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Addition of alkali to the hydrothermal–mechanochemical treatment of *Eucalyptus* enhances its enzymatic saccharification

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HIGHLIGHTS

- NaOH enhanced cleavage of ester bonds between lignin and hemicellulose.
- Hydrothermal–mechanochemical treatment with added NaOH produced finer nanofibers.
- Specific surface area of already fibrillated substrate increased by 76% with NaOH.
- Increased specific surface area correlated with increased enzymatic digestibility.

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ABSTRACT

The effects of alkali on hydrothermal–mechanochemical treatment (hydrothermal treatment combined with wet-milling) were examined with the aim of improving pretreatment of lignocellulosic biomass before enzymatic saccharification. After enzymatic saccharification, the highest glucose yield was obtained by autoclaving at 170 °C in the presence of 20% NaOH per substrate weight. The wood fiber was unraveled into finer nanofibers by hydrothermal–mechanochemical treatment, thus increasing the specific surface area of the substrate from 11 to 132 m^2/g. Adding 20% NaOH to the treatment further increased the specific surface area of the already fibrillated substrate by 76% (232 m^2/g) due to lignin removal and ester bond cleavage between lignin and hemicellulose. This increase in specific surface area was closely related to the increase in enzymatic digestibility; therefore, NaOH addition may have enhanced the effect of hydrothermal–mechanochemical treatment.

1. Introduction

Lignocellulosic biomass is an attractive and enormously abundant renewable resource whose efficient utilization is required for biorefinery applications, including bioethanol production. Therefore, the saccharides in lignocellulosic biomass (e.g., agricultural residue, energy crop, and forest residue) are of considerable interest for use as starting materials for sugar platform. However, cellulose and lignin, the principal structural components in lignocellulosic biomass, are recalcitrant compounds, being water-insoluble polymers with stable structures. Woody structure is often compared to that of ferroconcrete, with cellulose, hemicellulose, and lignin playing roles analogous to that of iron framework, reinforcing steel, and concrete, respectively. If sugars are to be obtained from cellulose via biodegradation, the cellulose (iron framework) must be broken down along with lignin (concrete) removal. Intact wood material cannot be digested by cellulolytic enzymes; therefore, it is necessary to conduct pretreatment to facilitate this.

To date, pretreatment studies have examined various chemical and physical processes. Physical pretreatment (e.g., milling, irradiation, hydrothermal treatment, and steam explosion) is known to increase the enzyme-accessible surface area by breaking up the wood into small pieces, improve reactivity by amorphization of highly crystalline cellulose, and break down and remove the lignin component. Hydrothermal treatment and steam explosion are often categorized as physico-chemical treatments because their behavior resembles acidic-treatment. They differ from milling treatment, which does not change components. In our laboratory, a hydrothermal–mechanochemical treatment was recently developed; this combines a hydrothermal treatment at 135–180 °C with wet-milling (Endo, 2010; Lee et al., 2010). This process does not require the raw materials to be dry because it is a wet process, and its dependency on raw material biomass species is low. High temperature treatment at over 200 °C produces enzyme inhibitors, such as furfural, because of over-decomposition; this increases the quantity of saccharification enzyme required. However, it is possible to reduce the amount of enzyme used by decreasing temperatures below 200 °C (Inoue et al., 2008), and by adding chemicals, such as alkali salts, to improve the efficiency of the

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hydrothermal–mechanochemical treatment, a further reduction in the required quantity of enzyme is expected.

Alkaline pretreatment is a favorable chemical approach that has several advantages versus other pretreatment processes; these include low operation cost, reduction in holocellulose degradation, and reduction in formation of enzyme inhibitors of downstream processing (Mosier et al., 2005). Sodium, potassium, calcium, and ammonium hydroxide are appropriate chemicals for alkaline pretreatment, but NaOH is used extensively. NaOH treatment of lignocellulosic biomass causes substrate swelling, decreased crystallinity, and lignin structure disruption (Balat, 2011). Studies on NaOH pretreatment of sorghum straw, cotton stalk, and other biomasses have been published, showing the effects of delignification by dilute 20% NaOH per substrate weight and subsequent enzymatic degradation (Silverstein et al., 2007; McIntosh and Vancov, 2010). In a study by Li et al. (2004), a combination of NaOH (12% per substrate weight) and homogenization was used to pretreat corn stover; consequently, enzymatic digestibility was 5-fold higher than that for NaOH-free controls.

A combination of pretreatments is expected to enhance enzymatic digestibility. Alkali treatment has often been conducted with hydrothermal treatment (Chen et al., 2009; Gupta et al., 2011; McIntosh and Vancov, 2011) or wet-milling (He et al., 2010; Lin et al., 2010). Dissolution of lignin by alkali, along with hemicellulose removal by hydrothermal treatment or reduction of particle size by wet-milling, facilitates disruption of the recalcitrant structure of lignocellulosic biomass. The combination of wet-milling treatment and hydrothermal treatment also improves enzymatic digestibility (Lee et al., 2010; Hideno et al., 2012). The lignocellulosic component network is readily decomposed during wet-milling, and hemicellulose solubilization is induced by hydrothermal treatment.

Here, we examined the influence of NaOH on the hydrothermal–mechanochemical treatment of hardwood *Eucalyptus* by evaluating the properties of pretreated substrates, including their enzymatic digestibility. NaOH treatment and hydrothermal–mechanochemical treatment, that is, alkali cooking of wood chips and refiner pulping, are widely used in the pulp and paper industry, and it is expected that applying these well-established processes could also be advantageous for bioethanol production.

2. Methods

2.1. Materials

Wood chips of *Eucalyptus globulus* were used as the raw material, and Accellerase DUET (Genencor, CA, USA), a cellulase cocktail derived from *Trichoderma reesei* was used for enzymatic saccharification. Both were provided by Oji Holdings Co., Tokyo, Japan. Sodium hydroxide, was used as the alkaline species, and was purchased from Wako Chemicals, Japan.

2.2. Hydrothermal–mechanochemical treatment with alkali

Eucalyptus chips (3 g), less than 3 mm in size, were mixed with 40 mL of distilled water or aqueous sodium hydroxide solutions of 5%, 10%, and 20% per weight of *Eucalyptus*. They were then soaked overnight. The hydrothermal treatment was performed using an autoclave (STP-3050VP; ALP, Japan) at 130 °C and 150 °C, and a reactor (Model 4565; Parr, IL, USA) at 170 °C for an hour. Wet ball-milling was directly applied to autoclaved samples. Ball-milling was performed for a total of 240 min at room temperature using a planetary micro mill (Pulverisette 7 premium line; Fritch, Germany). Samples were milled at 450 rpm in an 80 mL zirconium milling pot containing 30 zirconium spheres (ϕ = 10 mm). A cycle of 10 min of milling, followed by a 10 min pause, was used. After ball-milling treatment, samples were washed with distilled water until a neutral pH was achieved, and the water was completely exchanged with *t*-butyl alcohol to prevent cellulosic substrate aggregation due to sudden dewatering during lyophilization. Lyophilization was performed for a week to remove completely the *t*-butyl alcohol.

2.3. Enzymatic saccharification

Enzymatic saccharification was performed at 50 °C for 48 h using a reaction mixture (total volume, 25 mL) containing 0.3% pretreated *Eucalyptus*, an enzyme cocktail consisting of 12 FPU cellulase per gram of substrate and 0.1 M acetate buffer (pH 5.0). From the reaction mixture, 0.5 mL was periodically collected and centrifuged for 5 min at $1 \times 10^4 g$. The liberated sugars were quantified via a high-performance liquid chromatography (HPLC) system equipped with a refraction index detector (RI-2031 Plus; Jasco, Japan) and an Aminex HPX-78P column (Bio-Rad, CA, USA) at a flow rate of 0.6 mL/min at 80 °C. Ultrapure water was used as the mobile phase. Enzymatic digestibility was represented as the glucose yield, which was calculated using the following equation:

$$\text{Glucose yield}\ (\%) = [\text{weight of glucose liberated by enzymatic hydrolysis}\ (\text{mg})]/[\text{weight of maximum glucose obtained by sulfuric acid hydrolysis}\ (\text{mg})] \times 100$$

2.4. Quantification of chemical composition

The chemical composition of the pretreated material was determined. Klason lignin and sugar content were determined by sulfuric acid hydrolysis according to a previously described method (Sluiter et al., 2012) with some modifications. A 50 mg portion of dried sample was hydrolyzed with 0.5 mL of 72% (w/w) sulfuric acid for 60 min. Then, this solution was diluted to 4% (w/w) acid by adding 14 mL of distilled water. The mixture was autoclaved at 121 °C for 1 h, and the residue was filtered, dried at 105 °C for 24 h, and weighed as acid insoluble lignin. The 1 mL filtrate was neutralized with saturated barium hydroxide, and sugar products in solution were quantified by HPLC after filtering with a 0.2 μm membrane filter (PTFE; Advantec, Japan).

2.5. Measurement of specific surface area

The specific surface area of the sample was determined from the Brunauer–Emmett–Teller plot of nitrogen adsorption–desorption isotherms. The samples that were lyophilized with *t*-butyl alcohol were further dried at 105 °C for 6 h with degassing (BELPREP; Bel Japan, Japan) before setting up the measuring device. The measurement was performed using BELSORP-max (Bel Japan, Japan) at a temperature of −196 °C.

2.6. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectra were obtained using an FT-IR spectrophotometer (Spectrum GX; PerkinElmer, MA, USA) using the attenuated total reflectance (ATR) technique. Background spectra were measured in the absence of a sample. All the spectra were recorded in absorbance mode from 600 to 4000 cm^{-1} and averaged over 128 scans at room temperature. The resolution of the spectra was 4 cm^{-1}.