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Pretreatment on Corn Stover with Low Concentration of Formic Acid

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Bioethanol derived from lignocellulosic biomass has the potential to replace gasoline. Cellulose is naturally recalcitrant to enzymatic attack, and it also surrounded by the matrix of xylan and lignin, which enhances the recalcitrance. Therefore, lignocellulosic materials must be pretreated to make the cellulose easily degraded into sugars and further fermented to ethanol. In this work, hydrothermal pretreatment on corn stover at 195°C for 15 min with and without lower concentration of formic acid was compared in terms of sugar recoveries and ethanol fermentation. For pretreatment with formic acid, the overall glucan recovery was 89% and pretreatment without formic acid yielded the recovery of 94%. Compared with glucan, xylan was more sensitive to the pretreatment condition. The lowest xylan recovery of 55% was obtained after pretreatment with formic acid and the highest of 75% found following pretreatment without formic acid. Toxicity tests of liquor parts showed that there were no inhibitions found for both pretreatment conditions. After simultaneous saccharification and fermentation (SSF) of the pretreated corn stover with Baker's yeast, the highest ethanol yield of 76.5% of the theoretical was observed from corn stover pretreated at 195°C for 15 min with formic acid.

Keywords: Corn stover, formic acid, pretreatment, glucan recovery, fermentability, simultaneous saccharification and fermentation (SSF)

Enzymatic hydrolysis of lignocellulosic materials followed by ethanol fermentation is a very promising technology for bioethanol production [7, 11, 22, 33, 37]. In order to obtain higher hydrolysis rates, pretreatment on lignocellulosic materials is an essential prerequisite to loosen the biomass structure, thus improving the accessibility of enzymes to

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biomass [6, 31, 32]. Corn stover is one of the most abundant agricultural residues with low commercial value [12], and extensive work has been carried out on pretreatments for corn stover. Ammonia as a pretreatment reagent to remove lignin as well as swelling of biomass has been studied for the purpose of ethanol production [13–15]. Steam explosion on SO_2-impreganted corn stover has been systematically investigated under different temperatures, residence times and SO_2 concentrations [23–26]. Dilute sulfuric acid for corn stover pretreatment was also tested by several researchers [19]. However, hydrolysates from these pretreatments contain not only cellulose and fermentable sugars, but also a wide variety of degradation products such as HMF, furfural, phenols, of aromatic aldehydes, which are potential inhibitors on downstream microbial processes [5, 16] and thus reducing the overall efficiency for bioconversion of lignocellulosics to ethanol. Hydrothermal pretreatment is another promising technology in biomass utilization. A liquid hot-water pretreatment process (190°C for 15 min) on corn stover with pH control maximized the solubilization of the hemicellulose fraction as liquid-soluble oligosaccharides [21]. The minimization of complete hydrolysis to monosaccharides minimizes the subsequent degradation of these sugars to various aldehydes during pretreatment [2, 17, 36]. Carbonic acid was also utilized in liquid hot water pretreatments under 180°C to 220°C for 2 to 32 min, with pre-reaction carbon dioxide pressures of either 0 or 800 psig [27]. Compared with water-only pretreatment, carbonic acid was found to enhance the concentrations of xylose and furan compounds released from corn stover.

Formic acid, as an effective delignification media in a higher concentration (80%–100%), has been widely used in the papermaking industry [1, 8, 28, 30, 34]. Another study on degradation of hemicellulose with 20%–30% formic acid combined with acetic acid was investigated and the best condition was found with the mixture of formic acid/acetic acid/H_2O at the ratio of 30/60/10 [38]. In our previous hydrothermal pretreatment study, formic acid was found to be formed during pretreatment at 195°C, 15 min

and the production was around 40 g/kg corn stover. In the present study, formic acid was used for pretreatment of corn stover, and the concentration was set equally to that formed during our previous pretreatment (i.e., 40 g/kg corn stover).

MATERIAL AND METHODS

Feedstock Materials
Corn stover was harvested in September 2003 in Italy. The air-dried corn stove was milled to 2 mm particle size for the pretreatment experiments with Wiley mill, standard model No. 3. The composition of the dried raw corn stover was determined by a modified gravimetric method [9].

Pretreatment of Corn Stover
The pretreatment was performed on milled corn stover in a loop autoclave [3]. The autoclave was loaded with 60 g of milled corn stover (dry weight, DW) and 1 l of water. Two pretreatment conditions were chosen in the present study: 195°C, 15 min; and 195°C, 15 min, 40 g formic acid/kg raw corn stover. The gas phase of the autoclave was filled with N_2. All the pretreatments were duplicated.

After pretreatment, the materials were filtered into residue and liquor parts. Both parts were collected. The residues were dried and kept in a climate cabinet at 20°C and 65% relative humidity. The liquors were kept in the freezer for analysis.

Enzymatic Hydrolysis of the Residues
The enzymatic hydrolysis of the residues as well as the untreated corn stover were carried out at 50°C, pH 4.8, with a solid/liquid ratio of 1:50, and the enzyme (Cellubrix L, 96 IU FPA/ml; Novozymes A/S) dosage of 30 FPU/g DW. After 24 h, the samples were centrifuged and the supernatants were analyzed for their glucose and xylose contents by HPLC. All the experiments were performed in triplicates.

Toxicity Test of the Liquors
Toxicity tests were performed in 250-ml fermentation bottles with 100 ml of liquor obtained from pretreatment or 100 ml of water as control, 2 g of pure glucose, 0.2 ml of urea (24%, W/V), and 0.2 g of dry yeast (Batch No. 2366; De Danske Spritfabrikker A/S, Denmark). The gas phase of the bottles was charged with N_2 and the bottles were equipped with fermentation locks prefilled with glycerol. Simultaneous saccharification and fermentation was then carried out at 32°C and the amount of produced ethanol was determined as weight loss caused by CO_2 release. The final ethanol concentration was determined by HPLC.

Simultaneous Saccharification and Fermentation of the Residues
The residues were mixed with water (pH adjusted to 4.8) at a ratio of 13% (DW/v). Liquefaction was carried out at 50°C with 15 FPU enzyme/g DW. After 24 h, the mixture was first cooled down to 32°C, and then another 15 FPU enzyme/g DW, 0.2 g of dry yeast, and 0.2 ml of urea (24%) were supplemented. The gas phase of the fermentation bottles was filled with N_2 and the bottles were equipped with fermentation locks prefilled with glycerol. SSF proceeded at 32°C and the amount of produced ethanol was determined as weight loss caused by CO_2 release. The final ethanol concentration was determined by HPLC.

Analysis Methods and Calculations
Composition analysis of untreated corn stover and the residues. Duplicates of 0.5 g of residue and raw corn stover were dried at 105°C until constant dry weight (DW) was obtained. The ash content was determined by heating the dried materials to 550°C for 3 h.

The composition of the residues and untreated corn stover was determined by a two-step acid hydrolysis; that is the first hydrolysis at 30°C with 1.5 ml of H_2SO_4 (72%) per 0.16 g DW for 60 min, followed by 121°C for 1 h with supplementation of 42 ml of water. The mixture was then filtered, and the dried solid part subtracted from ash content was reported as Klason lignin. The amounts of sugars in the filtrate were determined by HPLC.

Composition of the liquors. The liquors filtered through 0.45-μm pore size were analyzed for HMF, furfural, and acetic acid by HPLC. One-step dilute H_2SO_4 of 4% at 121°C for 10 min was used to further hydrolyze the liquors to determine the cellulose and hemicellulose contents. The sugar content was analyzed by HPLC.

HPLC analysis. HMF and furfural were determined by HPLC (Shimadzu) using a Luna SU C 18 250×4.6 mm column (Phenomenex), a linear eluent gradient of methanol (10%–90%) at pH 3 with a flow rate of 0.7 ml/min, and a UV-detector (Shimadzu Corp., Kyoto, Japan) at 280 nm using authentic compounds as calibration standards.

The amounts of sugar monomers, acetic acid, and ethanol concentration were measured by HPLC using a Rezex ROA column (Phenomenex) at 63°C and 4 mM H_2SO_4 as eluent at a flow rate of 0.6 ml/min. A refractive index detector (Shimadzu Corp., Kyoto, Japan) was used.

Ethanol yield (Y_E). *S. cerevisiae* was used as the only fermenting microorganism in this work. Ethanol yield was calculated according to the glucan content.

$$Y_E = \frac{M_E}{M_{G_W} \times 0.51} \times 100\%$$

M_E: mass of ethanol measured by gravimetric method
M_{G_W}: glucan content in untreated/treated corn stover
0.51: the conversion constant of glucose to ethanol

RESULTS AND DISCUSSION

Composition of Raw Corn Stover and Residues after Pretreatment
Table 1 lists the average composition (dry basis) of raw corn stover. Glucan was the dominant component followed by xylan and Klason lignin. Arabinan, galactan, xylan, and mannan were reported as the main components of hemicellulose [20]. It varied with different biomass species. In wheat straw and grasses, hemicellulose normally contained

Table 1. Average chemical composition of raw corn stover.

Component	Percentage in weight (%)
Glucan	36.90±0.56
Xylan	20±0.73
Arabinan	3.07±0.38
Klason lignin	13.76±0.15
Ash	5.67±0.14

Table 2. Average chemical composition of the residues.

Component	Percentage in weight (%)	
	195°C, 15 min	195°C, 15 min, formic acid
Glucan	54.39±2.66	55.26±0.53
Xylan	9.29±1.13	4.58±0.02
Arabinan	0.41±0.04	0.19±0.01
Klason lignin	22.71±1.25	23.24±0.36
Ash	4.13±0.78	4.53±0.35

arabinan, galactan, and xylan [35], whereas mannan is only seen in hardwood and softwood [4, 35]. In the corn stover employed in the present study, arabinan accounted for a small proportion and there were no detectable mannan and galactan.

After pretreatment at 195°C with/without formic acid for 15 min, an increase of 17–19% in glucan content was found in residues compared with raw corn stover (Table 2). The lignin content also increased from 13% in raw corn stover to 22–24% in residues. The increases for both glucan and lignin were mainly due to the release of xylan into the liquor during the pretreatment process, leaving glucan and lignin in the residues. In the present study, the lowest xylan content of 5% was found in the residue from the more severe pretreatment condition (195°C, 15 min, formic acid).

Recoveries of Glucan and Xylan

Recovery of glucan or xylan, which was expressed as the sum of glucan or xylan recovered from both liquor and solid parts, is shown in Fig. 1. Apparently, most of the glucan had remained in the solid after pretreatment, accounting for 89% for pretreatment without formic acid and 82% for pretreatment with formic acid. Compared with the glucan in the solid, the glucan recovery from the
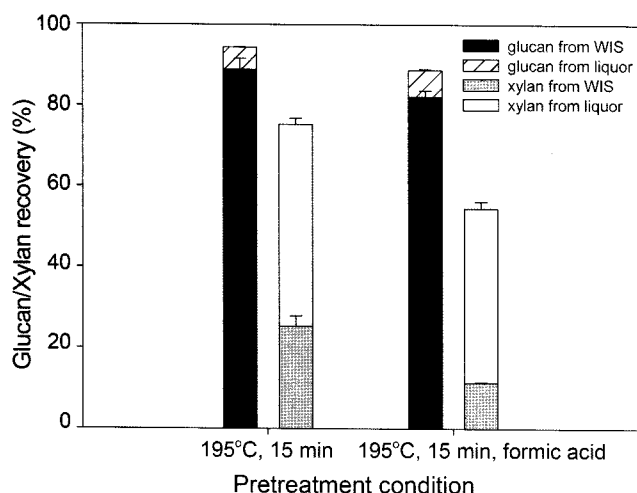
liquor for both pretreatment conditions was much less and there were only 5% and 7%, respectively. For xylan recovery, 50% was obtained from the liquor part under pretreatment without formic acid and 43% after pretreatment with formic acid. In addition, xylan recoveries from the solid part varied between 11% for pretreatment without formic acid and 25% for pretreatment with formic acid. In terms of total recovery, pretreatment without formic acid gave the highest glucan recovery of 94% and highest xylan recovery of 75%. Pretreatment with formic acid yielded less glucan recovery of 89% and xylan recovery of 55%. This indicated that pretreatment with formic acid was helpful in dissolving glucan, but the dissolved glucan was probably further degraded because of the harsher pretreatment condition. This made total glucan recovery from pretreatment with formic acid less than that from pretreatment without formic acid. Moreover, as stated above, total xylan recoveries from both pretreatments were much less than glucan recovery, especially for the pretreatment with formic acid. This indicated, in one side, that xylan was more sensitive to the pretreatment condition than glucan. On the other hand, it showed that the harsher the pretreatment condition was, the less xylan recovery was obtained.

Yields of Glucose and Xylose

Yield of glucose or xylose in the present study was defined as the sum of mono- and oligo- glucose or xylose from the pretreatment step and mono- glucose or xylose from the enzymatic hydrolysis step, as shown in Fig. 2. Obviously, most of the glucose was released in enzymatic hydrolysis step (Fig. 2) and xylose was mostly released in the pretreatment step. As for glucose yield, the two pretreatments gave a similar yield; that is 27.7 g/100 g raw corn stover following pretreatment without formic acid and 27.2 g/100 g with formic acid, representing 75.0% and 73.7% of glucose in

Fig. 1. Recoveries of glucan and xylan after pretreatment.
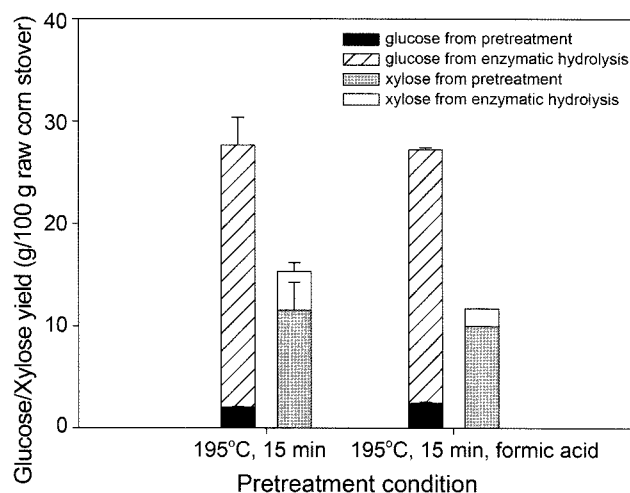
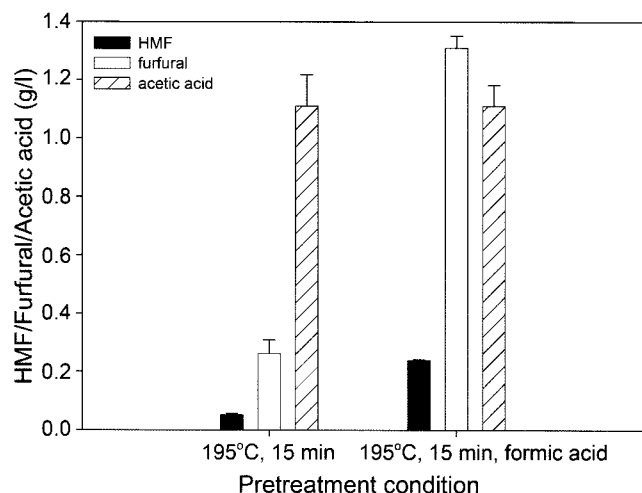**Fig. 2.** Yields of glucose and xylose after pretreatment and enzymatic hydrolysis.

Fig. 3. HMF, furfural, and acetic acid produced under different pretreatment conditions.

the raw corn stover, respectively. As for xylose yield, most of the xylose was dissolved into the liquor part, leaving less in the solid part for the subsequent enzymatic hydrolysis. Pretreatment without formic acid yielded 15.3 g of xylose from 100 g of raw corn stover and the other pretreatment condition produced 11.7 g of xylose, equivalent to 66.4% and 50.8% of theoretical, respectively.

Toxicity Test of Liquors

In the present study, higher temperature was employed in the pretreatment step, and inhibitors such as HMF, furfural, and acetic acid (Fig. 3) were produced. In order to test to what extent these inhibitors inhibited the proliferation and viability of Baker's yeast, ethanol fermentations were carried out and the ethanol yields are shown in Fig. 4. Compared with the reference solution, ethanol productions from liquors obtained from pretreatment were higher and no observable lag phase was seen for both liquors. This indicated that the
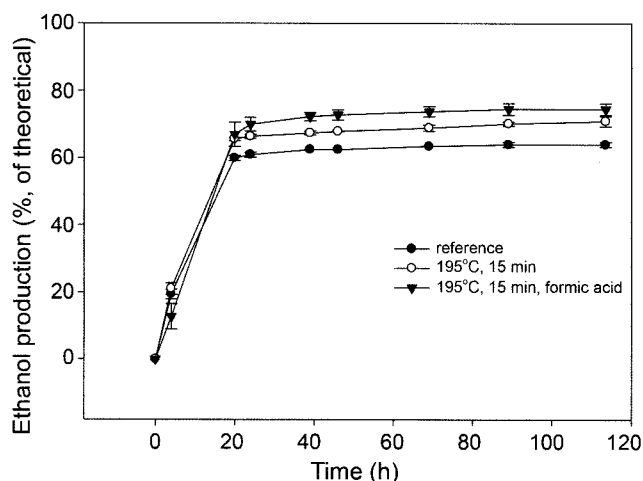

Fig. 4. Fermentability of liquors obtained from pretreatment.
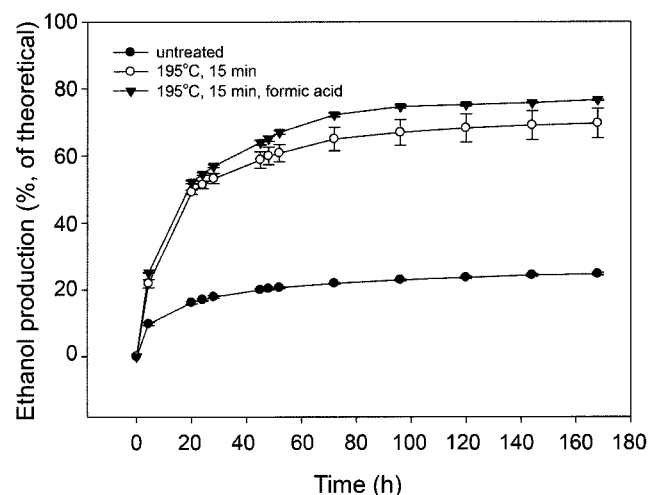

Fig. 5. SSF of the residues obtained from different pretreatment conditions.

inhibitors produced during the pretreatment process were not toxic to the Baker's yeast. After 12 h, the ethanol yields from both liquors were slightly higher than the reference solution. This was probably due to the enriched nutrients released in pretreatment into the liquors [10, 18, 29].

Simultaneous Saccharification and Fermentation of the Residues

The results of SSF carried out with pretreated and untreated corn stover are shown in Fig. 5. For both the pretreated corn stovers, the patterns of ethanol yields were similar; that is the ethanol yields increased rapidly during the first 2 days of SSF and thereafter increased slowly. The highest ethanol yield in 7 days was 76.5% of theoretical, observed from corn stover pretreated under 195°C, 15 min with formic acid. Fermentation from corn stover pretreated under 195°C, 15 min gave 69.6% of theoretical. Compared with these two pretreated corn stovers, the ethanol yield from untreated corn stover was as low as 24.6% of theoretical and remained approximately constant after 2 days.

No inhibition effects were found on corn stover for both pretreatments with and without formic acid. The ethanol production of 76.5% of the theoretical was obtained from corn stover pretreated by 195°C for 15 min with formic acid, higher than that from pretreatment without formic acid, which was 69.6%. This showed the formic acid was helpful in producing an easily degraded corn stover. Further optimization of the pretreatment on corn stover with formic acid will be investigated in the near future.

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