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Enhanced enzymatic hydrolysis of sugarcane bagasse by *N*-methylmorpholine-*N*-oxide pretreatment

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ABSTRACT

The cellulose dissolution solvent used in Lyocell process for cellulose fiber preparation, *N*-methylmorpholine-*N*-oxide (NMMO) monohydrate, was demonstrated to be an effective agent for sugarcane bagasse pretreatment. Bagasse of 20 wt% was readily dissolved in NMMO monohydrate at 130 °C within 1 h. After dissolution, bagasse could be regenerated by rapid precipitation with water as a porous and amorphous mixture of its original components. The regenerated bagasse exhibited a significant enhancement on enzymatic hydrolysis kinetic. Not only the reducing sugars releasing rate but also hydrolysis yield was enhanced at least twofold as compared with that of untreated bagasse. The cellulose fraction of regenerated bagasse was nearly hydrolyzed to glucose after 72 h hydrolysis with Cellulase AP3. The recycled NMMO demonstrated the same performance as the fresh one on bagasse pretreatment for hydrolysis enhancement. The regenerated bagasse was directly used in simultaneous saccharification and fermentation (SSF) for ethanol production by *Zymomonas mobilis*. No negative effect on ethanol fermentation was observed and ethanol yield approximately 0.15 g ethanol/g baggasse was achieved.

1. Introduction

Lignocellulosic biomass is by far the most abundant raw material can be used for biofuels production. Compared with starchy biomass, it is considered as a quite recalcitrant material due to its highly lignified and crystalline structure. The major components in lignocellulosic biomass are cellulose, hemicellulose, and lignin. The primary technical and economic challenge for the production of biofuels from lignocellulosic biomass mainly depends on converting the complex cellulosic and hemicellulosic carbohydrates into fermentable sugars which then can be fermented to biofuels by various microorganisms. In general, pretreatment of lignocellulosic biomass is required in the bioconversion process involving enzymatic hydrolysis and fermentation. The purpose of the pretreatment is to separate lignin and hemicellulose from cellulose, reduce cellulose crystallinity, and increase the porosity of the lignocellulosic materials so that cellulose hydrolysis can be significantly improved. Many pretreatment methods have been reported and several detailed review papers have been published (Sun and Cheng, 2002; Eggeman and Elander, 2005; Mosier et al., 2005). Generally, the pretreatment processes involve employing high temperature, pressure, acids or bases, and organic solvents to disrupt the lignin seal and cellulose crystalline structure of lignocellulosic material. Most of pretreatment methods have their drawbacks in large scale application. For example, the dilute acid process generates toxic byproducts, such as furfural and aldehydes, which not only significantly reduces the sugar yield but also poisons enzymatic hydrolysis and biofuels fermentation. Steam explosion, operated at high temperature and pressure to achieve fibrillation, requires costly capital investment for equipments. Organosolv method, using organic solvents at high temperature to dissolve the lignin, requires solvent recovery and high cost of capital investment.

Recently, the pretreatment involves using cellulose dissolution reagents, such as ionic liquids (Dadi et al., 2006), concentrated phosphoric acid (81%) (Wei et al., 1996; Zhang et al., 2006, 2007), NaOH/urea solution at cold temperature (Zhao et al., 2008) have been demonstrated to be very effective for enhancing cellulose hydrolysis. The operation conditions for these pretreatments are much milder (<100 °C and atmosphere pressure) as compared to the conventional pretreatment methods. *N*-Methylmorpholine-*N*-oxide (NMMO) is used as direct solvent for cellulose in the commercially Lyocell process as a modern industrial fiber-making technology (Fink et al., 2001). Main features of Lyocell process are the direct dissolution of cellulose without chemical derivatization and the almost complete recovery of the NMMO. NMMO is able to dissolve cellulose due to the high polarity of its N–O bond, which breaks the hydrogen bond network of the cellulose and forms new hydrogen bonds with the solute. The melting point of NMMO is at 170 °C, when hydrated with one molecule water (NMMO monohydrate; water content 13.3 wt%) its melting point decreases to 74 °C. The melt of NMMO monohydrate at elevated processing

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temperature about 100 °C is usually employed to dissolve cellulose. Since NMMO is a strong oxidant, in Lyocell process antioxidants such as propyl gallate (PG) is added with NMMO to stabilize the cellulose/NMMO mixture (Rosenau et al., 2002). Lignin, one of the main components in the lignocelluloses, is an irregular, cross-linked polymeric material composed of phenylpropanoid units (Adler, 1977). It has been demonstrated to be a potential radical scavenger and antioxidant (Catignani and Carter, 1982; Pan et al., 2006), and its stabilization effect in recycled polypropylene (Gregorova et al., 2005) has also been reported. The rich content of lignin in lignocelluloses should also play the role as anti-oxidants on stabilizing the lignocelluloses/NMMO mixture, if lignocelluloses could be dissolved by NMMO monohydrate. Therefore, the feasibility of applying the readily available cellulose dissolution solvent, NMMO monohydrate, to pretreat the lignocelluloses for enzymatic hydrolysis deserves a thorough study.

In this paper, sugarcane bagasse was used as the model lignocellulosic material to be pretreated by NMMO monohydrate at various conditions. The morphology and structure of the bagasse regenerated from pretreatment mixture were studied. The effect of pretreatment on enhancing bagasse hydrolysis by cellulase was investigated. Moreover, the regenerated bagasse was used directly in the simultaneous saccharification and fermentation (SSF) operation to investigate the possible adverse effect of NMMO pretreated bagasse on ethanol fermentation using *Zymomonas mobilis*.

2. Methods

2.1. Material

Sugarcane bagasse was collected from a local sugarcane juice shop in Taiwan. The collected bagasse was thoroughly washed with distilled water to remove residual soluble sugars. The washed bagasse was extensively homogenized by using a food processor and dry at 70 °C for 2 days. The dry bagasse powder with moisture content about 11.2% was screened and particle sizes between 30-mesh and 45-mesh were used for experiments. Micro-crystalline cellulose, Avicel (0.038 mm; moisture content ~6%) was obtained from SERVA Chem Co. (Heidelberg, Germany). Cellulase AP3 (8.5 FPU/g) from *Aspergillus niger* strain was supplied by Amano Enzyme Inc. (Nagoya, Japan). *N*-Methylmorpholine-*N*-oxide (NMMO) was obtained from Sigma (St. Louis, MO). Bacterial strain *Z. mobilis* for ethanol fermentation was obtained from BCRC (HsinChu, Taiwan). All other reagents and chemicals, unless otherwise noted, were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Bagasse pretreatment and regeneration

A 5% (w/w) bagasse or Avicel solution was prepared by combining of 0.25 g substrate with 4.75 g NMMO monohydrate in a 50 ml glass vial. For all other bagasse concentrations were prepared by changing the amount of NMMO monohydrate employed. The vial and the contents were heated in an oil bath at specific temperatures for various durations. The sample was stirred by a magnetic stirred bar on a magnetic stirring hotplate. About 25 ml deionized water was rapidly added to the stirred bagasse/NMMO mixture at the end of incubation. The stirring continued until the temperature cooled to room temperature. The mixture was then filtered with filter paper to collect the precipitated Avicel or bagasse. The filtrate collected was used for further NMMO recovery. The precipitate collected on the filter paper was washed by additional 150 ml deionized water. The washed precipitate was considered as regenerated bagasse or Avicel and subjected to cellulase hydrolysis.

2.3. Enzymatic hydrolysis

The regenerated Avicel or bagasse of 250 mg (based on initial load in pretreatment) was suspended in 25 ml of pH 4.8, 50 mM sodium citrate buffer supplemented with 0.02% sodium azide. Cellulase AP3 concentration of 5 FPU/g substrate was used in the hydrolysis reaction. The hydrolysis was carried out at 37 °C under magnetic stirring and the release of soluble reducing sugars was periodically measured by the DNS assay (Miller, 1959). Glucose was used as a standard for the reducing sugar measurement. The reducing sugars content in Cellulase AP3 preparation itself was also measured as the background concentration which was subtracted from that measured during hydrolysis reaction. A conversion factor of 1.11 was used to calculate the amount of glucose released from the amount of cellulose consumed.

2.4. Simultaneous saccharification and fermentation of regenerated bagasse

Simultaneous saccharification and fermentation of the regenerated bagasse was carried out at 30 °C in a 50 ml stirred vial containing 20 ml mixture of fermentation medium, Cellulase AP3 (5 FPU/g substrate), regenerated bagasse (50 g/l), and freshly grown *Z. mobilis* (1.5 g/l; dry weight). The composition of the fermentation medium was yeast extract (2.5 g/l), $(NH_4)_2HPO_4$ (0.25 g/l), $MgSO_4 \times 7H_2O$ (0.025 g/l) in pH 5.0, 0.1 M phosphate buffer.

2.5. Analysis

Sugars content in the hydrolysates was determined by an HPLC system equipped with an evaporative light scattering detector (ELSD; Alltech ELSD 2000). A 250 mm × 4.6 mm HYPERSIL HS APS column (Thermo Instrument Systems Inc., Runcorn, UK) was employed and the mobile phase was consisted of acetonitrile and distilled water (80/20) with a flow rate of 1 ml/min. The ELSD was operated with a nitrogen gas flow rate of 2 l/min and tube temperature at 80 °C. Cellulase activity was determined by the standard filter paper assay and expressed as filter paper units per gram of glucan (FPU) (Ghose, 1987). One FPU is defined as the enzyme that releases 1 μmol of glucose equivalents per minute from Whatman No. 1 filter paper. The composition of bagasse was determined using National Renewable Energy Laboratory (NREL) protocol (Sluiter et al., 2007). Scanning electron microscopy (SEM) of bagasse was analyzed by JEOL JSM-6390LV scanning electron microscope (JEOL Ltd., Japan). Fourier transformed IR (FTIR) of bagasse was measured by BIO-RAD FTS-3500 spectrometer. The spectra (4000–400 cm^{-1}) were recorded with a resolution of 4 cm^{-1} and 64 scans per sample. About 2 mg samples were prepared by mixing with 120 mg of spectroscopic grade KBr then pressed in a standard device using a pressure of 6000 psi to produce 13 mm diameter pellets. The background spectrum of pure potassium bromide was subtracted from that of the sample spectrum.

3. Results and discussion

3.1. Hydrolysis of regenerated Avicel

Avicel, a microcrystalline cellulose, was first employed to study the effectiveness of NMMO pretreatment on enhancing cellulose hydrolysis. At temperatures operated over 100 °C, 5% Avicel cellulose could be completely dissolved in NMMO monohydrate within 1 h. The regenerated Avicel collected from rapid precipitation was subjected to cellulase hydrolysis without further drying. The resulting hydrolysis reaction curves for untreated and regenerated