

# 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.  $\text{Fe}^{3+}$  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  $\text{Fe}^{3+}$  and the carboxyl group in the enzyme enlarged the enzyme's steric structure. The data on enzymatic kinetics displayed that  $\text{Fe}^{3+}$  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  $\text{FeCl}_3$  and  $\text{FeCl}_2$  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  $\text{NH}_4\text{Cl}$  and  $\text{MgCl}_2$  during pretreatment increases enzymatic digestibility and facilitates xylan removal.  $\text{FeSO}_4$  and  $\text{FeCl}_3$  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  $\text{FeCl}_3$  and  $\text{CuCl}_2$  are higher than those of samples pretreated with sulfuric acid.

However, literature is scarce on metal ion addition during mashing.  $\text{MgSO}_4$ ,  $\text{FeSO}_4$ , and other chemicals can increase the reducing sugar concentration for the enzymatic hydrolysis of rice straw (Ren *et al.* 2012).  $\text{Fe}^{2+}$  and  $\text{Cu}^{2+}$  promote the degradation of steam-exploded rice straw, whereas  $\text{Mg}^{2+}$  and  $\text{Fe}^{3+}$  inhibit the enzymatic hydrolysis of the same substrate (Li *et al.* 2015).  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  increase the sugar yield of corn stalk, and the corresponding yields are increased by 5.9 % and 4.8 %, respectively (Tong 2009).  $\text{Mg}^{2+}$  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  $\text{Fe}^{3+}$  metal ion.

In this work, the effects and mechanism of  $\text{Fe}^{3+}$  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<sup>2</sup>. 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<sup>3+</sup>, and substrate after enzymatic hydrolysis with added Fe<sup>3+</sup>) 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<sup>-1</sup> 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,  $\text{Fe}^{3+}$  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,  $\text{Fe}^{3+}$  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  $\text{Fe}^{3+}$  at 24 h and 36 h. The enzymatic hydrolysis conditions were as follows: mass concentration of cellulose was 3 g/L, mass concentration of  $\text{Fe}^{3+}$  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,  $\text{Fe}^{3+}$  and  $\text{Co}^{2+}$  improved the conversions. In particular,  $\text{Fe}^{3+}$  increased the reducing sugar conversion by 10.6%. However, beyond 0.4 g/L, the reducing sugar conversion decreased with rising ion concentration. Although  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Mg}^{2+}$  are all divalent cations,  $\text{Co}^{2+}$  and  $\text{Fe}^{2+}$  improved the conversions, whereas  $\text{Mg}^{2+}$  only slightly influenced the conversion. For the same cation  $\text{Mg}^{2+}$ , the effect of  $\text{MgSO}_4$  on the conversion was slightly greater than that of  $\text{MgCl}_2$ . Thus, the effects of metal ions not only depended on their charge but also on their types.

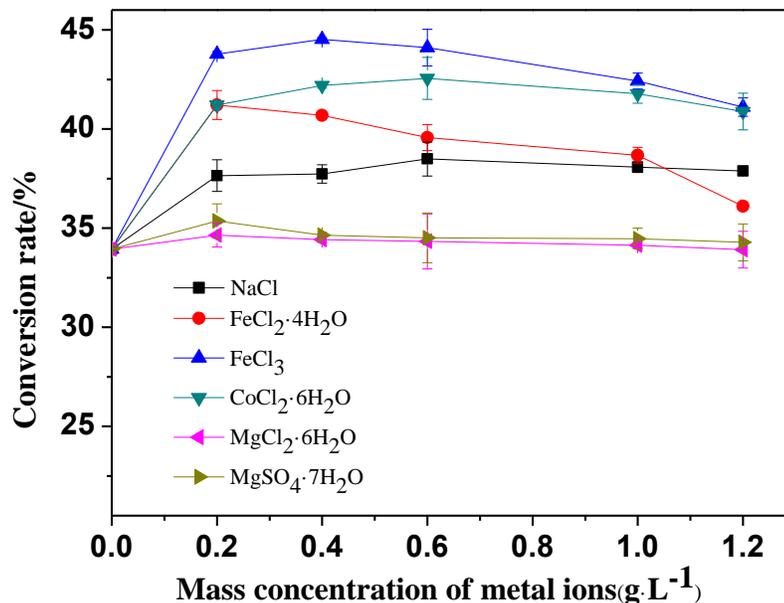
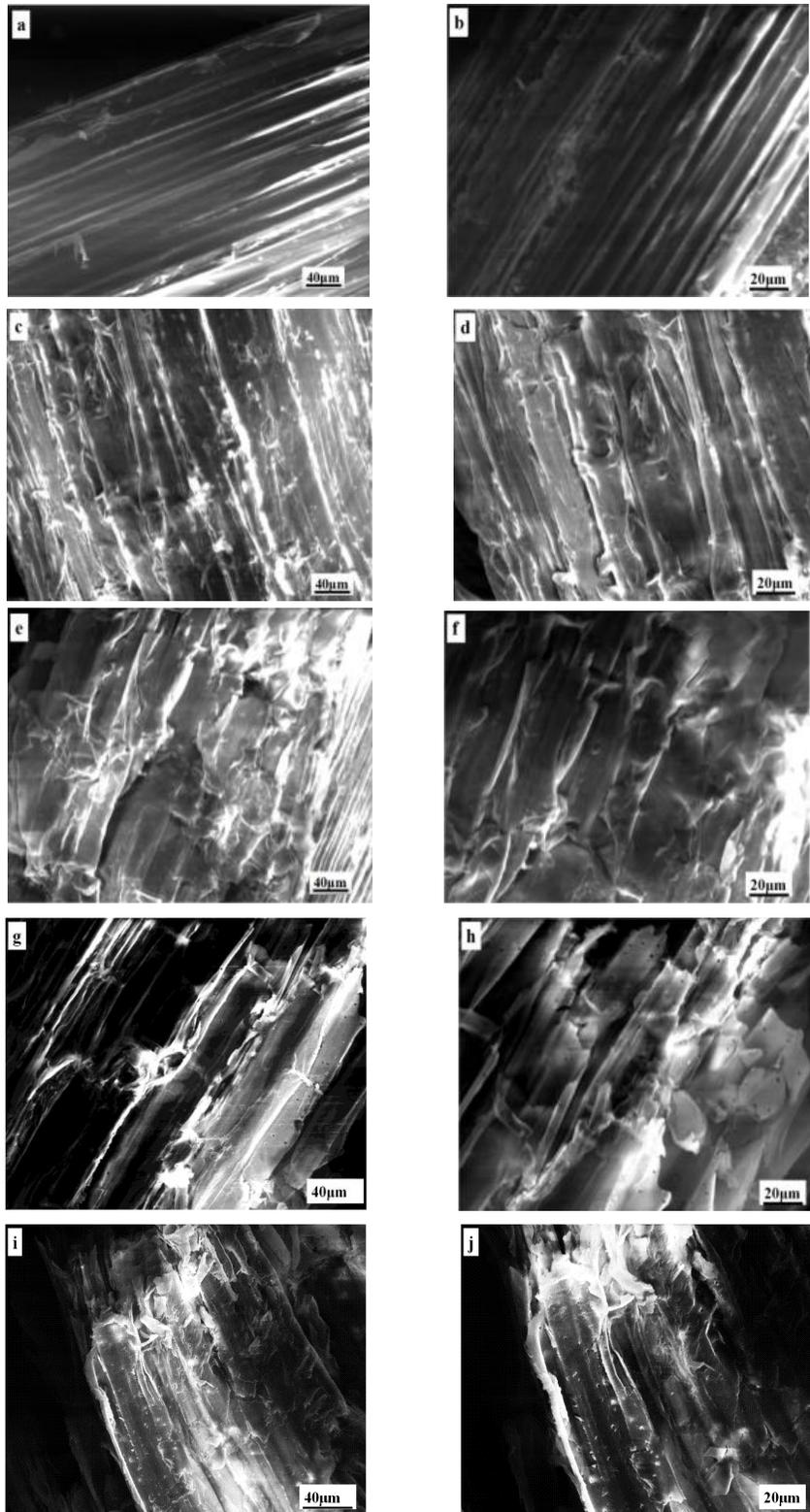


Fig. 1. Effects of different metal ions on the reducing sugar conversion

### 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<sup>3+</sup> has an important effect on enzymatic hydrolysis.



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

## 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  $\text{cm}^{-1}$  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  $\text{Fe}^{3+}$  addition (Fig. 3e) decreased relative to that in the absence of  $\text{Fe}^{3+}$  (Fig. 3d). This finding suggests that the added  $\text{Fe}^{3+}$  strongly degraded the intramolecular hydrogen bonding.

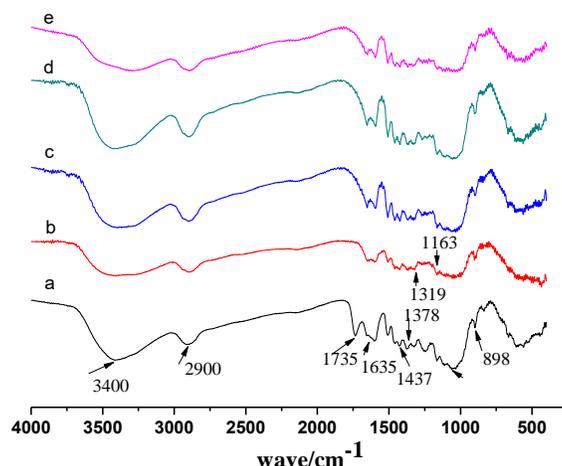
The absorption peak at 1735  $\text{cm}^{-1}$  corresponded to the uronic ester groups and acetyl groups of hemicelluloses or the ester linkage of the carboxylic group of ferulic and *p*-coumaric acid present in hemicellulose and lignin (Li *et al.* 2010). This peak disappeared in the cellulose-rich 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  $\text{cm}^{-1}$  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  $\text{cm}^{-1}$  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  $\text{cm}^{-1}$ , 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  $\text{cm}^{-1}$  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  $\text{Fe}^{3+}$ , and (e) substrate after enzymatic hydrolysis with added  $\text{Fe}^{3+}$

**Table 1.** Relative Absorption Intensity in the Infrared Spectra

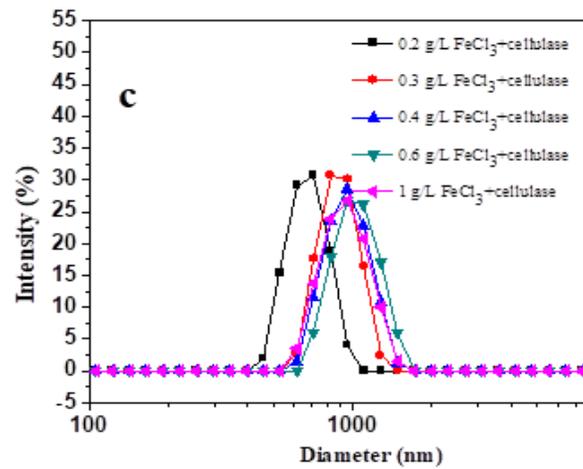
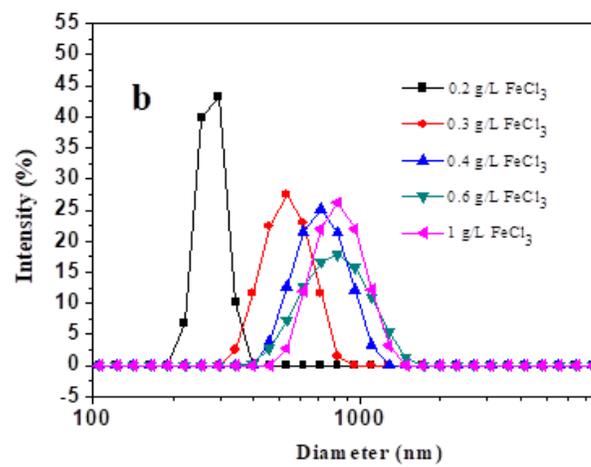
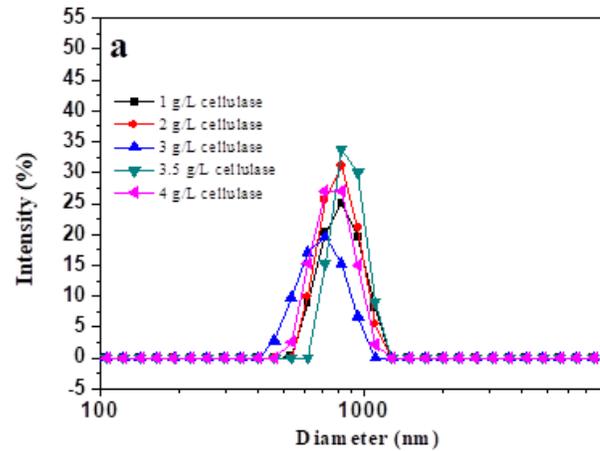
Wave number ( $\text{cm}^{-1}$ )	Raw Substrate	Alkali Pretreatment	Ultrasonic Pretreatment	Without $\text{Fe}^{3+}$	Added $\text{Fe}^{3+}$
3400	1.27	1.24	1.19	1.09	1.02
2900	0.90	1.11	1.05	0.87	1.12
1637	0.71	0.73	0.66	0.59	0.56
1432	0.92	0.99	0.97	0.96	0.95
1372	1.00	1.00	1.00	1.00	1.00
1164	1.29	1.23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

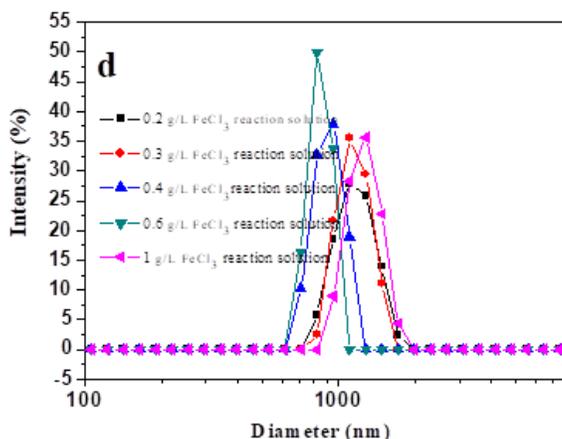
Note: The values correspond to the ratio of the absorbance of the corresponding wavenumber and the absorbance at  $1372 \text{ cm}^{-1}$  (Lu *et al.* 2005; Zhang *et al.* 2010b; Song *et al.* 2011).

### 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  $\text{Fe}^{3+}$  concentration, but when the concentration of  $\text{Fe}^{3+}$  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  $\text{Fe}^{3+}$  and 3 g/L cellulase showed similar trends with different  $\text{Fe}^{3+}$  concentrations (Fig. 4c). However, the particle size remained at 955 nm when  $\text{Fe}^{3+}$  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  $\text{Fe}^{3+}$  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  $\text{Fe}^{3+}$  was added.



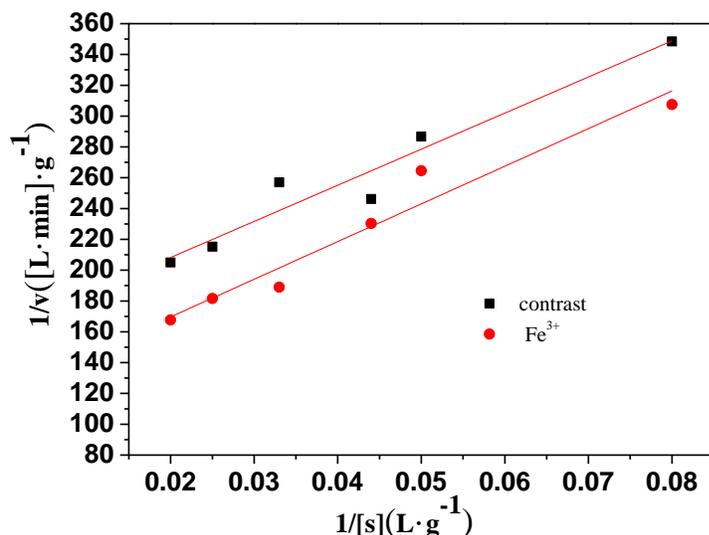


**Fig. 4.** Particle sizes in the (a) enzyme solution, (b) different concentrations of  $\text{Fe}^{3+}$  solution, (c) different concentrations of  $\text{Fe}^{3+}$  and 3 g/L enzyme mixture before enzymatic hydrolysis, and (d) different concentrations of  $\text{Fe}^{3+}$  and 3 g/L enzyme mixture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

### Effect of $\text{Fe}^{3+}$ on Enzyme Kinetics

Figure 5 and Table 2 show the effect of  $\text{Fe}^{3+}$  on enzyme kinetics.  $\text{Fe}^{3+}$  increased the maximum reaction rate and the Michaelis-Menten constant of the enzymatic reaction. Adding  $\text{Fe}^{3+}$  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  $\text{Fe}^{3+}$  reduced the affinity of cellulase and cellulose because  $\text{Fe}^{3+}$  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  $\text{Fe}^{3+}$  on enzyme kinetics

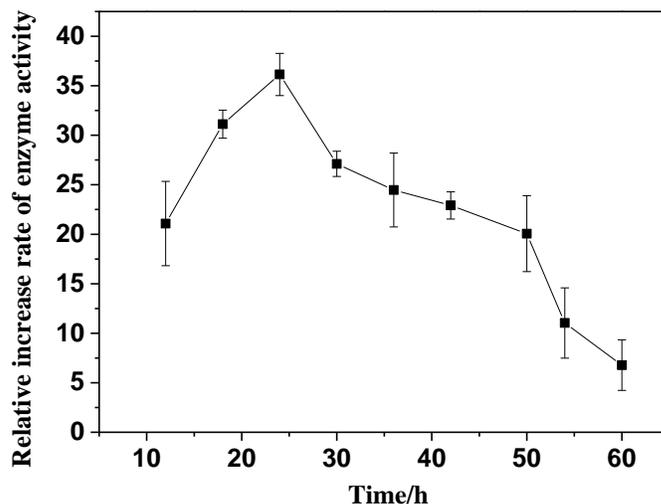
**Table 2.** Effect of Fe<sup>3+</sup> on Enzyme Kinetic Parameters

Sample	Millimeter equation	V <sub>max</sub> (g/L·min)	K <sub>m</sub> (g/L)	R <sup>2</sup>
Control	y = 2343.4x + 161.35	0.0062	14.52	0.9441
Added Fe <sup>3+</sup>	y = 2444.8x + 120.77	0.0083	20.24	0.9526

### Effect of Fe<sup>3+</sup> on Enzymatic Activity

The effect of Fe<sup>3+</sup> on the enzymatic activities of the enzymatic hydrolysis system is revealed in Fig. 6. Within the experimental time, Fe<sup>3+</sup> 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<sup>3+</sup> can significantly increase the activity of cellulase. Probably Fe<sup>3+</sup> 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<sup>3+</sup> 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<sup>3+</sup> on enzymatic activity

### Fluorescence Spectra

Fluorescence spectra of enzymatic hydrolysis solutions with and without Fe<sup>3+</sup> 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<sup>3+</sup>, 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<sup>3+</sup>; this shift was due to the combination of

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

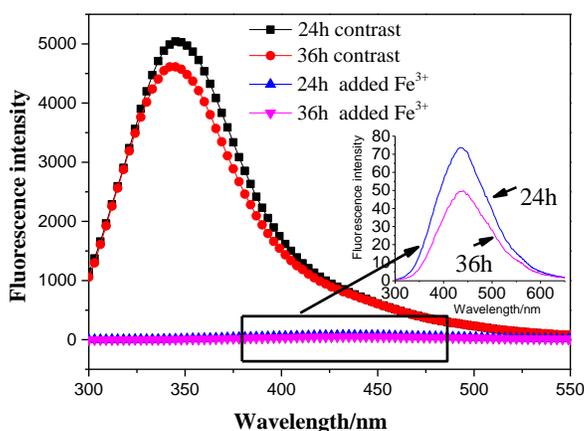


Fig. 7. Fluorescence spectrum of enzymatic hydrolysis solution

## 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  $\text{Fe}^{3+}$  and enzyme.
2.  $\text{Fe}^{3+}$  increased the maximum reaction rate and the Michaelis-Menten constant. The relative increase rate of enzyme activity rose initially and then diminished.

## ACKNOWLEDGMENTS

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