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Research paper

Characterization of cell wall structure in dilute acid-pretreated biomass by confocal Raman microscopy and enzymatic hydrolysis

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ABSTRACT

The chemical and ultrastructural properties of cell walls were investigated to determine the effect of dilute acid pretreatment on the hydrolysis of lignocellulosic biomass. Confocal Raman microscopy was used to gain a clear understanding of how dilute acid pretreatments destroy lignocellulosic cell walls. Total fermentable sugar (glucose and xylose) was high in oxalic acid hydrolysate (26.18 g/L) compared to that in sulfuric acid hydrolysate (24.34 g/L). Chemical composition of the pretreated biomass differed slightly according to the acid catalyst used. Oxalic acid pretreatment was effective for enzymatic hydrolysis, with 29.46 g/L of total fermentable sugar after 96 h. Optical microscopy showed that dilute acid pretreatment significantly changed cell wall structure, and broken and crushed cell walls could be clearly seen during pretreatment. Based on confocal Raman peak intensity, the ratio of lignin/cellulose [I(1600)/I(900)] was low for oxalic acid-pretreated biomass compared to sulfuric acid-pretreated biomass.

1. Introduction

The focus on lignocellulosic biomass conversion technology is on the rise, given its potential to yield fuels and bio-based chemicals. The use of lignocellulosic biomass as a replacement for fossil resources or other chemicals has immediate and far-reaching environmental benefits [1]. Thus, lignocellulosic biomass is undoubtedly a promising energy source, owing to its renewable nature and abundant supply.

Lignocellulosic biomass consists of cellulose (25%–50%), hemicelluloses (20%–35%), lignin (10%–30%), and some extractives. Cellulose, a homopolymer of glucose molecules, has a highly crystalline structure and a high degree of polymerization. In contrast, hemicellulose is an amorphous heteropolymer, and is thus comparatively easier to hydrolyze to simple sugars. Lignin consists of phenylpropanoid units covalently linked to hemicelluloses. It has remarkable resistance against chemical and microbial attack. Because of its recalcitrant properties, lignin is more difficult to process, extract, hydrolyze, or react than cellulose or hemicelluloses [2].

Because of the complex structure of lignocellulosic biomass, pretreatment is required, to improve the bioconversion process for fermentable sugars production [3]. Various pretreatment processes, including chemical, physical, physicochemical, and biological, have been suggested to reduce recalcitrance and improve the sugar yields of lignocellulosic biomass [4–7].

However, pretreatment efficiency differs considerably depending on the type of biomass and pretreatment method. Biomass properties related to the efficiency of enzymatic hydrolysis, such as cellulose crystallinity, degree of polymerization, acetylation degree of hemicelluloses, surface area, and lignin structure, are altered by pretreatment condition and biomass. Therefore, there is immense scope for research and development in exploring the potential of pretreatment for improving the efficiency and lowering the cost of such processes.

To date, most researchers have focused on hydrolysis yield, fermentable sugar production, or ethanol production to elucidate pretreatment efficiency. Some researchers have studied the interactions between pretreatment and enzymatic hydrolysis using microscopy or immunological method [8–10]. However, limited studies have investigated the effect of pretreatment with respect to the structure or ultrastructure of lignocellulosic biomass [11,12].

The effects of oxalic and sulfuric acid on lignocellulosic biomass

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pretreatment have been reported by some researchers [7,13,14]. However, the ultrastructural effects and overall mechanism of dilute acid pretreatment remain unknown. In this study, the pretreatment properties of a lignocellulosic biomass were investigated using oxalic acid and sulfuric acid pretreatments under the same reaction condition. These materials were then used for investigating changes in the micro and ultrastructural properties of the cell wall by using different microscopy approaches, including confocal Raman microscopy, in order to clearly understand how hydrothermal chemical pretreatments "open up" and destroy lignocellulosic cell walls. The present study should lead to a better understanding of the effects of oxalic and sulfuric acid pretreatments on lignocellulosic biomass, and allow us to relate the chemical treatments to changes in structure, thereby facilitating the optimization of the process.

2. Material and methods

2.1. Biomass

Yellow poplar (*Liriodendron tulipifera*) chips were used as the biomass in this study. The biomass was provided by the Korea Forest Research Institute. Prior to pretreatment, the biomass was cut into small blocks, of approximately 1.5 × 1.5 × 1.5 mm. The material was stored at 4 °C, with less than 10% moisture content.

2.2. Dilute acid pretreatment of biomass

Hydrolysis was performed in an oil bath with temperature and time control. Sulfuric acid and oxalic acid were used as acid catalysts in this study. The biomass and acid solutions were placed in a glass tube with a screw cap and then heated to the desired temperature. The biomass/acid solution ratio was 1:5 (w/w). The reaction temperature and time were 160 °C and 120 min, respectively. To provide the same acidic condition (pH 1.3), acid concentrations were 40 mM for sulfuric acid and 100 mM for oxalic acid. After the pretreatment, the hydrolysate was separated from the pretreated biomass by vacuum filtration, and pretreated biomass was washed with distilled water. The hydrolysate and pretreated biomass were stored at 4 °C for further analysis.

2.3. Analysis of hydrolysate and pretreated biomass

The concentration of sugars and fermentation inhibitors in the hydrolysate, such as furfural, 5-hydroxymethylfurfural (HMF), and organic acids were determined using HPLC (Waters 2695 system; Alliance, MA, USA) outfitted with an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA), and a refractive index detector (Waters 2414 system; Alliance, MA, USA). The analysis was performed with 5 mM H_2SO_4 as the mobile phase at an isocratic flow rate of 0.3 mL/min, for 55 min. All samples were properly diluted, and filtered through a 0.45 μm spin-filter before analysis to remove particles. Total phenolic compounds (TPC) were estimated colorimetrically by the Folin-Ciocalteu method using a standard curve related to absorbance at 760 nm [15].

The chemical compositions of the pretreated biomass and raw material were determined, using a laboratory analytical procedure of the National Renewable Energy Laboratory.

2.4. Sample preparation for microscopy

The untreated and pretreated biomass samples were fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde solution (in 0.05 M cacodylate buffer, pH 7.2) for 4 h at room temperature. The biomass was washed with 0.05 M cacodylate buffer (pH 7.2), after which dehydration was performed in an acetone series. Next, the samples were embedded in Spurr's resin. Semithin (0.5 μm) sections for microscopy analysis were prepared using an ultramicrotome, and mounted on slide glass.

2.5. Confocal Raman microscopy

Confocal Raman microscopy in a backscattering geometry was performed by using the NTEGRA Spectra system (NT-MDT, Russia) equipped with a piezoelectric sample scanner. A linear-polarized He–Ne laser operating at a wavelength of 632.8 nm was used as an excitation source. The laser beam was focused on samples by a 100X objective lens (numerical aperture = 0.9). The average power of the excitation laser was 3 mW, which was focused on a spot of approximately 429 nm in diameter. No irreversible laser-induced heating effects were detected during the measurements. All the Raman spectra were detected by a Newton electron multiplying charge-coupled-device (EMCCD) camera (Andor Technology, UK). The spectral resolution was approximately 1.2 cm^{-1}. For two-dimensional Raman maps, Raman spectra were obtained every 350 nm, using the *x-y* sample scanner. An acquisition time of 5 s was used to measure a representative Raman spectrum, at each point in the Raman maps.

2.6. Enzymatic hydrolysis of pretreated biomass

Enzymatic hydrolysis was performed, using Celluclast 1.5 L (cellulase), NS-50010 (β-glucosidase), and NS-22036 (xylanase) from Novozymes. Pretreated material (2 g dry weight) was transferred to a 125 mL Erlenmeyer flask, and 20 mL of 50 mM sodium citrate buffer (pH 5.0) was added. Appropriate amounts of cellulase (7.5 FPU/g substrate), β-glucosidase (11.25 CBU/g substrate), and xylanase (4 FXU/g substrate) were added. The flask was placed in a shaking incubator at 50 °C and 150 rpm, and incubated for 96 h. Samples were taken at 24, 48, 72, and 96 h, and the released sugar was analyzed using HPLC.

3. Results and discussion

3.1. Dilute acid pretreatment of biomass

The catalytic properties of sulfuric acid and oxalic acid on the degradation of the biomass compounds were investigated under the same pretreatment conditions. The concentrations of fermentable sugars contained in the hydrolysate are shown in Table 1. Xylose released from hemicelluloses was the most abundant sugar in the hydrolysate with both acid catalysts. Total fermentable sugar (glucose and xylose) in the oxalic acid hydrolysate was as high as 26.18 g/L, compared to that of sulfuric acid hydrolysate (24.34 g/L). This result was similar to that of a previous study [7,14]. Oxalic acid is a strong organic acid with a higher efficiency for hydrolysis than that of sulfuric acid, and this property is thought to yield superior selectivity for the hydrolysis of hemicelluloses [16–18]. The concentrations of fermentation inhibitors (furfural, HMF, acetic acid, and TPC) were similar or slightly higher in oxalic acid hydrolysate than in sulfuric acid hydrolysate. In particular, the acetic acid concentration was high in the oxalic acid hydrolysate because acetic acid released from hemicellulose was proportional to the xylose produced during pretreatment [19]. The total amounts of TCP, as determined by our analysis, did not differ under the examined conditions. This indicates that lignin degradation was not affected by the kind of acid catalyst used for pretreatment. However, the structural properties of lignin differed depending on the acid catalyst, as has been concluded in a previous study [14].