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ORIGINAL ARTICLE

Enzymatic hydrolysis of wood with alkaline treatment

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Abstract Pulverized samples of wood, cedar and eucalyptus were treated with 5 N NaOH solutions at 25–150 °C. Hemicellulose and lignin content in the samples decreased with increasing treatment temperatures, while the recovery of glucose was maintained at nearly 90 %. X-ray diffraction analysis showed that the content of the original cellulose I structure in the samples decreased with increasing temperature, and most of the cellulose in the sample treated at 150 °C was converted to cellulose II by mercerization. Enzymatic hydrolysis of the alkaline-treated samples was carried out at 37 °C using solutions comprising a mixture of cellulase and β-glucosidase. The samples treated at higher temperatures showed better enzymatic degradability. Treatment with an alkaline solution of lower concentration (1 N NaOH) at 150 °C was also used. Despite significant quantities of hemicellulose and lignin being removed, mercerization was not induced. The enzymatic degradability was much lower than that of the sample treated with a 5 N NaOH solution at 150 °C. Thus, treatment with concentrated alkaline solution at high temperature led to not only the removal of hemicellulose and lignin, but also to modification of the cellulose structure, which resulted in high efficiency of enzymatic saccharification of the wood samples.

Keywords: Alkaline pretreatment · Enzymatic hydrolysis · Cedar · Eucalyptus · Mercerization

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Introduction

Lignocellulosic biomass has attracted much attention as a resource of ethanol and various chemical products, because it is renewable and noncompetitive with feed/food production. For industrial application, however, inefficiency of the enzymatic hydrolysis of cellulose to monomeric sugars remains a serious issue. The accessibility of enzyme to cellulose is restricted in lignocellulosic biomass, where cellulose is surrounded by hemicellulose and lignin that comprise the complex structure of the cell walls. In addition, cellulose itself shows poor degradability, because native cellulose exists as crystalline microfibrils, which have a rigid crystalline form, cellulose I [1, 2].

Various pretreatments to improve the enzymatic efficiency of hydrolysis of lignocellulosic biomass have been proposed [3, 4]. Most of these pretreatments were aimed at the removal of hemicellulose and lignin. However, ever since Igarashi et al. [5] reported that conversion of cellulose I to cellulose III_I induced by ammonium treatment significantly enhanced the hydrolysis efficiency, studies of

the use of pretreatments to modify the cellulose crystals have also increased.

Alkaline treatment of cellulose, called mercerization, is one of the well-known treatments that induce the conversion of cellulose I. In the mercerization process, alkaline solution penetrates between cellulose chains to form a complex of cellulose, alkaline ions and water molecules. After washing with water, alkaline ions are washed away, yielding a cellulose hydrate called Na-cellulose IV. On drying, Na-cellulose IV is converted to the final structure of mercerization, cellulose II [6, 7]. Several studies on the hydrolysis efficiency of mercerized cellulose have been reported [8–10]. We have recently reported that mercerized cellulose, especially Na-cellulose IV, exhibits much higher degradability than cellulose I [11, 12]. Alkaline treatment, as a useful pretreatment of lignocellulosic biomass to remove hemicellulose and lignin, has also been studied. Although the concentration of alkaline solution used in these pretreatments is generally too low to induce mercerization, we nonetheless applied the same treatment, but with concentrated alkaline solution that could induce mercerization of cellulose to lignocellulosic biomass, bagasse, as cellulose samples [12]. The results of the study suggested that the alkaline treatment could lead to mercerization of cellulose in bagasse at the same time as the removal of hemicellulose and lignin, which indicates a significant improvement in enzymatic degradability.

In this study, we applied alkaline treatments to wood biomass, cedar and eucalyptus as softwood and hardwood biomass, respectively, at various temperatures. We then analyzed the chemical compositions and crystal structures of these samples after the treatments and carried out the enzymatic hydrolysis to characterize the effect on their degradability.

Materials and methods

Alkaline treatment of wood samples

We prepared alkaline-treated wood samples using different alkaline concentrations and temperatures.

Pulverized cedar (*Cryptomeria japonica*) and eucalyptus (*Eucalyptus camaldulensis*) (particle size 1.5–3 mm) were used as the wood samples in this study. The wood samples (1 g) were suspended in 25 mL of 5 N NaOH solutions. A suspension was incubated at 25 and 50 °C for 3 h, or it was inserted into a pressure vessel and heated in an oil bath at 100 and 150 °C for 1 h. The wood samples (1 g) were also treated in 25 mL of 1 N NaOH solutions at 150 °C for 1 h. After the alkaline treatments, the samples were thoroughly washed with deionized water until the supernatant reached neutral pH, by centrifugation. The alkaline-treated samples were stored in water until their use for enzymatic saccharification, or freeze dried for chemical composition analysis and X-ray diffraction analysis.

Chemical composition analysis

The contents of lignin and neutral sugars in the untreated and alkaline-treated wood samples were determined by the Klason method [13] and the alditol acetate method [14], respectively.

The samples (0.1 g) were hydrolyzed in 1 mL of 72 % (w/w) H_2SO_4 at room temperature for 4 h with occasional stirring by a glass rod. The reaction mixture was diluted with distilled water until the concentration of H_2SO_4 was 4 % and then autoclaved at 121 °C for 1 h. The solid residue of the hydrolyzed sample was collected by centrifugation, washed with distilled water and dried in an oven at 105 °C. The content of acid-insoluble lignin was then determined. The supernatant of the mixture was neutralized with $Ba(OH)_2$ solution to pH 5.5–6, and the precipitate was removed by centrifugation. Myoinositol was added to the supernatant as a gas chromatography standard, and this solution was reduced with $NaBH_4$ for 3 h. The reaction was terminated by addition of acetic acid until no more bubbles were generated. The reaction mixture was evaporated to dryness with a rotary evaporator, washed with methanol and evaporated to dryness again. After washing three times, the dried substance was treated with a moderate quantity of acetic anhydride at 120 °C for 3 h. The solution was analyzed by gas chromatography to determine the content of neutral sugars.

X-ray diffraction analysis

The untreated and alkaline-treated wood samples were pressed into disks, which were set in the goniometer of a diffractometer (RINT-2000, Rigaku, Japan). X-ray diffraction in reflection mode was carried out with monochromatic Cu Kα radiation ($\lambda = 1.5418$ Å), generated at 38 kV and 50 mA. The optical slit system comprised a divergence slit = 1.0°, a scattering slit = 1.0° and a receiving slit = 0.3 mm. The scanning mode parameters were the following: scattering angle $2\theta = 5$–30° and step in 2θ of $\Delta 2\theta = 0.1°$. The accumulation time for each step was $t = 10$ s.

Enzymatic hydrolysis

Cellulase (filter paper degrading unit; FPU) and β-glucosidase (cellobiase unit; CbU) activities were measured using the method of Ghose [15]. The untreated and alkaline-treated wood samples (100 mg) were incubated with cellulase from *Trichoderma reesei* (15 FPU/g-substrate,

Celluclast 1.5 L, Novozymes) and β-glucosidase from *Aspergillus niger* (80 CbU/g-substrate, Novozyme 188, Novozymes) in 10 mL of 50 mM sodium acetate buffer (pH = 4.5) at 37 °C using an end-over-end mixer (12 rpm). After incubation for 1, 2, 4, 8 and 24 h, 100 μL of the mixture was collected and centrifuged (15000 g × 3 min) to terminate the reaction. The concentration of glucose formed in the supernatant was determined using the Glucose CII-Test Wako reagent (Wako Pure Chemical Industries, Ltd., Japan). The absorbance at 505 nm was measured using a Shimadzu UVmini-1240 spectrophotometer. The saccharification ratio was calculated as the ratio of glucose content in the supernatant per glucan content in the untreated or alkaline-treated wood samples, determined by the chemical composition analysis.

Results and discussion

Changes in chemical composition with alkaline treatment

The contents of the five sugars, glucose, arabinose, xylose, mannose and galactose, and the Klason lignin in the solid remaining after alkaline treatment were determined for each wood sample (Fig. 1). Glucose and the other sugar components were mainly derived from cellulose and hemicellulose, respectively, although glucose was also partly derived from some hemicellulose components such as glucomannan. When the cedar and eucalyptus samples were treated with a 5 N NaOH solution at 25 °C, all the components decreased to 80–90 % of the untreated samples. The total recovery decreased gradually with an increase in the treatment temperature up to 100 °C. This resulted from a decrease in the unknown components and a slight decrease in the sugar components, except for glucose (hereafter called hemicellulose component). Thus, the content of glucose and lignin remained almost unchanged. However, the treatment at 150 °C induced drastic changes in the hemicellulose component and lignin. In the case of cedar, they decreased to approximately 60 % of the untreated sample. The decreases were even more remarkable in the case of eucalyptus. The content of the hemicellulose component and lignin was approximately 30 and 40 % of the untreated sample, respectively. Only the glucose recovery remained unchanged from approximately 90 %, even at 150 °C, for both cedar and eucalyptus. Treatment with a 5 N NaOH solution at higher temperature, in particular at 150 °C, resulted in the efficient removal of hemicellulose and lignin, while the glucose content was maintained.

The cedar sample treated with a 1 N NaOH solution at 150 °C had almost the same chemical composition as the sample treated with a 5 N NaOH solution at 150 °C. On the other hand, more than 60 % of the hemicellulose component and lignin remained in the eucalyptus sample treated with a 1 N NaOH solution at 150 °C, which is much more than in the eucalyptus sample treated with a 5 N NaOH solution at 150 °C. In the case of eucalyptus, in addition to the contribution of the high treatment temperature, the high concentration of NaOH solution also contributed to the efficient removal of hemicellulose and lignin.

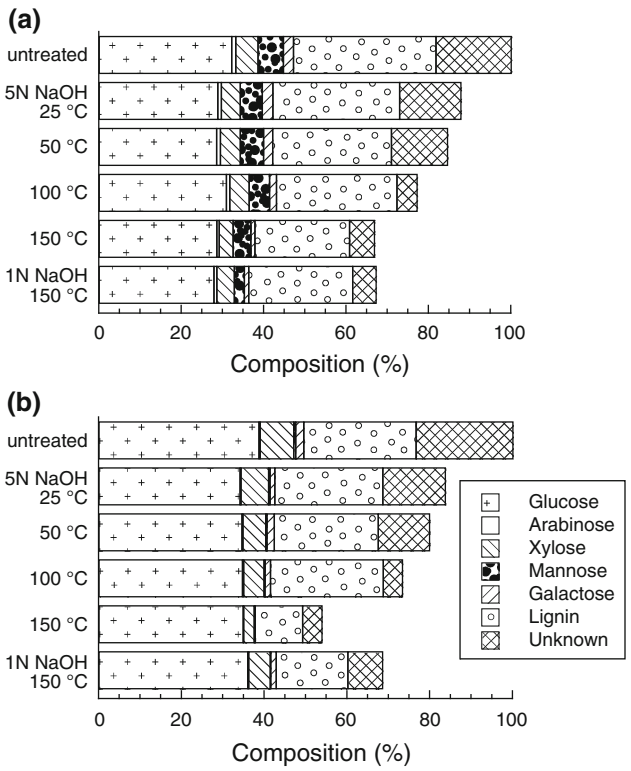

Fig. 1 Chemical composition of **a** cedar and **b** eucalyptus samples treated with NaOH solutions at various temperatures. The composition (%) of the treated samples indicates the proportion to the untreated samples

Crystal structure of cellulose in the wood samples

X-ray diffraction profiles of the untreated and alkaline-treated wood samples are shown in Fig. 2. Only the three typical peaks of cellulose I (2θ = 14.9°, 16.6° and 22.5°) were observed in the profiles of the untreated cedar and eucalyptus samples, but the three peaks from cellulose II (2θ = 12.2°, 20.1° and 21.9°) appeared faintly in the profiles of the samples treated with a 5 N NaOH solution at 25 °C. Although the intensity ratio of the cellulose II peaks slightly increased with an increase in the treatment temperature up to 100 °C, the intensity ratio increased rapidly from 100 to 150 °C. At 150 °C, more than half of cellulose I apparently converted to cellulose II. A 5 N NaOH

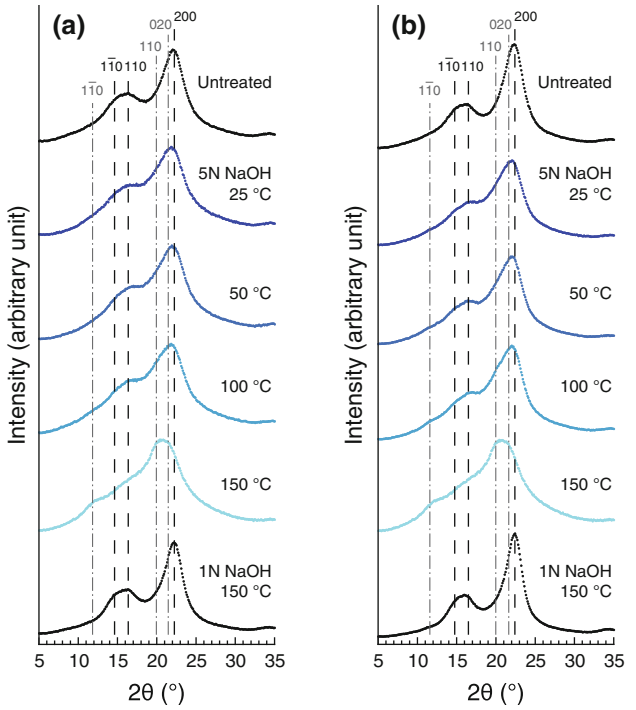

Fig. 2 X-ray diffraction profiles of **a** cedar and **b** eucalyptus samples treated with NaOH solutions at various temperatures. The *dashed lines* and the *dotted-dashed lines* indicate the positions of the peaks from cellulose I and cellulose II, respectively

solution essentially caused mercerization of cellulose readily, but hemicellulose and lignin could interfere with penetration of the NaOH solution into cellulose in the wood. Therefore, mercerization of cellulose rarely occurred in the samples treated with 5 N NaOH solutions at 25–100 °C, where most of the hemicellulose and lignin remained after alkaline treatment. However, heating at 150 °C in a 5 N NaOH solution removed hemicellulose and lignin significantly, which induced mercerization of cellulose. On the other hand, the profiles of the samples treated with a 1 N NaOH solution at 150 °C showed the typical pattern of cellulose I, although significant amounts of hemicellulose and lignin were removed as well as the samples treated with a 5 N NaOH solution at 150 °C. Because the low concentration of alkaline solution such as a 1 N NaOH solution could not induce mercerization even if there were no lignin and hemicellulose, the crystal form of cellulose remained unchanged throughout the alkaline treatment.

A 5 N NaOH solution was used to convert the crystal structure of native cellulose according to the precious study [12], but NaOH solutions of lower concentrations such as 3 N and 4 N NaOH solutions would also induce mercerization at least partly. In addition, these changes in the chemical composition and the crystal structure also depend on the particle size of the samples, thus the samples with smaller particle sizes such as nano-sized particles may be more affected by the alkaline treatment.

Enzymatic hydrolysis of wood samples

Enzymatic hydrolysis of the prepared wood samples was carried out using cellulase and β-glucosidase at 37 °C. Figure 3 shows the change in the saccharification ratio of the samples with time. The untreated wood samples were rarely hydrolyzed with the enzymes, and the saccharification ratios of both the cedar and eucalyptus samples at 24 h were less than 2 %. However, the alkaline treatment improved the saccharification efficiency. Furthermore, the samples treated at higher treatment temperature showed higher saccharification ratios at any time up to 24 h. The saccharification ratios of the cedar samples treated with a 5 N NaOH solution at 25, 50, 100 and 150 °C reached 26,

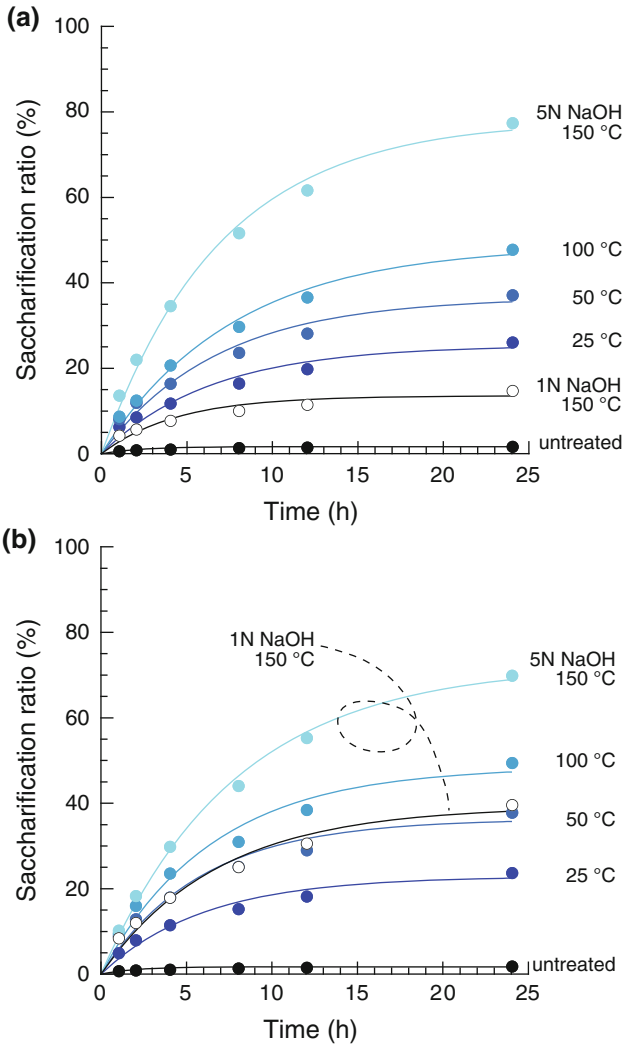

Fig. 3 Changes in the enzymatic saccharification ratios of **a** cedar and **b** eucalyptus samples with time

37, 48 and 78 %, respectively, and those of the eucalyptus samples reached 25, 38, 50 and 70 %, respectively, at 24 h. The samples treated with a 1 N NaOH solution at 150 °C also had improved saccharification ratios, but they were much lower than in the case of the samples treated with a 5 N NaOH solution at 150 °C. The saccharification ratios of the cedar and eucalyptus samples treated with a 1 N NaOH solution at 150 °C were 15 and 40 %, respectively, at 24 h.

The results of degradability of wood samples treated with a 5 N NaOH solution are in agreement with the amounts of remaining hemicellulose and lignin in the samples. The improvement in the accessibility of cellulose was obviously the important factor for the efficient hydrolysis of the wood samples. The samples treated with a 1 N NaOH solution at 150 °C, on the other hand, showed rather low degradability, although significant amounts of hemicellulose and lignin were removed as well as the samples treated with a 5 N NaOH solution at 150 °C. In the case of cedar, in particular, the difference between the saccharification ratios of the samples treated with 1 N and 5 N NaOH solutions at 150 °C was more than five times, while their chemical compositions were nearly the same. As mentioned above, there is a difference in the crystal structure of cellulose between the two samples, indicating that the efficient hydrolysis of the samples treated with a 5 N NaOH solution at 150 °C was achieved by modification of the cellulose crystal, combined with the removal of hemicellulose and lignin. Upon comparing the cases of cedar and eucalyptus, however, the degradability could not be described only based on the hemicellulose and lignin contents and the crystal structure of cellulose. There are probably other factors that also affect the hydrolysis efficiency, such as the distribution of the residual hemicellulose and lignin, the creation of degradation products by alkaline treatment and the surface conditions of the cellulose crystals.

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