

ST–WDM was the most effective pretreatment, not only for enzymatic saccharification, but also for fermentation by SSF and CBP, compared to ST or WDM alone, or the generally used pretreatment involving sulfuric acid-catalyzed HCWT. ST, using milder conditions, is known to prevent the generation of fermentation inhibitors and to facilitate the fibrillation during WDM, so reducing energy consumption and the need for additional cellulolytic enzymes. This effective pretreatment can markedly improve the sensitivity of biomass to enzymatic hydrolysis and subsequent fermentation. Therefore, ST–WDM may hold great potential for improving biofuel yields via both SSF and CBP.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.03.110.

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