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Reduction of Enzyme Dosage by Oxygen Delignification and Mechanical Refining for Enzymatic Hydrolysis of Green Liquor-Pretreated Hardwood

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Abstract In this study, a strategy to reduce enzyme dosage is evaluated by applying two post-treatments, oxygen delignification and mechanical refining. The sugar conversion for GL12 substrates was increased from 51.5% to 77.9% with post-treatments at the enzyme dosage of 10 FPU. When the amount of enzyme was reduced to 5 FPU with post-treatments, the conversion of 71.8% was obtained, which was significant higher than the conversion without any post-treatment using 10 FPU (51.5%). This clearly demonstrates the benefit of post-treatments that allows more than 50% of enzyme reduction at the same level of enzymatic conversion. Enzyme-accessible surface area and pore volume were evaluated by Simons' staining and DSC thermoporometry methods, and strong correlations were found with the sugar conversion.

Keywords Green liquor pretreatment · Post-treatment · Mechanical refining · Oxygen delignification · Reduction of enzyme dosage · Enzyme accessibility

Introduction

Lignocellulosic biomass has been a research and development focus as a feedstock for bioethanol production [29]. Woody biomass in particular can be harvested at any time, stores well for extended periods of time, and is not a food source. The lignocellulosic biomass has a complex structure and is inherently difficult to hydrolyze using biocatalyst, cellulolytic enzymes [2], and a pretreatment process is required to make substrates more accessible to enzymes and increase sugar conversion efficiency. Developing an effective pretreatment technology in conjunction with enzymatic hydrolysis is considered a primary challenge for commercialization of cellulosic bioethanol production [33]. Although several pretreatment methods have been developed, further research is necessary to identify a low-cost and efficient process that is financially viable for industrial aspects [10].

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Green liquor pretreatment has been proposed as an efficient process to pretreat woody biomass prior to enzymatic hydrolysis and fermentation [13, 32]. Green liquor, composed of sodium carbonate and sodium sulfide, is generated during the chemical recovery of a kraft pulping operation [13, 32]. This chemical recovery system has been practiced for several decades in pulping industry. Therefore, this particular pretreatment is well suited for repurposing a kraft pulp mill into a cellulosic ethanol mill. Green liquor has a high sulfide content and preferentially removes lignin, while preserving most of carbohydrates in biomass [13, 32]. Earlier work on green liquor pretreatment showed a marked improvement in sugar conversion as a result of delignification with a minimum carbohydrate loss [13, 32].

Despite substantial reduction in the cellulolytic enzyme cost, enzymatic hydrolysis still remains an expensive process and perhaps the most critical in the overall process [27]. Therefore, the amount of enzyme applied must be reduced to make the overall process more financially attractive. In this study, a chemical treatment and/or a mechanical treatment were applied to green liquor-pretreated hardwood to increase sugar conversion efficiency, thereby reducing the enzyme dosage.

Oxygen delignification has been used in the pulp and paper industry to remove a substantial fraction of lignin in bleaching processes [12], and it was reported that commercial oxygen delignification is capable of removing approximately 50% of lignin during the process [1]. One of the mechanisms of lignin reaction chemistry during oxygen delignification is phenolic delignification. The lignin reaction is initiated by a phenolate ion which is generated by a reaction of a phenolic hydroxyl group in lignin with alkali and the anion forms a reactive intermediate with oxygen. The intermediates then react with oxygen species radicals to form organic acids, carbon dioxide, and other small molecular organic products via side chain elimination, ring opening, and demethoxylation for lignin degradation [14]. In addition, covalent linkages between lignin and carbohydrates are involved in lignin reaction, and the cleavage of a bond between xylan and lignin would allow extensive delignification [7]. Oxygen delignification has been studied as a chemical treatment for biofuel production and can be applied to various types of pulps such as kraft, sulfite, and non-wood [6, 23].

Mechanical treatments such as chipping, milling, and refining improve the enzyme accessibility to cellulose through the increase in surface area available to enzymes, and particle size reduction [25]. The primary wall of a fiber is removed, intra-fiber bonds are broken, and fractures are generated throughout the cell wall during the mechanical treatments. Mechanical refining, in particular, has been used to develop paper strength in pulp and paper industry through fibrillation. During the refining process, the cellulosic fibers are mechanically treated in water, resulting in morphological and structural changes, and the fibers are fibrillated internally and externally [11]. These fibrillations caused by the high-shearing force of a refiner can significantly increase surface area [35], which facilitates enzyme access to cellulose. It is noted that mechanical treatment has been regarded as a non-viable option due to the requirement of the considerable amounts of energy and capital cost [2]. However, the benefit can be realized when the amount of enzymes is significantly reduced. In addition, chemical pretreatment increased specific surface of refined substrate with less energy consumption, and it indicated that chemical pretreatment prior to mechanical treatment can reduce the energy consumption of mechanical refining process [36].

The objective of this study was to investigate the effect of oxygen delignification and mechanical refining on enzymatic conversion of green liquor-pretreated hardwood and to verify the reduction of enzyme dosage when post-treatments are used. Two processes, oxygen delignification and mechanical refining, are named post-treatments in this study as these are performed between green liquor pretreatment and enzymatic hydrolysis. It also carried out to

determine key factors responsible for the improvement of sugar conversion when post-treatments were used. This study aims to develop post-treatment system that can reduce enzyme dosage.

Materials and Methods

Materials

Mixed hardwood chips (mainly poplar and maple) were obtained from a mill in the southeastern USA. The chips were screened in the range from 3/8 in. (9.5 mm) to 1 in. (25.4 mm), air-dried, and stored in the cold room at 4 °C until use. The compositional analysis of raw material is shown in Table 1.

Biomass Treatment

The green liquor (GL) used in all experiments was prepared by mixing 75% (*w*/*w*) of sodium carbonate and 25% (*w*/*w*) of sodium sulfide. The hardwood chips were pretreated using the GL at 160 °C for 1 h, which is equivalent to the kappa number of 400. The total titratable alkali (TTA) charge of 4%, 8%, and 12% (*w*/*v*) as Na_2O on oven-dried wood chips was applied for GL pretreatment, and the ratio of chips and liquor was 1:4 (*w*/*w*). These samples are called GL4, GL8, and GL12 in this study. All pretreatments were carried out in an M/K batch digester (M/K system Inc., Danvers, MA).

The portion of the pretreated hardwood chips was delignified using oxygen with 5% sodium hydroxide at 110 °C. The reaction was 1 hour at the oxygen pressure level of 100 psi. After pretreatment and oxygen delignification process, the chips were washed using sufficient amount of tap water to remove remaining chemicals and then defiberized three times using a disk refiner with disk a gap of 0.005 in. The disk refining was performed on all pretreated chips as a part of the pretreatment. Finally, the pretreated

Table 1 Recovery yields and chemical compositions of biomass substrates used in this study

Green liquor charge	Treatments	Yield (%)	Chemical compositions (%)											Total (%)
			Carbohydrates						Lignin			Ash	Ext	
			Glu	Xyl	Man	Gal	Ara	Sum	KL	ASL	Sum			
Raw materials			47.8	16.3	2.2	0.0	0.0	66.3	22.7	4.0	26.7	0.5	2.6	96.1
4%	GL-PT	89.5	45.7	14.5	1.7	0.0	0.0	61.9	20.2	2.6	22.8	ND	ND	84.7
	GL-PT&OD	80.7	48.0	14.0	1.6	0.0	0.0	63.6	14.4	2.9	17.3	ND	ND	80.9
8%	GL-PT	81.4	45.5	12.3	1.0	0.0	0.0	58.8	17.1	2.5	19.6	ND	ND	78.4
	GL-PT&OD	74.2	45.2	11.9	0.7	0.0	0.0	57.8	13.5	2.3	15.8	ND	ND	73.6
12%	GL-PT	81.9	46.6	12.5	0.6	0.0	0.0	59.7	15.9	2.3	18.2	ND	ND	77.9
	GL-PT&OD	75.6	47.2	12.0	0.6	0.0	0.0	59.8	10.6	1.9	12.5	ND	ND	72.3

GL-PT green liquor pretreatment, *OD* oxygen delignification, *Glu* glucan, *Xyl* xylan, *Man* mannan, *Gal* galactan, *Ara* arabinan, *KL* Klason lignin, *ASL* acid-soluble lignin, *Ext* extractives

Biomass substrates were prepared at various green liquor charges and oxygen delignification. All values are calculated on oven-dried weight of wood

substrates were centrifuged and stored in the cold room at 4 °C until use. The PFI refining was performed to improve the digestibility of green liquor-pretreated substrates. Pretreated substrates of 30 g (oven-dried basis) were disintegrated for 5 min, and the consistency of substrates was adjusted to 10% for the refining process [30]. Four different revolution counts were applied for the PFI refining in this study (2,000, 4,000, 6,000, and 8,000 revolutions).

Compositional Analysis

Contents of Klason lignin and acid-soluble lignin of the pretreated substrates were determined according to the procedures of NREL Chemical Analysis and Testing Standard Procedures [21]. The filtrate obtained from acid digestion was filtrated by 0.2-μm filter and analyzed by high-performance liquid chromatography (Dionex, Sunnyvale, CA) to quantify the amount of structural sugars. Sugars were separated through a Shodex SP0810 column at the temperature of 80 °C. Water was used as the eluant with the flow rate of 0.4 ml/min. A refractive index detector was used to quantify arabinose, galactose, glucose, xylose, and mannose in samples.

Enzymatic Hydrolysis

A commercial cellulase (NS 50013), β-glucosidase (NS 50010), and xylanase (NS 50014) provided from Novozymes (Franklinton, NC) were used for enzymatic hydrolysis. Cellulase dosages of 5 and 10 FPU/g biomass (2.42 and 4.85 mg dry matter/g biomass) were used. When 5 FPU was used, β-glucosidase and hemicellulase of 0.73 mg were used per gram biomass, and β-glucosidase and xylanase of 1.45 mg were dosed for 10 FPU of cellulase per gram biomass. All enzymatic hydrolysis were performed in 100 ml of 100 mM acetate buffer (pH 5.0) at a 5% (*w*/*v*) solids loading. The substrates and enzymes mixtures were incubated in a shaking water bath at 50 °C and 150 rpm for 48 h. The enzymatic conversion was determined in duplicate by weight loss during enzymatic hydrolysis, which was the amount of substrates enzymatically hydrolyzed for 48 h.

Enzyme-Accessible Surface Area

Porosity of biomass is characterized to estimate accessible surface to enzymes in solution. In order to measure the porosity, two independent methods were performed; Simons' staining method and thermoporometry method by differential scanning calorimeter (DSC).

For the Simons' staining method, the 1:1 staining method was used in this study [34]. For the preparation of dye solution, blue dye (DB) and orange dye (DO) were dissolved in nanopure water to a final concentration of 1% (*w*/*v*). For orange dye, only higher molecular fraction was used after the fractionation. The dye solution was then prepared with 1 ml of the dissolve DB, 1 ml of dissolved DO, 10 ml of 10% (*w*/*v*) NaCl, and 70 ml of water. For staining, a 25 mg of sample (dry weight) was placed in a bottle of the dye solution, and the bottle was incubated in a 75 °C shaking incubator at 200 rpm for 48 h. The stained samples were then filtered, rinsed with a minimum amount of distilled water, and stripped with 25% of aqueous pyridine at 45 °C for 18 h. The dye-stripping solution was then analyzed using a UV–vis spectrophotometer to determine the concentration of DO and DB (The maximum absorbance of DB and DO are at 624 and 455 nm,

respectively). The concentration of DO and DB dyes in the dye-stripping solution (C_O and C_B) was determined using the following two equations, Eqs. 1 and 2 (Lambert–Beer law for a binary mixture), that were solved simultaneously [5].

$$A_{455nm} = \varepsilon_{O/455} L C_O + \varepsilon_{B/455} L_{CB} \quad (1)$$

$$A_{624nm} = \varepsilon_{O/624} L C_O + \varepsilon_{B/624} L_{CB} \quad (2)$$

$$\left(\varepsilon_{O/455} = 35.62, \varepsilon_{B/455} = 2.59,\ \varepsilon_{O/624} = 0.19, \varepsilon_{B/624} = 15.62 \mathrm{Lg^{-1}cm^{-1}} \text{and L} = 1\ \text{cm}\right)$$

In addition to the Simons' staining method, the DSC thermoporometry method was applied to analyze the porosity of pretreated biomass [24]. A differential scanning calorimeter (Q100 DSC, TA Instruments) equipped with refrigerated cooling system was used, and pore volume distribution was determined by measuring the amount of water that has its melting temperature depressed at each isothermal step procedure. The samples were cooled to −40 °C and maintained for 5 min, and the temperature was then raised to −30 °C at 1 °C/min. The first segment (−40 °C to −30 °C) was used to determine the sensible heat of wet samples, assuming that there was no melting. Subsequent heating steps to slightly higher temperatures (−20, −15, −10, −6, −4, −2, −1.5, −1.1, −0.8, −0.5, −0.2, and −0.1 °C) were then performed in succession. In each step, the temperature was raised at 1 °C/min to the target temperature, and then, the sample was maintained isothermally until the heat flow returned to the baseline value. The heat absorbed during the heating and isothermal time period was calculated by integrating the endotherm. Thus, a melting enthalpy (H_m) is calculated by subtracting a sensible heat ($C_p \cdot \Delta T$) from a total heat (H_t) for each segment as shown in Eq. 3.

$$H_m = H_t - C_p \cdot \Delta T \quad (3)$$

The relationship between a pore diameter (D) and the depressed melting temperature (T_m) is described by Eq. 4, which reduces to the Gibbs–Thomson equation. The use of Eq. 4 is based on the assumption that the materials are not soluble in the water and its pore shape is cylindrical. Based on the equation, each melting temperature depression (ΔT) represents a specific pore diameter.

$$\Delta T = T_0 - T_m = (-4 \cdot T_0 \cdot \gamma_{1s} \cdot cos\theta)/D \cdot \rho \cdot H_f \quad (4)$$

where T_0 is the melting temperature of water (273.15 K), γ_{1s} is the surface energy at the ice–water interface (12.1 mJ/m^2), ρ and H_f are the density and the specific heat of fusion of freezing bound water, respectively, assumed to be the same as that of unbound water (1,000 kg/m^3, 334 J/g), D is the diameter of the pore, and ΔT is the melting temperature depression (K). Thus, water held in a smaller pore has a larger melting temperature depression.

Results and Discussion

Chemical Composition and Pretreatment Yields

Although some of the biomass components including xylan, mannan, and lignin were removed during the green liquor pretreatment and subsequent oxygen delignification

(Table 1), the overall pretreatment yield was found relatively high compared to other pretreatment methods. Dilute acid pretreatment yield is less than 60% at a similar reaction temperature (170 °C) to green liquor pretreatment [3]. However, the dilute acid pretreatment can increase total sugar recovery through the recovery of xylose released from the soluble fraction, and it has been reported that a higher sugar yield of 92.4% based on sugar in biomass can be achieved [8]. The soluble fraction containing the released sugars also contains inhibitory compounds generated during the dilute acid pretreatment [8], and thus, the soluble fraction must be neutralized and detoxified prior to fermentation. In the weak alkaline pretreatment such as green liquor pretreatment, the amount of released hemicellulose is significantly lower than other pretreatments. For example, the amount of xylan released during the green liquor pretreatment is 11~23% based on xylan amount in raw materials (Table 1). The green liquor pretreatment process has been designed without the recovery of released hemicellulose, which will simplify the process and reduce the total capital and operational costs. The SPORL (sulfite pretreatment to overcome recalcitrance of lignocellulose) process with 4% of sodium bisulfate on hardwoods showed the about 60% of solids yield and the conversion of cellulose was near completely [31]. The organosolv pretreatment also solubilizes a significant portion of biomass, creating a stream rich in sugar that must be utilized to make the process economical. Pan and coworkers reported a 53% solids yield after organosolv pretreatment; yield loss was the result of both lignin and carbohydrate solubilization [22].

When 4% green liquor was applied, the highest pretreatment yield of 89.5% was observed. With the increased green liquor charge, the pretreatment yield decreased and then leveled off. There was no difference in yield between 8% and 12% green liquor charge (Table 1). It was observed that the increased alkali charge during green liquor pretreatment produces a substrate with a lower residual lignin content [13, 32]. This partial lignin removal during green liquor pretreatment provides substrates more amenable to subsequent post-treatments and/or enzymatic hydrolysis. In this study, the residual lignin content also decreased with the increased green liquor charge. When 4% of green liquor was used, the lignin content decreased to 14.6% compared to raw materials. As the green liquor charge increased to 8% and 12%, the higher lignin removal was achieved, which were 26.6 and 31.8% compared to raw materials, respectively.

Oxygen delignification is well-known technology commonly used in a kraft pulping mill to selectively reduce the residual lignin content; hence, its application will not cause any excessive risk to the investor [32]. Lignin content in hardwood pretreated with 12% green liquor can be reduced another 30% by use of oxygen delignification (Table 1). More than 50% of lignin was removed based on lignin content in raw material, which indicates that green liquor pretreatment followed by oxygen delignification is the effective lignin removal process.

Under alkaline condition, it is well known that carbohydrates are degraded by both primary cell wall peeling reactions and random hydrolysis followed by secondary peeling reactions [26]. Although green liquor is also alkaline, it was reported that only the primary peeling reaction happened to degrade xylan and mannan under green liquor pretreatment conditions. It is because green liquor is mild alkaline and composed of sodium carbonate and sodium sulfide [13]. It should be noted that glucan, which is for a major sugar source for fermentation to ethanol, preserves in biomass structure and its yield in all cases is greater than 95% after both green liquor pretreatment and oxygen delignification. Therefore, the combination of green liquor pretreatment and oxygen delignification is considered an effective method not only to remove lignin from biomass, but also to preserve glucan in biomass.

Improvement of Enzymatic Hydrolysis by Post-Treatments

Enzymatic conversion was improved with the increased green liquor charge for the pretreatment (Fig. 1). When 10 FPU per gram of substrate of cellulase was used, the enzymatic conversion of green liquor-pretreated substrates without oxygen delignification increased from 29.4% for the green liquor charge of 4% to 51.5% for the green liquor charge of 12% (based on pretreated substrates) (Fig. 1b). When oxygen delignification was performed, the enzymatic conversion was improved higher, and those were from 42.8% to 62.2% (based on pretreated substrates). This suggests that additional lignin removal during oxygen delignification improves the efficiency of enzymatic hydrolysis. When 5 FPU of enzyme was used, enzymatic conversion of GL12 and oxygen-delignified substrate was 49.0% (Fig. 1a), and it could be converted to 60.5% of sugar recovery considering the carbohydrate contents in raw materials. This sugar recovery was found lower compared to other study using oxidative alkaline pretreatment. Chang and coworkers reported that 77% of sugar could be recovered from poplar wood, and this sugar recovery was obtained from poplar powder less than 10 mesh by oxidative lime pretreatment at 150 °C for 6 h [6]. The correlation between lignin content and enzymatic conversion was determined in Fig. 2. It is speculated that the decreased lignin amount in pretreated substrates facilitates enzyme access to cellulose, and therefore, enzymatic conversion was increased. It has been also suggested that median pores were formed in the structure during delignification [20].

The PFI refining process in this study significantly improved the enzymatic conversion at all conditions (Figs. 3 and 4). The enzymatic conversion of refined substrates at 6,000 PFI revolutions was 72% (based on pretreated substrates) at 5 FPU (Fig. 3), and it was higher than the enzymatic conversion of 62% for oxygen-delignified GL12 substrate without refining using 10 FPU of the enzyme dosage (Fig. 4). It clearly demonstrates the benefit of refining process, which is the possibility for the reduction of the enzyme dosage. According to the results, more than 50% of enzyme can be reduced to achieve the same enzymatic conversion. It was also found that the increase in enzymatic conversion was marginal at high PFI revolution, indicating that severe refining condition with high energy consumption was not necessary. The highest enzymatic conversion was 78%, and it was obtained from oxygen-delignified and refined substrates when 10 FPU of cellulase and 6,000 PFI revolutions were applied (Fig. 4b) based on pretreated substrates. The reason for the improvement of enzymatic hydrolysis by mechanical refining might be caused by the increase in specific surface area by the development of surface fibrillation [25]. During the

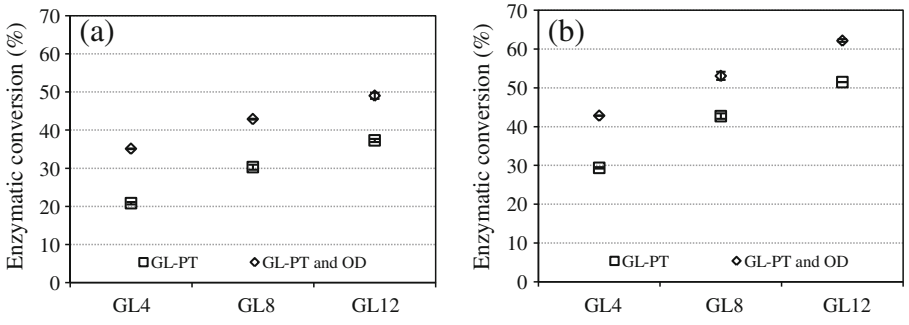

Fig. 1 Enzymatic conversion based on pretreated substrates with and without oxygen delignification at the various green liquor charges. Enzyme dosages of (**a**) 5 FPU and (**b**) 10 FPU were applied. *GL* green liquor, *GL-PT* green liquor pretreatment, *OD* oxygen delignification

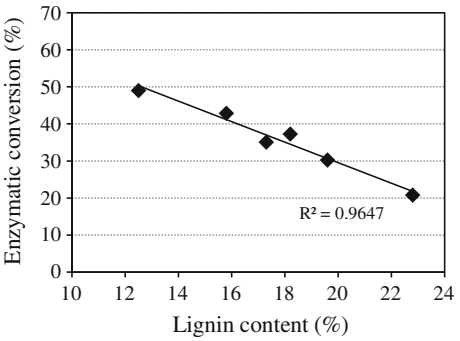

Fig. 2 Correlation between lignin content and enzymatic conversion based on pretreated substrates at enzyme dosage of 5 FPU

mechanical refining process, cellulosic fibers are mechanically treated in water, resulting in morphological and structural changes, and the fibers are fibrillated internally and externally [11]. These fibrillations, which are caused by the high-shearing force of a refiner, can increase the surface area significantly [35], and it facilitates enzyme access to cellulose [17]. Increase in green liquor charge and oxygen delignification also improved the enzymatic conversion. The enzymatic conversion of oxygen-delignified and refined (6,000 PFI revolution) substrates increased 54% from 46.6% to 71.8% as increasing the green liquor charge from 4% to 12%, and oxygen delignification enhanced the enzymatic conversion of refined GL12 substrates by 28% from 56.1% to 71.8%. When enzyme dosage increased 5 to 10 FPU, the enzymatic conversion of oxygen-delignified and refined GL12 substrates increased 8.5% from 71.8% to 77.9% (based on pretreated substrates). Considering the carbohydrate contents in raw materials, 78.9% of sugar was recovered from the substrates which pretreated by GL12, oxygen-delignified, and refined at 4,000 revolutions using only 5 FPU of enzyme. The high sugar recovery of 78.9% shows that the GL12 followed by post-treatments can recover the sugar effectively from hardwood without the recovery of released hemicellulose during GL pretreatment.

In order to calculate energy consumption by the PFI refining, an indirect method was applied based on a correlation between the energy consumption of the PFI mill and the Canadian Standard Freeness (CSF). This indirect method was reported using the bleached softwood pulp [15]. Although the correlation was obtained using a different pulp, the energy consumption of the PFI refining is determined by the revolution number, and thus,

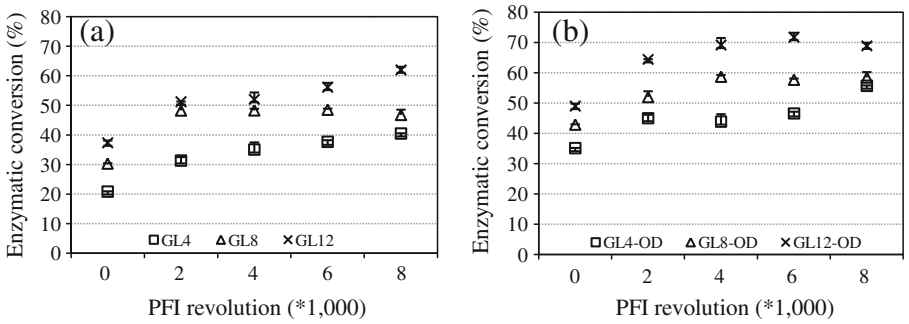

Fig. 3 Enzymatic conversion based on pretreated substrates with the enzyme dosage of 5 FPU at various refining energy inputs and green liquor charges, (**a**) without oxygen delignification and (**b**) with oxygen delignification

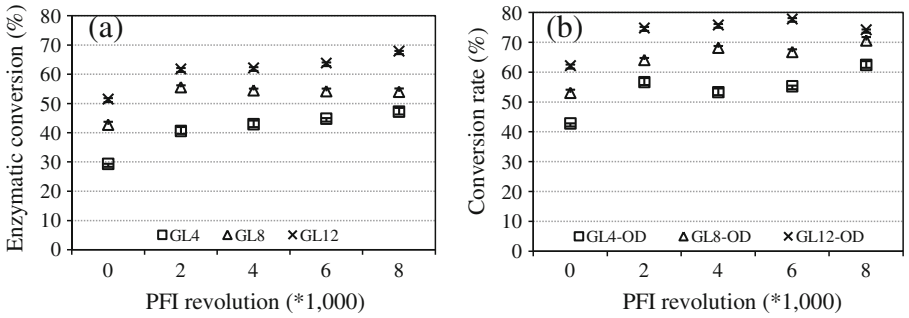

Fig. 4 Enzymatic conversion based on pretreated substrates with the enzyme dosage of 10 FPU at various refining energy inputs and green liquor charges, (**a**) without oxygen delignification and (**b**) with oxygen delignification

the correlation could be applied for green liquor-pretreated substrates in this study. Accordingly, the same bleached softwood pulp, which was used in the reference, was refined at 4,000 revolutions using a PFI refiner in this study, and the CSF of the refined pulp was measured to calculate the energy consumption. The CSF of the refined softwood pulp at 4,000 revolutions was 660 ml, and the energy consumption for making the refined pulp with the 660 ml of CSF was calculated at 188.5 kWh/ton based on the reference [15]. However, a PFI refiner is a lab scale refiner which consumes higher energy compared to a commercial scale refiner [18], and thus, a scale-up of refiner can reduce the specific energy. If an Escher–Wyss refiner, which is considered to give results indicative of commercial scale refining, is considered based on the reference correlation [15], the energy consumption can be reduced to 18.6 kWh/ton for 4,000 revolutions.

Enhancement in Enzyme Accessibility by Post-Treatments

An enzymatic hydrolysis is influenced by the various physicochemical properties of biomass, and enzyme-accessible surface area has been considered as one of the most important properties of substrates [9]. The BET (Bennett–Emmet–Teller) method that measures available surface area by nitrogen adsorption has been used to evaluate enzyme-accessible surface area. However, there are fundamental flaws to apply this technique to enzyme accessibility for two reasons. First, samples need to be dried prior to the measurement, and a drying process causes an irreversible physical collapse of cell wall structure through the aggregation of the cellulose chains [24]; thus, it does not provide a comparable result to accessible surface area in wet state [5]. Second, difference in sizes between nitrogen molecules and enzymes should be considered since smaller nitrogen molecules can access to nanopores that enzymes cannot enter [19]. Considering above reasons, three techniques were identified to evaluate enzyme accessibility: Simons' staining [5], DSC thermoporometry [24], and solute exclusion [28]. Two independent methods were utilized in the study.

The Simons' staining method has been used to assess the enzyme-accessible surface area of pulps, which were treated with wood decay fungi followed by chemical pulping, using the adsorption and desorption of dye [5]. It segregates the pores depending on their diameter using two different dyes which have a different affinity for the hydroxyl groups in biomass and thereby measures enzyme-accessible surface area in wet biomass. The large and orange-colored dye molecules can only penetrate the larger pores and adsorb in

preference to the small and blue-colored dye molecules as a result of their greater affinity for the hydroxyl groups in biomass [34]. The high molecule weight orange dye is mainly composed of two fractions which have 5–7 and 12–36 nm of hydrodynamic diameter [34], and the diameter of 5–7 nm is very similar to that of the catalytic core of *Trichoderma reesei* endoglucanase [9]. Therefore, the amount of adsorbed orange dye is a representative value for enzyme-accessible surface area, although the adsorption behavior onto biomass surface could be different compared to enzymes.

As the result of the Simons' staining, the total amount of adsorbed dye is increased in both sizes measured by orange and blue dyes (Fig. 5a). The amount of adsorbed orange dye, which could indicate enzyme-accessible surface area, increased 36% from 108 mg/g to 146 mg/g by 4,000 revolution of the PFI refining in green liquor-pretreated biomass. It was also found that the increase in the orange dye adsorption was marginal at high PFI revolution and the same trend was observed for enzymatic conversion efficiency. Thus, the strong correlation between the amount of adsorbed orange dye and enzymatic conversion was found (Fig. 6). It was well known that mechanical process fibrillates biomass

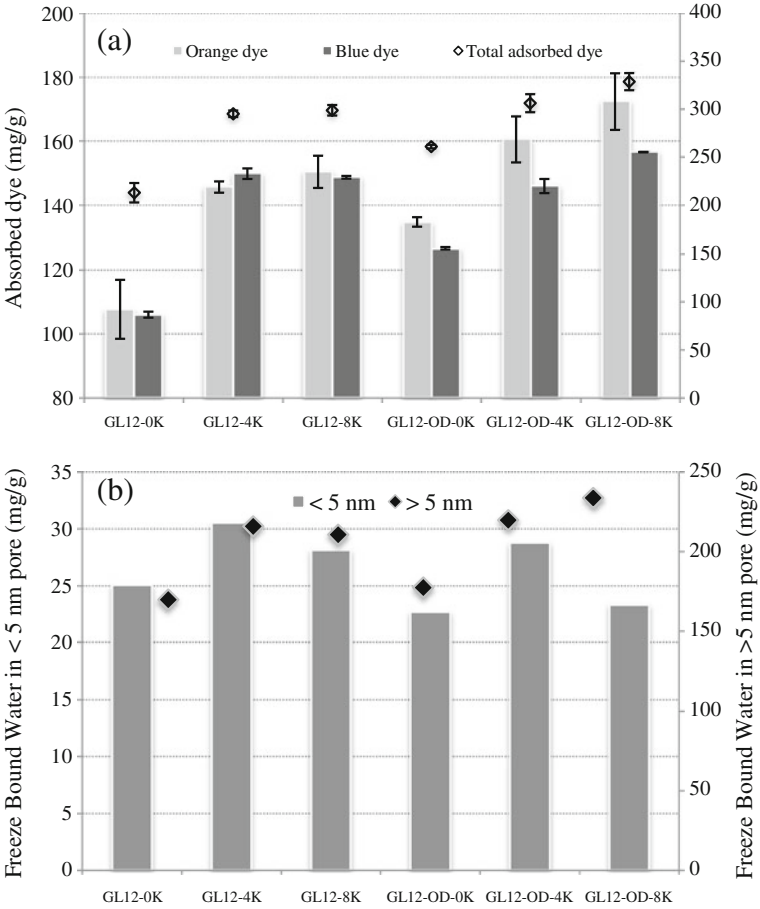

Fig. 5 Evaluation of enzyme accessibility through the Simons' staining and DSC thermoporometry methods: (**a**) amounts of adsorbed orange and blue dyes for the various pre- and post-treated biomass, (**b**) amounts of freezing bound water in pore volume for the various pre- and post-treated biomass

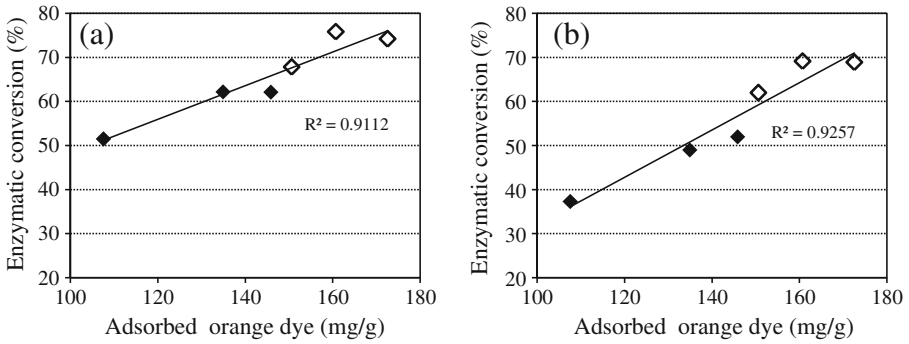

Fig. 6 A strong correlation between the adsorbed orange dye and enzymatic conversion calculated based on pretreated substrates for the enzyme dosages of (**a**) 10 FPU and (**b**) 5 FPU. *Solid* and *blank points* represent undelignified and delignified biomass samples, respectively

effectively by the shearing force and the fibrillation can open up the biomass cell wall structure through pore generation between cellulose microfibrils [16]. Therefore, the adsorption of orange dye, which was a representative of enzyme-accessible surface area, increased significantly through the fibrillation, and the increase in enzyme-accessible surface area can improve the enzymatic hydrolysis [4]. The PFI refining also fibrillated GL-pretreated biomass, and the fibrillation was observed using the FE-SEM (data not shown).

Although oxygen delignification increased 28% of the orange dye adsorption on green liquor-pretreated biomass without refining from 107.6 to 137.9 mg/g, the increase by the PFI refining was found higher. Increase in the orange dye adsorption was clearly demonstrated on the undelignified biomass. The 4,000 revolution of PFI refining increased 36% of the orange dye adsorption on the undelignified biomass from 107.6 to 145.8 mg/g; however, the increase on delignified biomass was only 19% from 134.9 to 160.7 mg/g. It was considered that the oxygen delignification already increased enzyme-accessible surface area in biomass, and thus, the increase in delignified biomass by the PFI refining was relatively low.

A DSC thermoporometry method has been used to investigate the pore size distribution of cellulose fibers at different moisture ratios [24]. Water held in the capillaries of porous materials has a depressed melting temperature because of the lower pressure at a curved interface in cavities. The melting temperatures depression has a reciprocal relationship with a pore diameter, and thus, pore volume can be evaluated.

The amount of freezing bound water, which indicates pore volume in biomass, is shown in Fig. 5b. Although larger pore volume, which has the diameter of more than 5 nm, increased by both oxygen delignification and the PFI refining, the greater increase by mechanical refining treatment was demonstrated. Oxygen delignification increased the larger pore volume, which can be a representative for enzyme accessibility, by 4.5% from 169.7 to 177.4 mg/g. Mechanical refining at 4,000 revolutions increased the amount by 27.3% from 169.7 to 216.0 mg/g. The increase in larger pore volume by the mechanical refining was also marginal at high PFI revolution. It should be noted that a similar trend were observed between the sugar conversion yield and enzyme accessibility measured by two fundamentally different methods, Simons' staining and DSC thermoporometry analyses. Therefore, it is concluded that post-treatments in this study enhanced sugar conversion yield through an increase in enzyme accessibility.

Conclusions

A strategy to reduce enzyme dosage by post-treatments on green liquor-pretreated hardwood was evaluated. A combination of green liquor pretreatment and oxygen delignification improved enzymatic hydrolysis through the lignin removal with minimum carbohydrate loss. Mechanical refining also enhanced enzymatic hydrolysis. The post-treatments increased sugar conversion to 79% using 5 FPU/g of enzyme dosage, and it was higher than that using 10 FPU/g without post-treatments. Therefore, post-treatments can reduce more than 50% of enzyme dosage to achieve the same level of sugar conversion. An increase in enzyme accessibility was demonstrated by Simons' stain and DSC thermoporometry methods.

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