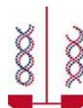

Journal of Bioscience and Bioengineering
VOL. 110 No. 4, 453–458, 2010

www.elsevier.com/locate/jbiosc

Enzymatic digestion of corncobs pretreated with low strength of sulfuric acid for bioethanol production

Prihardi Kahar,[1,*] Kazuo Taku,[1] and Shuzo Tanaka[1,2]

Asian Center for Environmental Research, Meisei University, 2-1-1 Hodokubo, Hino-shi, Tokyo 191-8506, Japan[1] *and Department of Environmental Systems, Faculty of Science and Engineering, Meisei University, 2-1-1 Hodokubo, Hino-shi, Tokyo 191-8506, Japan*[2]

Received 10 February 2010; accepted 6 May 2010
Available online 4 June 2010

In this study, the effect and the optimum pretreatment condition of corncobs using low strength of H_2SO_4 were investigated, in which H_2SO_4 was used to improve the enzymatic digestibility of corncobs for saccharification without degradation of sugars released. The optimum pretreatment condition was found to be the addition of 0.5% (vol./vol.) H_2SO_4 and autoclaving at 122 °C for 20 min. Under this condition, the structural integrity of corncob was altered to make cellulose microfibrils more accessible for cellulase enzymes, and the enzymatic digestion of corncobs could be significantly enhanced. A high yield of sugar, 80% (wt./wt.), could be obtained at a low enzyme dosage of 0.024 g enzymes/g cobs, when pretreated. As a result, the ethanol production was obviously improved by the pretreatment, i.e., the ethanol yield of 77% (wt./wt.) was obtained within 36 h in the SSF fermentation using *Saccharomyces cerevisiae* NBRC2114.

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[**Key words:** Acid pretreatment; Bioethanol; Corncobs; Enzymatic digestion; Sulfuric acid]

Biochemical conversion of lignocellulosic biomass through saccharification and fermentation is a major pathway for bioethanol production (1,2). In this approach, biomass is converted to fermentable sugars through enzymatic digestion by cellulase. The sugars are then converted to ethanol through fermentation. Lignocellulosic biomass is structurally complex, composed of crystalline cellulose (source of glucose) and amorphous hemicellulose (source of pentose such as xylose and arabinose; hexose such as glucose, galactose, and mannose) as its major sugar polymers (3,4). In the structure, hemicellulose matrices coat each fiber of cellulose microfibrils, building holocellulose structures inside, and severely protected by nonbiodegradable compounds such as lignin outside. The structures are recalcitrant to enzymatic degradation, since they rigidly pack to form a physical barrier to access holocellulose by cellulase (5). To digest them completely into sugars, a high dosage of expensive cellulase is required. Hence, the recalcitrance becomes one of the major barriers to the economical production of bioethanol from lignocellulosic biomass (6).

To overcome the problems, the pretreatment must be implemented before enzymatic digestion to alter structural integrity of biomass and also to allow cellulose microfibrils and hemicellulose matrices more accessible to cellulase for conversion to sugars (7,8). Among various techniques proposed in this direction, the chemical pretreatment using low strength of H_2SO_4 (0.1% to 1%, vol./vol.) has been commonly adopted to improve efficiently the enzymatic digestion because this method is cheap, safe (corrosion-free), easy to handle, and also feasible on a large scale (9). However, an elevated temperature (140–190 °C) is required to achieve high reaction rates and significant improvement on enzymatic digestion (10,11). Unfortunately, the formation of fermentation-inhibiting substances, such as furfural from the degradation of pentose and 5-hydroxymethyl furfural (5-HMF) from the degradation of hexose, occurs concomitantly with increasing temperature (12–14), leading to a significant reduction in total sugar recovery (15). Therefore, the formation of these substances has been recognized to prevent the chemical pretreatment from its commercial application (12).

In this study, we aimed to investigate conditions for the pretreatment of lignocellulosic biomass with low strength of H_2SO_4 to enable high digestion with a low enzyme dosage in the saccharification step. As a model, corncobs were used because they contain high amounts of hemicellulose (typically about 40%, wt./wt.). Under this condition, the strength of H_2SO_4 was evaluated at lower temperatures (105–128 °C), in the range to break the lignocellulosic structures but not to degrade them to sugars during pretreatment.

MATERIALS AND METHODS

Microorganism and chemicals *Saccharomyces cerevisiae* strain NBRC2114 obtained from NITE Biological Resource Center (NBRC), Chiba, Japan, was used for the fermentation. The yeast strain was maintained on YM agar plate containing the following (in 1 L distilled water): glucose, 10 g; bacto peptone, 5 g; bacto yeast extract, 3 g; bacto malt extract, 3 g; and bacto agar, 15 g. For fermentation, SD medium containing (in 1 L DW) yeast nitrogen (without amino acids and ammonium sulfate), 1.7 g; ammonium sulfate, 5 g; adenine sulfate, 20 mg, without carbon source was used. Here, the medium was prepared by passing the yeast nitrogen base and adenine sulfate

* Corresponding author. Tel.: +81 42 591 5615; fax: +81 42 591 5517.
E-mail address: pri@hino.meisei-u.ac.jp (P. Kahar).

1389-1723/$ - see front matter © 2010, The Society for Biotechnology, Japan. All rights reserved.
doi:10.1016/j.jbiosc.2010.05.002

solution through a sterilized membrane filter (pore size: 0.22 μm) into an autoclaved ammonium sulfate solution. The pH of the medium was adjusted to 5.0 by the addition of 5 N NaOH. All chemicals were purchased from Wako Chemicals (Tokyo, Japan).

Corncobs pretreatment Corncobs (obtained from local market in Tokyo, Japan) were sliced into small pieces and then sun-dried for 3 days before ball-milled with P-6 (Fretsch Co. Ltd, Germany). Ball-milled corncobs with a final particle size of about 5 mm were used throughout experiments. Corncobs were pretreated with H_2SO_4 solution at 0.5% and 5% (vol./vol.) and autoclaved at temperatures of 105 °C, 122 °C, and 128 °C for 20 min and 2 h, respectively. After cooling at room temperature, the pretreated corncobs were passed through the glass membrane filter to remove the H_2SO_4 solution and were then washed with 50 mM citric buffer solution (pH 5.0) to shift the substrate pH to around 5. The washed corncobs were soaked in 50 mM citric buffer (pH 5.0) before the saccharification and fermentation.

Saccharification and fermentation Saccharification by enzymatic digestion of 1, 10, 15, and 20 g-pretreated corncobs was carried out in Erlenmeyer flasks with a 100-mL working volume of 50 mM citric buffer solution (pH 5.0). The activity of Celluclast 1.5 L was 32 FPU and 16 CBU, while that of Novozyme 188 was 80 CBU per gram of enzymes. The digestion was started by adding a fixed amount of cellulase enzyme mixtures (per 1 L): 1.8 g Celluclast 1.5 L and 0.6 g Novozym 188 (obtained from Novozymes North America, Inc, NC) after preincubating at 50 °C for 30 min. The reaction was performed at 50 °C on an orbital shaker at 150 rpm for 3 days for the saccharification to completely occur. To avoid microbial contamination during saccharification, sodium azide was added at 1 mM. For further measurements, 1 mL of reaction mixture was placed in a 1.5-mL Eppendorf tube, and 10 μL of 5 N H_2SO_4 was immediately added to inactivate the cellulase enzymes.

The pretreated corncobs (10 g) were soaked in 200 mL of 50 mM citric buffer solution (pH 5.0) for 3 days and then used as a substrate for fermentation in an anaerobic 2-L jar fermentor (BEM Marubishi, Tokyo, Japan). For seed preparation, one single colony of NBRC2114 was used to inoculate 500 mL of baffled flask containing 100 mL of YM liquid medium after autoclaving at 122 °C for 20 min and was then aerobically cultured at 30 °C overnight. A 100-mL seed and 200-mL pretreated corncobs mixture was mixed with 700 mL of SD medium in jar fermentor. The fermentation was then started by the addition of cellulase enzyme mixtures. The solution of 5 N NaOH was used to maintain the pH culture at around 5. To keep the anaerobic condition at initial fermentation time, nitrogen gas was flowed through the sterilized membrane filter into the reactor.

Enzyme assay Filter paper activity of Celluclast 1.5 L and cellobiase activity of Novozyme 188 were determined according to the standard procedure recommended by the Commission on Biotechnology, IUPAC (16) and were expressed in filter paper units (FPUs) and cellobiose unit (CBU), respectively.

Avicelase activity of exoglucanase in the reaction mixtures was assayed by measuring the avicel-degrading rate from the reaction mixture. The sample (500 μL) was incubated for 30 min at 50 °C with 1% (wt./vol.) avicel (PH-101, Fluka, Ireland) in 3 mL of citric buffer (50 mM, pH 5.0). To inactivate the enzymes, the reaction mixture was immediately boiled for 5 min, and then residual avicel was removed by centrifuging at 6000 × g for 1 min. The amount of reducing sugar released was determined by high-performance liquid chromatography (HPLC). One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of glucose per minute. Protein concentrations were determined by the method of Bradford, with bovine serum albumin as a standard.

Analytical techniques The fermentable sugars containing major monosaccharides, such as glucose, xylose, and arabinose in the samples, were analyzed by HPLC fitted with a refractive index detector (RID-10A; Shimadzu, Tokyo). The column of ICSepICE-COREGEL-87H was used at a flow rate of 0.6 mL/min, with the elution program consisting of a isocratic elution with 5 mM H_2SO_4 buffer at 80 °C for 25 min per sample.

The amounts of aldehyde compounds such as furfural and 5-HMF in the samples were measured by HPLC fitted with UV–VIS detector (SPD-10A; Shimadzu). The column of INERTSIL ODS-3V (GL Sciences, Tokyo) was used at a flow rate of 0.5 mL/min, with the elution program consisting of a gradient elution with 20% (vol./vol.) of 50 mM phosphate buffer (pH 3.0) and 80% (vol./vol.) of acetonitrile at 25 °C for 20 min per sample. The same method was used for analyzing the phenol compounds such as 4-hydroxymethyl butyrate (4-HB) and vanillin.

The electron microscopic observation of pretreated corncobs was performed with FE-SEM (Hitachi S-4700 Type II; Hitachi, Tokyo) after the dried samples were placed on a conductive carbon tape and coated with Pt–Pd using a sputter coater (Hitachi E102 Ion Sputter; Hitachi) for 2 min at DC ± 20 mA.

RESULTS AND DISCUSSION

Effect of H_2SO_4 pretreatment on enzymatic digestibility of corncobs To study the effect of pretreatment on the enzymatic digestibility of corncobs, the pretreatment was conducted by

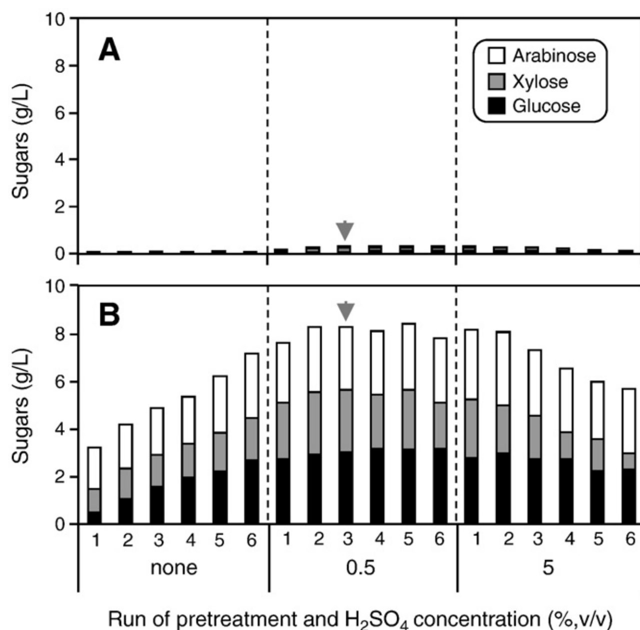

FIG. 1. Amount of sugars released during corncobs pretreatment without (A) and with digestion by cellulase enzyme mixtures consisting of 1.8 g Celluclast 1.5 L and 0.6 g Novozym 188 in 1-L corncobs suspension (B). Autoclaving conditions: run 1, 105 °C for 20 min; run 2, 105 °C for 2 h; run 3, 122 °C for 20 min; run 4, 122 °C for 2 h; run 5, 128 °C for 20 min; run 6, 128 °C for 2 h.

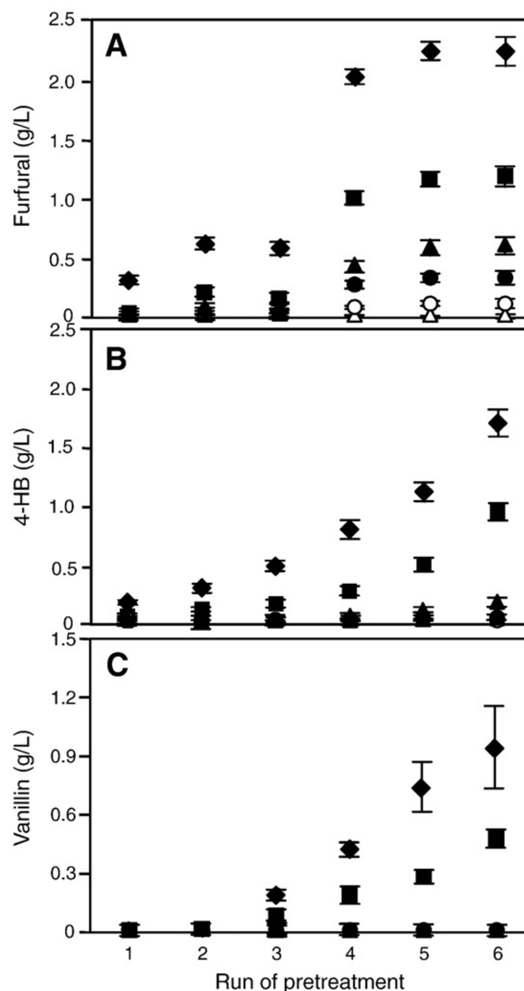

FIG. 2. Formations of furfural (A), 4-HB (B), and vanillin (C) during pretreatment of corncobs. Data were calculated from triplicate experiments. H_2SO_4 pretreatments were performed at concentration (%, vol./vol.) of 0 (open triangles), 0.1 (open cycles), 0.5 (closed cycles), 1 (closed triangles), 2 (closed squares), and 5 (closed diamonds) under the same autoclave condition as Fig. 1.

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