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Two-Step Hot-Compressed Water Treatment of Douglas Fir for Efficient Total Sugar Recovery by Enzymatic Hydrolysis

Hiroyuki Inoue,* Shinji Fujimoto, and Tsuyoshi Sakaki

The non-catalytic hydrothermal pretreatment of softwood is generally less effective for subsequent enzymatic hydrolysis. In this study, the efficacy of hot-compressed water (HCW) treatment of Douglas fir was investigated between 180 °C and 260 °C, allowing solubilization of the cellulose components. The enzymatic digestibility of cellulosic residues increased significantly under HCW conditions > 250 °C, and the enhanced glucan digestibility was closely related to the decomposition of the cellulose component. Combination of the first-stage HCW treatment (220 °C, 5 min) to recover hemicellulosic sugars with the second-stage HCW treatment (260 °C, 5 min) to improve cellulose digestibility gave a total sugar recovery of 56.2% based on the dried raw materials. This yield was 1.4 times higher than that from the one-step HCW-treated sample (260 °C, 5 min). Additionally, an enzymatic hydrolysate from the two-step HCW-treated sample exceeded 90% of the ethanol fermentation yield based on the total sugars present in the hydrolysates. These results suggest the potential of the two-step HCW treatment of softwood as a pretreatment technology for efficient total sugar recovery and ethanol production.

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INTRODUCTION

Lignocellulosic biomass, which includes agricultural residues, wood, and energy crops, is a sustainable feedstock for expanding ethanol production without affecting the food and feed markets (Sims *et al*. 2010). Cellulose and hemicellulose are major polysaccharide components within plant cell walls, providing fermentable sugars for the production of lignocellulosic ethanol by enzymatic hydrolysis using cellulosic enzymes. However, the digestion of these components is hindered by the complex structural and chemical mechanisms in plants (Himmel *et al*. 2007). Therefore, pretreatment to induce alteration or removal of the structural and compositional impediments in lignocellulosic biomass is necessary to render the polysaccharides susceptible to cellulosic enzymes.

Various methods utilizing milling, water, acids, bases, oxidizing agents, organic solvents, ionic liquids, and their combinations have been developed as suitable pretreatments based on the properties of raw materials (Mosier *et al*. 2005; Alvira *et al*. 2010). Among these methods, non-catalytic hydrothermal treatments employing water (*e.g*., steam and hot-compressed water (HCW) methods) are attractive in terms of lowering the capital costs and minimizing waste generation.

Water develops acidic characteristics at high temperatures, and the ion product of water, K_w, increases with temperature up to a maximum of 6.34×10^{-12} at 250 °C (Schacht *et al*. 2008). Under high temperature (approximately 200 °C) and pressure, water and steam partially dissolve hemicellulose and lignin to generate a reactive cellulosic residue for enzymatic hydrolysis. Furthermore, a two-step hydrothermal pretreatment technology has been proposed to maximize the recovery of fermentable sugars and to reduce the formation of degradation products of the biomass components. The first stage of treatment separates hemicellulosic sugars into a soluble fraction. The resulting cellulosic residue is then subjected to a second-stage of treatment under more severe conditions to enhance the enzymatic hydrolysis yield. For example, a two-step hydrothermal treatment of eucalyptus and giant cane (*Arundo donax*) gave higher sugar yields compared with one-step methods (Yu *et al*. 2010b; De Bari *et al*. 2013).

Non-catalytic hydrothermal treatment is highly effective for the pretreatment of hardwoods and herbaceous feedstocks (Yu *et al*. 2010a; Kim *et al*. 2013; Sun *et al*. 2014; Silva-Fernandes *et al*. 2015). For softwoods, these methods are generally less effective, so treatment using acid catalysts such as H_2SO_4 and SO_2 (Galbe and Zacchi 2002; Alvira *et al*. 2010) is employed to enhance the enzymatic hydrolysis yield. HCW treatment for softwood is performed at relatively low temperatures (< 180 °C) to enhance the effect of post-treatment by milling (Lee *et al*. 2010). In addition, HCW temperature has a larger influence than residence time on glucan hydrolysis yields from cellulosic residues in hardwoods (Yu *et al*. 2010b; Kim *et al*. 2014). Indeed, high HCW treatments at temperatures that allow the decomposition of cellulose (> 230 °C) appear to maximize enzymatic digestibility in cellulosic residues from hardwoods (Yu *et al*. 2010b; Kim *et al*. 2013). In contrast, the enzymatic digestibility of softwood residues obtained by more severe HCW conditions has not been clarified.

These observations prompted this study on the enzymatic digestibility of softwood residues obtained by high temperature HCW treatments. The efficacy of HCW treatment for the sugar recovery was examined in Douglas fir (DF), which is a major resource for commercial forest products such as lumber and paper and is a promising softwood species for biofuel production. The enzymatic digestibility of DF cellulosic residue increased significantly under HCW conditions above 250 °C. The total sugar recovery from DF was further improved by using the two-step HCW treatment method.

EXPERIMENTAL

Materials
Wood samples

DF and eucalyptus wood chips were kindly supplied by Kure Mill, Oji Paper Co. (Hiroshima, Japan). The chips were milled to pass a 2 mm screen and stored in dry conditions prior to use. The moisture contents of milled DF and eucalyptus were 2.25% and 2.8%, respectively. The contents of structural carbohydrates and acid insoluble lignin in the dried raw materials (DM) were determined based on the standard NREL laboratory analytical procedure (Sluiter *et al*. 2008), and the compositions (% dry weight) are shown in Table 1.

Table 1. Composition of Raw Material (% dry weight)

DM	Glucan	Xylan	Galactan	Alabinan	Mannan	Acid insoluble lignin
Eucalyptus	42.6	12.0	3.07	ND	ND	22.9
Douglas fir	46.0	4.95	3.63	1.70	13.7	28.2

Cellulosic enzymes

Acremonium cellulase (Meiji Seika Pharma Co., Tokyo, Japan) derived from *Talaromyces cellulolyticus* (formerly known as *Acremonium cellulolyticus* (Fujii *et al*. 2014) was used as a source of cellulase. Optimash BG (Genencor International, Palo Alto, CA, USA) and Cellulosin GM5 from *Aspergillus niger* (HBI Enzymes Inc., Hyogo, Japan) were used to supplement acremonium cellulase and to enhance xylan and mannan digestibility in eucalyptus and DF, respectively (Inoue *et al*. 2008, 2015).

**Methods**

One-step HCW treatment

A 14-mL stainless steel reactor (SUS316, 14.83 mm i.d. × 80 mm length) equipped with a thermocouple and a pressure gauge was used for the one-step HCW treatment. The sample (1 g) was further pulverized to a particle size of < 0.2 mm and charged to the reactor with water (9 mL). The reactor was purged with nitrogen gas at an initial pressure of 0.5 MPa, and the reactor was heated in a molten salt (KNO_3/$NaNO_2$/$NaNO_3$, 5:4:1) bath and the temperature maintained within 2 °C of the desired temperature (180 to 260 °C) with vertical shaking for 5 min. The reaction mixture reached the desired temperature within approximately 1 min. At the end of the reaction period, the reactor was cooled to 30 °C by immersion in a cool water bath.

The water-soluble (WS) fraction was recovered by filtration using a glass filter (5 to 10 µm) and stored as the "one-step HCW-treated WS sample (OSS)" (Fig. 1a). The water insoluble (WI) residual fraction was washed with distilled water, lyophilized, and stored as the "one-step HCW-treated WI sample (OSI)" (Fig. 1a). The one-step HCW treatment was repeated 5 times, and all samples were homogenized into a single lot. The reaction conditions for the one-step HCW treatment in this study are summarized in Table 2 along with the solid yields of the OSI samples.

The structural carbohydrate and acid insoluble lignin contents in the OSI were determined based on the standard NREL laboratory analytical procedure (Sluiter *et al*. 2008). The crystallinities of the OSI samples were measured using a Rigaku RINT-TTR3 X-ray diffractometer (Japan) with Cu Kα radiation at 50 kV and 300 mA, as has been described previously (Inoue *et al*. 2008). The diffraction spectra were taken using the θ–2θ method, and the crystallinity indexes (CrI) were calculated according to Eq. 1 (Segal *et al*. 1959),

$$\text{CrI (\%)} = [(I_{002} - I_{am})/I_{002}] \times 100 \qquad (1)$$

where I_{002} is the intensity of the crystalline peak at approximately 2θ = 22.5° and I_{am} is the intensity at 2θ = 18.7°.
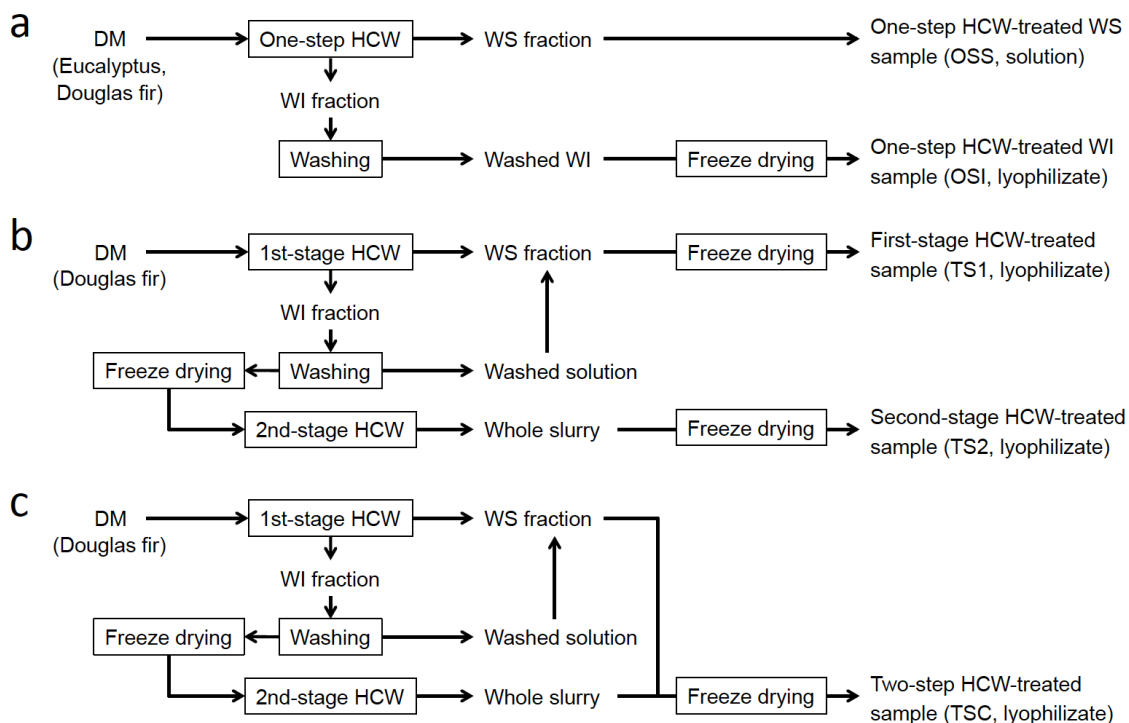
Fig. 1. Schematic of sample preparation by one-step HCW treatment (a), fist-stage and second-stage HCW treatments (b), and two-step HCW treatment (c)

Table 2. HCW Treatment Conditions and Solid Yields of Pretreated Samples

HCW Treatment	Temperature (°C)	Solid Yield (%)	
		Eucalyptus	Douglas fir
One-step	180	92.3	88.1
One-step	200	77.8	78.8
One-step	210	71.2	73.8
One-step	220	71.5	70.5
One-step	230	67.7	67.6
One-step	240	69.7	66.1
One-step	250	65.7	62.3
One-step	260	62.0	57.0
First-stage	220		26.2
Second-stage	250		66.6
Second-stage	260		65.1
Second-stage	270		57.8

Solid yields were calculated based on the weight of lyophilizate, OSI from one-step HCW, TS1 from first-stage HCW, and TS2 from second-stage HCW (Fig. 1).

First-stage and second-stage HCW treatment

A 20-mL stainless steel reactor (SUS316, 15.75 mm i.d. × 100 mm length) equipped with a thermocouple and a pressure gauge was used for the first-stage HCW

treatment. The DF sample (< 2 mm, 1.6 g) and water (14.4 mL) were charged to the reactor with nitrogen gas at an initial pressure of 0.5 MPa. The reactor was heated in an oil bath and maintained at 220 °C with horizontal shaking for 5 min. The temperature of the reaction mixture reached the desired temperature within approximately 2 min. At the end of the reaction period, the reactor was cooled to 30 °C by immersion in a cool water bath. The first-stage HCW treatment was repeated 5 times, and the samples were combined, as above. The WS fraction containing the washed solution from the WI fraction was lyophilized and stored as the "first-stage HCW-treated sample (TS1)" (Fig. 1b). The washed WI fraction was lyophilized, and the dried sample (1 g) applied in the second-stage HCW treatment at the desired temperature (240 to 270 °C) for 5 min in a 14-mL stainless steel reactor, according to the previously described procedure for the one-step HCW treatment. The slurry was recovered from the reactor, lyophilized, and stored as the "second-stage HCW-treated sample (TS2)" (Fig. 1b). The reaction conditions for the first-stage and the second-stage HCW treatments are summarized in Table 2 along with the solid yields for this stage.

Enzymatic hydrolysis

In standard assays for enzymatic hydrolysis of the HCW-treated samples, the lyophilized sample (50 μg, OSI, TS1, or TS2) or the liquid sample (0.8 mL, OSS) was used as the substrate, and hydrolysis was carried out at 45 °C for 72 h in the mixture (1 mL) containing a final concentration of 50 mM sodium acetate (pH 5.0). The lyophilized sample was hydrolyzed at a cellulase loading of either 10 or 40 FPU/g-substrate, while the liquid sample was hydrolyzed at a cellulase loading of 2.5 FPU/mL-substrate. Optimash BG, corresponding to 1 U of β-xylosidase activity, was added to the reaction mixture containing the eucalyptus substrate. Cellulosin GM5, corresponding to 0.35 U of β-mannosidase activity, was added to the reaction mixture containing the DF substrate.

The monomeric sugars in the hydrolysate were analyzed using the high-performance liquid chromatographs (HPLC) system described below. The sugar recovery yields, R_s, from DM were calculated according to Eq. 2:

$$R_s(\%) = 100 \times \frac{\text{Weight of monomeric sugar after enzymatic hydrolysis of pretreated sample} \times \text{solid yield (g)}}{\text{Weight of potential monomeric sugar in DM (g)}} \quad (2)$$

The enzymatic hydrolysis yields, Y_{eh}, of pretreated sample were calculated according to Eq. 3:

$$Y_{eh}(\%) = 100 \times \frac{\text{Weight of monomeric sugar after enzymatic hydrolysis of pretreated sample (g)}}{\text{Weight of potential monomeric sugar in pretreated sample (g)}} \quad (3)$$

Hydrolysis and fermentation of the two-step HCW-treated sample

DF (< 2 mm) was pretreated at 220 °C (first-stage) and 260 °C (second-stage) using a 20-mL and a 14-mL stainless steel reactor, respectively. The WS fraction obtained from the first-stage HCW treatment and the whole slurry obtained from the second-stage HCW treatment were combined, lyophilized, and stored as the "two-step HCW-treated sample (TSC)" (Fig. 1c). The two-step HCW treatments were repeated 5 times.

The enzymatic hydrolysis of TSC was carried out at 45 °C for 72 h in an aqueous solution (8 mL) containing the substrate (1.2 g) and an enzyme cocktail (see below). The initial pH of the reaction mixture was adjusted to pH 5.0 using 2 M sodium hydroxide solution. The TSC was hydrolyzed at a cellulase loading of 40 FPU/g-substrate

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supplemented with GM5, corresponding to a β-mannosidase activity of 7 U/g-substrate. Following enzymatic hydrolysis, the residue in the hydrolysate was removed by centrifugation. The pH of the supernatant was adjusted to pH 5.5 and filtered through a 0.22-μm polyethersulfone membrane (Thermo Scientific, Rockford, IL, USA) under sterile conditions.

The xylose-fermenting recombinant *Saccharomyces cerevisiae* MA-R4 was used in the fermentation experiments (Matsushika *et al*. 2009). This strain is a derivative from the industrial flocculent *S. cerevisiae* IR-2, and it expresses a single set of chromosomally integrated xylose-assimilating genes, namely *XYL1* (xylose reductase) and *XYL2* (xylitol dehydrogenase) from *Scheffersomyces stipitis*, and *XKS1* (xylulokinase) from *S. cerevisiae*. The MA-R4 strain was aerobically grown in a YPD (yeast extract, peptone, dextrose) medium for 24 h at 30 °C, and the washed cells (0.5 mL) were inoculated in the hydrolysate (4.5 mL) at a final concentration of 0.66 g dry cell/L, 1 g/L yeast extract, and 2 g/L peptone. Ethanol fermentation was performed in a closed bottle (13 mL) at 30 °C for 96 h with agitation at 100 rpm. Samples (0.1 mL) were removed from the broth at appropriate intervals and analyzed using the HPLC system described below.

HPLC analyses
Monomeric sugars and ethanol were analyzed using an HPLC system equipped with a refractive index detector (RI-2031Plus, JASCO, Tokyo, Japan) and an Aminex HPX-87P column (7.8 mm i.d. × 30 cm length, Bio-Rad, Hercules, CA, USA) with a Carbo-P micro-guard cartridge. Doubly deionized water was used as the mobile phase with a flow rate of 1.0 mL/min and a column temperature of 80 °C.

RESULTS AND DISCUSSION

Sugar Recovery from DF by One-Step HCW Treatment
Key factors affecting the effectiveness of the HCW process are temperature, residence time, and the combined effect of both temperature and time (Overend and Chornet 1987). In particular, the pretreatment temperature influences the pretreatment efficiency to a greater extent than pretreatment time in the commonly used severity function (Yu *et al*. 2010b; Kim *et al*. 2014). Thus, the efficacy of the HCW temperature for DF was evaluated between 180 °C and 260 °C with the enzymatic hydrolysis of both OSS and OSI samples prepared according to Fig. 1a. For cellulose decomposition at temperatures exceeding 230 °C (Sakaki *et al*. 2002), HCW treatments were performed at relatively short residence times (5 min). In addition, eucalyptus, which is highly susceptible to HCW treatment (Yu *et al*. 2010b; Silva-Fernandes *et al*. 2015), was also treated under the same conditions to compare the difference in sugar recovery between softwoods and hardwoods. The enzymatic hydrolysis of pretreated samples from eucalyptus and DF were supplemented with commercial hemicellulases, namely Optimash BG and Cellulosin GM, which exhibit high xylan- and mannan-hydrolyzing activities, respectively, to increase the monosaccharide recovery from the hemicellulose components.

The recovery of hemicellulosic sugars from the enzymatic hydrolysate of DF and eucalyptus OSS samples showed a similar tendency (Fig. 2). The highest yields of hemicellulosic sugars from DF OSS were obtained at 220 °C for 5 min (Fig. 2a), while those from eucalyptus were obtained at 210 °C for 5 min (Fig. 2b). Mannose was recovered as the major sugar in DF OSS with 115 mg/g-DM (Fig. 2a), corresponding to 76% of the

theoretical mannan. The glucose in the OSS recovered under these conditions was estimated to originate from glucomannan. To further examine the hemicellulose recovery, DF OSS from the samples treated for 10 min and 15 min at 210 °C were prepared. However, the mannose and xylose recovery was lower than that from the sample treated for 5 min at 210 °C (data not shown). In addition, the recovery of hemicellulosic sugars from the DF and from eucalyptus decreased at temperatures exceeding 230 °C and 220 °C, respectively, suggesting that these hemicellulosic sugars were further degraded to other compounds. In contrast, glucose recovery from DF and eucalyptus OSS remained constant and increased, respectively, under these conditions, suggesting that the cellulose component was partially solubilized.
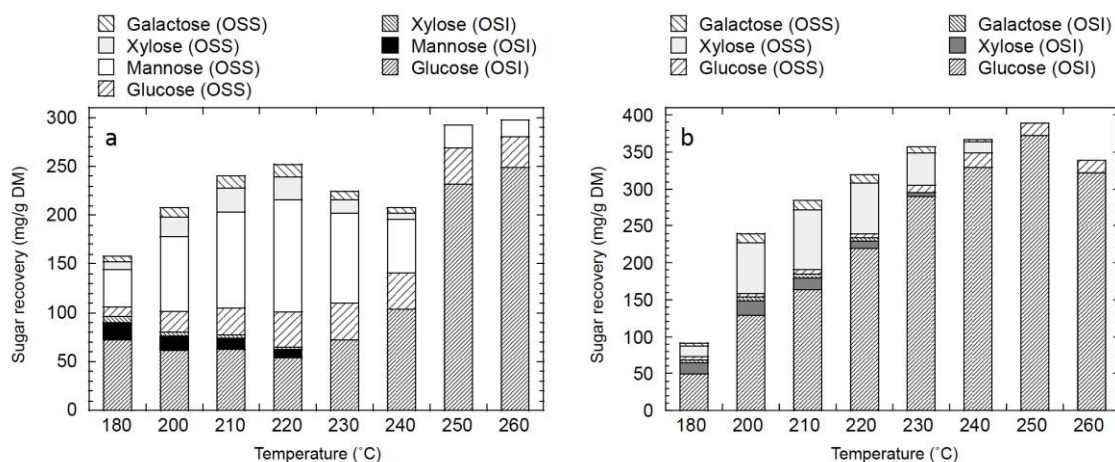

Fig. 2. Sugar recovery from DF (a) and eucalyptus (b) using the one-step HCW treatment and subsequent enzymatic hydrolysis. The sugar recoveries from OSI and OSS were based on the weight of potential monomeric sugar in DM.

Glucose recovery from DF OSI samples gradually decreased with increasing HCW temperature up to 220 °C (Fig. 2a), potentially related to loss of the residual glucomannan from OSI samples. In contrast, glucose recovery from eucalyptus OSI increased linearly with partial removal of hemicellulose at temperatures > 200 °C (Fig. 2b). Glucose recovery from the eucalyptus OSI reached 376 mg/g-DM with treatment at 250 °C for 5 min, corresponding to 79% of the theoretical glucan. These results support earlier suggestions that the non-catalytic hydrothermal treatment of softwood has little effect on the enzymatic digestibility of cellulosic residues (Alvira *et al* 2010; Lee *et al*. 2010). However, glucose recovery began to increase for the DF OSI samples treated above 230 °C in which the hemicellulose component was completely removed; the recovery was dramatically higher for DF OSI samples obtained at temperatures exceeding 250 °C (Fig. 2a). The pH values of DF OSS samples at 230 °C and 250 °C were pH 3.2 and pH 3.0, respectively. Furthermore, enzymatic hydrolysis of the DF OSI treated at 260 °C for 5 min gave the highest glucose recovery (248 mg/g DM), corresponding to 48.6% of the theoretical glucan. In addition, enzymatic hydrolysis of DF OSI samples treated at 260 °C and 270 °C with a shorter residence time (2 min) gave glucose recoveries of 205 and 249 mg/g-DM, respectively (data not shown), suggesting that the maximum sugar recovery of DF OSI was achieved at ~260 °C. These results indicate that HCW treatment is effective for sugar recovery from both softwood and hardwood. To the best of our knowledge, the

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enhancement of enzymatic digestibility of softwood residues by non-catalytic HCW treatment has not been previously reported.

The glucan contents and CrI values in the DF OSI samples increased with the HCW conditions up to 230 °C and 240 °C, respectively, due to removal of the hemicellulose component, and decreased significantly above 250 °C (Fig. 3). In contrast, the acid-insoluble lignin content increased continuously up to 260 °C (Fig. 3a). Interestingly, the enhancement of enzymatic digestibility of DF OSI was closely related to the decomposition of the cellulose component (Figs. 3 and 4). In addition, the glucan hydrolysis yields from DF OSI treated at 250 °C and 260 °C were 2.5- and 3.1-fold higher than that from DF OSI treated at 240 °C when a cellulase loading of 40 FPU/g-OSI was used for enzymatic hydrolysis (Fig. 4), with a glucan hydrolysis yield of 85.9% reached at 260 °C. These observations suggest that DF OSI treated above 250 °C may enable accelerated enzymatic hydrolysis through the changes in cellulose structure accompanying cellulose decomposition. HCW treatment of hardwood at high temperatures promoting cellulose decomposition increased the glucan hydrolysis yield in cellulosic residues (Kim *et al*. 2013, Yu *et al*. 2010b). In addition, as reported, the decomposition behavior of cellulose under hydrothermal treatment (250 °C) using a semi-continuous reactor is independent of the wood species used (Yedro *et al*. 2015). These results suggest that cellulosic residues in softwoods and hardwoods treated at high HCW temperatures are activated for enzymatic hydrolysis *via* the same mechanism, although the enzymatic digestibility of hardwood residues is accelerated sufficiently by hemicellulose removal.
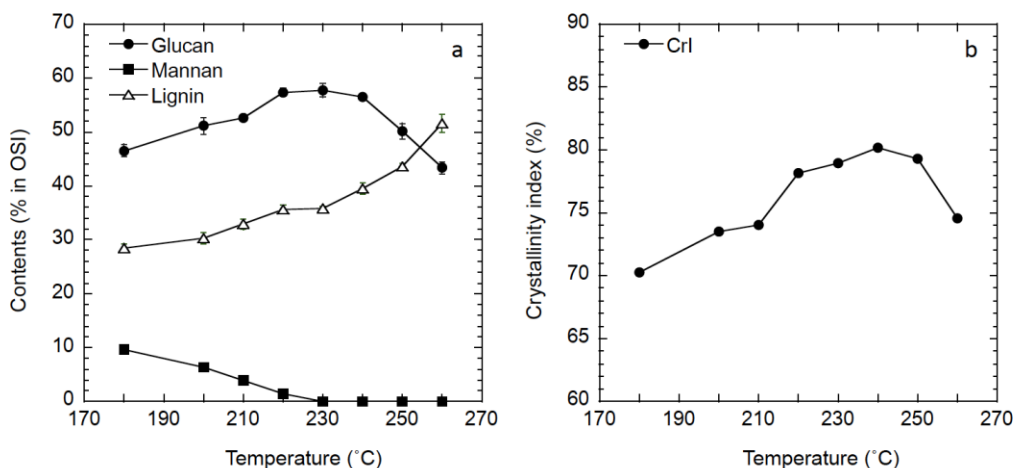
Fig. 3. Compositional analysis (a) and CrI (b) of DF OSI

Glucan hydrolysis yields from DF OSI samples treated at > 250 °C were considerably reduced at a cellulase loading of 10 FPU/g-OSI (Fig. 4), although this loading amount was sufficient for the efficient enzymatic hydrolysis of eucalyptus OSI (data not shown). These results indicate that the efficient hydrolysis of DF cellulosic residues requires a relatively high cellulase loading. Softwood lignin is known to limit the effective hydrolysis of the acid-impregnated steam-treated softwoods, and the use of a post-treatment step to remove or modify lignin is effective for obtaining high hydrolysis yields at low enzyme loadings (Várnai *et al*. 2010; Kumar *et al*. 2011). Furthermore, steam explosion pretreatment alters the lignin structure, leading to increased enzyme adsorption (Rahikainen *et al*. 2013). The lignin present in HCW-treated DF residues may also limit the effective enzymatic hydrolysis of the cellulosic residue.
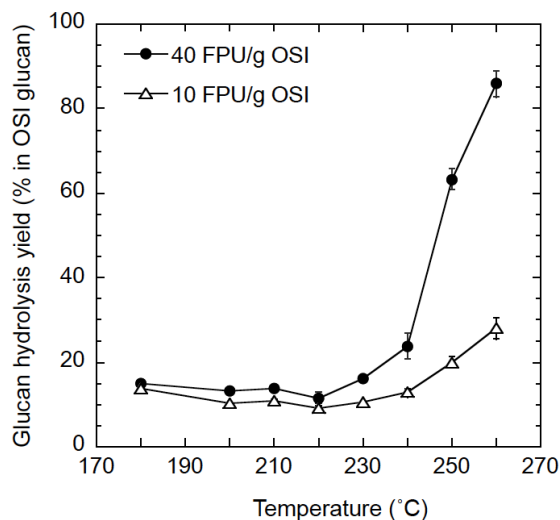
Fig. 4. Effect of cellulase loading for enzymatic digestibility of DF OSI

Improvement of Sugar Recovery Yields from DF by Combination of First-Stage and Second-Stage HCW Treatments

One-step HCW treatment of DF (260 °C for 5 min) yielded the highest total sugar recovery obtained from OSS and OSI samples despite the loss of a large fraction of hemicellulosic sugars (Fig. 2a). It was expected that the efficient recovery of hemicellulosic and cellulosic sugars of DF could be achieved by the combination of different HCW treatment conditions. Thus, TS1 and TS2 were prepared by first-stage and second-stage HCW, respectively, according to Fig. 1b, and their sugar recoveries were evaluated by enzymatic hydrolysis.

Based on results from the one-step HCW treatment, the first-stage HCW was performed at 220 °C for 5 min, and the mannose and xylose recovery yields from TS1 were 65.7% (100 mg/g DM) and 46.1% (26 mg/g DM), respectively (Table 3). However, mannose yield was slightly lower than that obtained from OSS at 220 °C (Fig. 2a). This may be due to the differences in the particle sizes of raw material and the reactors used for the one-step and first-stage HCW treatments. The cellulosic residues from the first-stage treatments were treated in the second-stage HCW between 250 °C and 270 °C for 5 min to increase the enzymatic digestibility. The glucose recovery yield of TS2 reached a maximum when the second-stage HCW treatment was performed at 260 °C for 5 min (Table 3). This yield was similar to that of OS1 treated at 260 °C. Second-stage treatment at 270 °C gave a significant drop in the glucose yield, suggesting quick decomposition of the cellulose component, even with a residence time of 5 min. No mannan was recovered in TS2 samples. In addition, the combined sugar recovery yield from TS1 and TS2 (260 °C) samples was estimated as 56.2% (427 mg/g DM), which is 1.4 times higher than that recovered by the enzymatic hydrolysis of OSS and OSI samples at 260 °C (Table 3). The improved sugar yield was primarily attributed to the difference in recovery of hemicellulosic sugars. These results indicate that two-step HCW treatment without excessive degradation of hemicellulose has an advantage over one-step HCW treatment for total sugar recovery from softwood.

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Table 3. Sugar Recovery by Enzymatic Hydrolysis of TS1 and TS2

Pretreated Sample	Sugar Recovery Yield (% DM)				Combined Yield (% DM)	Sugar Recovery (mg/g DM)
	Glucose	Xylose	Galactose	Mannose		
TS1 (220 °C)	6.4 ± 0.2	46.1 ± 2.5	43.4 ± 1.3	65.7 ± 2.3		
TS2 (250 °C)	32.1 ± 3.5	ND	ND	ND	44.8	340
TS2 (260 °C)	49.2 ± 2.5	ND	ND	ND	56.2	427
TS2 (270 °C)	18.8 ± 0.4	ND	ND	ND	35.8	272
OSS (260 °C)*	6.2 ± 0.1	ND	ND	11.5 ± 0.2		
OSI (260 °C)*	48.6 ± 1.7	ND	ND	ND	39.2	298

* Values estimated based on the data from Fig. 2a. Combined yield and sugar recovery were based on the weight of total sugars recovered by the enzymatic hydrolysis of TS1 and TS2 (or OSS and OSI).

Sugar Recovery and Ethanol Production from Douglas fir by Two-Step HCW Treatment

The two-step HCW treatment process was developed to recover a mixed sugar hydrolysate by the combined first-stage (220 °C for 5 min) and second-stage HCW (260 °C for 5 min) treatments and subsequent enzymatic hydrolysis (Fig. 1c). The TSC sample produced a solid yield of 89.5% and a total sugar yield of 72.7% (Table 4). Relatively row xylose (67.8%) and glucose yields (70.5%) in TSC suggest that xylan and glucan were decomposed to other compounds during the first- and second-stage treatments, respectively. The total sugar yield recovered by the enzymatic hydrolysis of TSC was 45.9% at a solid loading of 13% (*w/w*) (Table 4), and was lower than the combined yield (56.2%) of TS1 and TS2 (Table 3). This difference was mainly attributed to the low hydrolysis yield of glucan in TSC due to relatively high solid loadings of substrate. When enzymatic hydrolysis of TSC was performed with a low solid loading of 4.76% (*w/w*), the glucose and the total sugar recovery yield increased to 51.9% and 54.5% (414 mg/g DM), respectively, without significant changes in the recovery of hemicellulosic sugars (data not shown). These observations indicate that the glucan digestibility in HCW-treated DF was strongly influenced not only by enzyme loading, but also by substrate loading.

Table 4. Sugar Recovery from DF by Two-Step HCW Treatment and Subsequent Enzymatic Hydrolysis

Process	Sugar Recovery Yield (% DM)				Total Yield (% DM)	Sugar Recovery (mg/g DM)
	Glucose	Xylose	Galactose	Mannose		
Two-step HCW	70.5 ± 1.0	67.8 ± 4.9	85.3 ± 6.6	78.4 ± 4.1	72.7	552
Enzymatic hydrolysis*	40.8 ± 0.5	51.7 ± 3.0	31.0 ± 1.9	64.7 ± 1.5	45.9	349
	(57.9)	(76.2)	(36.3)	(82.6)	(63.1)	

*Enzymatic hydrolysis yield (%) based on TSC sugar contents are shown in parentheses

It should be noted that the enzymatic hydrolysis yield of galactose (36.3%) from TSC was significantly lower than those of other hemicellulosic sugars (Table 4). This implies that an enzyme related to galactose release was present in insufficient quantities in the enzyme cocktails used in this study. Softwood glucomannan has a branched α-1,6-linked galactose residue on the backbone consisting of mannose and glucose residues

(Lundqvist *et al.* 2003), and so an improved galactose hydrolysis yield through optimization of the enzyme composition is expected to lead to a further increase in the hydrolysis yields of mannose and glucose from hemicellulose.

Two-step HCW treatment of DF was compared with other pretreatment of softwoods. Two-step steam pretreatment of softwood impregnated with H_2SO_4 or SO_2 has total sugar yield of around 80% (Galbe and Zacchi 2002). Pretreatment using acid showed the higher sugar yield than our pretreatment. On the other hands, in a development of non-catalytic treatment, twin-screw extruder treatment of DF treated by HCW treatment (170 °C, 30 min) has a glucose recovery of 59% with total sugar production of 314 mg/g DM (Lee *et al.* 2010). Furthermore, wet explosion treatment (190 °C, 30 min, 7.5% O_2) of DF has been recently reported at pilot scale, and the glucose recovery of 63.3% with total sugar production of 361 mg/g DM was achieved (Biswas *et al.* 2015). These results show that two-step HCW treatment is comparable to other pretreatment of softwood without chemical additives, although the HCW conditions and process needs to be optimized in future applications.
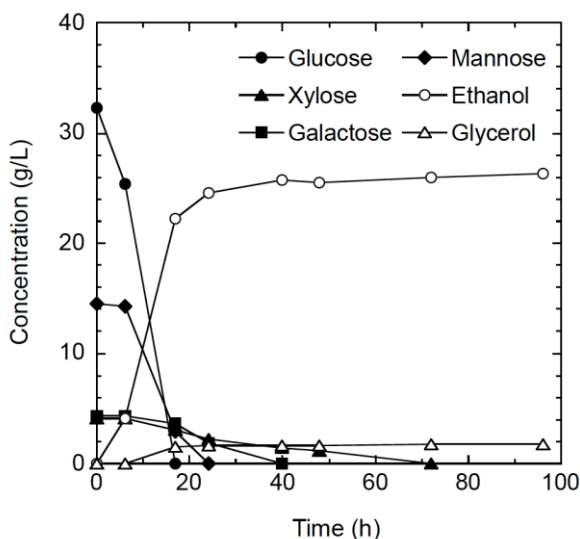
Fig. 5. Ethanol fermentation of enzymatic hydrolysate from DF TSC

Finally, ethanol productivity from the TSC hydrolysate was evaluated using xylose-fermenting recombinant *S. cerevisiae* MA-R4. Xylose-fermenting recombinant yeast is generally used for the ethanol production from hardwood that contains relatively high xylose content. Although the xylose content in the TSC hydrolysate was only 8.5% of the total sugar contents, it should not be ignored in the efficient ethanol fermentation of softwood. In the ethanol fermentation of the hydrolysate, glucose, mannose, galactose, and xylose were consumed within 18 h, 24 h, 40 h, and 72 h, respectively, with 26.3 g/L ethanol being produced at 96 h (Fig. 5). The fermentation yield was calculated as 93.3% based on the total sugars in the hydrolysate, and the ethanol productivity from DF was estimated as 166 mg/g DM in the process. These results suggest the potential of two-step HCW treatment as a pretreatment technology for ethanol production from softwood.

CONCLUSIONS

1. A non-catalytic HCW treatment was demonstrated to be applicable as a pretreatment technology for sugar recovery from DF. The enzymatic digestibility of the DF cellulosic residue was enhanced at high temperatures promoting cellulose decomposition.

2. Compared to the one-step HCW treatment, the combination of different HCW conditions to recover the both hemicellulosic and cellulosic sugars improved the total sugar recovery from DF. The combined two-step HCW treatment gave total sugar recoveries comparable to other chemical-free softwood pretreatments.

3. The TCS hydrolysate containing high hemicellulosic sugar contents was suitable for ethanol production using the xylose-fermenting recombinant *S. cerevisiae*, indicating the potential of the two-step HCW treatment as a pretreatment technology for efficient ethanol production from softwood.

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