EFFECT OF SULFITE PRETREATMENT TO OVERCOME THE RECALCITRANCE OF LIGNIN (SPORL) ON ENZYMATIC SACCHARIFICATION OF CORN STALK

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In order to maximize the glucose yield in the subsequent enzymatic hydrolysis, corn stalk was pretreated with sulfite to overcome the recalcitrance of lignocellulose (SPORL) under different operational conditions (pretreatment temperature, bisulfite charge, and pH). The parameters optimized included sodium bisulfite charge from 0 to 7% (w/w) on od dry (od) substrate, pretreatment temperature 160 to 190 °C, and pH 2.2 to 6.7 required in the process. The results indicated that after the SPORL pretreatment of corn stalk with 7% bisulfite at 180 °C for 30 min, about 69% and 62% of enzymatic conversion efficiency and glucose yield were achieved, respectively, with emzyme loading of about 5 FPU cellulase per gram of cellulose plus β-glucosidase after 72 h hydrolysis. Temperature had a positive effect on enzymatic hydrolysis. The enzymatic conversion efficiency was reached 81.04% with 7% sodium bisulfite at 190 °C for 30 min. The pH of pretreatment liquor plays a crucial role in enhancing enzymatic digestibility of SPORL substrate.

Keywords: Corn stalk; SPORL; Pretreatment; Enzymatic saccharification/hydrolysis; Sodium bisulfite

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INTRODUCTION

Lignocellulose is produced in enormous quantities by photosynthesis in nature, and it is characterized by its abundance, renewability, and low cost of production (Alvira *et al*. 2010). Conversion of lignocellulose to biofuels has drawn much attention due to the global energy crisis and environmental pollution. Ethanol is one of the most attractive biofuels, and it can be produced from lignocellulose via a sugar platform consisting of three consecutive steps: pretreatment, saccharification (enzymatic hydrolysis), and fermentation (Sun et al. 2002; Wang et al. 2011; Mosier et al. 2005). However, high polymerization of cellulose and lignin existence seriously inhibits enzymatic hydrolysis (Hendriks et al. 2009; Alvira et al. 2010). To address this problem, many pretreatment technologies such as alkaline, lime, dilute acid, hot water, ammonia, steam explosion, and organosolv pretreatments have been proposed (McMillan, 1996; Sun et al. 2002; Mosier et al. 2005; Pan et al. 2005). However, some critical issues have remained unresolved, such as low conversion efficiency of enzymatic hydrolysis except for the organosolv process, intensive energy consumption, and high cost (Silverstein et al. 2007; Kim et al.

2005; Zhu et al. 2009). SPORL could help in this respect by alleviating lignocellulose recalcitrance. It has a good adaptability to lignocellulosic biomass materials including spruce, pine, eucalyptus, poplar and wheat straw, where the enzymatic conversion rate can reach more than 90% (Wang et al. 2009; Zhu et al. 2009; Tian et al. 2010; Zhu, et al. 2011; Yang, et al. 2011).

In this paper, corn stalk was pretreated by SPORL, and its effect on enzymatic saccharification was investigated.

EXPERIMENTAL

Materials

Corn stalk was kindly provided by a farm located in the Northeast of China after harvest. The leaves and husks were striped from the stalks, and the stalks were air-dried and then maintained at room temperature. Before pretreatment, the stalks were chopped and then screened to obtain particle in the size range between 2 and 3 cm in length. The chopped corn stalk was washed thoroughly with water to remove some adsorbent dust, then filtered and air-dried. The sample was stored in a plastic bag and kept at room temperature for use in all tests, and the moisture content was determined.

Commercial enzymes, cellulase NS50013 and β-glucosidase NS50010 with an activity of 70 FPU and 250 CBU per gram were provided by Novozymes (China) Investment Co., Ltd.

All chemical reagents including tetracycline hydrochloride, sodium bisulfite, phloroglucinol, acetic acid, sulfuric acid, and hydrochloric acid were of analytical grade.

Sulfite Pretreatment

The moisture content in the raw feedstock was 8.31% (w/w). The pretreatment liquid was prepared by mixing sodium bisulfite with distilled water according to experimental requirements (if necessary, add sulfuric acid or NaOH to adjust pH value). The standard industry practices for preparing sulfite pulping liquor using sulfur and counter-ion oxide (Bryce et al. 1980) can be readily adapted and applied to the SPORL.

The pretreatment was performed in sealed stainless steel 1.5 L pressure vessels using an air bath digester (manufactured in TaiXing, China), where 100 g (by oven dry weight) of corn stalk fractions were immersed in 400 mL of pretreatment liquid and mixed for some time before raising the temperature. These 1.5 L vessels were mounted inside of the larger pressure vessel and heated via the external steam bath while rotating at a certain speed to provide mixing during pretreatments. After preparation, the system was warmed up to the predetermined temperature in about 150 min and maintained for additional 30 min. This procedure was performed at different temperatures and pretreatment liquid compositions. After reaction, the pretreatment spent liquor was separated from the residual solid material (cellulose pulp) by fitration and collected for analysis. The pretreated solid was then directly transferred to disk refiners for size reduction under atmospheric conditions with washing until the final pH value was neutral, and then collected after filtering in 100% polyester cloth. Substrate yield was determined from the measured solid wet weight and moisture content of the washed

substrate. The pretreated solid (substrate) from filtration and certain drying were kept at 4 ^{o}C for the subsequent chemical analysis and enzymatic saccharification.

Enzymatic Saccharification

Commercial cellulase and β-glucosidase preparations were used as received. Enzymatic hydrolysis of pretreated substrate was conducted at 50 ^{o}C in a rotary shaker incubator (Tianjin Uno Instruments, Model HNY 200) at 200 rpm in a 100 mL Erlenmeyer flask with rubber stoppers on the top. SPORL-pretreated substrate equivalent to 2 g dry weight was loaded into the flask with 50 mL of 0.1 M sodium acetate buffer for maintaining the pH (4.8) in the reaction. 1.4 mL diluted cellulase with equivalent activity loading of 5 FPU cellulase per gram of cellulose based on the measured glucan contents of the substrates was used for enzymatic hydrolysis. Cellulase was supplemented with β-glucosidase to avoid product inhibition caused by cellobiose accumulation (the dosage of β-glucosidase was 10% of the volumetric cellulase addition, equivalent to 1.75 CBU/g cellulose). Approximately 0.4 mL tetracycline hydrochloride solution with a concentration of 10 g/L was added to control the growth of microorganisms and prevent consumption of liberated sugars during saccharification. Hydrolysates were sampled periodically for glucose analysis, and the hydrolysis process was terminated by heating the tube in a boiling water bath for 5 min. All experiments were performed in duplicate, and the results presented here are an average of two replications.

Analytical Methods

The carbohydrate and lignin contents in the original materials and SPORL-pretreated substrates were determined using concentrated acid hydrolysis (with addition of 72% H_2SO_4) at room temperature followed by dilute acid hydrolysis at 121 ^{o}C to hydrolyse the cellulose and hemicellulose, according to the analytical procedure recommended by NREL (Sluiter et al. 2004, 2008). The remaining acid-insoluble (Al) lignin was weighed, and the concentration of acid-soluble (AS) lignin was determined by UV spectrophotometer from the adsorption at 205 nm against a 4% H_2SO_4 blank (Dence, 1992). Cellulose and hemicellulose content were represented by glucan and pentosan.

For quick analysis in the enzymatic saccharification experiments, glucose was determined using a commercial biochemical analyzer (SBA-40D D-glucose assay tester). The instrument has an auto-calibration procedure that calibrates the system every ten tests, and its precision is about 2% based on manufacturer specifications. The samples were diluted with deionized water, and injected into the analyzer. The injection volume was 20 μL. The yield of glucose was obtained from the glucose concentration in the enzymatic hydrolysate, and the enzymatic conversion efficiency was calculated based on the above experimental analysis. Cellulose conversion refers to enzymatic hydrolysis of cellulose to glucose in a substrate, whether chemically pretreated or not.

The concentration of hemicelluloses hydrolyzate (the major components was pentosan) in the pretreatment spent liquor were analyzed by UV spectrophotometer at 553 nm using an external standard (Yu et al. 2007). The effluent was centrifuged to obtain the supernatant, which was used as analysis samples after dilution. Approximately 1 mL samples were measured into 25 mL tubes, and 10 mL colorimetric reagent was added. After mixing, the tubes were submerged in boiling water for 10 min and finally

diluted with 14 mL ultra-pure water and then analyzed by UV spectrophotometer at 553 nm. Each data point was averaged from two replicates.

RESULTS AND DISCUSSION

In order to evaluate the effects of the pretreatment process on subsequent enzymatic hydrolysis, the solids after pretreatment were hydrolyzed by cellulase of 5 FPU/g substrate with β-glucosidase supplementation for 72 h.

Effect of Bisulfite Charge on Enzymatic Saccharification

The effect of sodium bisulfite charge on enzymatic conversion efficiency and glucose yield are shown in Fig.1. At the pretreatment temperature of 180 ^{o}C and retention time 30 min, there was an optimal bisulfite charge about 7%. At this loading, substrate enzymatic conversion efficiency and glucose yield were about 69.4% and 62.44% through 72 h hydrolysis, respectively. On the other hand, when the loading was reduced to zero, the enzymatic conversion efficiency and glucose yield were the lowest, less than 50.74% and 45.85%, respectively. This suggests that increasing bisulfite charge has a significant effect for the enzymatic digestibility of a SPORL substrate.

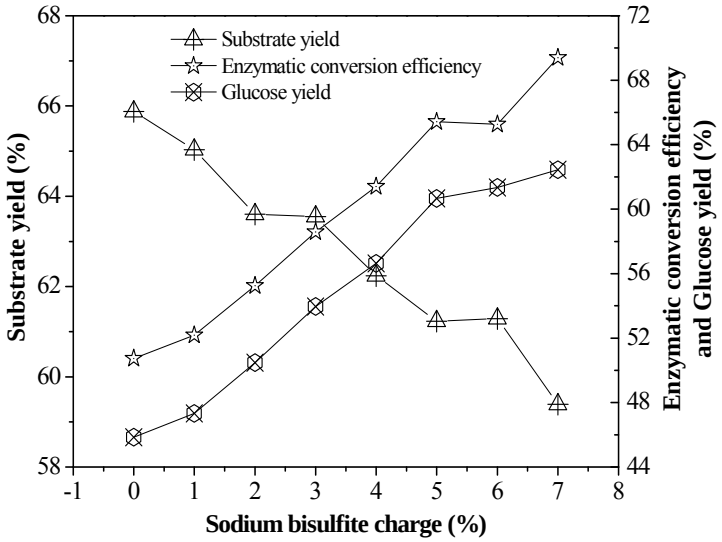

Fig. 1. Effect of bisulfite charge on enzymatic conversion efficiency, the yield of glucose, and the yield of substrate

The concentration of acid-soluble lignin and hemicelluloses hydrolysates (calculated from the amount of the dissolved lignin and pentosan in the SPORL liquor estimated by an UV method) were measured to further analyze the effect of SPORL on enzymatic digestibility over the course of bioconversion. As shown in Fig. 2, the reduction of lignin, based on a comparison between the concentration of acid-soluble lignin in the initial sample with zero bisufite charge pretreatment and the concentration of acid-soluble lignin in the sample with 7% loading pretreatment, ranged from 7.636% to 18.783%.

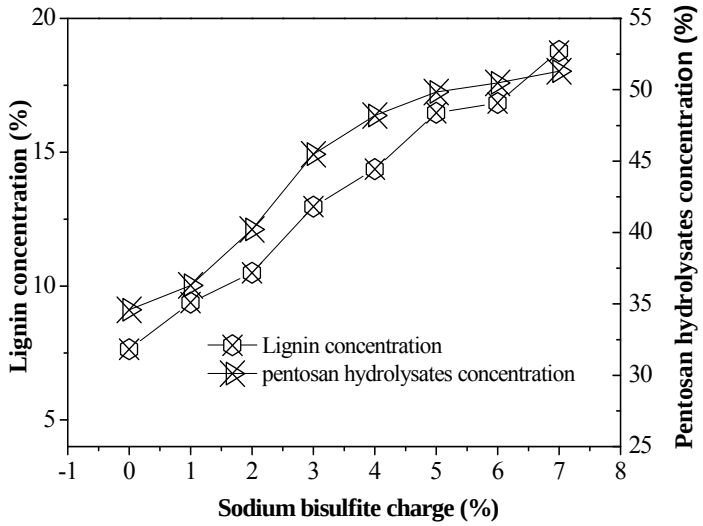

Fig. 2. Effect of bisulfite charge on concentration of lignin and pentosan hydrolysates in liquid phase

The results indicated that the addition of bisulfite resulted in dissolution of a portion of the lignin. The probably reason is that sodium bisulfite introduced sulfonic groups at the lignin benzylic carbons, which may (1) partially depolymerize and dissolve the lignin and (2) increase the hydrophilicity of the residual lignin that is retained in the pretreated substrate. The partial delignification and sulfonation of lignin may contribute to the improved digestibility of the SPORL substrate.

The pentosan content, which makes up the largest portion of hemicelluloses in the corn stalk, is the most important with respect to the effectiveness of pretreatment. Bisulfite pretreatment effectively solubilized 34.57 g/L (54.48%) of the pentosan for the least severe pretreatment (0%, 30 min, 180 ℃) and 51.30 g/L (80.85%) for the most severe treatment (7%, 30 min, 180 ℃) (Fig. 2). This indicated that an increase in the concentration of sodium bisulfite significantly improved solubilization of pentosan.

The degradation of hemicelluloses, coupled with solubilization of lignin created favorable conditions for enzymatic hydrolysis, and substantial amounts of cellulose molecules were exposed on the substrate surface, which allows the enzymes greater access so that they can become adsorbed to the cellulose and then accelerate the hydrolysis reaction. This is reflected in the relative high (ca. 70%) enzymatic conversion efficiency during enzymatic hydrolysis of the pretreated corn stalk despite the low enzyme dosage employed.

Effect of Pretreatment Temperature on Enzymatic Saccharification

The effect of temperature (160 to 190 ^{o}C) on enzymatic hydrolysis of SPORL-pretreated corn stalk was investigated. Figure 3 shows the release of glucose and enzymatic conversion efficiency after 72 h for various temperatures. Glucose yield as well as enzymatic conversion efficiency increased with the increase of pretreatment temperature. The glucose yield increased from 26.79% to 71.91% (168.42% increase) and the enzymatic conversion efficiency increased from 30.18% to 81.04% (168.52%

increase), when the temperature increased from 160 °C to 190 °C. The results clearly showed that temperature had a pronounced individual effect on the hydrolysis rate. The higher the pretreatment temperature was, the more glucose was produced.

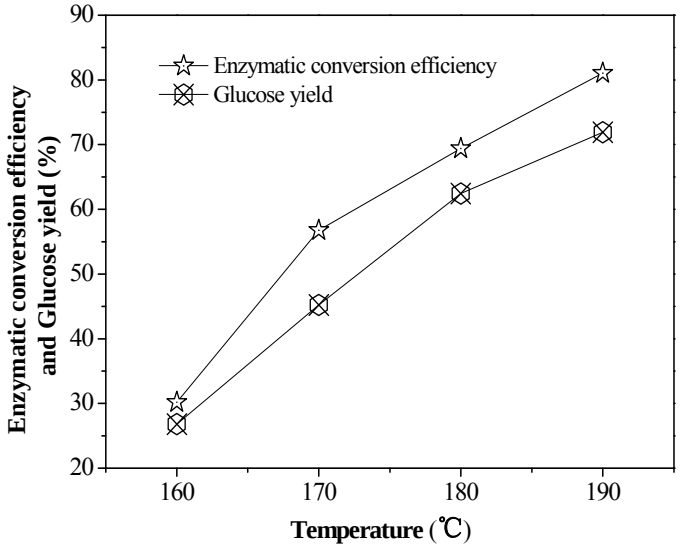

Fig. 3. Effect of pretreatment temperature on enzymatic conversion efficiency and glucose yield

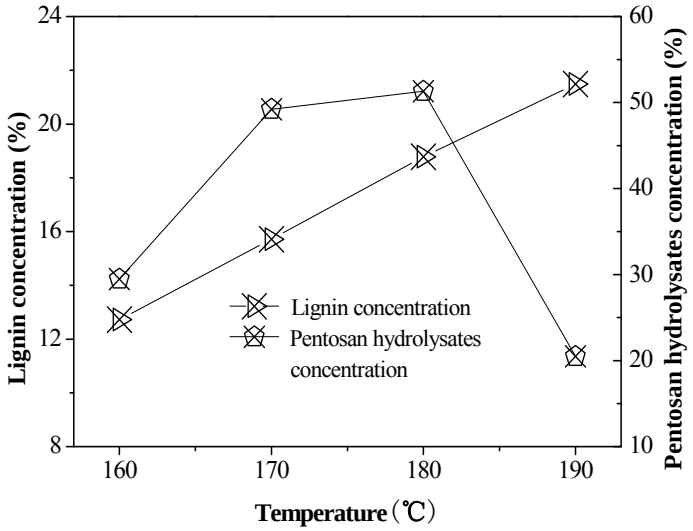

Fig. 4. Effect of pretreatment temperature on concentration of lignin and pentosan hydrolysates in liquid phase

Analyses of the effect of temperature on the lignin dissolution and hemicelluloses degradation are summarized in Figs. 4 and 5. Temperature had dramatic effects on removal of lignin and hemicelluloses. As shown in Fig. 4, the concentration of acid-soluble lignin at 160 °C and 190 °C were about 12.73 g/L and 21.48 g/L, respectively, indicating that temperature increase can significantly accelerate the solubilization of lignin. Meanwhile, the pentosan hydrolysates concentration in liquid first increased and then decreased with increasing pretreatment temperature, and the maximum value 51.30

g/L (80.85% solubilization rate) peaked at 180 °C. Subsequently the variation of percentage of pentose retained in pretreated substrate was analyzed, as shown in Fig .5. Xylan in the residue decreased with temperature, which meant that the hemicelluloses removal increased with temperature increase. The plausible reasons for this behavior were: under such high temperature, glycosidic linkages of cellulose are broken and cellulose starts to convert to water-soluble compounds and further to glucose. Molecules of the carbohydrates, especially pentosan hydrolyzates, are unstable at high temperature and a portion is further degraded, The carbohydrates not detected in pretreated liquid fractions are inferred to have degraded to furans and other degradation products such as acetic acid (Wang et al. 2009), which results in the reduction of the remaining content of the pentosan hydrolysates in the liquid phase.

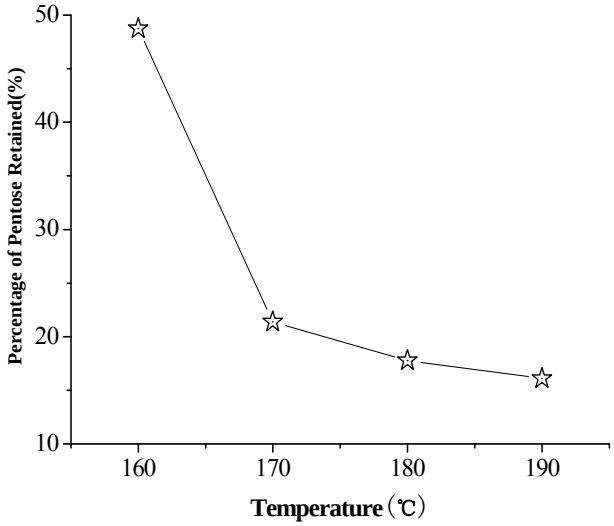

Fig. 5. Effect of pretreatment temperature on percentage of pentose retained in substrate

As discussed above, the higher the pretreatment temperature, the more adequately the raw materials were pretreated, and the higher enzymatic saccharification efficiencies were achieved. However, under a severe high temperature condition, the concentration of pentosan hydrolysates in liquid phase decreased rapidly, and part of the hydrolysates were further converted to furans and acetic acid, etc. The production of these degradation products have a greater negative impact on the subsequent fermentation processes. Moreover, high-temperature pretreatment would increase production cost. Considering these factors, the following experiments were carried out at a fixed pretreatment temperature at 180 °C to investigate the effect of pH in SPORL process on enzymatic hydrolysis of corn stalk.

Effect of Pretreatment pH on Enzymatic Saccharification

The effect of pH (2.2 to 6.7) of pretreatment liquor on enzymatic conversion efficiency and glucose yield after 72 h enzymatic hydrolysis is shown in Fig. 6. At a given sodium bisulfite charge of 7% (pH 4.7) on od substrate, the optimal pH was 6.7 (the experiment pH was adjusted with sulfuric acid or sodium hydroxide), at which both enzymatic conversion efficiency and glucose yield were maximized. The maximized

conversion efficiency and glucose yield were about 70.19% and 63.80%, respectively. Glucose yield and enzymatic conversion efficiency increased sharply with pH varying from 4.2 to 4.7, and basically leveled off from 4.7 to 6.7.

Pretreatment conducted at pH 2.2 removed only 18 g/L (39.17% lignin removal efficiency) of lignin (acid-soluble lignin) (Fig. 7). The enzymatic conversion efficiency of the resulting substrate was 52.80% (Fig. 6), which is lower than the 63.80% achieved at higher pH of 6.7. The high lignin removal 23.86 g/L (51.92% lignin removal efficiency) achieved at lower acid charges is likely attributable to enhanced lignin sulfonation. As shown in Fig. 7, hemicellulose can be completely removed (about 92.02% pentosan degradation efficiency) at pH 2.2. Lignin (acid-soluble lignin) showed an increased trend with the pH of pretreatment liquor increase.

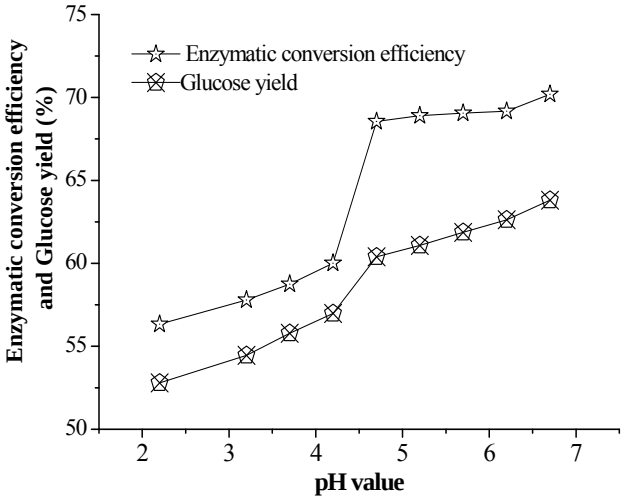

Fig. 6. Effect of pH on enzymatic conversion efficiency and glucose yield

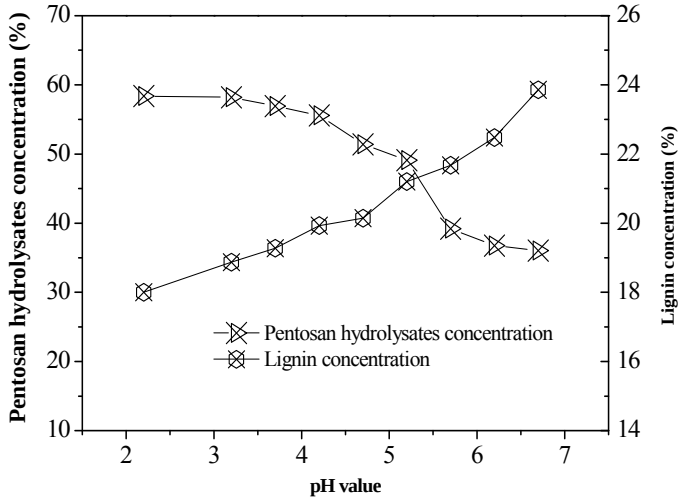

Fig. 7. Effect of pH on concentration of lignin and pentosan hydrolysates in liquid phase

As discussed above, increasing the pH of pretreatment liquor accelerated the delignification, which also increased cellulose conversion efficiency of SPORL substrate. However, it depressed the dissolution of hemicelluloses; this may be due to the fact that hemicelluloses are more readily hydrolyzed under low pH conditions. Previous studies have reported that cellulose hydrolysis improves with increasing lignin removal, although differences have been reported in the degree of lignin removal needed (Converse 1993; Lu et al. 2002). Yang and Wyman reported that condensed lignin can adsorb protein from aqueous solutions, and that lignin removal should improve the hydrolysis performance by reducing nonspecific adsorption of cellulase enzymes (Yang et al. 2004).

CONCLUSIONS

Pretreatment of corn stalk by SPORL was effective in enhancing enzymatic digestibility. Under the conditions used in this study, (i.e., pretreatment liquor pH 2.2 to 4.7, sodium bisulfite charge 7% on substrate, 30 min reaction time at 180 ^{o}C), the 90% removal of hemicellulose and half dissolution of lignin can be easily achieved. About 70% enzymatic conversion efficiency was obtained after 72 h enzymatic hydrolysis at an enzyme loading of 5 FPU/g of cellulose under 7% bisulfite charge at 180 ^{o}C for 30 min. Increasing the bisulfite charge and pretreatment temperature can increase hemicellulose and lignin removal (Figs. 2 and 4). However, under high loading and temperature, hemicellulose can further degrade to fermentation inhibitors such as furfural and HMF during SPORL pretreatment. Therefore, reasonable pretreatment conditions (temperature 180 ^{o}C, 7% bisulfite charge and pH 4.2 to 4.7) can be recommended. Furthermore, high value utilization of ligninsulfonate and hemicellulose sugars in sulfite spent liquor has been in commercial practice. SPORL offers a potentially excellent co-product pathway for biomass conversion in terms of industry infrastructure and commercial markets.

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