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Enhanced Enzymatic Hydrolysis of Lignocellulose by Ethanol-Assisted $FeCl_3$ Pretreatment

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The pretreatment and saccharification processes are both the bottlenecks of lignocellulose bioconversion. Pretreatment plays an important role in the saccharification efficiency. In this paper, ethanol-assisted $FeCl_3$ pretreatment was developed to reduce lignocellulose resistance and to enhance the hydrolysis efficiency. The effect of various pretreatment conditions, such as the concentration of $FeCl_3$ and ethanol, pretreatment temperature and time, on the chemical compositions and physical structures of lignocellulose were investigated by scanning electron microscope (SEM), X-ray diffraction (XRD) and Fourier infrared spectrum (FTIR). The increase in the concentration of $FeCl_3$ and ethanol could both boost the degradation of hemicellulose and delignification, respectively. The optimized conditions were obtained, i.e. the concentration of $FeCl_3$ is 0.1 mol/L, the concentration of ethanol is 60 % (v/v), 120 °C and 60 min. The ratio of cellulose conversion at solid loading of 10 % after pretreatment under the optimized conditions reached 96.8 %. And we found that the solution of $FeCl_3$ provided significantly acidic environment, which promoted the degradation of hemicellulose when the pH reached a certain degree (pH 2.5). The iron ions could be absorbed by the pyran ring of carbohydrate to form complex, which may trigger the degradation of carbohydrates and delignification. Our work suggested that the ethanol-assisted $FeCl_3$ pretreatment was very competitive for biomass bioconversion.

1. Introduction

The depletion of fossil energy makes the development of renewable energy has become a key issue in the world. The techno-economic and environmental impacts of second-generation ethanol from lignocellulose were widely concerned and studied (Bonomi et al., 2016). The pretreatment and saccharification processes are both the bottlenecks of lignocellulose bioconversion (Huang et al., 2017). The biomass recalcitrance caused by the chemical composition and physical structure of lignocellulose makes it very difficult to be degraded. Pretreatment plays an important role in the saccharification efficiency. Various pretreatment processes have been developed to promote the enzymatic efficiency by altering the structure or chemical composition of the substrate. The increase in cellulose accessibility by dilute acid pretreatment is mainly attributed to the hydrolysis of hemicellulose. In alkaline pretreatment, the intermolecular ester bonds cross-linking hemicellulose and lignin are saponified, resulting in delignification of lignocellulose. It is believed that the internal bonds in lignin are hydrolyzed as well as lignin-hemicellulose bonds during organosolv pretreatment, thus increasing the adsorption of cellulase enzymes onto substrates and enhancing the saccharification efficiency (Su et al., 2017).

It was verified that inorganic salts, such as $FeCl_3$, could improve the enzymatic hydrolysis yields through hemicellulose degradation. Pretreatment using $FeCl_3$ is a method similar to dilute acid pretreatment, but it has a more positive effect on improving the saccharification efficiency, especially when combined with organic pretreatment (Lopez-Linares et al., 2013). (Kim et al., 2010) used $FeCl_3$ as a catalyst instead of H_2SO_4 to

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assist organosolv pretreatment (170 °C, 60 min, 50 % w/w ethanol), and the bioconversion of barley straw at a low solid loading of 1 % increased from 50 % to 89 %. However, the mechanisms why the ethanol-assisted $FeCl_3$ pretreatment can improve enzyme access are not explicit. Meanwhile harsher pretreatment conditions will lead to further hydrolysis of monosaccharides into furan, hydroxymethyl furfural and other inhibitors. It is not conducive to the effective bioconversion, especially for saccharification and fermentation at high solid loading, which is very important to commercial production of ethanol.

The main objective of the present study was to evaluate the effects of ethanol-assisted $FeCl_3$ pretreatment on the saccharification efficiency at high solid loading (10 %). In order to analyze the mechanism of the pretreatment process, chemical compositions and physical structures of lignocellulose were investigated after pretreatment. In addition, the moderate pretreatment conditions were optimized to fulfill the high solids saccharification process.

2. Materials and methods

2.1 Materials

Air-dried corncobs were pre-milled and screened to a size of 20 – 80 meshes. The commercial cellulases (GC220) was purchased from Genencor International (CA, USA). The average activities of GC220 were 123 filter paper unit (FPU)/mL. Glucose, xylose and arabinose were purchased from Sigma. All other reagents were of analytical grade and were obtained from commercial sources.

2.2 Ethanol-assisted $FeCl_3$ pretreatment

The pretreatment was performed in hydrothermal synthesis reactor with a capacity of 200 mL. The reactor was manufactured with stainless steel and can be maintained at a certain reaction pressure. Mixture of 50 %(v/v) aqueous ethanol and inorganic catalyst ($FeCl_3\cdot 6H_2O$) of 0.1 mol/L was used for the pretreatment, and the air-dried corncobs were mixed with the above mixture at a solid-to-liquid ratio (S:L) of 1:6 (w:v). The pretreatment temperature was set at 120 °C for 60 min without the ramping time to reach the target temperature. The various pretreatment conditions were applied to optimize the pretreatment performance. After treatment, the corncobs were separated by filtering and washed with distilled water to remove the residual chemical reagents until the pH was neutral.

2.3 Enzymatic hydrolysis

The pretreated corncobs (2 g) were added into a citrate buffer (50 mM, pH 4.8) at a solid loading of 10 % (w/v). All the enzymatic hydrolysis experiments were carried out in a rotary shaker at 50 °C and 150 rpm. Unless otherwise stated, enzyme loading for hydrolysis was 30 FPU per gram of glucan. 300 μL aliquots were taken out at different time and centrifuged to collect the supernatants. The contents of sugars in the supernatant were determined by high performance liquid chromatography (HPLC).

2.4 Compositional analysis

Compositional analysis of all lignocellulosic samples was performed by standard methods (NREL laboratory analytical procedure LAP-002, 2008). Soluble sugars, such as glucose, xylose, were quantified by a HPLC system (Agilent, USA) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 65 ^{o}C. The mobile phase was 0.005 mol/L sulfuric acid, and the flow rate was 0.6 mL/min. All the samples before injection were centrifuged and filtered through 0.45 μm membranes.

2.5 Microscopic analysis

SEM (Hitachi High-technologies CO., Japan) analysis was performed to investigate morphological changes in corncobs during pretreatment. Air-dried samples were prepared by adhibiting them on a specimen stub using carbon tape. Then the samples were sputter coated with Pt by sputter coater prior to imaging with scanning electron microscope using 5.0 kV accelerating voltage.

FTIR spectrum detection of lignocellulose is often used to represent the change of the chemical bond in corncobs during pretreatment. Corn cob samples were mixed with potassium bromide in 1:100 and pressed into sheets. Then the samples were scanned from 4,000 cm^{-1} to 500 cm^{-1}.

2.6 Cellulose crystallinity

Cellulose crystallinity (CrI) was measured by the X-ray diffractometer (X-Pert, PANAlytical CO., Holland) operating at 30 kV and 30 mA. The samples were scanned at 12 ^{o}/min from 5 ^{o} to 50 ^{o}. The crystalline index was calculated according to the method of (Segal et al., 1959).

3. Results and discussion

3.1 Effects of pretreatment temperature and time

Figure 1a displays that the decomposition of hemicellulose and lignin were enhanced with the increase in the pretreatment temperature at a certain range of temperature (80 °C ~ 120 °C). After pretreatment at 120 °C, the percentage of cellulose in the substrate increased significantly, which was 2 times higher than that pretreated at 80 °C. When the pretreatment temperature increased from 120 °C to 160 °C, the components of pretreated corncobs were almost the same. As shown in Figure 1b, when the pretreatment temperature increased from 80 °C to 120 °C, the glucose yield of pretreated corncobs increased significantly from 31.4 % to 88.4 % after 96 h hydrolysis. And the conversion of cellulose slowly climbed to 97.5 % by increasing the pretreatment temperature to 160 °C. Taking into account that high temperature can easily lead to the formation of fermentation inhibitors, and the reaction vessel materials and energy consumption were put forward higher requirements, we selected 120 °C as the suitable pretreatment temperature.

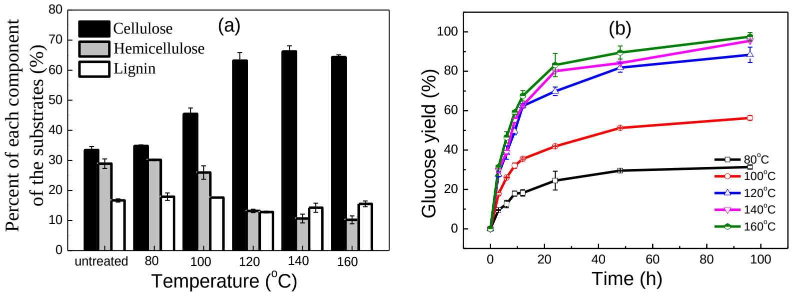

Figure 1: Effect of pretreatment temperature on the substrate composition (a); and the hydrolysis yield of the pretreated corncobs (b).

During the pretreatment, the hemicellulose in the substrate was degraded to xylose and arabinose, and part of the lignin was degraded or dissolved as the pretreatment time prolonged (Figure 2). Most of the inorganic salt-catalyzed ethanol pretreatment methods achieved optimal fractional effects within 30 min in relevant reports (Zhao et al., 2011). When the pretreatment was prolonged from 20 min to 120 min, the yield of glucose was increased from 82.9 % to 95.8 % after 96h enzymatic hydrolysis. As shown in Figure 2b, the pretreatment time had little influence on the hydrolysis efficiency.

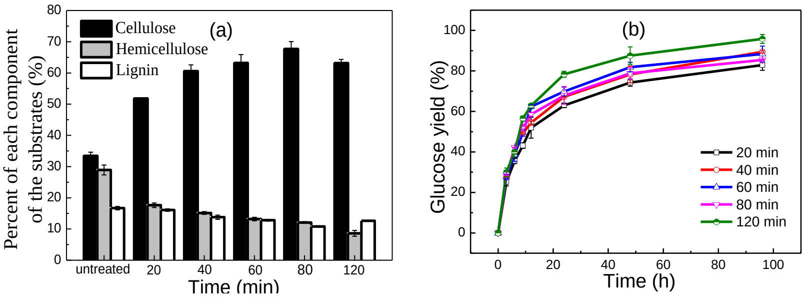

Figure 2: Effect of pretreatment time on the substrate composition (a); and the hydrolysis yield of the pretreated corncobs (b).

3.2 Effect of the concentration of ferric chloride

Figure 3 illustrates the effect of the concentration of ferric chloride on the fractionation and enzymatic efficiency of the corncobs. When the concentration of ferric chloride was 0.01 mol/L and the volume fraction of ethanol was 50 % in the pretreatment solution, we found the glucose yield of pretreated corncobs was about twice that of untreated corncobs (24.8 %) after 96h hydrolysis (Figure 3b), even though the composition of these two substrates was almost the same (Figure 3b). The reason was supposed to be the structural changes of substrates after pretreatment, which increased the accessible area of cellulase and promoted enzymatic hydrolysis of the substrates. When the concentration of ferric chloride increased to 0.05 mol/L, the pretreatment solution was acidic (pH = 2.3). After pretreatment, the hemicellulose content of the substrate decreased, indicating that the acidic environment can promote hemicellulose hydrolysis. If the concentration of ferric chloride increased to 0.2 mol/L, the yield of glucose after hydrolysis climbed to 92.8 %. Although the higher concentration of ferric chloride (c_{FeCl_3}=1.0 mol/L) can greatly enhance the removal of hemicellulose, no significant increase in the hydrolysis efficiency was found. The reason might be that high concentrations of ferric chloride led to further degradation of hemicellulose to furan, while a portion of cellulose was degraded to

5-hydroxymethylfurfural, both of which have an inhibitory effect on enzymatic hydrolysis of cellulose.

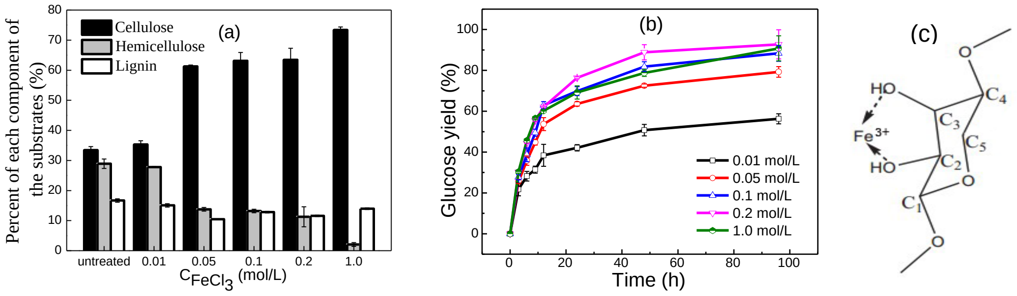

Figure 3: Effect of the $FeCl_3$ concentration on the substrate composition (a); the hydrolysis yield of the pretreated corncobs (b); and the complex of xylan-Fe^{3+} (c).

As shown in Figure 3c, polar polysaccharide molecules (such as xylan) in lignocellulose have activating properties. Hydroxyl groups attached to C2 and C3 in the pyran ring could capture metal ions to form complexes. The metal ions make the bond angle and bond length between the C atom and the O atom in the pyran ring become larger, leading to the ring-opening reaction between C1 and C2, and promote the degradation of the glycosidic bond to monosaccharide or oligosaccharide. (Yang et al., 2006) proposed that the "ionic force" between the metal ion and the pyran ring can induce the degradation of polysaccharide and produce various products with different linkages. Under acidic conditions, protons can trap electrons to weaken bond energy and promote chemical bond cleavage. When the acidity reaches a certain degree ($pH<2.5$), inorganic metal ions provide more empty orbitals, and promote hemicellulose hydrolysis and lignin removal. Table 1 shows the effect of pH-triggered $FeCl_3$-catalyzed ethanol pretreatment with acid addition. Increasing the concentration of $FeCl_3$, or decreasing the pH of the solution can significantly improve the bioconversion efficiency. However, when the acidity of the solution was strong, the change in ferric chloride concentration has little effect on the saccharification efficiency. It can be inferred that the pretreatment process is a combination of ferric chloride concentration and the acidity of the solution.

Table 1: The chemical components and saccharification yield of different pretreated corncobs ("" refers that the pH is adjusted to the specified value using HCl)*

c_{FeCl_3}(mol/L)	pH	Cellulose(%)	Hemicellulose(%)	Lignin(%)	Glucose yield(%)
0.05	1.0*	74.1	8.6	6.4	90.4
0.01	1.0*	68.3	9.0	5.5	86.1
0.01	2.0*	47.8	22.6	14.3	67.6
0.05	2.0*	61.3	15.1	10.6	81.9
0.01	2.8	35.3	27.8	15.1	50.7
0.05	2.3	61.3	13.7	10.5	79.2

3.3 Effect of ethanol concentration

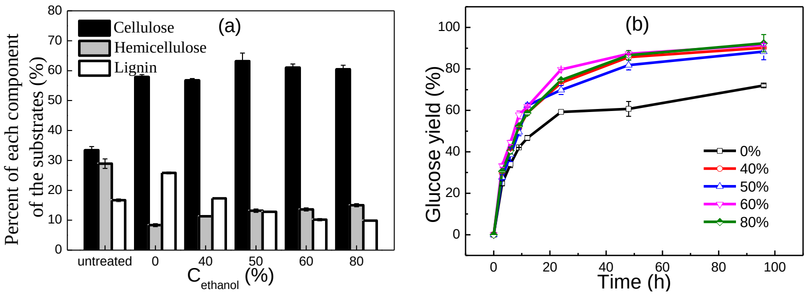

Figure 4: Effect of the ethanol concentration (v/v) on the substrate composition (a); and the hydrolysis yield of the pretreated corncobs (b).

As shown in Figure 4a, the pretreatment of corn cob substrate with 0.1 mol/L $FeCl_3$ solution and no ethanol could lead to the degradation of most hemicellulose in the substrate. After pretreatment, the glucose yield of pretreated corncobs increased rapidly to 72.0 % (Figure 4b). This further reflected the significant role of the ferric chloride concentration on the pretreatment. When the volume fraction of ethanol in the pretreatment solution was increased to 60 %, the lignin content was reduced remarkably. The dissolution or degradation of hydrophobic lignin in the substrate reduced the competitive adsorption of lignin to the cellulase, therefore enhancing the enzymatic efficiency of the substrates to 96.8 %.

3.4 Structural analysis

Figure 5a illustrates that the structure of untreated corn cob was fluffy. After pretreated with 0.1 mol/L ferric chloride aqueous solution (Figure 5b), the degradation of hemicellulose in corncob resulted in the collapse of the substrate structure, the deepening of the surface gully and the decrease of the particle size. As shown in Figure 5c, when the volume fraction of ethanol in the pretreatment solution was 50 %, some droplets appeared on the surface of corncob. Lignin-polysaccharide complexes or lignin dissolved in ethanol might attached to the surface of the substrate to form spherical precipitates when the temperature decreased after pretreatment (Koo et al., 2012).

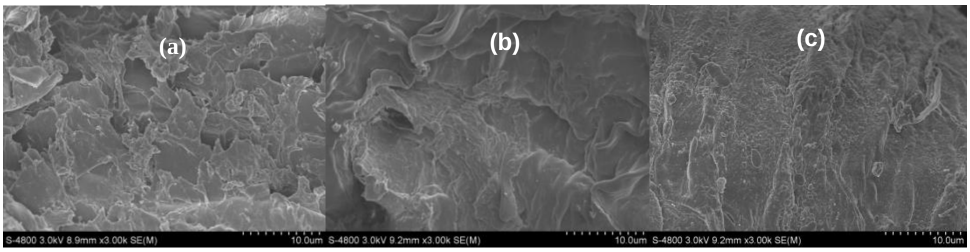

Figure 5: SEM images of corncob samples, (a) Untreated corncobs; (b) Pretreated corncobs with 0.10 mol•L^{-1} FeCl$_3$; (c) Pretreated corncobs with 0.10 mol•L^{-1} FeCl$_3$, 50 % (v/v) ethanol.

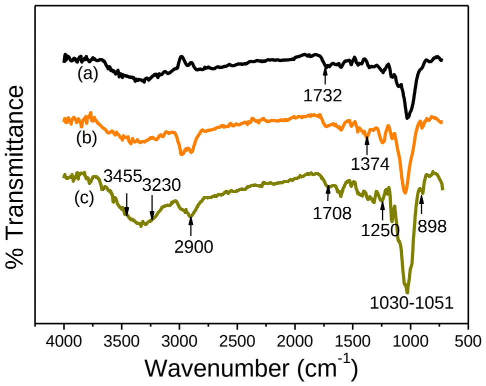

Figure 6: FTIR spectra of corncob before and after the pretreatment process. (a) control; (b) $c_{ethanol}$ = 50 % (v/v); (b) $c_{ethanol}$ = 50 % (v/v), c_{FeCl_3} = 0.1 mol/L.

The effects of ethanol and ferric chloride were analyzed by FTIR spectra. As shown in Figure 6, 1,051 cm^{-1} and 1,374 cm^{-1} represent the cyclic ether linkages and phenolic hydroxyl groups in lignocellulose, respectively. This indicates that the increase in the volume fraction of ethanol could promote the acid-catalyzed cleavage of α and β-ether bonds in lignin (Figure 6b). When the concentration of ferric chloride was 0.2 mol/L, the absorption peak of ether linkage (C-O-C) at 1,250 cm^{-1} was weakened, which indicated that ferric chloride could promote the interaction of ether bond between lignin and polysaccharide. While the ester bond absorption peak at 1,732 cm^{-1} weakened, indicating that ferric chloride can break the ester bond between lignin and polysaccharide (Figure 6c).

The peaks at 3,455 – 3,230 cm^{-1} represent the intramolecular or intermolecular hydrogen bonds in cellulose, and the peak at 898 cm^{-1} is the characteristic peak of the β-glycosidic bond between cellulose and hemicellulose, indicating that after pretreatment cellulose II or amorphous cellulose appears in the substrate. As shown in Figure 6b, the 2,900 cm^{-1} absorption peak represents the C-H stretching vibration of the cellulose, whereas 1,057 cm^{-1} represents the C-O stretching bond in the cellulose. The cellulose in the lignocellulosic substrate has a crystalline structure, while the lignin and hemicellulose displayed amorphous structures. The crystallinity of the substrate is related to the cellulose content and the specific structure of the cellulose crystals (Haque et al., 2013). The internal crystalline cellulose is exposed after pretreatment, which is manifested as the increase in the crystallinity of the substrate (Table 2). This enhanced the specific adsorption of the substrate to the cellulase and promoted the enzymatic hydrolysis of the substrate. When the pretreatment temperature was 120 °C, ferric chloride concentration was 0.1 mol/L and ethanol volume fraction was 60 %, the relative crystallinity of corncob was 65.3 % and the conversion of cellulose reached 96.8 %.

Table 2: The relative crystallinity of different substrates

Substrate	Pretreatment conditions				CrI / %
	T / ºC	t / min	c_{FeCl3} / mol·L^{-1}	$c_{ethanol}$ / %	
Corncobs	-	-	-	-	39.4
Corncobs	120	60	0.10	50	59.0
Corncobs	120	60	0.01	50	50.5
Corncobs	120	60	0.20	50	64.1
Corncobs	120	60	0.10	0	58.8
Corncobs	120	60	0.10	60	65.3

4. Conclusions

This work indicated that ethanol-assisted $FeCl_3$ pretreatment could effectively break the recalcitrance of lignocellulose. The acidic environment and ionic force provided by ferric chloride solution were conducive to the decomposition of hemicellulose and the rupture of the bond between lignin and polysaccharide. Ethanol was proved to change the structure of lignin and improve the pretreatment performance in relatively mild conditions. We have selected the appropriate pretreatment conditions to treat the corncobs and hydrolyze them at high solids loading (10 %) for 96 h. The cellulose conversion of pretreated corncobs reached 96.8 %, which showed an absolute superiority to the saccharification results of other substrates at low solid loading.

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