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Application of ammonia pretreatment to enable enzymatic hydrolysis of hardwood biomass

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ABSTRACT

Ammonia pretreatment greatly improves enzymatic hydrolysis of grass biomass, but is reported to be ineffective for hardwood biomass. Here, we examined the effectiveness of ammonia pretreatment of biomass from six hardwood species with different contents of xylan and lignin. Ammonia pretreatment greatly improved enzymatic hydrolysis of polysaccharides in birch and willow, but was less effective for acacia, eucalyptus, and poplar. The effectiveness of ammonia pretreatment increased with xylan content but decreased with lignin content of the hardwood species. By adding a recombinant xylanase to the commercial enzyme digestion cocktail, the yield of enzymatic hydrolysis of ammonia-pretreated birch biomass was improved to a similar level to that obtained with grass biomass. Our results indicate that enzymatic hydrolysis of biomass from hardwood species having a relatively high xylan/lignin ratio can be achieved with a xylanase-enriched enzyme cocktail after ammonia pretreatment.

1. Introduction

Plant biomass is the most abundant carbon-neutral renewable resource on earth. In order to produce biofuels such as ethanol and other value-added chemicals from plant biomass, structural polysaccharides such as cellulose and xylan must be converted into monosaccharides such as glucose and xylose. For this purpose, enzymatic hydrolysis is generally preferred to acid hydrolysis. However, thermochemical pretreatment is still required to obtain higher yields of the sugars, and various pretreatment techniques using acids or alkalis under hydrothermal conditions have been investigated [1,2]. One of the most promising approaches for grass biomass is pretreatment with liquefied ammonia, and this methodology has been extensively investigated [3–5]. The ammonia cleaves ester bonds in plant biomass and generates amides [6,7]. We have demonstrated that ammonia pretreatment also alters the polymorphic form of crystalline cellulose with a low water content, transforming the natural crystalline form (cellulose I) to cellulose III_I [8], which is far more susceptible to enzymatic degradation [9]. Based on those results, we applied ammonia pretreatment for the enzymatic hydrolysis of grass biomass, *Erianthus ravennae*, and achieved glucose and xylose yields > 80% within 24 h at an initial enzyme/biomass ratio of 1/100 [10]. However, in contrast, ammonia pretreatment was reported to be ineffective for enzymatic hydrolysis of hardwood biomass derived from acacia and poplar [11–13]. So far, there has been no report of successful application of ammonia pretreatment to hardwood biomass.

The aim of the current study was to examine the feasibility of using ammonia pretreatment to enable hydrolysis of hardwood biomass. Specifically, we compared the effects of ammonia pretreatment on the chemical composition and enzymatic hydrolysis of six species of hardwood, including the reported species, acacia and poplar.

2. Materials and methods

2.1. Ammonia pretreatment of hardwood biomass

Wood blocks from acacia (*Acacia mangium*), beech (*Fagus crenata*), white birch (*Betula platyphylla*), eucalyptus (*Eucalyptus camaldulensis*), poplar (*Populus sieboldii*), and willow (*Salix pet-susu*), and samples of grass (*Erianthus ravennae*), were ground in a Wiley mill (W140; Ikeda Scientific Co., Ltd, Tokyo, Japan) and then sieved to obtain 40-mesh-passed meal. Each sample was subjected to Soxhlet extraction with benzene-ethanol (2:1 v/v) and then dried by evaporation of the solvents and used for experiments as untreated biomass.

Abbreviations: FTIR, Fourier-transform infrared spectrometer; HPLC, high-performance liquid chromatography

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Untreated biomass was loaded into a 120 mL steel pressure vessel (TVS-N2; Taiatsu Techno Corp., Tokyo, Japan), which was cooled in a water bath set to −13 °C. Ammonia gas was slowly introduced into the vessel until the sample was completely immersed in liquid ammonia. The vessel was sealed, heated to 140 °C for 1 h, and then cooled to room temperature, and the ammonia gas was released. Each sample was air-dried overnight at room temperature, and used for further experiments as ammonia-treated biomass.

2.2. Chemical analysis of biomass

Chemical analysis of untreated and ammonia-treated samples was conducted, and their neutral sugars, organic acids, lignin, and nitrogen compositions were compared.

Neutral sugar content was determined according to the two-stage sulfuric acid hydrolysis method [14] with slight modifications. For neutral sugar analysis, approximately 0.1 g of each dried sample was dissolved in 1 mL 72% sulphuric acid for 1 h in a 30 °C water bath. Each mixture was quantitatively diluted by adding 28 mL distilled water (final sulphuric acid concentration, 4%), and each solution was incubated for 1 h at 121 °C. The resulting hydrolysate was filtered through a 0.2-μm high-performance liquid chromatography (HPLC)-certified filter (GE Healthcare, Chicago, IL, USA). The neutral sugars obtained by acid hydrolysis were analyzed by HPLC (Prominence, Shimadzu Cooperation, Kyoto, Japan) on SP0810 columns (Showa Denko K. K., Kanagawa, Japan), with a charged aerosol detector (Corona Veo RS; Thermo Fisher Scientific, MA, USA). Neutral sugars were eluted with acetonitrile/water 13.0/87.0 (v/v) at a flow rate of 0.5 mL min^{-1}. Analytical-grade L-arabinose, D-galactose, D-glucose, D-mannose (all from Wako Pure Chemical Industries, Ltd., Osaka, Japan), and D-xylose (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were used as standards for quantification of neutral sugars.

Acid-insoluble lignin (Klason lignin) content was estimated according to the Klason method [15], except for sulphuric acid treatment, which was the same as used in the neutral sugar analysis.

For the quantification of glucuronic acid and acetic acid, approximately 0.1 g of each dried sample was dissolved in 1 mL 72% sulphuric acid for 1 h in a water bath at 30 °C. Each mixture was diluted with 10 mL distilled water (final sulphuric acid concentration, 10%), and the resulting solution was incubated for 1 h at 121 °C. The hydrolysate was filtered as described above and subjected to separation on an HPLC system equipped with KC-811 and SH-1821 columns (Showa Denko) and a UV-2075 detector (Shimadzu). Acetic acid and glucuronic acid were eluted with 3 mM sulphuric acid aqueous solution at a flow rate of 1.0 mL min^{-1}. Analytical-grade acetic acid and glucuronic acid (Wako) were used as standards for quantification.

The nitrogen content of untreated and ammonia-treated samples was estimated using a total nitrogen analyzer, Nitrogen Detector TN-2100H (Mitsubishi Chemical Analytech Company Ltd., Mie, Japan), according to the manufacturer's instructions. Analytical-grade pyridine and toluene (Wako) were used as standards for quantification.

2.3. Spectroscopic analysis of biomass

Untreated and ammonia-treated biomasses were formed into tablets and analyzed using a Nicolet iN10 attenuated-total-reflection, Fourier-transform infrared (FTIR) spectrometer (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) over the range of 4000–400 cm^{-1}.

X-Ray diffraction images of untreated and ammonia-treated samples were collected on a RINT 2200 goniometer equipped with a one-dimensional position-sensitive proportional counter (Rigaku Co., Tokyo, Japan). Nickel-filtered Cu Kα radiation generated at 38 kV and 50 mA was collimated with a 1.0-mm-wide collimator. The diffraction profiles were recorded from 5° to 30°.

2.4. Enzymatic hydrolysis of biomass

Untreated and ammonia-treated samples were used as substrates for enzymatic hydrolysis. Equal amounts of Cellic® CTec (Novozymes, Bagsvaerd, Denmark) and Cellic® HTec (Novozymes) were mixed and used as the enzyme cocktail. Untreated and ammonia-treated samples (1.0% w/v) were incubated with the enzyme cocktail in 1 mL of 50 mM sodium acetate pH 4.5 at 37 °C and 15 rpm at an initial enyzme/biomass (E/B) ratio of 1/25. The reaction mixtures were collected at 1, 2, 4, 8, 24, and 48 h and the amounts of D-glucose and D-xylose formed by enzymatic reactions was measured using a Glucose CII-Test Wako reagent (Wako) and a D-Xylose Assay Kit Test (Megazyme International Ireland, Ltd., Wicklow, Ireland). Each conversion data point represents the mean of three measurements with the standard deviation.

Enzymatic hydrolysis of ammonia-treated biomass from birch wood was also performed with the above enzyme cocktail plus recombinant enzyme (glycoside hydrolase family 11 endo-1,4-β-Xylanase from *Neocallimastix patriciarum* or glycoside hydrolase family 43 β-xylosidase from *Bacillus pumilus*, Megazyme International Ireland) at an initial E/B ratio of 1/250. The conditions were the same as described above.

3. Results and discussion

3.1. Effects of ammonia pretreatment on chemical composition of biomass

First, we examined the effects of ammonia pretreatment on the chemical composition, crystal structure, bonding state, and enzymatic hydrolysis of biomass from six hardwood species, compared with grass biomass (*Erianthus*). The results of the analysis of the cellulose and xylan (calculated from glucose and xylose yields, respectively, as anhydrous conversion), lignin, and nitrogen contents of untreated and ammonia-treated samples are summarized in Table 1.

The biomass recovery after ammonia treatment was more than 95% by weight. Significant loss of lignin was observed from the grass biomass after ammonia treatment, but not from the hardwood samples. In previous studies, ammonia pretreatment of corn stover and poplar was reported to cleave ester bonds in the biomass [6,7]. The major chemical changes caused by ammonia treatment should be cleavage of glucuronoyl and acetyl ester linkages on the main chain of xylan. In all cases, the content of nitrogen increased and the content of acetyl group decreased after ammonia treatment, suggesting that acetyl substitution of xylan was eliminated. A slight decrease in the content of glucuronoyl group was also observed. Fig. A1 shows the relationship between organic acid content and xylan content. There was a good correlation between the contents of acetyl group and xylan in all hardwood samples (Fig. A1a), whereas there was no clear correlation between the contents of glucuronoyl group and xylan contents of all hardwood samples (Fig. A1b).

The content of cellulose in hardwood samples was in the range of 33–41%, being generally higher than that in grass biomass (33%). There were substantial differences in the contents of xylan and lignin among hardwoods. In the untreated biomasses, birch and willow had higher xylan content (16.4% and 14.4%, respectively) and lower lignin content (21.3% and 23.0, respectively) than the other hardwoods did. In contrast, acacia and eucalyptus showed higher lignin content (30.5% and 27.0%, respectively) and lower xylan content (8.3% and 10.2%, respectively) than other hardwoods did. Beech showed moderate lignin and xylan contents (23.7% and 13.6%, respectively) compared with the two groups mentioned above. A similar tendency was also observed with the ammonia-treated biomasses.

3.2. Spectroscopic analysis of untreated and ammonia-treated biomasses

Fig. 1 shows FTIR spectra of untreated and ammonia-treated samples. In all cases, the absorption peak near 1745 cm^{-1} disappeared and that around 1245 cm^{-1} was reduced after ammonia treatment. These