Effects and Mechanism of Metal Ions on Enzymatic Hydrolysis of Wheat Straw after Pretreatment

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The effects and mechanism on the enzymatic hydrolysis of lignocellulose by metal ions were investigated by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), Fluorescence Spectra**,** and enzymatic kinetics and activity analyses of the enzymatic hydrolysis system. Fe^{3+} exerted the best enzymatic hydrolysis effect on pretreated wheat straw, and the highest reducing sugar conversion was 44.5%. SEM and FTIR indicated that alkali and ultrasonic pretreatments partially removed the lignin and hemicellulose from lignocellulose. DLS and fluorescence spectra results revealed that the electrostatic interaction between Fe^{3+} and the carboxyl group in the enzyme enlarged the enzyme's steric structure. The data on enzymatic kinetics displayed that Fe^{3+} increased the maximum rate of reaction by 33.9% and the Michaelis-Menten by 5.72 g/L. The enzyme activity increase rate rose initially and then diminished, and the maximum increase rate was 36.1%.

Keywords: Metal ions; Cellulose; Enzymatic hydrolysis

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INTRODUCTION

Lignocellulose accounts for nearly half of the total bioresources in the world. The utilization and transformation of lignocellulose are highly important in solving many global problems, such as the energy crisis, resource shortage, environmental issues, and food safety. Cellulose degradation and conversion have become hot research topics in the field of science and technology (Farrell *et al*. 2006; Balat 2011).

The hydrolysis of lignocellulose to fermentable sugar is usually conducted by acid or enzyme. Acid hydrolysis is constantly associated with high pressure, low glucose yield, and undesirable furfural byproducts; it is expensive and not environment friendly (Suganuma *et al*. 2008; Rinaldi and Schüth 2009). Therefore, hydrolytic efficiency is improved and costs are reduced by optimizing the method through various additives. Adding metal ions has achieved valuable progress. Adding $FeCl_3$ and $FeCl_2$ to acid solutions improves sugar yield (Yan and Ren 1999; Nguyen and Tucker 2002).

Enzymatic hydrolysis has increasingly attracted much attention because of its mild reaction conditions, high selectivity, environmental friendliness, and low byproduct amount (Paljevac *et al*. 2007; Kristensen *et al*. 2007; Fang *et al*. 2010; Liu *et al*. 2011). However, the main problems of enzymatic hydrolysis are the high enzyme-level requirement, high cost, low enzyme activity, lack of economical and feasible fermentation technology, and low conversion rate.

Metal ions are employed to promote enzymatic hydrolysis at two stages, namely,

pretreatment and enzymatic hydrolysis. There are substantial reports on the addition of metal ions during pretreatment (Liu and Wyman 2006; Li *et al*. 2009; Liu *et al*. 2009; Chen *et al*. 2010; Jing *et al*. 2011; Kamireddy *et al*. 2013; Kang *et al*. 2013). Adding NH_4Cl and $MgCl_2$ during pretreatment increases enzymatic digestibility and facilitates xylan removal. $FeSO_4$ and $FeCl_3$ aid hemicellulose removal from corn stalk and increase glucose yield from enzymatic hydrolysis by altering the composition and structure of corn stalk. Under the same conditions, the enzymatic digestibility yields of samples in the presence of $FeCl_3$ and $CuCl_2$ are higher than those of samples pretreated with sulfuric acid.

However, literature is scarce on metal ion addition during mashing. $MgSO_4$, $FeSO_4$, and other chemicals can increase the reducing sugar concentration for the enzymatic hydrolysis of rice straw (Ren *et al*. 2012). Fe^{2+} and Cu^{2+} promote the degradation of steam-exploded rice straw, whereas Mg^{2+} and Fe^{3+} inhibit the enzymatic hydrolysis of the same substrate (Li *et al*. 2015). Mg^{2+} and Ca^{2+} increase the sugar yield of corn stalk, and the corresponding yields are increased by 5.9 % and 4.8 %, respectively (Tong 2009). Mg^{2+} inhibits the ineffective adsorption of cellulase by lignin, and the substrate is wheat straw pretreated with dilute acid (Akimkulova *et al*. 2016). Strong ion chelators and polyethylene glycols mitigate the inhibition (Tejirian and Xu 2010).

Thus it can be seen that the effects of different metal ions on different cellulose substrates significantly differ, and the effects of metal ions on enzymatic catalysis are affected by many factors. Therefore, in order to get more regular achievement in this respect, investigating the effects of metal ions on enzymatic hydrolysis is very important. In addition, although the effects of inorganic salts on the cellulase hydrolysis have been studied, few studies have investigated the mechanism of the cellulase hydrolysis by metal salts, especially about Fe^{3+} metal ion.

In this work, the effects and mechanism of Fe^{3+} metal ion on enzymatic hydrolysis of wheat straw after pretreatment were investigated by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), Fluorescence spectra, and enzymatic kinetics and enzymatic activity analyses of the enzymatic hydrolysis system. This work aims to clarify the effects of metal ions on enzymatic hydrolysis and the mechanisms of such effects.

EXPERIMENTAL

Materials

Wheat Straw was obtained from South China University of Technology. The materials were milled until passage through a 2 mm sieve and then screened to obtain the 40 to 60 mesh fraction. The straw was composed of 40.4% cellulose, 24.5% hemicellulose, 13.0% lignin and 7.2% ash. The cellulase was purchased from Beijing Solarbio Technology Corporation (Beijing, China), and the filter paper enzyme activity was 46 FPU/g.

Pretreatment of Wheat Straw

The alkali pretreatment operation was as follows. Wheat straw fractions at ranging from 40- to 60-mesh were placed in 3% sodium hydroxide aqueous solution for 48 h at room temperature. The solid liquid ratio was 10:1 (10 mL of sodium hydroxide solution and 1 g of wheat straw). The samples were washed continuously with water until

they reached a pH of 7.

Ultrasonic pretreatment was performed on an ultrasonic cleaner (Elmasonic P60H, Shenzhen Feite Li Technology Co. Ltd, Shenzhen, China) 30 min after alkali pretreatment. The frequency is 37 KHz, and the power density is 1W/cm^2. The other operations were the same as the alkaline pretreatment.

The wet wheat straws were dried to constant weight. Substrate after alkali pretreatment was composed of 65.8% cellulose, 12.0% hemicellulose, 9.2% lignin and 5.6% ash. Substrate after ultrasonic pretreatment was composed of 69.4% cellulose, 11.8% hemicellulose, 8.2% lignin, and 4.4% ash, and the experimental method based on literature (Soest 1963; Mo 2008).

Enzymatic Hydrolysis

All enzymatic hydrolysis experiments were carried out in an oscillator at 150 rpm equipped with a constant temperature thermostat (IS-REDA, Suzhou Jiemei Electronic Co Ltd, Suzhou, China). All experiments were performed in duplicate under the same conditions, and their average values and standard deviations were calculated.

Analytical Method for the Reducing Sugar

The reducing sugar concentrations were determined by the DNS method (Zhang and Yu 2017), *i.e.*, the reduction of 3,5-dinitrosalicylic acid by the reducing sugar in the presence of bases and heat. The process produces a red-brown product. The amount of reducing sugar is directly related to the concentration of this red-brown product and is determined through a UV-visible light spectrophotometer (UV-3600, Shimadzu, National Tsing Hua University (Beijing) Technology Co., Ltd, Beijing, China) (Shi 2004). The reducing sugar conversion rate was calculated by Eq. 1,

$$\text{The reducing sugar conversion}(\%) = \frac{\text{reducing sugar produced(g)}}{\text{substrate(g)}} \times 100 \quad (1)$$

SEM

The substrates (which included the non-pretreated substrate, alkali-pretreated substrate, ultrasonically pretreated substrate, substrate after enzymatic hydrolysis without added Fe^{3+}, and substrate after enzymatic hydrolysis with added Fe^{3+}) were observed using a scanning electron microscope (TESCAN VEGA II, S.R.O. Corporation, Brno, Czech Republic) at an acceleration voltage of 20 kV. Prior to analysis, the samples were mounted on an aluminum stub by using double-sided tape and coated with Au (JEOL JFC-1600 Auto Fine Coater, Tokyo, Japan) under vacuum.

FTIR

The FTIR spectra of the substrates were acquired using a Nicolet Is10 spectrophotometer (Thermo Scientific, Waltham, USA). The substrates were mixed with KBr and measured within the wavenumber range of 400 to 4000 cm^{-1} with an accumulation of 32 scans.

DLS

DLS measurements were performed with a Malvern Instrument Zetasizer Nano (Malvern, UK), equipped with a 22 mW He–Ne laser operating at a wavelength of 632.8 nm. The solutions were filtered through 450 nm filters. The scattering angle was 173°.

Kinetics of Enzyme-Catalyzed Reactions

The Michaelis-Menten equation was used to obtain the kinetics of enzyme-catalyzed reactions (Eq. 2),

$$V = V_{max}\, s / (K_m + s) \quad (2)$$

where s is the mass concentration of the substrate (g/L), V is the starting rate (g/(L·min)), V_{max} is the maximum reaction rate (g/(L·min)), and K_m is the Michaelisian constant (g/L). The enzymatic hydrolysis conditions were as follows: buffer pH of 4.8, cellulase mass concentration of 3 g/L, Fe^{3+} mass concentration of 0.3 g/L, and enzymatic hydrolysis time and temperature of 24 h and 50 °C, respectively.

Enzymatic Activity of Enzymatic Hydrolysis

The enzymatic activity of enzymatic hydrolysis was obtained by the relative increase in enzyme activity, as calculated using Eq. 3,

$$\text{The enzyme activity increase rate} / \% = \frac{\text{enzyme activity with } Fe^{3+} - \text{enzyme activity without } Fe^{3+}}{\text{enzyme activity without } Fe^{3+}} \times 100 \quad (3)$$

The enzymatic hydrolysis conditions were as follows: cellulose mass concentration of 3 g/L, Fe^{3+} mass concentration of 0.3 g/L, and substrate liquid mass fraction of 1.25 %.

Fluorescence Spectra

An F-4600 spectrofluorometer was used to measure enzymatic hydrolysis solutions without and with Fe^{3+} at 24 h and 36 h. The enzymatic hydrolysis conditions were as follows: mass concentration of cellulose was 3 g/L, mass concentration of Fe^{3+} was 0.3 g/L, and substrate liquid mass fraction was 1.25 %. The emission spectra of 300 to 650 nm were recorded for the excitation wavelength of 280 nm.

RESULTS AND DISCUSSION

Effect of Different Metal Ions

The effects of different metal ions on enzymatic hydrolysis at 50 °C are shown in Fig. 1. These metal ions demonstrated positive influences on enzymatic hydrolysis that depended on the metal ion concentrations. For example, at the concentration of 0.4 g/L, Fe^{3+} and Co^{2+} improved the conversions. In particular, Fe^{3+} increased the reducing sugar conversion by 10.6%. However, beyond 0.4 g/L, the reducing sugar conversion decreased with rising ion concentration. Although Co^{2+}, Fe^{2+}, and Mg^{2+} are all divalent cations, Co^{2+} and Fe^{2+} improved the conversions, whereas Mg^{2+} only slightly influenced the conversion. For the same cation Mg^{2+}, the effect of $MgSO_4$ on the conversion was slightly greater than that of $MgCl_2$. Thus, the effects of metal ions not only depended on their charge but also on their types.

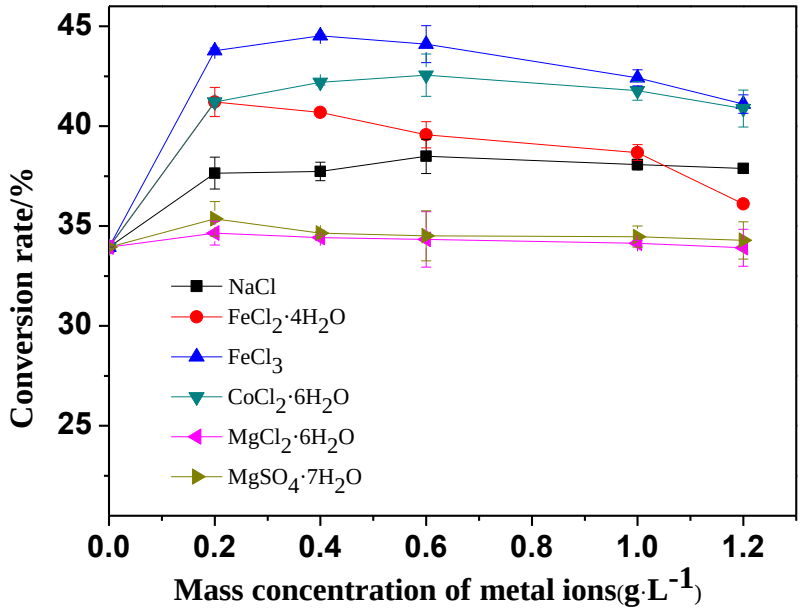


Fig. 1. Effects of different metal ions on the reducing sugar conversion

Surface Morphology

The morphological changes of substrates were examined by SEM to obtain insights into the structural modification of the surface. Figure 2 shows the SEM micrographs of non-pretreated, pretreated, and after enzymatic hydrolysis substrates. The images were amplified by factors of 800 and 1200. The non-pretreated substrates (micrographs a and b) showed compact and rigid structure. However, the substrates became a rough and rich fraction after alkali pretreatment (micrographs c and d), and the substrates became looser after ultrasonic pretreatment (micrographs e and f) (Li *et al*. 2010). Compared with the non-pretreated substrates, pretreated substrates appeared looser and exhibited more internal surfaces. It can be inferred that the partial removal of hemicelluloses and lignin destroyed the network formed by cellulose - hemicelluloses - lignin, leading to the disruption of the hydrogen bond between cellulose. The looser structure and increased surface area of pretreated substrates increase the accessibility of enzyme and allow for cellulase to penetrate, absorb, and hydrolyze the lignocellulose more easily, thus promoting the enzymatic hydrolysis efficiency (Zhang and Lynd 2004; Hendriks and Zeeman 2009).

A comparison of the micrographs taken before and after enzymatic hydrolysis showed many holes in the substrate after enzymatic hydrolysis, and micrographs i and j reveal more holes than micrographs g and h. This illustrates that Fe^{3+} has an important effect on enzymatic hydrolysis.

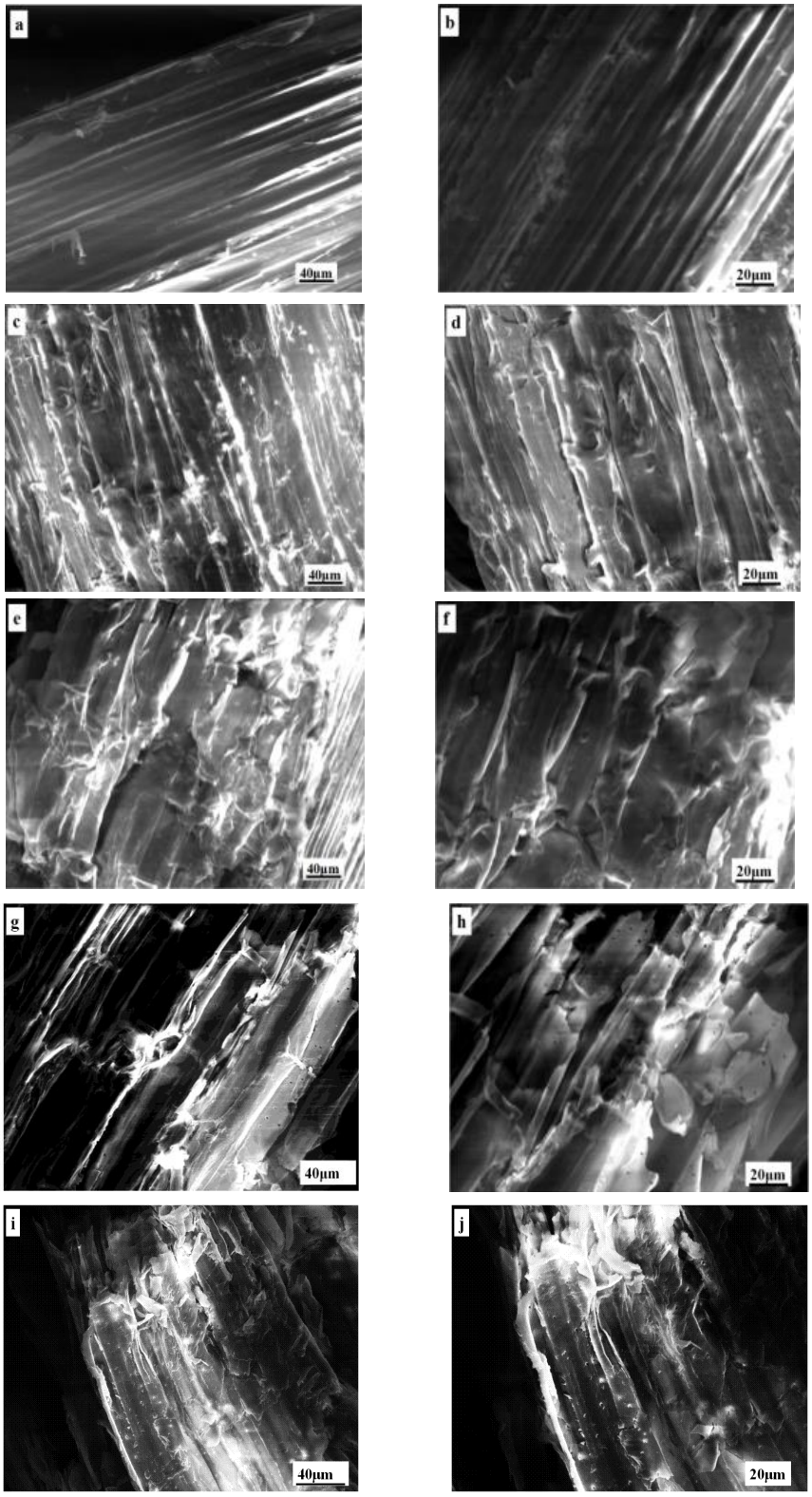


Fig. 2. SEM micrographs of (a,b) non-pretreated substrate ×800 and ×1200, (c,d) alkali-pretreated substrate ×800 and ×1200, (e,f) ultrasonically pretreated substrate ×800 and ×1200, (g,h) substrate after enzymatic hydrolysis without added Fe^{3+} ×800 and ×1200, and (i,j) substrate after enzymatic hydrolysis with added Fe^{3+} ×800 and ×1200

FTIR Spectra

The FTIR spectra of different substrates are shown in Fig. 3. The relative intensities of absorption peaks of infrared spectra are shown in Table 1. The absorption peaks in the region near 3500 to 3300 cm^{-1} corresponded to the characteristic absorption peak of the intramolecular hydrogen bonding, and the relative intensity of the absorption peak decreased with substrate change. This result indicates that the hydrogen bonding is increasingly destroyed. Alkali pretreatment (Fig. 3b) and ultrasonic pretreatment (Fig. 3c) resulted in the breakdown of hydrogen bonds in the cellulose molecule. The relative intensities of the absorption peaks under Fe^{3+} addition (Fig. 3e) decreased relative to that in the absence of Fe^{3+} (Fig. 3d). This finding suggests that the added Fe^{3+} strongly degraded the intramolecular hydrogen bonding.

The absorption peak at 1735 cm^{-1} corresponded to the uronic ester groups and acetyl groups of hemicelluloses or the ester linkage of the carboxylic group of ferulic and *p*-coumaric acid present in hemicellulose and lignin (Li *et al.* 2010). This peak disappeared in the cellulose-rich fractions. Such alterations imply that the ester bonds were cleaved after alkali pretreatment (Fig. 3b), ultrasonic pretreatment (Fig. 3c), and enzymatic hydrolysis (Figs. 3d and 3e).

The absorption peak at 1637 cm^{-1} corresponded to the absorbed water in the substrates (Lu *et al.* 2004; Li *et al*. 2010). The absorption peak was split into two peaks after pretreatment and enzymatic hydrolysis (Fig. 3). The relative intensities of the absorption peaks decreased, as shown in Table 1, and this reduction indicated a decrease in water absorption.

The characteristic absorption peak at 1432 cm^{-1} corresponded to the structure of cellulose, and the relative intensity of absorption peak did not change considerably after pretreatment and enzymatic hydrolysis. This result revealed that the macromolecular structure of cellulose did not change drastically.

The absorption peak of the C–O–C groups from lignocellulose appeared at 1164 cm^{-1}, and the relative intensity of the absorption peak decreased after pretreatment and enzymolysis (Table 1). This result was achieved because the lignin content decreased.

Peaks from 1058 to 1060 cm^{-1} corresponded to the stretching vibrations of the C=O groups from hemicellulose and lignin, and the relative intensity of the absorption peak decreased after pretreatment and enzymolysis (Table 1). This result indicated that the hemicellulose and lignin contents decreased after pretreatment and enzymatic hydrolysis.

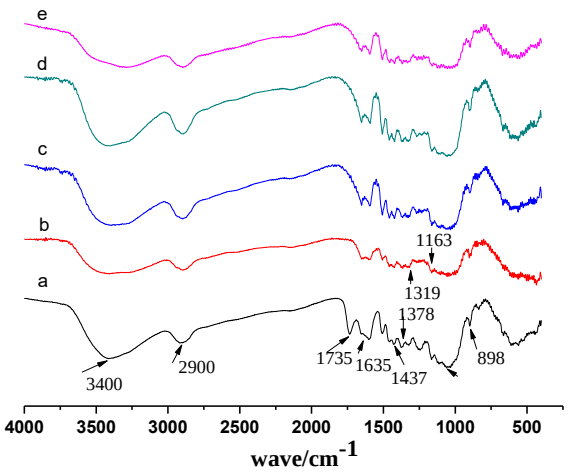


Fig. 3. FTIR spectra of the (a) non-pretreated substrate, (b) alkali-pretreated substrate, (c) ultrasonically pretreated substrate, (d) substrate after enzymatic hydrolysis without added Fe^{3+}, and (e) substrate after enzymatic hydrolysis with added Fe^{3+}

Table 1. Relative Absorption Intensity in the Infrared Spectra

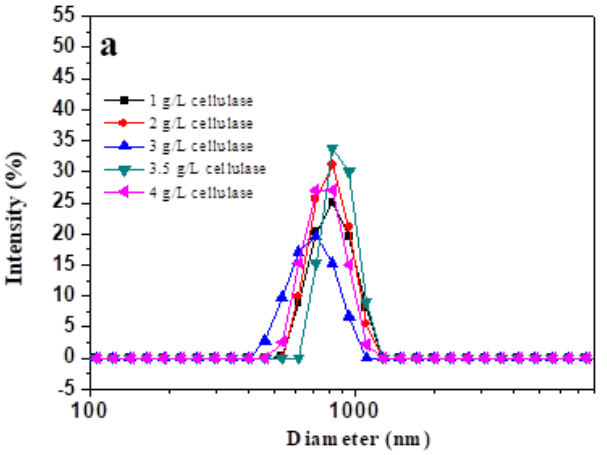
Wave number (cm^{-1})	Raw Substrate	Alkali Pretreatment	Ultrasonic Pretreatment	Without Fe^{3+}	Added Fe^{3+}
3400	1.27	1.24	1.19	1.09	1.02
2900	0.90	1.11	1.05	0.87	1.12
1637	0.71	0.73	0.66	0.59	0.56
1432	0.92	0.99	0.97	0.96	0.95
1372	1.00	1.00	1.00	1.00	1.00
1164	1.29	1.23	1.20	1.19	1.07
1058–1060	1.50	1.32	1.29	1.28	1.13

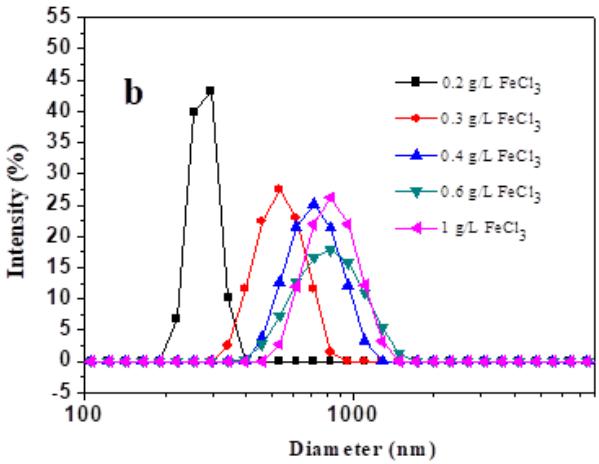
Note: The values correspond to the ratio of the absorbance of the corresponding wavenumber and the absorbance at 1372 cm^{-1} (Lu *et al*. 2005; Zhang *et al*. 2010b; Song *et al*. 2011).

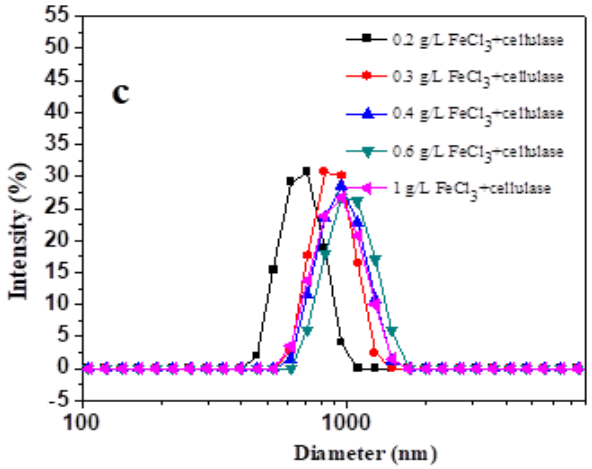
Hydrated Particle Size

The DLS measurements of the hydrated particle size are presented in Fig. 4. The hydrated particle sizes of the different concentrations of cellulases were similar, and the average hydrated particle sizes were about 825 nm (Fig. 4a). The particle size increased with increasing Fe^{3+} concentration, but when the concentration of Fe^{3+} was 0.6 g/L and 1 g/L, the particle size was constant at 825 nm (Fig. 4b). The hydrated particle sizes in the mixture of different concentrations of Fe^{3+} and 3 g/L cellulase showed similar trends with different Fe^{3+} concentrations (Fig. 4c). However, the particle size remained at 955 nm when Fe^{3+} concentration exceeded 0.4 g/L. The particle sizes were between 1100 and 1300 nm after enzymatic hydrolysis in Fig. 4d and were larger than that before enzymatic hydrolysis.

An enzyme molecule is a specific 3D structure consisting of helical folded sheets and unfolded amino acids. The electrostatic interaction between Fe^{3+} and the carboxyl group in the enzyme reduced the electrostatic repulsion between the carboxyl groups and thus enlarged the enzyme's steric structure. Therefore, the particle sizes enlarged when Fe^{3+} was added.


a
1 g/L cellulase
2 g/L cellulase
3 g/L cellulase
3.5 g/L cellulase
4 g/L cellulase
Intensity (%)
Diameter (nm)


b
0.2 g/L FeCl3
0.3 g/L FeCl3
0.4 g/L FeCl3
0.6 g/L FeCl3
1 g/L FeCl3
Intensity (%)
Diameter (nm)


c
0.2 g/L FeCl3+cellulase
0.3 g/L FeCl3+cellulase
0.4 g/L FeCl3+cellulase
0.6 g/L FeCl3+cellulase
1 g/L FeCl3+cellulase
Intensity (%)
Diameter (nm)

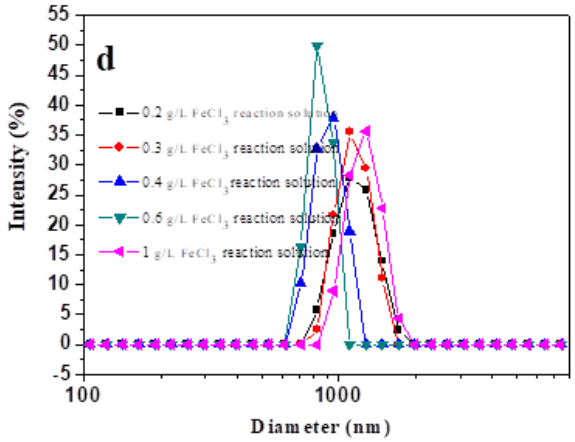


Fig. 4. Particle sizes in the (a) enzyme solution, (b) different concentrations of Fe^{3+} solution, (c) different concentrations of Fe^{3+} and 3 g/L enzyme mixture before enzymatic hydrolysis, and (d) different concentrations of Fe^{3+} and 3 g/L enzyme mixture after enzymatic hydrolysis

Effect of Fe^{3+} on Enzyme Kinetics

Figure 5 and Table 2 show the effect of Fe^{3+} on enzyme kinetics. Fe^{3+} increased the maximum reaction rate and the Michaelis-Menten constant of the enzymatic reaction. Adding Fe^{3+} also raised the maximum reaction rate by 33.9% and the Michaelis-Menten constant by 5.72 g/L.

The Michaelis–Menten constant K_m represents the affinity between substrate and enzyme, and the K_m value is inversely proportional to the affinities of the enzyme and substrate. Adding Fe^{3+} reduced the affinity of cellulase and cellulose because Fe^{3+} adsorbed onto the lignins and occupied the adsorption position of cellulose (Akimkulova *et al*. 2016 and Liu *et al*. 2010). As a result, the ineffective adsorption of cellulase decreased; hence, the enzyme activity and enzymatic intermediates of combination and dissociation were increased. The apparent K_m value increased, but with decreased concentration of the intermediate product. This effect was beneficial to the enzymatic reaction, which was then promoted to the positive reaction direction. Ultimately, the dissociation of the intermediate product was enhanced (Zhang *et al*. 2010a).

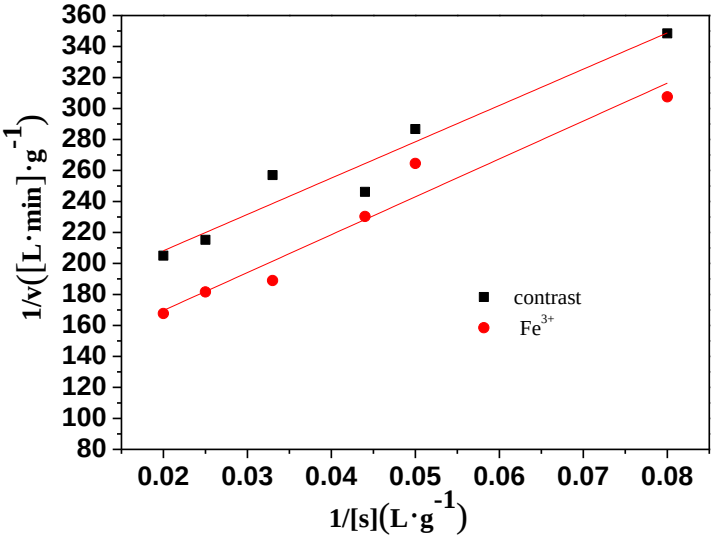


Fig. 5. Effect of Fe^{3+} on enzyme kinetics

Table 2. Effect of Fe^{3+} on Enzyme Kinetic Parameters

Sample	Millimeter equation	V_{max} (g/L·min)	K_m (g/L)	R^2
Control	y = 2343.4x + 161.35	0.0062	14.52	0.9441
Added Fe^{3+}	y = 2444.8x + 120.77	0.0083	20.24	0.9526

Effect of Fe^{3+} on Enzymatic Activity

The effect of Fe^{3+} on the enzymatic activities of the enzymatic hydrolysis system is revealed in Fig. 6. Within the experimental time, Fe^{3+} promoted the enzymatic activity, and the relative increase rate of enzyme activity rose initially and then diminished. The highest relative increase rate of enzyme activity appeared at 24 h, and the maximum increase rate was 36.1%.

Fe^{3+} can significantly increase the activity of cellulase. Probably Fe^{3+} is the activator of cellulase, and changes the conformation of cellulase protein, so that the number of active enzyme increases and reduces in non-productive enzyme adsorption. As a consequence, the cellulase enzyme activity increases (Li *et al.* 2009; Wang *et al.* 2012; Vasconcellos *et al.* 2016). At the same time, Fe^{3+} played a bridging role between the active site of cellulase and substrate, and promoted the combination of active site of cellulase and substrate, thus accelerating the reaction and improving the enzymatic hydrolysis efficiency.

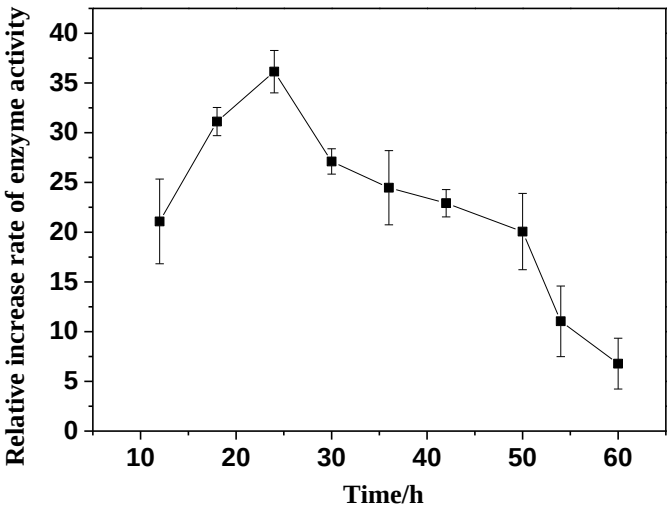


Fig. 6. Effect of Fe^{3+} on enzymatic activity

Fluorescence Spectra

Fluorescence spectra of enzymatic hydrolysis solutions with and without Fe^{3+} are shown in Fig. 7. Because 280 nm was the maximum absorption wavelength of cellulase, so 280 nm is used as the excitation wavelength. The emission spectrum of 300 to 650 nm reflected the change of the molecular conformation of enzyme combined with others (Wu *et al.* 2008). In Fig. 7 it is apparent that the wavelengths of the maximum fluorescence intensity of the enzyme were 346.4 nm (24 h) and 344.80 nm (36 h) without Fe^{3+}, which were the emission spectrum of tryptophan and tyrosine in cellulase (Podestá and Plaxton 2003; Sułkowska *et al.* 2004). The wavelengths were shifted to 435.90 nm (24 h) and 439.40 nm (36 h) with the addition of Fe^{3+}; this shift was due to the combination of

cellulase molecules and iron ions. In addition, due to the fluorescence quenching effect of Fe^{3+}, the fluorescence intensities were obviously lower than those without Fe^{3+}.

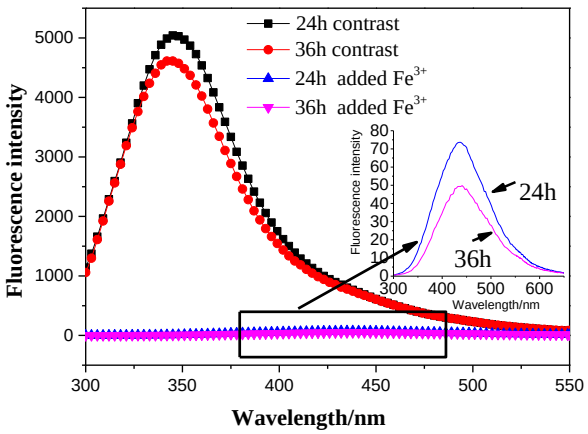


Fig. 7. Fluorescence spectrum of enzymatic hydrolysis solution

CONCLUSIONS

1. Different metal ions had different effects on enzymatic hydrolysis of pretreated wheat straw. The hemicellulose and lignin can be partially removed by alkali and ultrasonic pretreatment. DLS and fluorescence spectra results revealed that there was interaction between Fe^{3+} and enzyme.
2. Fe^{3+} increased the maximum reaction rate and the Michaelis-Menten constant. The relative increase rate of enzyme activity rose initially and then diminished.

ACKNOWLEDGMENTS

This work was funded by the National Key R&D Program of China (No 2017YFB0308701), the National Natural Science Foundation of China (Nos. 21203005, 21676003), the Science and Technology Program Key Project of Beijing Municipal Commission of Education (KZ201510011010), and the transformation of scientific and technological achievements-promotion plan project (TJSHG201510011020).

REFERENCES CITED

Akimkulova, A., Zhou, Y., Zhao, X., and Liu, D. (2016). "Improving the enzymatic hydrolysis of dilute acid pretreated wheat straw by metal ion blocking of non-productive cellulase adsorption on lignin," *Bioresource Technology* 208, 110-116. DOI: 10.1016/j.biortech.2016.02.059

Balat, M. (2011). "Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review," *Energy Conversion & Management* 52(2), 858-875. DOI: 10.1016/j.enconman.2010.08.013

Chen, Y., Dong, B., Qin, W. J., and Xiao, D. G. (2010). "Xylose and cellulose

fractionation from corncob with three different strategies and separate fermentation of them to bioethanol," *Bioresource Technology* 101(18), 7005-7010. DOI: 10.1016/j.biortech.2010.03.132

Fang, X., Qin, Y. Q., Li, X. Z., Wang, L. S., Wang, T. H., Zhu, M. T., and Qu, Y. B. (2010). "Progress on cellulase and enzymatic hydrolysis of lignocellulosic biomass," *Chinese Journal of Biotechnology* 26(7), 864-869. DOI：10.13345/j.cjb.2010.07.021

Farrell, A. E., Plevin, R. J., Turner, B. T., Jones, A. D., O'Hare, M., and Kammen, D. M. (2006). "Ethanol can contribute to energy and environmental goals," *Science* 311(5760), 506-508. DOI: 10.1126/science.1121416

Jing, Z., Zhang, H. M., Zheng, R. P., Lin, Z. X., and He, H. (2011). "The enhancement of pretreatment and enzymatic hydrolysis of corn stover by $FeSO_4$ pretreatment," *Biochemical Engineering Journal* 56(3), 158-164. DOI: 10.1016/j.bej.2011.06.002

Hendriks, A.T.W.M., and Zeeman, G. (2009). "Pretreatments to enhance the digestibility of lignocellulosic biomass," *Bioresource Technology* 100(1), 10-18. DOI: 10.1016/j.biortech.2008.05.027

Kamireddy, S. R., Li, J., Tucker, M., Degenstein, J., and Ji, Y. (2013). "Effects and mechanism of metal chloride salts on pretreatment and enzymatic digestibility of Corn stover," *Industrial & Engineering Chemistry Research* 52(5), 1775-1782. DOI: 10.1021/ie3019609

Kang, K. E., Park, D. H., and Jeong, G. T. (2013). "Effects of NH_4Cl and $MgCl_2$ on pretreatment and xylan hydrolysis of miscanthus straw," *Carbohydrate Polymers* 92(2), 1321-1326. DOI: 10.1016/j.carbpol.2012.10.019

Kristensen, J. B., Börjesson, J., Bruun, M. H., Tjerneld, F., and Jørgensen, H. (2007). "Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose," *Enzyme & Microbial Technology* 40(4), 888-895. DOI: 10.1016/j.enzmictec.2006.07.014

Li, D. Y., Gong, D. C., Tian, Y. H., and Yu, Y. (2009). "Study on the effects of metal ions on cellulase activity," *Liquor-making Science & Technology* 2009(6), 40-42. DOI: 10.13746/j.njkj.2009.06.001

Li, D. Y., Tian, Y. H., and Gong, D. C. (2015). "Effects of metal ion on the hydrolysis of steam-exploded straw by cellulase," *Hubei Agricultural Sciences* 54(3), 546-549. DOI: 10.14088/j.cnki.issn0439-8114.2015.03.010

Li, L., Sun, J., Min, L., Wang, S., Pei, H., and Zhang, J. (2009). "Enhanced enzymatic hydrolysis and structural features of corn stover by $FeCl_3$ pretreatment," *Bioresource Technology* 100(23), 5853-5858. DOI: 10.1016/j.biortech.2009.06.040

Li, M. F., Fan, Y. M., Xu, F., Sun, R. C., and Zhang, X. L. (2010). "Cold sodium hydroxide/urea based pretreatment of bamboo for bioethanol production: Characterization of the cellulose rich fraction," *Industrial Crops & Products* 32(3), 551-559. DOI: 10.1016/j.indcrop.2010.07.004

Liu, C., and Wyman, C. E. (2006). "The enhancement of xylose monomer and xylotriose degradation by inorganic salts in aqueous solutions at 180 °C," *Carbohydrate Research* 341(15), 2550-2556. DOI: 10.1016/j.carres.2006.07.017

Liu, H., Zhu, J. Y., and Fu, S. Y. (2010). "Effects of lignin-metal complexation on enzymatic hydrolysis of cellulose," *Journal of Agriculture &Food Chemistry* 58(12), 7233-7238. DOI: 10.1021/jf1001588

Liu, L., Sun, J., Cai, C., Wang, S., Pei, H., and Zhang, J. (2009). "Corn stover pretreatment by inorganic salts and its effects on hemicellulose and cellulose

degradation," *Bioresource Technology* 100(23), 5865-5871. DOI: 10.1016/j.biortech.2009.06.048

Liu, Y. Y., Sun, J. S., Pei, H. S., Liu, L., and Duan, K. H. (2011). "Research progress on improving the efficiency of enzymatic hydrolysis of lignocellulose," *China Brewing* 30(5), 16-20.

Lu, J., Shi, S.L., Xing, X.G., Yang, R.N. (2004). "Pretreatment of lignocellulose by sodium hydrolysis," *Journal of Cellulose Science and Technology* 12(1), 1-6. DOI: 10.16561/j.cnki.xws.2004.01.001

Lu, J., Shi, S. L., Yang, R. N., Niu, M. H., and Song, W. J. (2005). "Modification of reed cellulose microstructure and IT change in enzymatic hydrolysis of reed pulp," *Transactions of China Pulp and Paper* 20(2), 85-90. DOI: 10.3321/j.issn:1000-6842.2005.02.021

Mo, D. (2008). "Study on the effect of surfactants on enzymatic hydrolysis of rice straw," Hunan University.

Nguyen, Q. A., and Tucker, M. P. (2002). "Dilute acid/metal salt hydrolysis of lignocellulosics," U.S. Patent 6,423,145 B1.

Paljevac, M., Primožič, M., Habulin, M., Novak, Z., and Knez, Ž. (2007). "Hydrolysis of carboxymethyl cellulose catalyzed by cellulase immobilized on silica gels at low and high pressures," *Journal of Supercritical Fluids* 43(1), 74-80. DOI: 10.1016/j.supflu.2007.05.006

Podestá, F. E., and Plaxton, W. C. (2003). "Fluorescence study of ligand binding to potato tuber pyrophosphate-dependent phosphofructokinase: Evidence for competitive binding between fructose-1,6-bisphosphate and fructose-2,6-bisphosphate," *Archives of Biochemistry & Biophysics* 414(1), 101-107. DOI: 10.1016/S0003-9861(03)00157-7

Ren, T. B., Yin, S. Y., Ma, X. Q., Zhang, L. L., Su, Y., and Song, A. D. (2012). "The effect of chemical inducers on enzymatic hydrolysis of rice straw," *Journal of Agro-Environment Science* 31(1), 206-211.

Rinaldi, R., and Schüth, F. (2009). "Acid hydrolysis of cellulose as the entry point into biorefinery schemes," *Chemsuschem* 2(12), 1096-1107. DOI: 10.1002/cssc.200900188

Shi, Z. H. (2004). "Study on rapid measuring soluble sugar by DNS method," *Journal of Tongren Vocational Institute* 2(2), 34-36.

Soest, V. P. J. (1963). "Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin," *Journal - Association of Official Analytical Chemists* 49, 546-551.

Song, Y. K., Huang, W., Yao, Q. H., Chen, L. H., Liu, W. J., and Li, Y. R. (2011). "Chemical composition and cellulosic structure of straw from transgenic rice (*Oryza sativa*) plants expressing broad-spectrum antifungal proteins," *Journal of Biosafety* 20(2), 160-165.

Suganuma, S., Nakajima, K., Kitano, M., Yamaguchi, D., Kato, H., Hayashi, S., and Hara, M. (2008). "Hydrolysis of cellulose by amorphous carbon bearing SO_3H, COOH, and OH groups," *Journal of the American Chemical Society* 130(38), 12787-12793. DOI: 10.1021/ja803983h

Sułkowska, A., Równicka, J., Bojko, B., Pożycka, J., Zubik-Skupień, I., and Sułkowski, W. (2004). "Effect of guanidine hydrochloride on bovine serum albumin complex with antithyroid drugs: fluorescence study," *Journal of Molecular Structure* 704(1), 291-295. DOI: 10.1016/j.molstruc.2003.12.065

Tejirian, A., and Xu, F. (2010). "Inhibition of cellulase-catalyzed lignocellulosic hydrolysis by iron and oxidative metal ions and complexes," *Applied & Environmental Microbiology* 76(23), 7673-7682. DOI: 10.1128/AEM.01376-10

Tong, M. (2009). "Application of microwave and additives to the pretreatment and enzymatic hydrolysis of plant fiber materials," *Nanjing Forestry University*.

Vasconcellos, V. M., Tardioli, P. W., Giordano, R. L., and Farinas, C. S. (2016). "Addition of metal ions to a (hemi)cellulolytic enzymatic cocktail produced in-house improves its activity, thermostability, and efficiency in the saccharification of pretreated sugarcane bagasse," *New Biotechnology* 33(3), 331-338. DOI: 10.1016/j.nbt.2015.12.002

Wang, G., Zhang, X. W., Wang, L., Wang, K. K., Peng, F. l., and Wang, L. S. (2012). "The activity and kinetic properties of cellulases in substrates containing metal ions and acid radicals," *Advances in Biological Chemistry* 2(4), 390-395. DOI: 10.4236/abc.2012.24048

Wu, B., Wang, L. S., and Gao, P. J. (2008). "Study on the molecular configuration and conformation changes of nitrobenzene fiber two glycoside (PNPC) binding to exotangent cellulase I (CBHI)," *Science in China Press* 38(6), 527-535.

Yan, Y. J., and Ren, Z. W. (1999). "Continuous catalytic hydrolysis of cellulose," *Acta Energiae Solaris Sinica* 20(1), 55-58.

Zhang, J., and Yu, Y. (2017). "Study on the interaction between cellulase and surfactants," *Tenside Surfactants Detergents* 54(3), 206-213. DOI: 10.3139/113.110493

Zhang, M., Su, R., Wei, Q., and He, Z. (2010a). "Enhanced enzymatic hydrolysis of lignocellulose by optimizing enzyme complexes," *Applied Biochemistry & Biotechnology* 160(5), 1407-1414. DOI: 10.1016/0021-9673(95)00728-8

Zhang, N., Jiang, J.C., Fang, J., Zhang, Y., Yu, L. (2010b). "The effect of low energy ion implantation on crystallinity structure and morphological structure of cellulose," *Journal of Radiation Research and Radiation Processing* 28(4), 215-219.

Zhang, Y. H. P., and Lynd, L. R. (2004). "Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Non complexed cellulase systems," *Biotechnol. Bioeng.* 88(7), 797-824. DOI: 10.1002/bit.20282

Article submitted: October 29, 2017; Peer revision completed: December 30, 2017;
Revisions accepted: February 13, 2018; Published: February 16, 2018
DOI: 10.15376/biores.13.2.2617-2631