Improving the Efficiency of Biomass Pretreatment and Enzymatic Saccharification Process by Metal Chlorides

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A series of metal chlorides with different valences were used during biomass pretreatment and enzymatic saccharification. After pretreatment, the solid substrate (SS), pretreatment liquor (PL), and conversion yield of cellulose (CYC) were characterized. The results showed that the monovalent salts, NaCl and KCl, as well as the divalent salts, $MgCl_2$ and $FeCl_2$, could promote the enzymatic saccharification to a certain extent, while $CaCl_2$ had little influence on the enzymatic saccharification and cellulase activity, and $ZnCl_2$ had an inhibitor effect on them. For a trivalent salt, $FeCl_3$, the removal rate of hemicellulose and the CYC could come up to a high value. The hemicellulose degradation was mainly related to the valences of metal ions. The cellulose models, the enzymatic saccharification, and the enzyme activity assay results showed that most of the metal chlorides had promoter action toward the enzymatic hydrolysis and cellulase activity, with the exception of $ZnCl_2$. Moreover, the metal ions, especially with high valences, had local effects, which could intensify the potentiation or inhibition impacts on the cellulase activities.

Keywords: Metal chlorides; Pretreatment; Enzymatic saccharification; Cellulase activity

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INTRODUCTION

Cellulose, the most abundant organic compound on the earth, is one of the three main components in plant cell walls. Cellulose is a linear, unbranched, and natural polymer consisting of 500 to 10000 glucose units, and the long polymer chains are parallel to each other, forming microfibers through hydrogen bonds. Among the hydroxyl groups and oxygen-containing groups within the same, or adjacent molecules, the hydrogen bonds are easily generated and allow many molecules to form a crystal structure. This dense structure makes the cellulose degradation become difficult, and a pretreatment process is normally conducted for the lignocellulose prior to enzymatic saccharification.

Metal chlorides are divided into three types, alkaline metal chloride, alkaline-earth metal chloride, and transition metal chloride (Kang *et al*. 2013). According to the electronic theory of acid and alkali, a Lewis acid is defined as the atom, molecule, ion, or atomic cluster that can accept the external electron pairs, namely the electron-pair acceptor. Thus, the metal chlorides are Lewis acids. For the pretreatment using metal chlorides, the dense structures among cellulose, hemicellulose, and lignin can be destroyed. A proportion, even

all of the hemicellulose, can be removed, and the physical-chemical structure can be changed, such as decreasing the crystallinity and degree of polymerization of the cellulose, increasing the porosity and specific surface area of the raw material, *etc*. (Liu and Wyman 2006; Sathitsuksanoh *et al*. 2012; Zhang *et al.* 2016). To date, the actions of the metal chlorides in biomass pretreatment and enzymatic hydrolysis are unclear. This study examined the characteristics of the metal chlorides in biomass pretreatment and enzymatic hydrolysis, which can provide an overall evaluation for the biomass conversion process.

EXPERIMENTAL

Materials

Eucalypt chips were milled and screened to obtain a fraction of 40 to 60 mesh size. Cellulase (Celluclast 1.5 L, 50.97 FPU/mL) and β-glucosidase (Novozyme 188, 1290.69 CBU/mL) were obtained from Novozymes (Beijing, China). All other reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

Pretreatment with Metal Chlorides

The pretreatment process was conducted in an oil bath, where 5 g of eucalyptus sample was placed into a steel reactor (effective volume 45 mL) with a solid/liquid ratio of 1:6. The metal chloride concentration, pretreatment time, and pretreatment temperature were as follows: 0.3 mol/L of Cl^-, 20 min, and 160 °C, respectively. After pretreatment, the reactor was placed into cold water to terminate the reaction. The solid residue from the reactor was filtered with an 80-mesh screen. The filtrate designated as the PL was analyzed to quantify the glucose and xylose contents. The filter residue designated as the SS was washed with deionized water (100 mL) three times. The SS was analyzed to quantify the cellulose, hemicellulose, and lignin contents and then used for enzymatic hydrolysis.

Enzymatic Hydrolysis

A total of 2 g of the SS was placed into a beaker flask and diluted with a buffer solution (sodium acetate / acetic acid, 0.1 mol/L, pH = 4.8) to a concentration of 2.5% (all based on the oven dried SS, o.d.). Then, 10 FPU/g of cellulase, 15 CBU/g of β-glucosidase, and 3 drops of ethyl acetates were added into the beaker flask. The beaker flask was sealed and put into a shaker for 48 h at 50 °C with a rotational speed of 150 rpm. After hydrolysis, the beaker flask was heated for 5 min at 90 °C in a water bath to deactivate the enzymes.

For the enzymatic hydrolysis, using cellulose model as substrate, the absorbent cotton was also milled and screened to obtain a fraction of 40 to 60 meshes. The metal chlorides were added into the hydrolysis system with a range of 0.05 mol/L to 1.0 mol/L. In particular, sodium acetate and acetic acid were used to readjust the buffer solution (pH 4.8). Other hydrolysis conditions were the same as that of the SS.

Characterization of the Raw Material, the SS, and Hydrolysate

For the raw material and the SS, the NREL LAP method was used (Mosier *et al*. 2005). The composition of the raw material was as follows: glucan 47.86%, xylan 15.50%, arabinan 1.03%, and lignin 29.15%. The SS yield was calculated as the mass ratio of the SS and eucalyptus chips.

The PL and enzymatic hydrolysate were centrifuged at 10000 rpm for 10 min and filtered through a membrane filter (0.22 μm) prior to detecting. The HPLC (Agilent 1260)

equipped with a Bio-Rad Aminex HPX-87H (300 × 7.8 mm) column was used, and detecting conditions were as follows: column temperature 55 ºC, differential detector, mobile phase H_2SO_4 (0.05 mol/L) with a flow rate of 0.6 mL/min, and a sampling volume of 10 μL. The removal rates of cellulose and hemicellulose were calculated as their loss ratios during the pretreatment. The CYC was calculated as follows:

$$CYC = 0.9 * (\text{glucose in enzymatic hydrolyzate}) / (\text{cellulose in the SS}) \quad (1)$$

Determination of Cellulase Activity

The filter paper activity (Fpase) for cellulase was determined using a buffer solution (pH 4.8) and the DNS method (Ghose 1987; Sluiter *et al.* 2012). The original cellulase was diluted with the buffer solution prior to detecting. The specific dilution factor, achieved by releasing 2 mg of glucose in the Fpase detection, was used to investigate the influence of metal chlorides on the activity. Consequently, the metal chlorides were dissolved into the buffer solution and formed a serious of solutions (pH 4.8), which replaced the buffer solution, that were used for activity detection.

RESULTS AND DISCUSSION

Pretreatment using Different Metal Chlorides

The raw materials were pretreated under the same conditions, and the addition of metal chlorides was based on the same Cl^- concentration. For comparison, the pretreatments using H_2O (blank sample) and HCl were conducted. The results are shown in Figs. 1 and 2.

In Fig. 1, the pretreatment using HCl had the highest CYC (96.3%) and the lowest SS yield (39.5%). When the monovalent and divalent salts KCl, NaCl, $CaCl_2$, and $MgCl_2$ were used, the SS yields were all about 85%. However, the SS yields of the pretreatment using $ZnCl_2$ and $FeCl_2$ decreased, indicating that the degradation impact of $ZnCl_2$ and $FeCl_2$ on the raw material was more severe. The pretreatment using divalent salts had a higher CYC than that of the monovalent salts. The pretreatment using $MgCl_2$ not only retained a higher SS yield but also obtained a higher CYC (40.0%), demonstrating that the $MgCl_2$ was more suitable for enzymatic hydrolysis. For pretreatment using the trivalent salt $FeCl_3$, the SS yield was further decreased, while the CYC was as high as 67.9% and was increased 7.74 times compared to the blank sample, meaning that the Fe^{3+} ions were extremely helpful for enzymatic saccharification, rather than the Fe^{2+} ions. Though the CYC was very high for the pretreatment using HCl, the removal rate of cellulose came up to 39.5%, indicating that a great amount of the cellulose was degraded into the PL. The pretreatment using metal chlorides mainly caused the hemicellulose degradation compared to the lignin and cellulose degradation, especially for the pretreatments using $CaCl_2$, $MgCl_2$, $ZnCl_2$, $FeCl_2$, and $FeCl_3$, where their hemicellulose losses were all over 30% and cellulose losses were less than 10%. This demonstrated that pretreatment using trivalent and divalent salts were more beneficial for the hemicellulose degradation than that of the monovalent salts. When $FeCl_3$ was used in pretreatment, the hemicellulose and cellulose losses were as high as 75.7% and 12.4%, respectively. Though higher losses could reduce the cellulose mass in the SS, the conversion efficiency (based on the raw material) of the pretreatment using $FeCl_3$ was still higher than that of the other salts. Based on that analysis, the $FeCl_3$ and $MgCl_2$ were more suitable for pretreatment and followed enzymatic saccharification.

For the PL, glucose and xylose concentrations were analyzed (Fig. 2). Specifically, the cellulose and hemicellulose degradation products not only contained monosaccharides, but also contained a series of oligosaccharides. Generally, pretreatment using metal chlorides had a weaker influence on cellulose than hemicellulose, and the glucose content in the PL was correspondingly lower than that of the xylose. When the monovalent, bivalent, and trivalent salts were used in the pretreatment, the xylose contents were about 1.0 mg/mL, 3.9 to 5.7 mg/mL, and as high as 9.1 mg/mL, respectively. Considering the removal rate of hemicellulose, it demonstrated that the hemicellulose degradation was related to the valence of metal ions. That point was also confirmed by the previous research done by Liu *et al.* (2006), who treated xylose and xylotriose using KCl, NaCl, $CaCl_2$, $MgCl_2$, and $FeCl_3$. The results showed that the degradation of these two types of sugars could be accelerated by these inorganic salts, with the order of $FeCl_3$> $MgCl_2$> $CaCl_2$> KCl> NaCl.

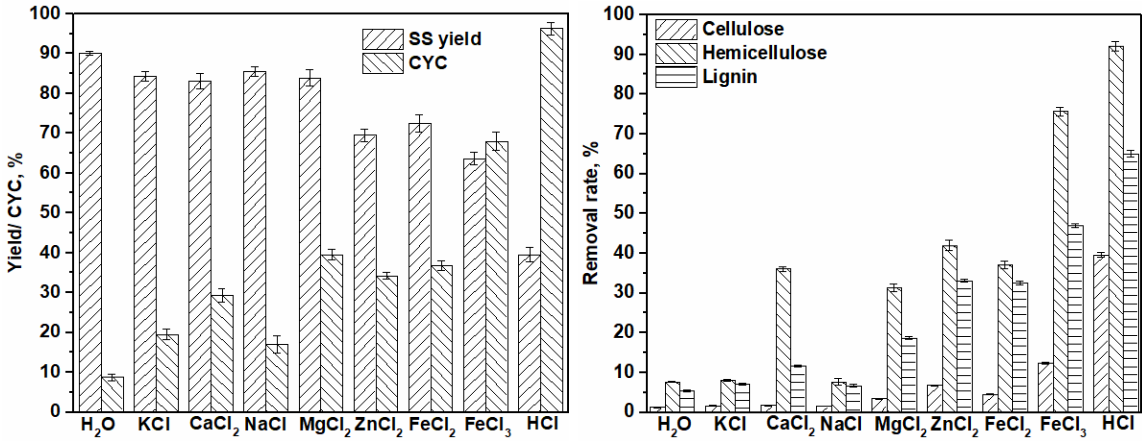

Fig. 1. Effects of the pretreatment using metal chlorides on the SS and the CYC

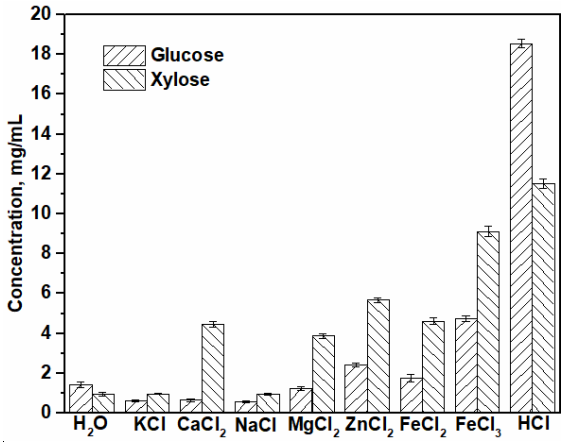

Fig. 2. Characterization of the PL from the pretreatment using metal chlorides

Enzymatic Hydrolysis using Metal Chlorides

Even though the SS had been washed sufficiently, a portion of the metal ions from the metal chlorides still remained absorbed or chelated onto its surface. To research these metal ions influence, the metal chlorides were added into the enzymatic hydrolysis system, and the cellulose models absorbent cotton was used as substrate. The results are shown in Fig. 3.

Compared to the blank sample, the increased rate of the CYC was more than 20% when the addition of monovalent salts KCl and NaCl came up to 1 mol/L. For the divalent salts, when 1 mol/L of the $MgCl_2$ and $FeCl_2$ were added into the hydrolysis processes, the CYC was increased 56.2% and 115.2%, respectively. However, the $CaCl_2$ had little effect on the enzymatic hydrolysis process, and the $ZnCl_2$ severely restrained the hydrolysis process. When 1 mol/L of $ZnCl_2$ was used, the CYC was decreased by as much as 59.7%. This indicated that the divalent salts had different impacts on enzymatic saccharification, though their metal ions had the same valences. For the trivalent salt $FeCl_3$ (1 mol/L), the CYC was increased 233.1%, which could greatly facilitate the hydrolysis process. Thus, the enzymatic hydrolysis process was obviously changed by adding suitable metals ions into the hydrolysis system, especially adding Fe^{3+} ions. Among these metal chlorides, the changes of enzymatic hydrolysis efficiency were not related to the valence of metal ions, which were mainly caused by the metal ions itself.

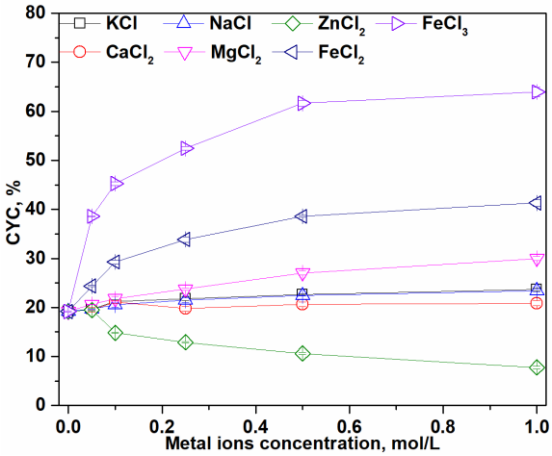

Fig. 3. Effects of the metal chlorides on the enzymatic saccharification

Cellulase Activity Assay using Chloride Salts

The metal chloride solutions were used, instead of the buffer solution, for the cellulase activity assay. The results are shown in Fig. 4.

Some metal ions, by serving as an activator, can impact the cellulase activity to a certain extent, and some enzymes also need the metal ion or metal complex to arouse their activities. When the concentration of KCl, $CaCl_2$, NaCl, $MgCl_2$, $ZnCl_2$, $FeCl_2$, and $FeCl_3$ came up to 1 mol/L in the system, the cellulase activities were increased 13.6%, 0.6%, 14.1%, 7.3%, -5.8%, 10.2%, and 17.5%, respectively. Similar to the hydrolysis impacts, the cellulase activity could be promoted by KCl, NaCl, $MgCl_2$, $FeCl_2$, and $FeCl_3$, whereas it could be inhibited by $ZnCl_2$ and not affected by the $CaCl_2$. Unlike other metal ions, Zn^{2+} is a necessary ion for some enzymes to make up the active center and maintain spatial structure. However, it also could form a coordinate bond with other electronic centers and thereby intercept the combination of enzymes and substrate. Thus, the Zn^{2+} mainly had a blocking effect on the cellulase activity (McCall *et al.* 2000).

Moreover, Table 1 shows the growth rates of the CYC and cellulase activity when 1 mol/L of metal chlorides were added into the enzymatic hydrolysis and activity detection processes, respectively. The NaCl and KCl had a certain promoter action on the cellulase activity and enzymatic hydrolysis, where the growth rate of the CYC was only 1.6 to1.7 times higher than that of the cellulase activity. When the divalent salts $CaCl_2$, $MgCl_2$, and

$FeCl_2$ were used, the growth rate of the CYC was about 7.0 to 11.0 times higher than that of the cellulase activity. The inhibition effects of the $ZnCl_2$ on enzymatic hydrolysis were also more severe than on the cellulase activity. For the trivalent salt $FeCl_3$, the biggest gap of the growth rate between the CYC and cellulase activity appeared. This showed that the cellulase activity was improved less than the CYC under the same metal ions concentrations, especially the metal ions with high valences. Further analysis found that the absorbent cotton with polar hydroxyl on the surface could be ionized in water, which had negative charges (Horvath and Lindstrom 2007). Unlike the FPase detection, the milled absorbent cotton had a larger specific surface area. The metal ions from the dissolved metal chlorides could be attracted onto the cellulose surface, causing their surface concentration to be raised. Hence, the local effects of the metal ions could strengthen the promotion or inhibitory influences on the cellulase activity. Because of the low valences of the Na^+ and K^+ ions, the promotion action of NaCl and KCl were weak compared to the other metal ions. Thus, it was beneficial for enzymatic saccharification to select suitable metal ions to be added into the hydrolysis system.

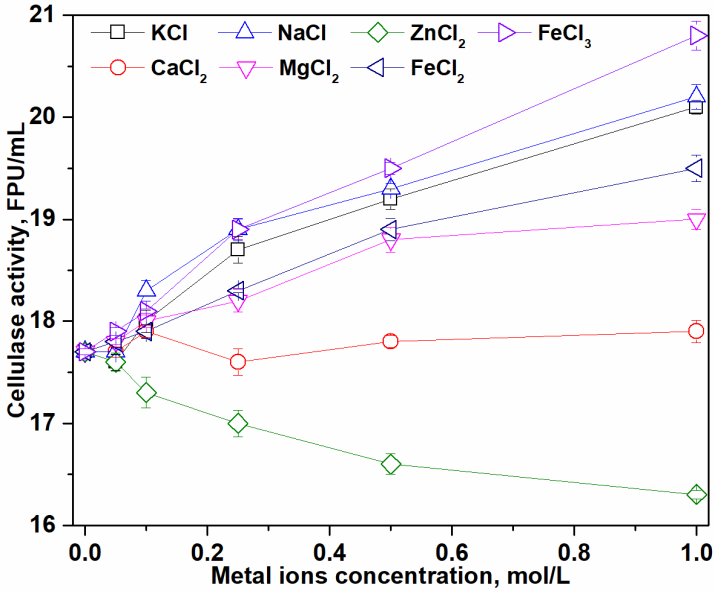

Fig. 4. Effects of the metal chlorides on the cellulase activity

Table 1. Changes of the CYC and Cellulase Activity by Adding Metal Chlorides

	CYC, %		Cellulase activity	
	Measured value	Growth rate[a]	Measured value, FPU/mL	Growth rate,%
Blank	19.20	-	17.7	-
KCl	23.68	23.3	20.1	13.6
NaCl	23.40	21.9	20.2	14.1
$CaCl_2$	20.88	8.8	17.9	1.1
$MgCl_2$	29.99	56.2	19.0	7.3
$ZnCl_2$	7.74	-59.7	16.3	-7.9
$FeCl_2$	41.32	115.2	19.5	10.2
$FeCl_3$	63.96	233.1	20.8	17.5

[a] Based on the blank sample.

CONCLUSIONS

1. The pretreatment using metal chlorides mainly resulted in hemicellulose degradation, where the removal rate of hemicellulose followed a sequence of trivalent salts ($FeCl_3$) > divalent salts ($CaCl_2$/$MgCl_2$/$ZnCl_2$/$FeCl_2$) > monovalent salts (KCl/NaCl), and the $MgCl_2$ and $FeCl_3$ were more suitable for enzymatic hydrolysis.
2. Most of the metal chlorides had promoter actions for the enzymatic saccharification and cellulase activity, while the $ZnCl_2$ could inhibit those two processes.
3. The metal ions, especially those with high valences, had local effects that could accelerate the enhancement or inhibition actions on the cellulase activities.

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REFERENCES CITED

Ghose, T. K. (1987). "Measurements of cellulase activities," *Pure App. Chem.* 59, 257-268. DOI: 10.1351/pac198759020257

Horvath, A. E., and Lindstrom, T. (2007). "Indirect polyelectrolyte titration of cellulosic fibers – Surface and bulk charges of cellulosic fibers," *Nord. Pulp Pap. Res. J.* 22, 87-92. DOI: 10.3183/NPPRJ-2007-22-01-p087-092

Kang, K. E., Park, D. H., Jeong, G. T. (2013). "Effects of NH_4Cl and $MgCl_2$ on pretreatment and xylan hydrolysis of miscanthus straw," *Carbohyd. Polym.* 92, 1321-1326. DOI: 10.1016/j.carbpol.2012.10.019

Liu, C., and Wyman, C. E. (2006). "The enhancement of xylose monomer and xylotriose degradation by inorganic salts in aqueous solutions as 180 °C," *Carbohyd. Res.* 341, 2550-2556. DOI: 10.1016/j.carres.2006.07.017

McCall, K. A., Huang, C. C., and Fierke, A. A. (2000). "Function and mechanism of zinc metalloenzymes," *J. Nutr.* 130, 1437S-1446S.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., and Ladisch, M. (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass," *Bioresource Technol.* 96, 673-686. DOI: 10.1016/j.biortech.2004.06.025

Sathitsuksanoh, N., George, A., and Zhang, Y. P. (2012). "New lignocellulose pretreatments using cellulose solvents: A review," *J. Chem. Technol. Biotechnol.* 88, 169-180. DOI: 10.1002/jctb.3959.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D.

(2012). *Determination of Structural Carbohydrates and Lignin in Biomass* (NREL/TP-510-42618), National Renewable Energy Laboratory, Golden, CO.

Zhang, W., Sathitsuksanoh, N., Barone, J. R., Renneckar, S. (2016). "Enhanced enzymatic saccharification of pretreated biomass using glycerol thermal processing (GTP)," *Bioresource Technol.* 199, 148-154. DOI: 10.1016/j.biortech.2015.08.141.

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