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Ethanol Production from H_2SO_3-Steam-Pretreated Fresh Sweet Sorghum Stem by Simultaneous Saccharification and Fermentation

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Abstract The present work presents an alternative approach to ethanol production from sweet sorghum: without detoxification, acid-impregnated fresh sweet sorghum stem which contains soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose) was steam pretreated under mild temperature of 100 °C. Simultaneous saccharification and fermentation experiments were performed on the pretreated slurries using *Saccharomyces cerevisiae*. Experimentally, ground fresh sweet sorghum stem was combined with H_2SO_3 at dosages of 0.25, 0.50, and 0.75 g/g dry matter (DM) and steam pretreated by varying the residence time (60, 120, or 240 min). According to enzymatic hydrolysis results and ethanol yields, H_2SO_3 was a powerful and mild acid for improving enzymatic digestibility of sorghum stem. At a solid loading of 10% (*w*/*v*) and acid dosage of 0.25 g/g DM H_2SO_3 at 100 °C for 120 min, 44.5 g/L ethanol was obtained after 48±4 h of simultaneous saccharification and fermentation. This corresponded to an overall ethanol yield of 110% of the theoretical one, based on the soluble carbohydrates in the fresh sweet sorghum stem. The concentrations of hydroxymethylfurfural and furfural of the sulfurous acid pretreated samples were below 0.4 g/L. Ethanol would not inhibit the cellulase activity, at least under the concentration of 34 g/L.

Keywords Ethanol · Steam pretreatment · Simultaneous saccharification and fermentation · SSF · Sweet sorghum · Sulfurous acid (H_2SO_3)

Introduction

Sweet sorghum has the potential of becoming a useful energy crop [1, 2]. It has been evaluated in several recent reports as an alcohol fuel crop with a promising future. The primary advantages of sweet sorghum are: (1) its high ethanol productivity, 3,500 l/ha to 5,600 l/ha per year; (2) its adaptability to diverse climate zones and soil conditions; and (3)

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its reduced need for nitrogen fertilizer and water when compared with more conventional crops such as corn.

Sweet sorghum is a high biomass and sugar yielding crop [3]. It contains approximately equal quantities of soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose) [4], and has been considered as an important source for the production of fuel ethanol. While the soluble carbohydrates are easily converted to ethanol, process economy calls for the utilization of the insoluble ones.

Liu et al. investigated the fermentation of stem juice, which contained most of the soluble carbohydrates, using immobilized yeast cells [5]. The residual bagasse was hydrolyzed with acid or enzyme to soluble oligosaccharides followed by ethanol fermentation [6]. It was also reported that one or more microorganisms carry out simultaneous hydrolysis and fermentation of the total carbohydrates to ethanol in the same bioreactor [7]. This approach achieved 68.6% of the theoretical yield based on total polysaccharides and exceeded that based on oligosaccharides of sorghum stem by 53.7%. However, it took almost 6 days to complete the fermentation and the process was relatively complex.

To fully and effectively utilize fresh sweet sorghum stem as a feedstock for ethanol production, optimal pretreatment is required to render the cellulose fibers more amenable to the action of hydrolytic enzymes. A variety of physical (comminution, hydrothermolysis), chemical (acid, alkali, solvents, ozone), physico-chemical (steam explosion, ammonia fiber explosion), and biological pretreatment techniques have been developed to improve the accessibility of enzymes to cellulosic fibers [8]. Acid pretreatment involves the use of sulfuric, phosphoric acid, or hydrochloric acids to remove hemicellulose components and expose cellulose for enzymatic digestion [9–10]. Agricultural residues such as corncobs and stovers have been found to be particularly well-suited to dilute acid pretreatment [11].

This study investigated sulphurous acid pretreatment and enzymatic treatment of fresh sorghum stem to improve ethanol yields and productivity rates. It was therefore initiated to: (1) investigate the effect of sulfurous acid on fresh sweet sorghum stem, (2) identify pretreatment parameters which provide the highest cellulose to glucose conversion during subsequent enzymatic hydrolysis and ethanol yield, and (3) optimize the factors for high ethanol yield including solid loading, enzyme loading, and the time for enzyme adding. It aims to cut down the long fermentation period and maintain the high ethanol yield.

Materials and Methods

Biomass

Sweet sorghum “Rio” harvested in October 2006 was kindly provided by Prof. Wang (Chinese Academy of Agriculture Engineering, China). Leaves and husks were stripped from the fresh stem by hand. The stem was cut to pass a 1 mm sieve using a speed disintegrator which was bought in supermarket and stored in sealed plastic bags at −20 °C until use. The composition of carbohydrates in the fresh stem was (in g/100 fresh stem): glucose, 3.0; fructose, 4.5; sucrose, 8.0; glucan, 4.6; and xylan, 2.6.

Analysis

Dry-matter content was determined by drying samples in an oven at 80 °C until constant weight was obtained. Reducing sugars were determined using the 3,5-dinitrosalicylic acid

method [12, 13], which were given as glucose equivalents. The solutions after the pretreatment steps and all samples from the acid and the enzymatic hydrolysis, fermentation, and simultaneous saccharification and fermentation (SSF) were analyzed by high performance liquid chromatography (HPLC) using a chromatograph equipped with a refractive index detector. Glucose, mannose, arabinose, galactose, and xylose were analyzed with a Shimadzu LC-10AD chromatograph (Shimadzu Corporation, Kyoto, Japan) using an Aminex HPX-87P column (Bio-Rad, Hercules, USA) at 80 °C, using water as eluent, at a flow rate of 0.5 ml min^{-1}. Cellobiose, glucose, arabinose, lactic acid, glycerol, acetic acid, ethanol, hydroxymethylfurfural (HMF), and furfural were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, USA) at 65 °C using 5 mmol l^{-1} H_2SO_4 as the eluent, at a flow rate of 0.5 ml min^{-1}. All samples were filtered through a 0.20 μm filter before HPLC analysis.

Pretreatments

Pretreatments were performed by H_2SO_3 (0.25, 0.50, and 0.75 g/g dry matter (DM)) at a solid loading of 10% (*w*/*v*). Treatments were performed in triplicate at 100 °C for residence time of 60, 120, and 240 min. Representative samples of the pretreated slurries were withdrawn and analyzed. The samples were separated by filtration into a solid residue and a liquid. The liquid fraction was analyzed for soluble sugars and degradation products including glucose, fructose, xylose, lactic acid, glycerol, acetic acid, ethanol, HMF, and furfural.

Electron Microscopic Scanning

The sorghum stem was studied with an electron microscope. The samples were soaked in 3.5% (*v*/*v*) glutaraldehyde for 6 h, and dried by treatment with 50, 70, 90, 95, and 100% (*v*/*v*) ethanol, followed by overnight retention of the samples in a desiccator for the removal of moisture.

Microorganism and Inoculum Cultivation

The laboratory mutant strain of *Saccharomyces cerevisiae* AF37X was used throughout the experiments. The yeast strain was maintained in MY medium whose composition (in g/L) was: glucose, 20; yeast extract, 3; polypeptone, 5; malt extract, 3; agar, 20. In all cases, cultures were maintained at 37 °C for 24 h and then stored at 4 °C. Subculturing was done every 2 months. The composition of the preculture medium for yeast (in g l^{-1}) was: glucose, 10; sucrose, 10; yeast extract, 3; polypeptone, 5; malt extract, 3. All the media were adjusted to pH 6.5 and autoclaved at 116 °C for 20 min before use. Two loops from yeast slants were used to inoculate 100 ml of the preculture medium in 250 ml Erlenmeyer flasks and cultivated on a rotary shaker (180 rpm) at 37 °C for 20 h.

Simultaneous Saccharification and Fermentation

The SSF experiments were performed with unwashed slurry of pretreated fresh sorghum stem in 250-mL flasks. The pH of the slurries was neutralized with solutions of NaOH to 7. No sterilization and additional nutrients were employed. A commercial cellulase mixture (200 FPU/mL), supplied by China Textile Research Institute, was employed with the loading of 60 FPU/g DM in SSF. Filter paper activity was measured according to the

method described by Ghose [14]. The yeast cell suspension was added to the flasks to a concentration of 5 g dry yeast cells/L. The SSF experiments were cultivated on a rotary shaker (180 rpm) at 37 °C. Samples were withdrawn at frequent intervals during the entire experiment, and analyzed for ethanol, sugars, glycerol, acetic acid, and lactic acid.

Results and Discussion

Effect of Pretreatments

Electron Microscopic Scanning

In naturally occurring cellulosic substrates, carbohydrate-rich macrofibrils are surrounded by a lignin seal forming a complex structural matrix that is resistant to enzymatic attack [15]. Principal substrate factors which have been correlated with pretreatment effectiveness include cellulose pore volume, hemicellulose and lignin removal [16], and cellulose crystallinity [17].

The structure of the untreated sorghum stem was shown in Fig. 1a, which was very compact. The cellulose was surrounded by lignin so tightly that no free cellulose can be seen. After the pretreatment, acids broke the matrix structure of the lignocellulose into more accessible cellulose, which was demonstrated by the scanning electronic microscope Fig. 1b. After acid pretreatment, the compact sorghum stem was destroyed and turned into fibers, which were separated from each other.

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed on unwashed slurry of pretreated fresh sorghum stem to assess the effect of pretreatment and to determine the potential sugar yield.

All hydrolysis experiments were carried out at 37 °C for 3 days in 250-mL flasks and cultivated on a rotary shaker (180 rpm). In each flask 10 g DM (based on the original stem) of pretreated material, 3 ml cellulase, and 0.1 mol/L sodium acetate buffer (pH 5) were added to a total volume of 100 ml. The sodium acetate buffer was not used in SSF.

Dilute acid pretreatment of lignocellulosic biomass is one of the most effective pretreatment methods which predominantly affect hemicellulose. The pretreatment stage is

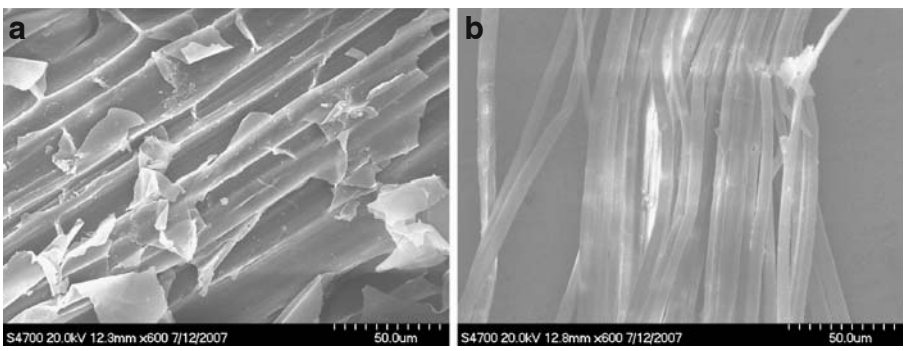

Fig. 1 Scanning electron microscopy of unpretreated and acid pretreated sorghum stem **a** unpretreated, **b** pretreated with H_2SO_3 with a concentration of 0.25 g/g DM by 120 min

Table 1 Total reducing sugar concentrations from the sulfurous acid pretreatments.

Time (min)	Sulfurous acid concentration (g/g DM)		
	0.25	0.50	0.75
60	89.1	83.5	79.9
120	94.2	91.1	83.6
240	95.7	95.9	86.2

evaluated by the total amount of reducing sugars existed in the liquid phase after enzymatic hydrolysis and the ethanol yield in SSF. The total reducing sugars existed after the enzymatic hydrolysis were shown in Table 1. During the pretreatment of H_2SO_3, the reducing sugar content was increased from 89.1 g/L (60 min, 0.25 g/g DM) to 95.7 g/L (240 min, 0.25 g/g DM) and 79.9 g/L (60 min, 0.75 g/g DM) to 86.2 g/L (240 min, 0.75 g/g DM). Higher dosage of H_2SO_3 leaded to lower total reducing sugar contents but higher amount of by-products.

Composition of Sugars and Inhibitors

Sugar degradation products, such as HMF, furfural, levulinic acid and formic acid, together with phenolic compounds derived from degraded soluble lignin, are formed during steam pretreatment. These are examples of compounds that are inhibitory to various degrees in the subsequent SSF step [18–21]. They are analyzed as a measure of the quantity of by-products produced, as elevated concentrations of these compounds indicate the presence of other compounds, which may be inhibitory in the succeeding process steps. The sugar composition and by-products of the typical samples, which were pretreated with an acid dosage of 0.25 g/g DM, are illustrated in Figs. 2 and 3.

The initial concentrations of HMF, furfural and acetic acid in the SSF experiments at typical conditions are shown in Fig. 3. The concentrations of HMF and furfural of the three H_2SO_3 pretreated samples were below 0.4 g/L. For the concentration of acetic acid, there were no major differences. Larsson et al. [22] showed that low concentrations of acetic acid, formic acid, and levulinic acid (for acetic acid below approximately 6 g/L) favor ethanol production from softwood.

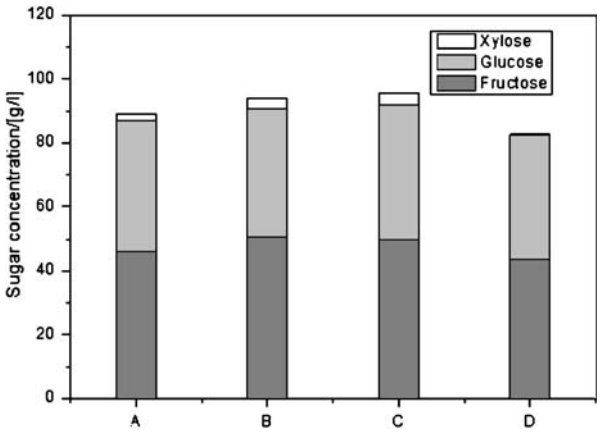

Fig. 2 Concentration of fructose, glucose and xylose in the liquid fraction after enzymatic hydrolysis. (Pretreatment time: *A* 60 min, *B* 120, *C* 240, *D* without pretreatment)

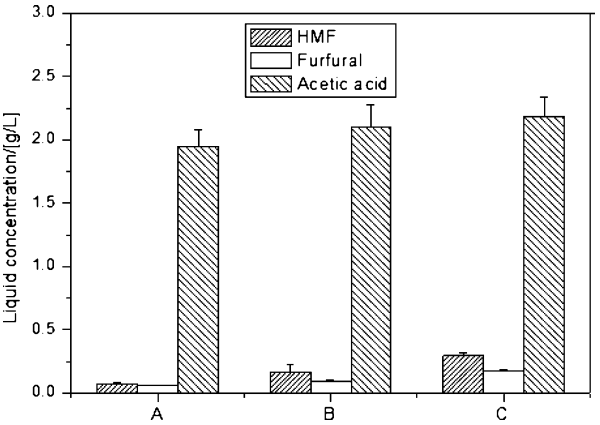

Fig. 3 Concentration of HMF, furfural and acetic acid in the liquid fraction after pretreatment. (Pretreatment time: *A* 60 min, *B* 120, *C* 240)

Effect of Pretreatment on Ethanol Yield

Based on the amount of soluble carbohydrates contained in the initial stem, the ethanol concentration was 37.7 g/L (or 7.6 g/100 g fresh sorghum stem), which was 93% of the theoretical yield (40.5 g/L or 8.1 g/100 g fresh sorghum stem). The use of H_2SO_3 as an acid catalyst in the pretreatment step resulted in the substrate giving the highest ethanol concentrations of 44.5 g/L (9.0 g/100 g fresh sorghum stem), which exceeded the theoretical value by 10% (Fig. 4). It can, not only promote the enzymatic hydrolysis with little by-products, but also provide anaerobic conditions to enhance the ethanol fermentation. On the other hand, superfluous sulfurous acid had inhibitory effects on yeast cells. As shown in Fig. 4, ethanol concentrations increased as the pretreatment time prolonged, and decreased as the H_2SO_3 concentration increased. These results suggest that H_2SO_3 can be used as pretreatment catalyst to improve the enzymatic hydrolysis of sorghum stem. Optimal conditions for hydrolysis of sorghum stem were 0.25 g/g DM H_2SO_3 at 100 °C for 120 min, which yielded a solution with 45.5 g ethanol/L.

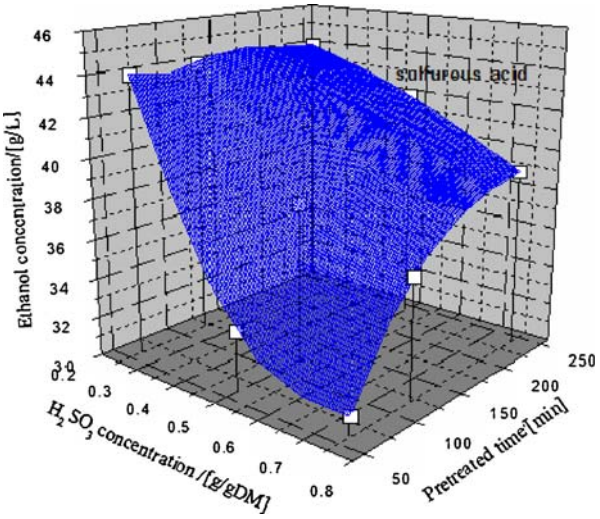

Fig. 4 Ethanol concentrations of the sulfurous acid pretreated fresh sorghum stem after SSF

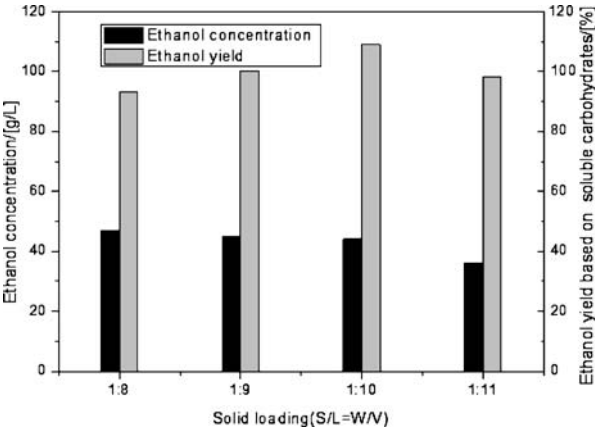

Fig. 5 Effect of solid loading on ethanol concentration and ethanol yield based on soluble carbohydrates

Simultaneous Saccharification and Fermentation

During fermentation of pretreated fresh sorghum stem by SSF, the process was first optimized with regard to the solid loading. The results (Fig. 5) showed that ethanol concentration and yield were inversely related. Thus, at the loading of 1:10, an optimum ethanol yield of 108.9% based on soluble carbohydrates, corresponding to 8.82 g ethanol/100 g fresh sorghum stem was obtained. It is worthy of noting that as the solid loading dropped from 1:8 to 1:10, the fermentation system changed from solid (without free water) to liquid and the cellulase can travel more freely which leads to catalyze more effectively. On the other hand, too much liquid (water) also can reduce the cellulase activities. When the loading reached 1:11, the ethanol yield dropped to 98.2%. So solid loading of 1:10 was optimum in this fermentation system.

Soluble carbohydrates as the products of cellulase can inversely inhibit the hydrolytic activity of cellulase. Meanwhile, Ghosh et al. showed that hydrolytic activity of *Trichoderma reese QM9414* cellulase was inhibited by ethanol concentrations above 0.75% (*v*/*v*) [23]. Consequently, the same amount of enzyme was delivered at the fermentation time of 0 h (α) and 12 h (β), when the ethanol concentrations were 0 and 34.2 g/L. For comparison, no enzyme was added for the control (γ).

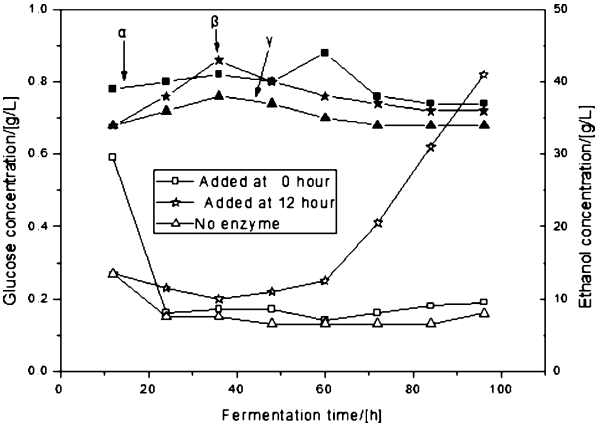

Fig. 6 Effect of cellulase delivering time on ethanol production. Enzyme was delivered at the fermentation time of 0 h (glucose (*unfilled square*), ethanol (*filled square*)) and 12 h (glucose (*unfilled star*), ethanol (*filled star*)). For comparison, no enzyme was delivered (glucose (*unfilled triangle*), ethanol (*filled triangle*))

As seen in Fig. 6, because of the cellulase, the ethanol concentration of α at 12 h was 38.1 g/L, which was 11.2% higher than that of β and γ. Meanwhile, the concentration of residual glucose was also 2.14 times higher. After cellulase was delivered at 12 h, the ethanol concentration of β increased sharply to 44.0 g/L which was the same as that of α and 10% higher than that of γ. The residual glucose of β increased steeply after 60 h, but the ethanol concentration decreased slowly, the trend of which was the same as α and γ.

Though the cellulase delivering time had influence on residual glucose, no difference of maximum ethanol yield was observed between α and β. So, ethanol would not inhibit this cellulase activity, at least under the concentration of 34 g/L.

Conclusion

Steam pretreatment of H_2SO_3-impregnated fresh sweet sorghum stem resulted in some degree of lignocellulose destroying. Meanwhile, the enzymatic hydrolysis and the SSF experiments showed that a high overall reducing sugar yield (123.4%) and ethanol yield (110%) based on soluble oligosaccharides were obtained from fresh sweet sorghum stem which were pretreated at a solid loading of 10% (*w*/*v*), acid dosage of 0.25 g/g DM H_2SO_3, and at 100 °C for 120 min. Under such mild conditions, the fermentation cycle was decrease to 50 h without removing the by-products, such as HMF and furfural. Ethanol wouldn't inhibit this cellulase activity, at least under the concentration of 34 g/L. Thus, the cellulase can be delivered with inoculum together. A higher ethanol concentration was obtained after SSF which decrease the energy demand and water consumption in the downstream processing.

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