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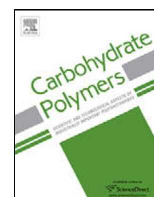

Integrated chemical and multi-scale structural analyses for the processes of acid pretreatment and enzymatic hydrolysis of corn stover

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ABSTRACT

Corn stover was pretreated with acid under moderate conditions (1.5%, w/w, 121 °C, 60 min), and kinetic enzymolysis experiments were performed on the pretreated substrate using a mixture of Celluclast 1.5 L (20 FPU/g dry substrate) and Novozyme 188 (40 CBU/g dry substrate). Integrated chemical and multi-scale structural methods were then used to characterize both processes. Chemical analysis showed that acid pretreatment removed considerable hemicellulose (from 19.7% in native substrate to 9.28% in acid-pretreated substrate) and achieved a reasonably high conversion efficiency (58.63% of glucose yield) in the subsequent enzymatic hydrolysis. Multi-scale structural analysis indicated that acid pretreatment caused structural changes via cleaving acetyl linkages, solubilizing hemicellulose, relocating cell wall surfaces and enlarging substrate porosity (pore volume increased from 0.0067 cm^3/g in native substrate to 0.019 cm^3/g in acid-pretreated substrate), thereby improving the polysaccharide digestibility.

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1. Introduction

Lignocellulose biomass is mainly composed of cellulose, hemicellulose, and lignin. Although carbohydrate-enriched lignocellulose biomass is likely to become the primary feedstock of biofuels obtained by biochemical conversion, the structural and compositional features of lignocellulose biomass create a protective barrier and prevent enzymatic depolymerization of structural polysaccharides (Sanderson, 2011). To improve the enzyme hydrolysis conversion efficiency, some pretreatment methods such as comminution (Zhu, Wang, Pan, & Gleisner, 2009), hydrothermal (Wan, Zhou, & Li, 2011), and thermomechanical pretreatments (Pierre, Maache-Rezzoug, Sannier, Rezzoug, & Maugard, 2011; Pierre, Sannier, et al., 2011), have been explored. The dilute acid pretreatment is one of the most efficient pretreatments for lignocellulose biomass (Kim, Seo, Kim, & Han, 2015). Pretreatment with dilute acid offers the following attractive features: ease of use, a higher reaction rate and a readily produced purified product. Biochemical conversion typically consists of two key steps: pretreatment and enzymatic hydrolysis (Chundawat, Beckham, Himmel, & Dale, 2011). Accordingly, the combination of dilute acid pretreatment and enzymatic hydrolysis has been widely applied for the biochemical conversion of lignocellulosic biomass (Gao et al., 2014; Kapoor et al., 2015; Tan & Lee, 2015).

A number of previous studies have investigated the chemical and structural changes occurring in lignocellulose during dilute acid pretreatment. For example, Kumar, Mago, Balan, and Wyman (2009) pretreated corn stover with dilute acid and then measured the chemical composition and identified structural changes in the pretreated substrate using X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and X-ray Photoelectron Spectroscopy (XPS). Similarly, Li et al. (2010) dilute acid pretreated switchgrass and characterized the material using XRD, SEM, FTIR, Raman spectroscopy and chemical methods. As enzymolysis is the key step after pretreatment in the biochemical conversion of lignocellulose, it is also very important to chemically and structurally characterize an enzymatically hydrolyzed substrate. Some studies have attempted to explore such changes in lignocellulose during both acid pretreatment and enzymatic hydrolysis. For example, Hsu, Guo, Chen, and Hwang (2010) not only performed a chemical analysis on sugar and an inhibitor during the acid pretreatment and enzymatic hydrolysis of rice straw but also characterized their structural features using FTIR, XRD, and Specific Surface Area/Pore Volume (SSA/PV). It should be noted that rice straw is a challenging substrate for cellulosic

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conversion because it contains a high ash content, which can interfere with the dilute acid pretreatment and affect enzyme activity (Yu & Chen, 2010). A similar study was carried out by Chandel et al. (2014) in which several imaging techniques (SEM, Transmitted Light Microscopy (TLM), FTIR, Fourier Transform Near-Infrared (FT-NIR), Raman spectroscopy, and Nuclear Magnetic Resonance (NMR)) were used to investigate the structural changes induced during the acid pretreatment and enzymatic digestion of sugarcane bagasse.

Corn stover, which has different chemical and structural characteristics than rice straw, is a likely feedstock for cellulosic conversion in many geographic areas, whereas bagasse is generally only used in parts of the world where sugarcane is grown. In the present study, dilute acid pretreatment and enzymatic hydrolysis of corn stover were carried out, and the material was characterized using chemical and structural analyses. To our knowledge, this is the first work in the literature combining numerous chemical and structural techniques for a global comprehensive approach to reveal chemical and structural changes at the molecular level during the acid pretreatment and enzymatic digestion of corn stover.

2. Materials and methods

2.1. Feedstock and chemicals

Corn stover was collected in 2013 from the Shangzhuang agronomy farm of the China Agricultural University, located in Beijing, China. The corn stover was air dried and milled to coarse particle size (~1–2 cm). Then it was dried in a forced-air oven at 45 °C for 48 h and milled to a size less than 1 mm in a RT-34 hammer mill (Rong Tsong Precision Technology Co., Taiwan). The milled material was sieved by a JH-300A sieve shaker fitted with a 40-mesh screen (Jiahe Machinery Co., Henan province, China). The samples were stored in a sealed plastic bag at room temperature before use. All of the chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA), Beijing Chemical Works (Haidian district, Beijing, China), and J&K Scientific Ltd. (Chaoyang district, Beijing, China).

2.2. Assay methods involving enzymes

Celluclast 1.5 L (cellulase) and Novozyme 188 (β-glucosidase) were used for the enzymatic hydrolysis of corn stover. The filter paper unit (FPU) activity of cellulase was determined according to the National Renewable Energy Laboratory (NREL) Analytical Procedure (Adney & Baker, 2008). The cellobiase unit (CBU) activity of β-glucosidase was determined using the methodology published by Kim et al. (2013). The enzyme protein content was determined by the Bradford method (Bradford, 1976). The protein content of Celluclast 1.5 L and Novozyme 188 were 35.3 mg protein/mL and 27.4 mg protein/mL, respectively.

2.3. Pretreatment and enzymatic hydrolysis

Corn stover at a solid-to-liquid ratio of 1:10 was mixed with 1.5% (w/w) dilute sulfuric acid and autoclaved at 121 °C for 60 min to generate the acid-pretreated sample. The water-insoluble solid was washed thoroughly with distilled water to establish a neutral pH. Enzymatic hydrolysis of the acid-pretreated solid sample was performed in a 50 mL Erlenmeyer flask containing 2 g (dry matter) of the solid sample and 38 mL of citrate buffer (50 mM, pH 4.8). The sample in citrate buffer was supplemented with cellulase loadings (Celluclast 1.5 L: 20 FPU/g dry substrate equal to 8.41 mg protein/g dry substrate; Novozyme 188: 40 CBU/g dry substrate equal to 2.36 mg protein/g dry substrate). Enzymatic hydrolysis was allowed to proceed at 50 °C at 150 rpm in an incubator shaker (model SHA-B(A), Kexi instrument, Jiangsu province, China), and samples were collected at different hydrolysis times (0.5 h, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h).

2.4. Cellulase adsorption

Cellulase absorption on native and acid-pretreated substrates were performed in 10 mL centrifuge tubes containing 5 mL 0.05 M citrate buffer (pH 4.8) with several enzyme loadings (4.78–34.76 mg/g substrate, 2%, w/v, dry basis). The mixture of substrate and enzyme was incubated for 2 h in a shaking water bath at 100 rpm and 4 °C to avoid hydrolysis. Substrate blanks without enzyme was also run in parallel. After incubation, all the samples were centrifuged at 4 °C for 10 min in a refrigerated centrifuge at 4000 × g. The supernatant was decanted to measure the protein concentration by the Bradford method (Bradford, 1976). Bound enzyme was calculated by subtracting the free cellulase concentration from the initial enzyme concentration loaded to each reactor. Bound enzyme concentration was correlated with free enzyme concentration using the following Langmuir equilibrium isotherm:

$$E_b = \frac{E_{\max} K_a E_f}{1 + K_a E_f} \quad (1)$$

where E_b is the bound enzyme (mg/g substrate), E_f is the concentration of free enzyme in solution (mg/mL), E_{max} is the maximum adsorption capacity (mg/g substrate), and K_a is the equilibrium constant (mL/mg).

The Langmuir adsorption constants (E_{max} and K_a) on native and acid-pretreated substrates were obtained by nonlinear regression of their adsorption data. Distribution coefficient (K_r in mL/g), another constant from Langmuir adsorption isotherm, could be used to estimate the relative affinity of enzyme for substrates (Zhang & Lynd, 2004). The distribution coefficient can be calculated by $K_r = E_{max} \times K_a$.

2.5. Chemical and structural analysis

2.5.1. Chemical analysis

The carbohydrate and lignin contents of the solid samples (native and acid-pretreated substrates) were determined using the analytical procedures in NREL/TP-510-42618 (Sluiter et al., 2011). The constituent concentrations in the dilute acid and enzymatic hydrolysates were measured by high-performance liquid chromatography (HPLC) (Hitachi L-7200 with a refractive index detector L-2490, Hitachi Ltd., Tokyo, Japan). The concentrations of glucose, xylose, arabinose, and cellobiose were determined by an HPLC system equipped with a BP-800 Pb^{2+} column. The mobile phase was ultrapure water. The HPLC column was operated at 80 °C with a mobile phase flow rate of 0.6 mL/min. The injection volume was 20 μL, and the elution time per injection was 40 min. The concentrations of furfural, HMF and acetic acid were determined by an HPLC system equipped with a BP-800 H^{+} column. The mobile phase was 5 mM sulfuric acid in ultrapure water. The HPLC column was operated at 55 °C with a mobile phase flow rate of 0.6 mL/min. The injection volume was 20 μL, and the elution time per injection was 50 min. All of the samples were filtered through a 0.22-μm filter prior to analysis. The amount of total reducing sugars was estimated using a spectrophotometer (UV 2550, Shimadzu, Kyoto, Japan) following the dinitrosalicylic acid (DNS) method of Miller (Miller, 1959).

2.5.2. Structural analysis

The SEM analysis was performed using a Hitachi S-3400 scanning electron microscope (Hitachi, Tokyo, Japan). The specimens were prepared for SEM inspection by adhering each sample to carbon glue followed by plating with Pt. Representative images were acquired with an accelerating voltage of 15–20 kV.

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