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## Hydrogen Peroxide-Assisted Sodium Carbonate Pretreatment for the Enhancement of Enzymatic Saccharification of Corn Stover

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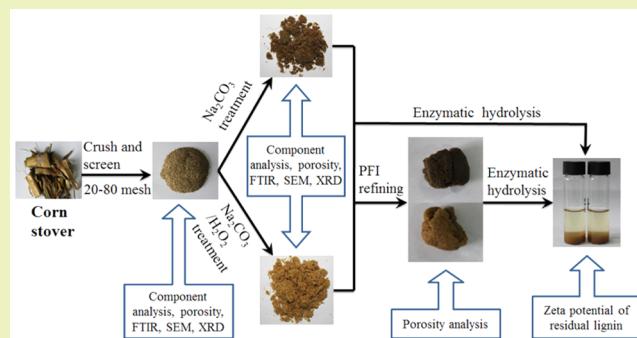
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**ABSTRACT:** In this work, the effectiveness of hydrogen peroxide-assisted sodium carbonate (HSC) pretreatment of corn stover to produce fermentable sugars was investigated. It was found that the addition of  $H_2O_2$  (a green oxidative agent) in  $Na_2CO_3$  pretreatment could enhance the lignin removal and increase the hydrophilicity of the residual lignin, leading to the improved enzymatic digestibility and the yield of fermentable sugars. Also, results showed that lignin removal (in the range of 12–65%) was positively and linearly correlated with final total sugar yields. Post PFI refining could further improve the enzyme accessibility by increasing porosity of substrates. After HSC pretreatment (40 wt %  $Na_2CO_3$  and 15 wt %  $H_2O_2$ , 120 °C for 60 min) and enzymatic hydrolysis, the final total sugar yield could achieve 79%, which was about 10% higher in comparison with the conventional  $Na_2CO_3$  pretreatment under the same conditions. HSC pretreatment will have less environmental impact and less barriers for large scale production.

**KEYWORDS:** Pretreatment, Sodium carbonate, Hydrogen peroxide, Corn stover, Enzymatic hydrolysis/saccharification



### INTRODUCTION

Lignocellulosic agricultural residues as the green and sustainable feedstock are deemed as one of the most strategically important raw materials for sugar platform biorefinery on a large-scale production because they are inexpensive, abundant and rich in carbohydrates.<sup>1</sup> For instance, the annual production of agricultural residuals (including corn stover, wheat straw, rice straw, etc.) are over 700 million tons in China. Thus, biofuels or biomaterials produced from agricultural residues have become a hotspot in the energy and material fields and have been extensively studied in recent years.<sup>2,3</sup> Because the enzymatic degradation efficiency of polysaccharides in raw lignocelluloses is low, their effective utilization for enzymatic hydrolysis and fermentation purposes requires a green and cost-effective pretreatment process to destroy the natural recalcitrance of lignocelluloses (for instance, to break the lignin seal and reduce cellulose crystallinity).<sup>4,5</sup>

Among various pretreatment technologies such as physical, chemical, biological, and the combined pretreatment methods (e.g., Alkaline Twin-Screw Extrusion (ATSE)<sup>6</sup> and Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose (SPORL)<sup>7</sup>), alkali-based pretreatment is one of the most promising approaches, particularly for the treatment of herbaceous crops and agricultural residues.<sup>8</sup> Alkali-based pretreatment can efficiently remove lignin by cleaving the

cross-linking bonds between carbohydrates and lignin, generate less inhibitors, and achieve high final total sugar yields compared to liquid hot water pretreatment and dilute acid pretreatment.<sup>5</sup> Also, alkali-based pretreatment can be integrated into alkali-based pulp mills by utilizing the existing and mature pulping equipment, well developed chemical recovery and wastewater treatment system, to reduce carbon emission and gain more benefits for the mills.<sup>9</sup>

Sodium hydroxide as a strong base is commonly used in alkali-based pretreatment, but NaOH can cause severe degradation of lignocelluloses (particularly for hemicelluloses), and the recovery of NaOH achieved through the combustion of black liquor and the followed causticization stage is quite expensive. Recently, sodium carbonate as a weak and inexpensive base was used to pretreat lignocelluloses, as it could cause less degradation of carbohydrates and its recovery does not need causticization.<sup>10,11</sup> Thus, the use of  $Na_2CO_3$  could lower the cost of pretreatment process. Yet, the lignin removal of  $Na_2CO_3$  pretreatment was inefficient even with high  $Na_2CO_3$  loading due to its weaker alkalinity, resulting in a low digestibility of  $Na_2CO_3$  pretreated substrate. Hence, to promote

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delignification, a chemical agent like  $\text{Na}_2\text{S}$  ( $\text{Na}_2\text{CO}_3 + \text{Na}_2\text{S}$ , namely green liquor pretreatment),<sup>12,13</sup>  $\text{Na}_2\text{SO}_3$ ,<sup>14</sup> or  $\text{O}_2$  ( $\text{Na}_2\text{CO}_3 + \text{O}_2$ , i.e., wet oxidation pretreatment)<sup>15,16</sup> was applied to ameliorate the effectiveness of  $\text{Na}_2\text{CO}_3$  pretreatment. However, a majority of hemicelluloses were dissolved during wet oxidation treatment due to the high temperature (180–210 °C) and high pressure (1.2 MPa), and the presence of S would lead to the complexity of the spent liquor handling process and environmental issues if the process scale is small.<sup>11</sup> Therefore, selecting a more suitable and green chemical agent was needed to improve the effectiveness of  $\text{Na}_2\text{CO}_3$  pretreatment.

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is a very weak acid and green oxidative agent, which is commonly applied for pulp bleaching.<sup>17</sup> In alkaline conditions, hydrogen peroxide is dissociated to generate the hydroperoxy anion ( $\text{HOO}^-$ ), which is the main oxygen species to degrade lignin by reacting with the quinone structures of lignin, and the double bonds or carbonyl group of side chains in lignin.<sup>18</sup> Thus, the lignin is fragmented and its solubility is increased, leading to the improved lignin removal. It has been reported that the addition of  $\text{H}_2\text{O}_2$  in  $\text{NaOH}$  pretreatment (i.e., alkaline hydrogen peroxide pretreatment (AHP)) could promote delignification,<sup>19</sup> and both the glucose and xylose yields of AHP treated corn stover were clearly higher compared to the ammonia fiber expansion (AFEX) treated corn stover.<sup>20</sup>

However, to our knowledge, the  $\text{H}_2\text{O}_2$ -assisted  $\text{Na}_2\text{CO}_3$  (HSC) pretreatment has not been explored. Hence, in the present study, the performance of HSC pretreatment of corn stover for the production of fermentable sugars was investigated in comparison with  $\text{Na}_2\text{CO}_3$  (SC) pretreatment. Standard enzymatic hydrolysis conditions with 2 wt % solid loading were conducted to evaluate the effectiveness of HSC pretreatment, and the structural changes of pretreated corn stover was characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), etc.

## EXPERIMENTAL SECTION

**Material.** Corn stover was obtained from a village of Qingdao, China. The air-dried corn stover was cut into pieces with a length of 3–5 cm, and then ground and screened to get the particles between 20 and 80 meshes. After that, the screened corn stover was collected and stored in the sealed plastic bags at room temperature for moisture balance. The composition of corn stover is listed in Table 1.

Cellulase (Celluclast 1.5 L) and xylanase (from *Thermomyces lanuginosus*) were purchased from Sigma-Aldrich China Inc.  $\beta$ -glucosidase was kindly gifted by Qingdao Vland Biotech Group Co., Ltd. The activities of cellulase,  $\beta$ -glucosidase and xylanase were 52 FPU/mL, 300 IU/mL, and 365 U/g, respectively, as tested following the IUPAC standards.<sup>21</sup> Congo red was obtained from BASF China Co. Inc. All other chemicals (e.g., hydrogen peroxide (30 wt %)) were bought from Sinopharm Chemical Reagent Co. Ltd. and used as received.

**$\text{H}_2\text{O}_2$ -Assisted Sodium Carbonate (HSC) Pretreatment.** HSC pretreatment experiments were carried out in a cooking digester (PL1-00, Xianyang TEST Equipment Co. Ltd., Xianyang, China). The cooking digester was equipped with four small cooking tubes inside with the volume of 1 L for each. For each small tube, 40 g of corn stover (oven-dried basis) was added and mixed with the desired amount of  $\text{Na}_2\text{CO}_3$  (varied from 10 to 60 wt %) and  $\text{H}_2\text{O}_2$  (loaded from 0 to 25 wt %). The chemical dosage was based on the oven-dried weight of corn stover and the solid-to-liquid ratio for pretreatment was 1:8. The temperature was elevated from room temperature to 120 °C in 30 min, and the heating duration was 60 min at 120 °C. During pretreatment, the cooking digester was rotated with the speed of 1

rpm. Upon completion of pretreatment, the small tubes were immediately cooled down with tap water. Then the pretreated samples were washed in a Nylon bag (300 mesh) to neutralize pH and stored at 4 °C for further tests. For comparison,  $\text{Na}_2\text{CO}_3$  pretreatment was performed under the same conditions using the same procedure as described above.

**Mechanical Refining Treatment.** To enhance further enzymatic digestibility, the pretreated and washed corn stover was refined in a PFI mill (a standard laboratory pulp refining instrument) (PL11-00, Xianyang TEST Equipment Co., Ltd., Xianyang, China) at 10 wt % of refining consistency. The refining revolution number, revolution speed, and refining gap were 4000, 1400 rpm, and 0.24 mm, respectively.

**Enzymatic Hydrolysis.** The enzymatic hydrolysis of the pretreated and refined samples was conducted at 2% (w/v) solid loading in a 40 mL bottle containing 0.02% sodium azide and 50 mM sodium citrate buffer (pH 4.8). The enzymes loading were 36 FPU cellulase/g-glucan, 5 IU  $\beta$ -glucosidase/g-substrate, and 5 U xylanase/g-substrate. The hydrolysis temperature was controlled at 50 °C in an air bath incubator with shaking at 120 rpm. The hydrolysates were sampled and filtered (through a 0.22  $\mu\text{m}$  membrane) at the required time for sugar analysis.

**Composition Analysis of Corn Stover.** The chemical composition of corn stover was determined by the use of the National Renewable Energy Laboratory (NREL) procedure with the standard two steps of sulfuric acid hydrolysis.<sup>22</sup> The monosaccharides in hydrolysate were tested by a high-performance liquid chromatography (HPLC, Model 1200, Agilent, USA) equipped with a Bio-Rad Aminex HPX-87P column at 75 °C. The mobile phase used was ultrapure water with the flow rate of 0.6 mL/min. The calibration and blank test were performed using the standard sugar solutions with known concentrations to correct the losses of sugar destruction during acid hydrolysis. The lignin, extractives and ash contents of corn stover were also measured following NREL test procedures. The effectiveness of HSC pretreatment was evaluated by the following variables:

$$R_{\text{solid}} (\%) = (M_{\text{pretreated biomass}} (\text{g}) / M_{\text{original biomass}} (\text{g})) \times 100\% \quad (1)$$

$$R_{\text{glucan/xylan}} (\%) = (R_{\text{solid}} \times C_{\text{glucan/xylan of pretreated biomass}}) / C_{\text{glucan/xylan of raw biomass}} \times 100\% \quad (2)$$

$$D_{\text{lignin}} (\%) = 1 - (R_{\text{solid}} \times C_{\text{lignin of pretreated biomass}}) / C_{\text{lignin of raw biomass}} \times 100\% \quad (3)$$

$$Y_{\text{glucan}} (\%) = M_{\text{glucose in hydrolyzate}} \times 0.9 / M_{\text{glucan in raw biomass}} \times 100\% \quad (4)$$

$$Y_{\text{xylan}} (\%) = M_{\text{xylose in hydrolyzate}} \times 0.88 / M_{\text{xylan in raw biomass}} \times 100\% \quad (5)$$

$$Y_{\text{sugar}} (\%) = ((M_{\text{glucose in hydrolyzate}} \times 0.9 + M_{\text{xylose in hydrolyzate}} \times 0.88) / M_{\text{glucan and xylan in raw biomass}}) \times 100\% \quad (6)$$

where  $R_{\text{solid}}$  was the percentage of solid recovery,  $R_{\text{glucan/xylan}}$  was the recovery rate of glucan or xylan,  $D_{\text{lignin}}$  was the lignin removal rate,  $Y_{\text{glucan}}$ ,  $Y_{\text{xylan}}$ , and  $Y_{\text{sugar}}$  were the sugar yields of glucan, xylan, and final total sugar (glucan plus xylan), respectively, after pretreatment and enzymatic saccharification.  $C$  was the content of the corresponding component in biomass (%) and  $M$  was the mass of the corresponding substance (g). All experiments were carried out at least in duplicate and the average value was reported.

**Characterization of Corn Stover before and after Pretreatment.** The FTIR spectra of corn stover before and after pretreatment were determined on a FTIR spectrometer (Nicolet 6700, Thermo Fisher Scientific Inc.). The samples were prepared by mixing 2 mg of freeze-dried sample with 200 mg of KBr. The resolution of spectra was

**Table 1.** Effect of Pretreatment on Solid Recovery and Chemical Composition of Corn Stover

sample ID	chemical dosage (%)			final pH <sup>b</sup>	main chemical composition (%)		
	Na <sub>2</sub> CO <sub>3</sub>	H <sub>2</sub> O <sub>2</sub>	R <sub>solid</sub> (%) <sup>a</sup>		glucan	xylan	lignin
0 <sup>c</sup>			100		33.08 ± 0.23	17.62 ± 0.13	17.33 ± 0.02
1	10	0	71.31	8.82	43.71 ± 0.73	22.03 ± 0.03	20.31 ± 0.15
2	20	0	63.43	9.39	49.96 ± 0.54	23.70 ± 0.17	16.48 ± 0.02
3	30	0	61.12	9.66	51.55 ± 0.28	24.29 ± 0.18	13.63 ± 0.19
4	40	0	60.89	9.82	52.29 ± 0.17	25.23 ± 0.23	12.72 ± 0.08
5	50	0	59.55	9.91	52.65 ± 0.50	23.35 ± 0.17	12.70 ± 0.31
6	60	0	59.52	9.96	50.86 ± 0.13	23.22 ± 0.11	12.40 ± 0.10
7	10	15	73.23	8.37	43.42 ± 0.22	21.15 ± 0.46	20.59 ± 0.11
8	20	15	67.19	8.42	47.39 ± 0.16	21.66 ± 0.04	16.47 ± 0.07
9	30	15	60.86	8.8	51.84 ± 0.53	22.46 ± 0.05	12.36 ± 0.21
10	40	15	60.48	9.09	52.19 ± 0.17	22.29 ± 0.13	11.98 ± 0.11
11	50	15	59.14	9.26	53.02 ± 0.92	22.47 ± 0.71	11.06 ± 0.22
12	60	15	56.98	9.42	53.81 ± 0.20	22.96 ± 0.10	11.24 ± 0.14
13	40	10	60.67	9.37	51.0 ± 0.17	23.2 ± 0.66	12.6 ± 0.20
14	40	20	59.74	8.99	50.6 ± 0.07	22.7 ± 0.34	11.7 ± 0.11
15	40	25	59.43	8.87	50.7 ± 0.25	22.7 ± 0.36	11.3 ± 0.23

<sup>a</sup>R<sub>solid</sub> is the percentage of solid recovery after pretreatment. <sup>b</sup>Final pH is the pH of spent liquor after pretreatment. <sup>c</sup>Sample 0 is the raw corn stover, which also contained 2.08 ± 0.47% arabinan, 19.23 ± 0.41% extractives (hot water-soluble and ethanol soluble material), and 6.18 ± 0.02% ash.

4 cm<sup>-1</sup> in the range of 400–4000 cm<sup>-1</sup>, and 32 scans were carried out for each sample.

The SEM analyses of the samples were conducted on a scanning electron microscopy (S-4800, Hitachi, Japan), to analyze the microstructure changes and surface features of corn stover. The freeze-dried samples were coated with platinum and analyzed with the accelerating voltages from 3.0 to 8.0 kV.

The crystallinity index (CrI) of corn stover was examined by an X-ray diffractometer (D8 ADVANCE, Bruker Co., Germany). The scanning speed was 0.5°/min in the scattering angle (2θ) from 10 to 80°, and the Ni-filtered Cu Kα radiation was generated at 80 mA and 40 kV. The CrI of corn stover was calculated by the flowing formula according to the empirical method:<sup>23</sup>

$$CrI = (I_{002} - I_{am})/I_{002} \times 100\% \quad (7)$$

where  $I_{002}$  was the diffraction intensity of the 002 peak at  $2\theta = 22.5^\circ$ , and  $I_{am}$  was the diffraction intensity at  $2\theta = 18.7^\circ$ .

**Porosity Analysis of Substrate by Dye Adsorption.** It has been known that the porosity of substrate can be semiquantified by dye adsorption method and the porosity test of corn stover samples were measured as reported previously.<sup>24</sup> The dye used in the present work was Congo red with the molecular size of 2.6 nm. The amount of dye adsorbed by substrates (mg dye/g-substrate) was tested based on the decrease of dye concentrations after dyeing and the data was fitted to the Langmuir adsorption isotherm to calculate the maximal adsorbed amount of dye.

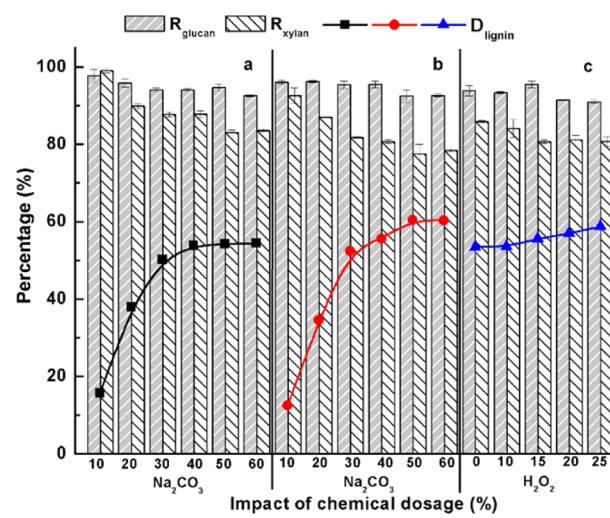
**ζ-Potential Measurement of Hydrolysis Lignin Residues.** The ζ-potential of the hydrolysis lignin residues were measured following the previously reported procedure.<sup>25</sup> Briefly, after enzymatic hydrolysis, the hydrolysate and solid residue were separated. Then, a small amount of lignin residues were added in a buffer solution (50 mL of 50 mM sodium citrate, pH 4.8) and the lignin concentration was 0.04 wt %. The lignin solution was mixed with a shaking incubator at 50 °C and 200 rpm for 30 min. After that, the lignin solution was allowed to stand for 60 min and the supernatant was tested using a ζ-potential analyzer (JS94J2, Zhongchen Digital Technology Equipment Co., Ltd, Shanghai, China). All ζ-potential measurements were carried out in duplicate with at least seven readings in each experiment, and the averages were reported.

## RESULTS AND DISCUSSION

### Effect of HSC/SC Pretreatment on Chemical Composition and Carbohydrate Recovery.

Table 1 shows the

impact of HSC/SC pretreatment on solid recovery and the main chemical composition changes of corn stover. As can be seen, after SC/HSC pretreatment, the solid recovery was clearly decreased, whereas the glucan and xylan content of pretreated corn stover was obviously increased, which was due to the removal of major part of extractives, ash, and part of lignin. Also, both the solid recovery and lignin content decreased with the increasing of Na<sub>2</sub>CO<sub>3</sub> charge. For example, with respect to SC pretreatment, when Na<sub>2</sub>CO<sub>3</sub> charge increased from 10 to 40 wt %, the solid recovery decreased from about 71% to 61%, and the lignin content of pretreated corn stover reduced from about 20% to 13%. Correspondingly, the recovery rates of glucan and xylan decreased about 3% and 10%, respectively, and the delignification rate improved approximately 2.3-fold, as presented in Figure 1. This was because alkali charge was one of the most important factors affecting the pretreatment



**Figure 1.** Effect of chemical dosage of HSC/SC pretreatment on sugar recovery and lignin removal. (a, SC pretreatment; b, HSC pretreatment (fixed H<sub>2</sub>O<sub>2</sub> dosage: 15 wt %); c, HSC pretreatment (fixed Na<sub>2</sub>CO<sub>3</sub> dosage: 40 wt %)).

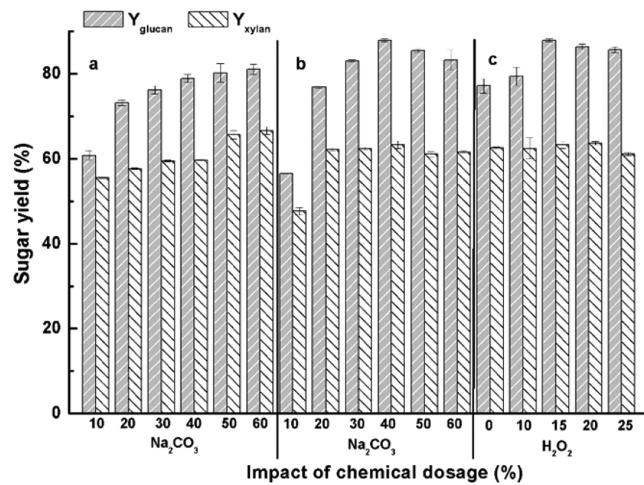
effectiveness and the strengthened alkali could enhance the degradation of lignin and carbohydrates (e.g., peeling reaction, particularly for xylan).<sup>18,26</sup> According to Figure 1a, after SC pretreatment with 30 wt % Na<sub>2</sub>CO<sub>3</sub> about 50% of lignin could be removed, which was better compared to the rice straw (about 40% lignin removal) pretreated under similar conditions<sup>11</sup> and the corn stover (14.7% lignin removal) pretreated by hydrogen peroxide (50% H<sub>2</sub>O<sub>2</sub>, 60 °C for 1 h).<sup>27</sup> In addition, 40 wt % of Na<sub>2</sub>CO<sub>3</sub> charge (i.e., 0.46 M Na<sub>2</sub>CO<sub>3</sub>) was sufficient to achieve the highest lignin removal for SC pretreatment, as shown in Figure 1a.

Table 1 and Figure 1 also exhibit that the addition of H<sub>2</sub>O<sub>2</sub> in SC pretreatment could facilitate lignin removal and the delignification rate increased with the increase of H<sub>2</sub>O<sub>2</sub> loading. For instance, the delignification rate of the sample 15 (pretreated with 40 wt % Na<sub>2</sub>CO<sub>3</sub> and 25 wt % H<sub>2</sub>O<sub>2</sub>) was about 13% higher compared to the control (sample 4 in Table 1). This was because the reactive species (e.g., hydroperoxy anion (HOO<sup>-</sup>) and hydroxyl radical (HO<sup>·</sup>)) generated by H<sub>2</sub>O<sub>2</sub> decomposition could oxidize and degrade lignin, thus increasing lignin solubility and promoting lignin removal.<sup>27,28</sup> But these reactive species could also degrade carbohydrates (mainly hemicelluloses), resulting in a lower xylan recovery after HSC pretreatment (Figure 1c).

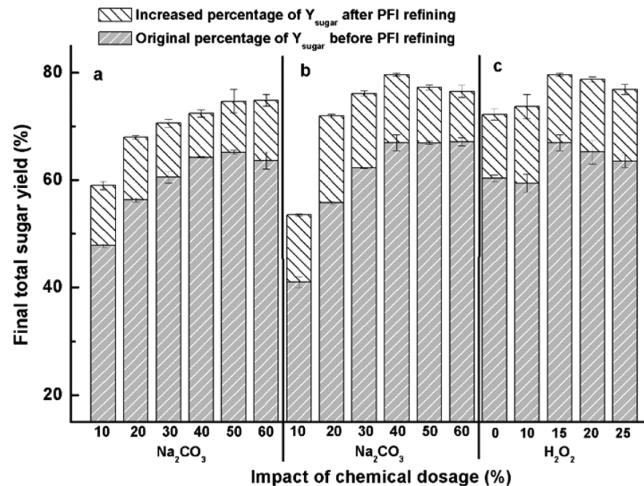
It is shown in Figures 1a,b that, however, when Na<sub>2</sub>CO<sub>3</sub> charge was less than 20 wt %, the lignin removal for the HSC pretreated samples were slightly lower in comparison with the SC pretreated samples under the same conditions. The reason could be that, on the one hand, the addition of H<sub>2</sub>O<sub>2</sub> could slightly reduce the pH of cooking liquor because H<sub>2</sub>O<sub>2</sub> was a weak acid. For example, the pH values of cooking liquor were 11.20 and 11.26 when Na<sub>2</sub>CO<sub>3</sub> dosages were 10 and 20 wt %, respectively. When 15 wt % H<sub>2</sub>O<sub>2</sub> was added, the corresponding pH of cooking liquor were decreased to 10.11 and 10.34, respectively. After HSC pretreatment, the corresponding pH of spent liquor were 8.37 and 8.42, respectively, which were also lower than the corresponding pH of spent liquor from SC pretreatment with the same Na<sub>2</sub>CO<sub>3</sub> dosages (8.82 and 9.39 for sample 1 and 2, respectively) (Table 1). The lower pH could reduce the alkaline degradation of lignin and carbohydrates.<sup>18</sup> On the other hand, the pH is one of the most key variables for efficient application of H<sub>2</sub>O<sub>2</sub>.<sup>29</sup> It has been reported that H<sub>2</sub>O<sub>2</sub> could only react with the aliphatic part of lignin, whereas in alkaline conditions with relatively high temperatures (e.g., 90 °C), H<sub>2</sub>O<sub>2</sub> could attack the phenolic rings of lignin, opening rings and adding carboxylic groups to lignin macromolecular.<sup>30</sup> Therefore, to achieve an efficient action of H<sub>2</sub>O<sub>2</sub>, the Na<sub>2</sub>CO<sub>3</sub> dosage should be no less than 20 wt % (i.e., 0.23 M Na<sub>2</sub>CO<sub>3</sub> concentration), based on the results obtained.

#### Enzymatic Digestibility of HSC/SC Pretreated Corn Stover.

Shown in Figure 2 is the impact of chemical dosage of HSC/SC pretreatment on glucan and xylan yields for the PFI refined samples. As can be seen from Figure 2a, both glucan (60.7–82.1%) and xylan yields (55.5–63.6%) gradually went up with the increase of Na<sub>2</sub>CO<sub>3</sub> dosage for SC pretreatment. Correspondingly, the final total sugar yield also increased when Na<sub>2</sub>CO<sub>3</sub> dosage was increased (Figure 3a). This was mainly because more alkali added (Na<sub>2</sub>CO<sub>3</sub>) could facilitate lignin removal, thus improving the digestibility of pretreated substrate.<sup>5,11</sup> Figure 2b presents that the addition of H<sub>2</sub>O<sub>2</sub> (15 wt %) could boost glucan and xylan yields when Na<sub>2</sub>CO<sub>3</sub> dose was higher than 20 wt %. Yet, both glucan and xylan yields



**Figure 2.** Impact of chemical dosage of HSC/SC pretreatment on sugar yield for PFI refined samples. (a, SC pretreatment; b, HSC pretreatment (fixed H<sub>2</sub>O<sub>2</sub> dosage: 15 wt %); c, HSC pretreatment (fixed Na<sub>2</sub>CO<sub>3</sub> dosage: 40 wt %)).



**Figure 3.** Impact of chemical dosage of HSC/SC pretreatment on final total sugar yield. (a, SC pretreatment; b, HSC pretreatment (fixed H<sub>2</sub>O<sub>2</sub> dosage: 15 wt %); c, HSC pretreatment (fixed Na<sub>2</sub>CO<sub>3</sub> dosage: 40 wt %)).

reduced with the overcharge of Na<sub>2</sub>CO<sub>3</sub> (over 40 wt %) (Figure 2b) or H<sub>2</sub>O<sub>2</sub> (over 15 wt %) (Figure 2c) because of the overdegradation of carbohydrates. Hence, the suitable Na<sub>2</sub>CO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> dosages for HSC pretreatment were 40 and 15 wt %, respectively. Under such conditions, the glucan and xylan yields could reach 88% and 63%, respectively. The corresponding final total sugar yield was 79% (Figure 3b,c), which was about 10% higher compared to SC pretreatment under the same conditions. This total sugar yield was also clearly higher than the corn stover (70%) pretreated by green liquor pretreatment (14 wt % Na<sub>2</sub>CO<sub>3</sub>, 40% sulfidity, 140 °C).<sup>12</sup> In addition, the appropriate temperature and cooking duration of HSC pretreatment were 120 °C and 60 min, respectively, according to our previous tests (data not shown). Higher temperature (higher than 140 °C) could resulted in the sever degradation of sugars, particularly for xylan.<sup>18</sup> The temperature of HSC pretreatment was lower than that of hot water pretreatment (150–230 °C) and dilute acid pretreatment (150–190 °C).<sup>5,8</sup> Thus, the process cost could be reduced.

Figure 3 also shows the impact of PFI refining on final total sugar yields. It was seen that, after PFI refining, the final total sugar yields improved about 8–18%, which was in agreement with the previously reported results (10–20%).<sup>31</sup> This was mainly due to the increased porosity and specific surface area resulted from the further modifications (e.g., size reduction, fibrillation, cell wall delamination, etc.) of refined fibers, leading to the improved enzyme accessibility.<sup>4,24</sup> In addition, the final sugar yield improvement (9–18%) achieved by PFI refining for HSC pretreatment was statistically higher compared to the SC pretreated samples (8–12%) (Figure 3). This was probably because the enhancement of enzymatic digestibility achieved by post mechanical treatment was depended on the severity of chemical pretreatment and the properties of substrates.<sup>4,32</sup> Compared with SC pretreated corn stover, HSC pretreated corn stover had lower residual lignin content (Table 1), and the residual lignin had higher hydrophilicity because the  $\zeta$ -potential ( $-18.37 \pm 1.10$  mV) of the residual lignin of HSC pretreated corn stover (sample 10 in Table 1) was lower than that ( $-13.42 \pm 1.32$  mV) of SC pretreated corn stover (sample 4 in Table 1). The increased hydrophilicity of lignin could reduce the nonspecific binding of cellulase.<sup>25</sup>

**Impact of Lignin Removal on Final Total Sugar Yield.** Lignin is one of the most key barriers for enzymatic saccharification of lignocelluloses and its content, structure and distribution have a big impact on the efficiency of enzymatic hydrolysis.<sup>2</sup> The removal of lignin could increase the porosity of biomass and reduce the nonspecific bonding of cellulase, thus ameliorating the enzymatic digestibility.<sup>4,25</sup> Figure 4 displays that the lignin removal (in the range of

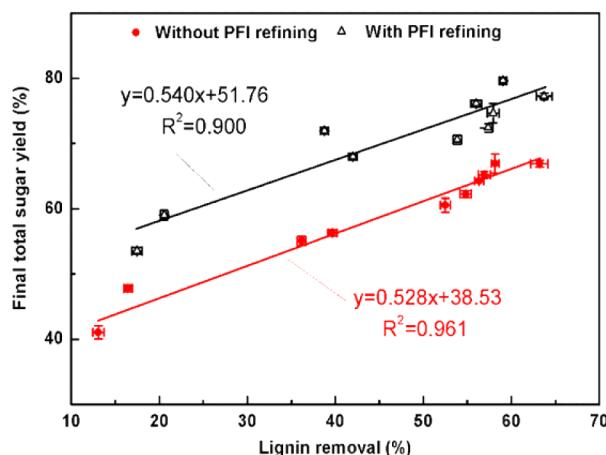


Figure 4. Correlation between final total sugar yields and lignin removal.

12–65%) was linearly and positively correlated with the final total sugar yields. Similar results were also reported previously for the green liquor pretreatment of corn stover<sup>12</sup> and the  $\text{Na}_2\text{CO}_3$ – $\text{Na}_2\text{SO}_4$  pretreatment of rice straw.<sup>14</sup> Furthermore, again, post PFI refining could further boost the final total sugar yields (Figure 4), which was in line with the results shown in Figure 3.

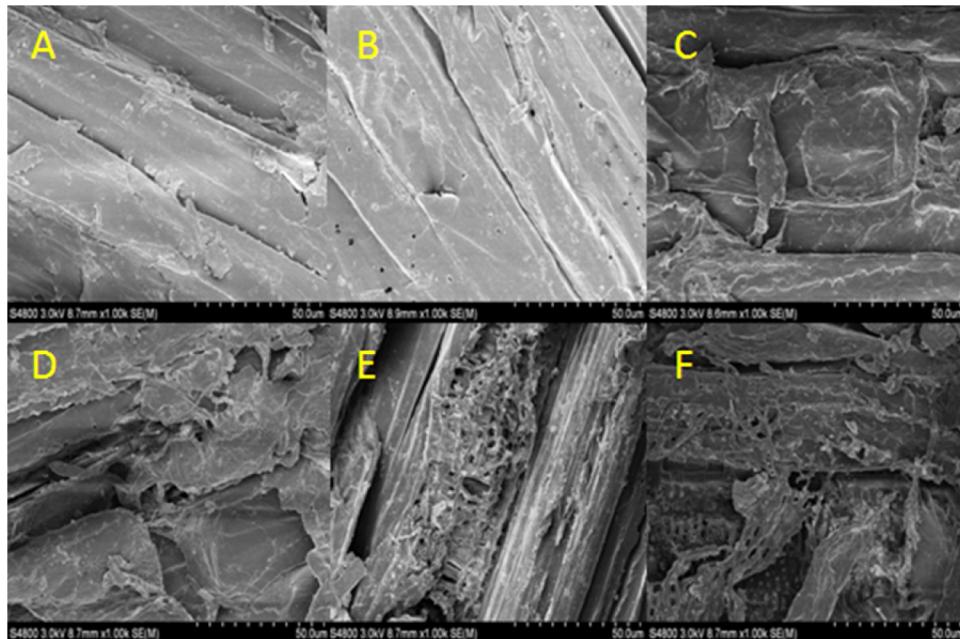
**Characterization of Corn Stover after HSC/SC Pretreatment.** To investigate more detailed physical and chemical changes of corn stover before and after HSC pretreatment, SEM, porosity, FTIR, and XRD analyses were conducted. SEM images of raw and pretreated corn stover are shown in Figure 5. As can be seen, the untreated corn stover (Figure 5A) had an

even, smooth, and flat surface, implying a rigid and highly ordered surface structure. Yet, after SC pretreatment, pores were generated probably due to the partial removal of extractives, ash, and lignin (Figure 5B); the fiber surface was fragmented and splintered (Figure 5C,D) and became looser and coarser with the increase of  $\text{Na}_2\text{CO}_3$  loading. Comparable results were also reported by Xu et al.<sup>5,24</sup> With the supplement of  $\text{H}_2\text{O}_2$ , the surface of corn stover fiber was broken into lamellar fragments. The specific surface area and porosity were obviously increased (Figure 5E,F). In fact, as measured by dye adsorption method, the porosity ( $308 \pm 17$  mg/g) of the corn stover pretreated by 40 wt %  $\text{Na}_2\text{CO}_3$  and 15 wt %  $\text{H}_2\text{O}_2$  was obviously higher compared to the one pretreated by 40 wt %  $\text{Na}_2\text{CO}_3$  ( $239 \pm 9$  mg/g), as well as the raw corn stover ( $173 \pm 23$  mg/g). This was the consequence of the addition of  $\text{H}_2\text{O}_2$ , leading to the promoted lignin removal (Table 1 and Figure 1) and possible relocation of lignin due to the oxidation reaction of  $\text{H}_2\text{O}_2$ .<sup>18</sup>

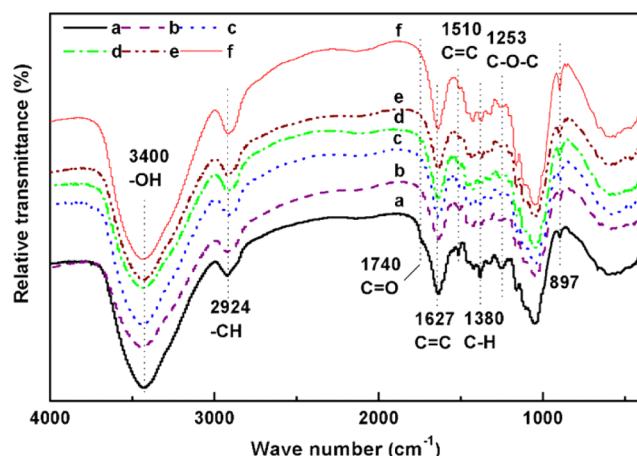
Shown in Figure 6 are the FTIR spectra of corn stover before and after pretreatment. The broad band located around  $3400 \text{ cm}^{-1}$  corresponded to the –OH stretching vibration and the band at about  $2924 \text{ cm}^{-1}$  was due to the asymmetric stretching vibration of methylene groups.<sup>33</sup> The shoulder peak at  $1740 \text{ cm}^{-1}$  was associated with the  $\text{C}=\text{O}$  conjugates of acetyl and feruloyl groups in hemicelluloses,<sup>34</sup> and its disappearance or decreased intensity in the pretreated samples was resulted from the partial degradation of hemicelluloses (e.g., deacetylation).<sup>32</sup> The peak at  $1627 \text{ cm}^{-1}$  was related to the absorption of  $\text{C}=\text{C}$  in lignin and it was also overlapped by the bending vibration of hydroxyl groups ( $1640 \text{ cm}^{-1}$ ) from the adsorbed water molecules.<sup>4,33</sup> The increased intensity of the peaks at  $897 \text{ cm}^{-1}$  ( $\beta$ -glucosides, in cellulose) and  $1158 \text{ cm}^{-1}$  ( $\text{C}—\text{O}—\text{C}$  stretching in cellulose),<sup>5</sup> as well as the reduced intensity of the peaks at  $1627 \text{ cm}^{-1}$  ( $\text{C}=\text{C}$  in lignin),  $1510 \text{ cm}^{-1}$  ( $\text{C}=\text{C}$  in lignin),  $1380 \text{ cm}^{-1}$  (aromatic skeletal vibration; CH in-plane deformation), and  $1253 \text{ cm}^{-1}$  (aryl ring breathing mode; CO stretching)<sup>35</sup> indicated the removal of lignin after HSC pretreatment.

$\text{CrI}$  is another influential factor affecting the enzymatic digestibility of lignocelluloses.<sup>36</sup> As shown in Figure 7, the  $\text{CrI}$  of raw corn stover was 0.429, whereas the  $\text{CrI}$  of the pretreated sample was clearly increased with the strengthening of pretreatment. For instance, the  $\text{CrI}$  of the corn stover pretreated by 40 wt %  $\text{Na}_2\text{CO}_3$  and 15 wt %  $\text{H}_2\text{O}_2$  was 0.518, which was about 4%, and 14% higher compared to the one pretreated by 40 wt %, and 10 wt %  $\text{Na}_2\text{CO}_3$ , respectively. The increase of  $\text{CrI}$  after HSC pretreatment was mainly due to the partial removal of amorphous components like lignin and hemicelluloses.<sup>5,37</sup> These results were also in accordance with the data presented in Table 1 and FTIR analysis (Figure 6).

**Impact of Hydrolysis Duration on Sugar Yields.** Glucose and xylose yields as the function of enzymatic hydrolysis time are exhibited in Figure 8a,b, respectively. It was seen that the efficiency of enzymatic saccharification in the first 24 h (particularly in the first 8 h) was higher in comparison with the longer saccharification duration. This was in agreement with the previous results,<sup>5,37</sup> which was due to the fact that the gradually increased sugar concentration during saccharification could generate feedback inhibition effect.<sup>38</sup> Figure 8 also shows that the enzymatic digestibility of HSC pretreated samples was clearly higher than that of SC pretreated samples. After 24 h hydrolysis, the glucose yield for HSC pretreated samples was about 14% higher compared to the SC

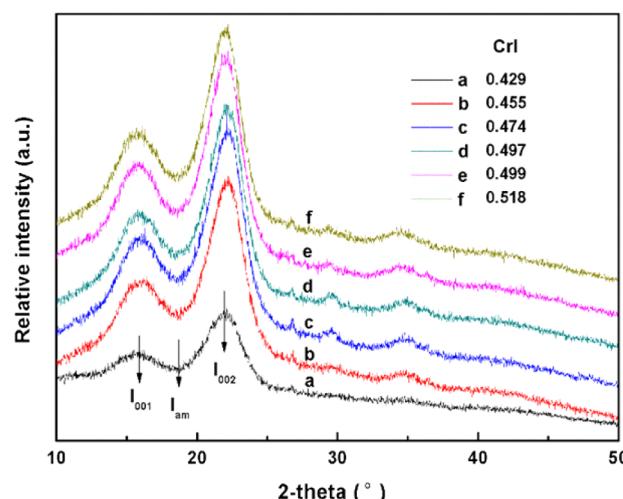


**Figure 5.** SEM images of corn stover (A, raw corn stover; B, pretreated with 10 wt %  $\text{Na}_2\text{CO}_3$ ; C, pretreated with 20 wt %  $\text{Na}_2\text{CO}_3$ ; D, pretreated with 40 wt %  $\text{Na}_2\text{CO}_3$ ; E, pretreated with 40 wt %  $\text{Na}_2\text{CO}_3$  and 10 wt %  $\text{H}_2\text{O}_2$ ; F, pretreated with 40 wt %  $\text{Na}_2\text{CO}_3$  and 15 wt %  $\text{H}_2\text{O}_2$ ).



**Figure 6.** FTIR spectra of corn stover (a, raw corn stover; b, pretreated with 10 wt %  $\text{Na}_2\text{CO}_3$ ; c, pretreated with 20 wt %  $\text{Na}_2\text{CO}_3$ ; d, pretreated with 40 wt %  $\text{Na}_2\text{CO}_3$ ; e, pretreated with 40 wt %  $\text{Na}_2\text{CO}_3$  and 10 wt %  $\text{H}_2\text{O}_2$ ; f, pretreated with 40 wt %  $\text{Na}_2\text{CO}_3$  and 15 wt %  $\text{H}_2\text{O}_2$ ).

pretreated samples. This was mainly because HSC pretreated samples contained less lignin (Table 1). Furthermore, the oxidation of lignin by  $\text{H}_2\text{O}_2$  could increase the hydrophilicity of the residual lignin in substrate and this was verified by  $\zeta$ -potential tests of the residual lignin after enzymatic saccharification, as mentioned above. The enhanced hydrophilicity could lower the nonspecific bonding of cellulase,<sup>25,39</sup> leading to a higher enzymatic digestibility. PFI refining could further improve the sugar yields mainly due to the increased porosity and specific surface area of substrate after mechanical treatment.<sup>5,24</sup> As measured by dye adsorption, the porosity of HSC pretreated corn stover (40 wt %  $\text{Na}_2\text{CO}_3$  and 15 wt %  $\text{H}_2\text{O}_2$ ) after refining increased from  $308 \pm 17$  to  $334 \pm 9$  mg/g. In addition, xylose yields of SC pretreated samples were higher compared to the HSC pretreated samples after 96 h of hydrolysis (Figure 8b). This was due to the fact that SC

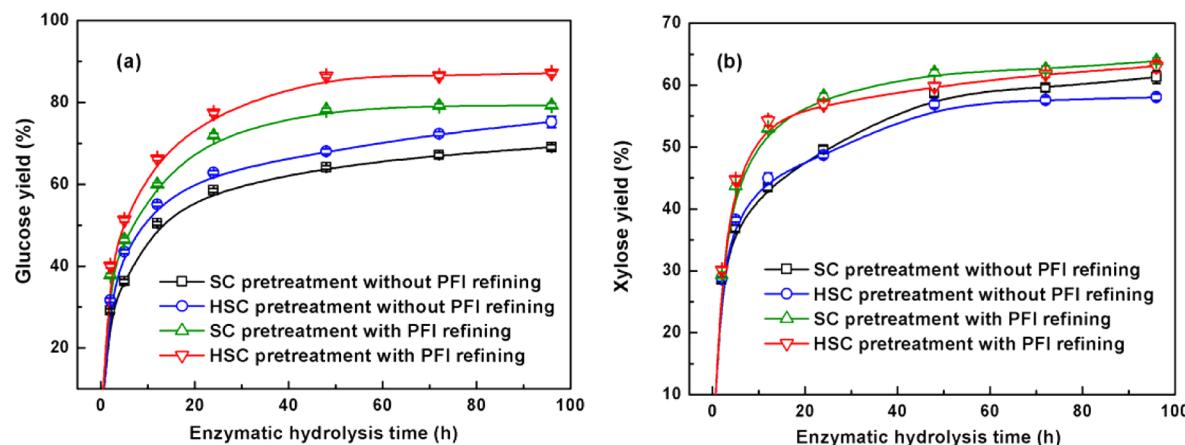


**Figure 7.** XRD patterns of corn stover (a, raw corn stover; b, pretreated with 10 wt %  $\text{Na}_2\text{CO}_3$ ; c, pretreated with 20 wt %  $\text{Na}_2\text{CO}_3$ ; d, pretreated with 40 wt %  $\text{Na}_2\text{CO}_3$ ; e, pretreated with 40 wt %  $\text{Na}_2\text{CO}_3$  and 10 wt %  $\text{H}_2\text{O}_2$ ; f, pretreated with 40 wt %  $\text{Na}_2\text{CO}_3$  and 15 wt %  $\text{H}_2\text{O}_2$ ).

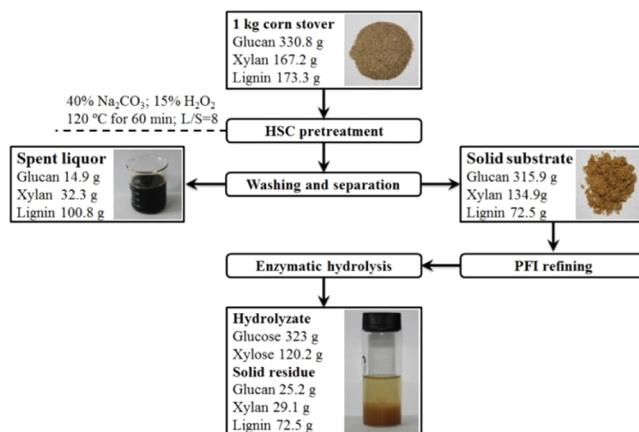
pretreated samples had higher content of xylan ( $25.23 \pm 0.23\%$ ) than HSC pretreated samples ( $22.29 \pm 0.13\%$ ) (Table 1). Yet, PFI refining could compensate the xylose yield loss after 96 h of saccharification, as shown in Figure 8b.

#### Mass Balance of HSC Pretreatment of Corn Stover.

Figure 9 shows the mass balance of HSC pretreatment and enzymatic hydrolysis of corn stover. 1 kg of milled and screened corn stover (with the particle size between 20 and 80 meshes) was treated by HSC pretreatment with 40 wt %  $\text{Na}_2\text{CO}_3$  and 15 wt %  $\text{H}_2\text{O}_2$  at  $120^\circ\text{C}$  for 60 min. After pretreatment, the solid and liquid was separated, and the solid yield was about 60 wt %. Then, the recovered solid was washed with tap water, and the washed solid substrate was subjected to PFI refining to modify fibers and further increase the enzymatic digestibility. After that,

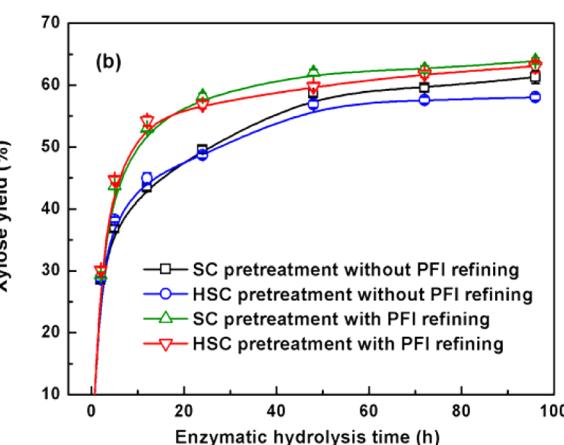


**Figure 8.** Glucose (a) and xylose (b) yields as the function of enzymatic hydrolysis time. (The chemical doses were 40 wt %  $\text{Na}_2\text{CO}_3$  and 15 wt %  $\text{H}_2\text{O}_2$  for HSC pretreatment, and 40 wt % for SC pretreatment.)



**Figure 9.** Mass balance of HSC pretreatment and enzymatic hydrolysis.

the refined corn stover was hydrolyzed to fermentable sugars by enzymes. As presented in Figure 9, 323 g of glucose and 120 g of xylose were obtained after HSC pretreatment and enzymatic hydrolysis from 1 kg of corn stover. The corresponding final total sugar yield was about 79% based on the total sugar in original corn stover, and this was about 10% higher in comparison with the one pretreated by SC pretreatment under the same conditions. This yield was also comparable to the total sugar yields achieved by alkaline sulfite pretreatment (78%) at the total alkali charge of 12 wt % with the ratio of  $\text{Na}_2\text{SO}_3$  to  $\text{NaOH}$  1.2<sup>26</sup> and the modified alkali pretreatment (80%) with 11 wt %  $\text{NaOH}$ , 0.1 wt % anthraquinone and 2 wt % sodium lignosulfonate.<sup>5</sup> In addition, after HSC pretreatment with 40 wt %  $\text{Na}_2\text{CO}_3$  (i.e., 29.3 g/L total titratable alkali) and 15 wt %  $\text{H}_2\text{O}_2$ , the total titratable alkali (based on  $\text{Na}_2\text{O}$ ) in spent liquor was about 12.5 g/L, indicating that over half of alkali was consumed after pretreatment. But the residual peroxide was not detectable in spent liquor, indicating that all peroxide was decomposed in HSC pretreatment. But the spent liquor containing residual alkali still could be reused at least three times (data not shown) to lower the  $\text{Na}_2\text{CO}_3$  consumption and the concentrated spent liquor could go to chemical recovery system to generate power and recover  $\text{Na}_2\text{CO}_3$  by combustion. A causticization stage is not needed compared to  $\text{NaOH}$  pretreatment. Lignin could also be recovered by spray drying and further modified to value added products like lignin-based



adhesives to gain more benefits.<sup>40</sup> The added  $\text{H}_2\text{O}_2$  is a green chemical. Its decomposition products are  $\text{H}_2\text{O}$  and  $\text{O}_2$ , which have no negative impact on the environment. On the other hand, diethylene triamine penta-acetic acid (DTPA), ethylene diamine tetra-acetic acid (EDTA), silicate, or alkaline earth metal ions (e.g.,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ) could be used as a stabilizer to reduce wasteful decomposition of peroxide.<sup>17</sup> It was found that a 1–2% increase of final total sugar yields could be obtained by adding 0.3 wt %  $\text{MgO}$  or 0.2% DTPA in HSC pretreatment. Yet, for the data reported above in this work, no peroxide stabilizer was used in HSC pretreatment. Finally, HSC pretreatment could be integrated into an alkali-based pulp mill as well to utilize partially the skilled workers, existing pulping equipment (e.g., cooking reactor, disc refiner), wastewater treatment and chemical recovery systems. Therefore, the capital cost of HSC pretreatment could be further lowered, and there would be fewer barriers to do large-scale production although more research work is needed (e.g., further optimization of HSC pretreatment by lowering  $\text{H}_2\text{O}_2$  dose, and the enzymatic hydrolysis at high-solid loadings).

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### Author Contributions

The paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

### Notes

The authors declare no competing financial interest.

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