Enhanced Enzymatic Hydrolysis of Poplar after Combined Dilute NaOH and Fenton Pretreatment

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Five types of pretreatment processes were investigated to confirm the enhancement of the enzymatic hydrolysis of poplar. These processes included a hot water pretreatment, a calcium oxide pretreatment, NaOH extraction at low temperature, a Fenton reaction, and a combined dilute NaOH and Fenton pretreatment. The combined dilute NaOH and Fenton pretreatment was found to be the most effective pretreatment process. After enzymatic hydrolysis for 72 h, 74% of the cellulose recovery yield was obtained when the poplar substrates were pretreated with 2% NaOH at 75 °C for 3 h, followed by 20 mmol/g of H2O2 (30%) and 0.2 mmol/g of FeSO4·7H2O for a Fenton reaction period of 12 h. The cellulose recovery yield was approximately five-fold greater than that of the untreated sample directly processed by enzymatic hydrolysis. Furthermore, microscopic observations of changes in the surface structure of the pretreated residue were correlated with the enhancement of the enzymatic hydrolysis of cellulose. In conclusion, the combined dilute NaOH and Fenton pretreatment shows high potential for future application.

Keywords: Combination pretreatment; Poplar; NaOH extraction; Fenton reaction; Enzymatic hydrolysis

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INTRODUCTION

As the availability of oil-based non-renewable resources continues to dwindle, and the negative impact of fossil fuels on the environment is increasingly recognized, worldwide energy demand for sustainable development is rapidly increasing (Kato et al. 2014). Lignocellulosic biomass feedstocks, like sugarcane bagasse (Li et al. 2016; Mesquita et al. 2016), corn stover (Liu et al. 2009a; Wang et al. 2016), switchgrass (Li et al. 2010), and wheat straw (Qiu and Chen 2012; Coimbra et al. 2016), have already attracted much attention on the subject. However, the complex net structure of cellulose, hemicelluloses, and lignin hinders further utilization of lignocellulosic materials (Zhou et al. 2014; Jung et al. 2015). Its highly recalcitrant nature affects liquid penetration, enzyme activity, and enzyme accessibility (Jain and Vigneshwaran 2012; He et al. 2015). Thus, an appropriate pretreatment process involved in the enzymatic hydrolysis is necessary to keep enough cellulose intact while simultaneously removing as much hemicellulose and lignin as possible (Sun and Chen 2008; Qing et al. 2014). Different pretreatment processes, such as steam explosion (Qiu and Chen 2012), acid pretreatment (Yang et al. 2013), alkaline peroxide pretreatment (Yamashita et al. 2010), and fungal pretreatment (Wan and Li 2011), have been investigated. However, these pretreatment methods still are not cost-effective, high-efficiency, or environment-friendly (Jung et al. 2013, 2014).
A NaOH pretreatment showed an increase in cellulose conversion after enzymatic hydrolysis, attaining values close to 69% of theoretical glucose yield (Negro et al. 2015). However, the experiment was carried out at a high temperature of 110 °C. Therefore, it is necessary to investigate NaOH pretreatment at lower temperatures.

Previous studies have shown that the Fenton (i.e., iron and H₂O₂) reaction is an effective technology for lignocellulose pretreatment. Cotton fibers have been shown to be decayed completely by Fenton’s reagent (Jain and Vigneshwaran 2012), and Fenton’s reagent is effective on garden biomass (Bhanage et al. 2015). After enzymatic hydrolysis for 72 h, 35.41% of the glucose and 61.44% of the reducing sugars were obtained from the corn stover that had been pretreated with Fenton reagent and then dilute NaOH extraction (He et al. 2015). Previous studies have shown that Fenton’s reaction does not need harsh conditions such as a high concentration of chemicals, a high temperature, or high pressure (Kato et al. 2014; Jung et al. 2015). Current knowledge is that H₂O₂ itself is a very powerful oxidant that proceeds through a radical mechanism, and Fe²⁺ ions present in the pretreatment solution serve as a catalyst. The Fenton reaction involves the oxidation of Fe²⁺ to Fe³⁺ by H₂O₂ and then the reduction of Fe³⁺ to Fe²⁺, as given in Eqs. 1 and 2 (Arantes et al. 2012), simultaneously bringing about two kinds of oxygen radicals, HO* and HOO* (Arantes et al. 2011; He et al. 2015). These oxygen radicals initiate chain reactions for oxidizing lignocellulosic components (He et al. 2015).

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{HO}^* \tag{1}
\]

\[
\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{H}^+ + \text{HOO}^* \tag{2}
\]

In this study, we focused on combined dilute NaOH and Fenton pretreatment. The sugar content in the pretreated liquid and the enzymatic hydrolysate were measured to evaluate the enhancement of the hydrolysis. Then, the optimized reaction conditions were established. Moreover, the surface structural changes in the pretreated residue were studied with scanning electron microscopy.

**EXPERIMENTAL**

**Materials**

*Poplar preparation*

Poplar was obtained from the Forestry research fields at Beijing Forestry University (Beijing, China). After the poplar was cut into small pieces, it was oven-dried to bring down the moisture content, then subsequently ground and sifted to obtain a powder with an average particle size of less than 1 mm. The dry sample was kept at -20 °C for future use. Through composition analysis, the composition of dry poplar was determined to be as follows: 49.10% glucan, 17.30% xylan, 21.0% lignin, 1.0% arabinan, and 0.4% galactan.

*Enzyme and reagents*

The enzymes, cellulase, and xylanase used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cellulase activity was expressed in terms of 1.0x6.0 cm filter paper activity (FPA) using Whatman No. 1 as the substrate. One unit (U) of FPA corresponded to 1 μmol glucose formed per minute during hydrolysis, and one activity unit of xylanase is defined as the quantity of xylanase required to liberate 1.0 μmol xylose from
1.0 mg/mL xylan per minute during hydrolysis (Batool et al. 2015). In the following, the activities of cellulase and xylanase were 42 FPU/g and 16 U/g, respectively. Chemical reagents used were of analytic grade and were purchased from Shanghai Lingfeng Reagents Co. and Shanghai Demo Medical Tech Co. (Shanghai, China).

Methods

Pretreatment of poplar with hot water, calcium oxide, and NaOH

One gram of dry poplar was mixed together with 15 mL of deionized water, 15 mL of 2% calcium oxide, or 15 mL of 2% NaOH in tubes to conduct a hot water pretreatment at 121 °C for 1 h, a calcium oxide pretreatment in boiling water for 1 h, or a NaOH pretreatment at 65 °C in a water bath for 2 h, respectively. When these reactions were complete, the contents in each tube were separated into two parts: the liquid hydrolysate, which was used for the analysis of the monomeric and oligomeric sugar content by high-performance liquid chromatography (HPLC); and the solid residue, which was washed with deionized water repeatedly and then recovered for subsequent enzymatic hydrolysis or structural feature analysis.

Fenton pretreatment of poplar

The pretreatment was performed in 50-mL tubes. The tube was first loaded with 1 g of dry poplar, 10 mL of deionized water, using hydrochloric acid to adjust pH 4. And Fenton’s reagent was added by mixing H₂O₂ (30%) and FeSO₄·7H₂O in different ratios of the amount of substance (10:1, 20:1, 50:1, 100:1, 125:1, and 150:1). Then the tube was kept away from light for 12 h to completely perform the Fenton reaction. After the pretreatment was complete, the mixed contents were separated into the residue and reaction liquid using a 200-mesh sieve. The liquid was recovered for an analysis of the monomeric and oligomeric sugar content by HPLC, and the remaining residue was washed repeatedly with 10% oxalic acid to eliminate the absorbed Fe ions, followed by rinsing thoroughly with deionized water to remove possible acid and recovery for subsequent enzymatic hydrolysis or structural feature analysis.

Combined dilute NaOH and Fenton pretreatment of poplar

The combined pretreatment was performed in a centrifugal 50-mL tube. First, 1 g of dry poplar and 15 mL of 2% NaOH were mixed completely in a tube reactor, which was then incubated in a 65 °C water bath for 2 h. When the reaction was over, the tube reactor was cooled, followed by filtration via a 200-mesh sieve to separate the mixture into the solid residue and liquid hydrolysate. The liquid hydrolysate was recovered for subsequent quantitative determination, while the solid residue was rinsed thoroughly with deionized water and subjected to Fenton’s reaction.

Enzymatic hydrolysis of poplar

In the following, U/g and FPU/g indicate activity of xylanase and cellulase, respectively.

One gram of poplar and 16 U/g xylanase were mixed with 30 mL of acetate buffer (PH 5.0) in a 50-mL centrifugal tube. Also, 40 μL of cycloheximide and 80 μL of tetracycline hydrochloride were added to sterilize the interior of the tube reactor to prevent the pH value from changing (Liu et al. 2009b). The reaction mixture was placed in a 70 °C water bath for 1 d. When the reaction was complete, the tube reactor was cooled, and 42 FPU/g cellulase was added to perform the hydrolysis reaction at 50 °C for 72 h. Finally,
the product was filtered, the enzymatic hydrolysate was recovered, and the residue was rinsed thoroughly with deionized water and recovered.

**Analytical procedures**

The monomeric glucose and xylose concentrations were measured via an HPLC system (Agilent 1200, Palo Alto, CA) equipped with a refractive index (RI) detector, and the corresponding protection column. A Rezex RPM (Phenomenex, CA) column was used for quantitative measurement of the sugar concentrations. The mobile phase of the column was HPLC-grade water, at a flow rate of 0.6 mL/min, with a column temperature of 65 °C.

The cellulose and hemicellulose contents in the initial poplar material, as well as the remaining glucan and xylan in the residue, were determined by the National Renewable Energy Laboratory (NREL) standard analytical procedure (Qing et al. 2014). A two-step acid hydrolysis method was used, during which these components were correspondingly hydrolyzed into glucose and xylose and were then quantified by HPLC. The percent cellulose/hemicellulose conversion was calculated as the following equations,

\[
\text{Cellulose conversion rate} (%) = \frac{C_1 \times V \times 0.9}{p_{s1}} \times 100% \tag{3}
\]

\[
\text{Hemicellulose conversion rate} (%) = \frac{C_2 \times V \times 0.88}{p_{s2}} \times 100% \tag{4}
\]

where \(C_1/C_2\) is the concentration of glucose/xylose released during pretreatment or enzymatic hydrolysis (mg/mL), \(V\) is total volume of the reaction system (mL), 0.9 is the conversion factor for glucose to equivalent cellulose, 0.88 is the conversion factor for xylose to equivalent hemicellulose, and \(p_{s1}/p_{s2}\) is the potential glucose/xylose in the initial poplar (mg).

The liquid fraction after two-step acid hydrolysis, pretreatment, or enzymatic hydrolysis, was autoclaved with 4% sulfuric acid for 1 h at 121 °C to degrade oligomeric glucose or xylose into monomeric sugars (Jung et al. 2015). Sugar standards containing known concentrations were also autoclaved for the same time, at the same acid concentration, to correct the factors that influence hydrolysis loss. The oligomeric sugar contents in the liquid fraction were calculated as follows:

\[
\text{Oligomeric sugar (g)} = \text{Total sugar (g) in the hydrolysate calibrated for degradation} - \text{monomeric sugars (g) that are in the hydrolysate before autoclaving} \tag{5}
\]

The NaOH pretreatment conditions were statistically evaluated by applying statistical methodology viz. analysis of variance (ANOVA) for process optimization (Bhang et al. 2015).

**Scanning electron microscopy (SEM) of pretreated poplar**

Samples of pretreated and untreated poplar were fixed onto a specimen stub, using double-coated tape, to determine the surface structural changes. Then, the samples were sprayed with AuPd (Kim and Lee 2005; Mosier et al. 2005; Zeng et al. 2007) and imaged with a KYKY-2800 scanning electron microscope (KYKY Technology Development Ltd., China) operated at an accelerating voltage of 10 kV.
RESULTS AND DISCUSSION

Effects of Pretreatment Process on Enzymatic Hydrolysis of Cellulose and Hemicellulose

Lignocellulose pretreatment affects the cellulose removal rate and the sugar yield (Liu et al. 2009a). A previous study showed that the cellulose that was lost during the pretreatment was amorphous or para-crystalline cellulose (Wang et al. 2013). This correlated with not only the changes in the cellulose structural characteristics (Ju et al. 2013), but also the positive effect on enzyme accessibility. This is why the glucose content in the enzymatic hydrolysate increased with the increase of cellulose loss in the pretreated liquid fraction, except in the Fenton group (Fig. 1a).

After pretreatment, the composition changes of un-pretreated, NaOH-treated, Fenton-treated, and NaOH plus Fenton treated poplar were 68.4% (33.4% cellulose + 35.0% hemicellulose; Table 1), 68.0% (36.3% cellulose + 31.7% hemicellulose), 63.3% (35.1% cellulose + 28.2% hemicellulose), and 73.4% (41.5% cellulose + 31.9% hemicellulose), respectively. These results reveal the synergy of the NaOH and Fenton reagent (i.e., FeSO₄·7H₂O and H₂O₂, in this study).

Table 1 also indicates that the weight loss of poplar in pretreatment was mainly caused by the elimination of lignin and other soluble components rather than by polysaccharide degradation. Figure 1 shows that the combined pretreatment also consistently gave the highest enzymatic hydrolysis efficiency of cellulose (60.72%), followed by NaOH treatment (42.86%), and non-pretreatment gave the lowest (11.4%). In addition, some authors have reported that most solubilized xylose was present in oligomeric form in the pretreated liquid (Cao et al. 2012), and our results supported this fact: more oligomeric xylose was found in the pretreated liquid than monomeric xylose (Fig. 1b). Figure 1 also shows that 20% of xylose was obtained from the enzymatic hydrolysate after the combined pretreatment, which was the highest yield among the five kinds of pretreatment processes.

Given the sugar yield after enzymatic hydrolysis, the combined pretreatment, that is, dilute NaOH extraction and subsequent Fenton reaction, was established as the optimal pretreatment process in this study.

Table 1. Effects of each Pretreatment on Solid Recovery and Cellulose and Hemicellulose Composition Changes in Poplar

<table>
<thead>
<tr>
<th>Pretreatment Process</th>
<th>Solid Recovery (%)</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>100</td>
<td>33.4</td>
<td>35.0</td>
</tr>
<tr>
<td>H₂O</td>
<td>92.3</td>
<td>35.8</td>
<td>32.4</td>
</tr>
<tr>
<td>CaO</td>
<td>88.9</td>
<td>36.4</td>
<td>32.6</td>
</tr>
<tr>
<td>NaOH</td>
<td>82.1</td>
<td>36.3</td>
<td>31.7</td>
</tr>
<tr>
<td>Fenton</td>
<td>86.5</td>
<td>35.1</td>
<td>28.2</td>
</tr>
<tr>
<td>NaOH + Fenton</td>
<td>66.2</td>
<td>41.5</td>
<td>31.9</td>
</tr>
</tbody>
</table>
Fig. 1. Enzymatic hydrolysis of (a) cellulose and (b) hemicellulose with various pretreatment processes.

In Fig. 1, X1 (Y1)/X2 (Y2) is the monomeric/oligomeric glucose (xylose) in the pretreated liquid; X3/Y3 is the glucose/xylose in the enzymatic hydrolysate; X4/Y4 is the potential glucose/xylose remaining in the residue after enzymatic hydrolysis. The cellulose and hemicellulose removal rate (i.e., loss) in the pretreatment phase = X1+X2, Y1+Y2, respectively. The cellulose/ hemicellulose recovery yield (i.e., enzymatic hydrolysis efficiency) = X3/Y3.
Enhanced Enzymatic Hydrolysis of Cellulose by Control of NaOH Extraction Conditions

Figure 2a shows the enhancement of the enzymatic hydrolysis of poplar that had been pretreated by dilute NaOH (0.5% to 2.5%) at 65 °C for 2 h and the subsequent Fenton’s reaction (H₂O₂ 1 mL/g, FeSO₄ • 7H₂O 30 mg/g) for 12 h. It demonstrates that when the NaOH concentration rose from 0.5% to 2.0%, both the cellulose removal rate and the enzymatic hydrolysis efficiency gradually increased, attaining the highest glucose yield of 61% at a concentration of 2.0%.

When the NaOH concentration was further increased to 2.5%, the hydrolysis efficiency decreased by 5%, and more than 20% of the cellulose was lost. This can be explained by the fact that 2.0% NaOH concentration provides an efficient removal of lignin, which is one of the main obstacles for the enzymatic hydrolysis of cellulose (Banerjee et al. 2001).

The NaOH extraction time is a relatively important parameter in the pretreatment. Figure 2b shows the results of the enzymatic hydrolysis of poplar pretreated by dilute NaOH, with 2% concentration, at 65 °C for 1 to 5 h, and the subsequent Fenton reaction. With an increase in treatment time, the enzymatic hydrolysis efficiency of cellulose first increased, achieving the highest value of 65% at 3 h. By continuously increasing the treatment time, the monosaccharide presented in the NaOH-pretreated liquid increased and the enzymatic hydrolysis efficiency of the cellulose decreased. This was because with an increase in pretreatment time, more ester and ether bonds between lignin and cellulose were broken with some cellulose dissolved in NaOH-pretreatment solution, resulting in percent cellulose in residue after pretreatment reduced. Therefore, it was found that the optimal dilute NaOH extraction time was 3 h.

Figure 2c shows the results of the enzymatic hydrolysis of poplar pretreated with dilute NaOH, with 2% concentration, at temperatures of 55, 65, 75, 85, and 95 °C for 3 h, and the subsequent Fenton’s reaction. The data indicate that NaOH extraction at 75 °C increased the enzymatic hydrolysis efficiency by 15.6% compared with extraction at 55 °C, and only 7.6% of the potential glucose was seen in the residue. When the extraction temperature increased up to 95 °C, there was a marked reduction in the enzymatic hydrolysis efficiency, and more than 25% of the cellulose remained in the residue. What made the lower glucose yield in this temperature is that the denaturation of enzymes at 95 °C and the degradation of partial cellulose. Given the relatively high enzymatic hydrolysis efficiency and the small amount of cellulose remaining in the residue, the optimal NaOH extraction temperature was determined to be 75 °C.

The ANOVA for the degradation of cellulose is shown in Table 2, and the ANOVA results indicate that the effects of ideal NaOH treatment conditions were significant (P = <0.01) for cellulose degradation. Current knowledge is that NaOH pretreatment causes a partial solubilization of hemicellulose, resulting in a partial extraction of the xylan component, which favors the accessibility of cellulase to cellulose. In addition, NaOH could also decrease lignin content, increase enzyme effectiveness by eliminating nonproductive adsorption sites and by increasing access to cellulose (Cao et al. 2012). Our results show that appropriate NaOH extraction conditions lead to the removal of more hemicellulose and lignin. That is why in the combined pretreatment, the cellulose recovery yield first increased and then decreased with increasing NaOH concentration, treatment time, and temperature.
Fig. 2. Effects of NaOH treatment conditions on enzymatic hydrolysis of pretreated poplar, where enzymatic hydrolysis was carried out under the following conditions: (a) pretreatment by dilute NaOH (0.5% to 2.5%) at 65 °C for 2 h and the subsequent Fenton reaction for 12 h; (b) poplar pretreated by dilute NaOH (2%) at 65 °C for 1 to 5 h and the Fenton reaction for 12 h; (c) pretreatment by dilute NaOH (2%) at 55 to 95 °C for 3 h and the Fenton reaction for 12 h. X1/X2 is the monomeric/oligomeric glucose in the NaOH extraction liquid; X3 is the loss of the cellulose in the Fenton pretreatment liquid; X4 is the monomeric glucose in the enzymatic hydrolysate; and X5 is the potential glucose remaining in the residue. The cellulose removal rate in the NaOH extraction phase = X1 + X2, and the cellulose recovery yield = X4.
Table 2. ANOVA of the NaOH Pretreatment Conditions

<table>
<thead>
<tr>
<th>Terms</th>
<th>Mean square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic hydrolysis efficiency (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$X_1^*$</td>
<td>.006</td>
<td>97107.284</td>
<td>.000</td>
</tr>
<tr>
<td>$X_2^*$</td>
<td>.000</td>
<td>982.232</td>
<td>.000</td>
</tr>
<tr>
<td>$X_3^*$</td>
<td>.000</td>
<td>116.660</td>
<td>.000</td>
</tr>
<tr>
<td>The monomeric glucose in the NaOH extraction liquid (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$X_1$</td>
<td>.000</td>
<td>5731.413</td>
<td>.001</td>
</tr>
<tr>
<td>$X_2$</td>
<td>.000</td>
<td>19.161</td>
<td>.003</td>
</tr>
<tr>
<td>$X_3$</td>
<td>.001</td>
<td>5409.33</td>
<td>.000</td>
</tr>
<tr>
<td>The oligomeric glucose in the NaOH extraction liquid (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$X_1$</td>
<td>.000</td>
<td>263.337</td>
<td>.004</td>
</tr>
<tr>
<td>$X_2$</td>
<td>.015</td>
<td>528.366</td>
<td>.000</td>
</tr>
<tr>
<td>$X_3$</td>
<td>.018</td>
<td>208.949</td>
<td>.000</td>
</tr>
</tbody>
</table>

$X_1^*$, $X_2^*$ and $X_3^*$ were defined as NaOH concentration, NaOH treatment time, NaOH treatment temperature, respectively.

Enhanced Enzymatic Hydrolysis of Cellulose by Control of Individual Component Concentrations of Fenton’s Reagent

To clarify the effects of different ratios of H$_2$O$_2$ and FeSO$_4$·7H$_2$O involved in the Fenton’s reagent, 5 to 30 mmol/g H$_2$O$_2$ and 0.05 to 0.4 mmol/g FeSO$_4$·7H$_2$O were investigated. In Fig. 3, it is clear that the enzymatic hydrolysis efficiency increased significantly as the ratio increased from 10:1 to 100:1. That is because an appropriate concentration of H$_2$O$_2$ will help sustain the reaction for a longer period of time by continuously generating more hydroxyl radicals, and a much higher concentration of Fe$^{2+}$ can lead to radical scavenger generation and induce a decrease in the degradation rate of cellulose (Michalska et al. 2012).

![Fig. 3. Effects of different $[\text{H}_2\text{O}_2]/[\text{Fe}^{2+}]$ ratios on enzymatic hydrolysis of cellulose](image-url)
Figure 3 also indicates that an increase in H$_2$O$_2$ concentration during pretreatment led to a more obvious increase in cellulose degradation in contrast to increasing Fe$^{2+}$ concentration. As the ratio increased continuously, the enzymatic hydrolysis efficiency decreased. This is consistent with others’ reports (Bhange et al. 2015). Considering about these, the ratio 100:1, that is, 20 mmol/g of H$_2$O$_2$ and 0.2 mmol/g of FeSO$_4$·7H$_2$O seems to work the best for a high enzymatic hydrolysis efficiency of cellulose.

Thus, the Fenton reagent media containing 20 mmol/g of H$_2$O$_2$ (30%) and 0.2 mmol/g of FeSO$_4$·7H$_2$O was used for the combined pretreatment of poplar.

**Surface Structure Characteristics of Pretreated Poplar**

A previous study adopted an X-ray diffraction assay to visualize the crystallinity changes of cotton fiber (Zhang et al. 2015). In this study, untreated and pretreated samples were observed using a scanning electron microscope to confirm the enhancement of the enzymatic hydrolysis resulting from surface structure changes, which can be seen in Fig. 4. As shown in Fig. 4a, the surface of the untreated poplar is smooth, contiguous, and flat. After 72 h of enzymatic hydrolysis, there was only a slight crimp on the surface of the un-pretreated poplar, shown in Fig. 4b. Both the pretreatment and the enzymatic hydrolysis can lead to holes on the surface of poplar. The edges of the holes could be parts of the lignin framework with some of the remaining cellulose. The lignin framework would further reduce to only a few fragments, or even disappear, after enzymatic hydrolysis when the pretreatment severity was greater (Liu et al. 2009b). These results illustrate that the pretreatment is vital in the destruction of the integral structure of the biomass.
Fig. 4. SEM images of (a) untreated poplar (500x); (b) untreated poplar after enzymatic hydrolysis (500x); (c) NaOH pretreated poplar (500x); (d) NaOH pretreated poplar after enzymatic hydrolysis (500x); (e) Fenton pretreated poplar (500x); (f) Fenton pretreated poplar after enzymatic hydrolysis (500x); (g) NaOH and Fenton pretreated poplar (500x); and (h) NaOH and Fenton pretreated poplar after enzymatic hydrolysis (500x)

Pretreatment can also increase the effective absorbability of cellulase (Ding et al. 2012), which certainly enhances the enzymatic hydrolysis. The formation of holes on the surface of the sample was consistent with previous observations (Kohlmann et al. 1996) that hypothesized that the enzymatic hydrolysis of cellulose is dominated by a tunneling mechanism, that is, the enzyme complex acts on the cellulose by penetration (Zhou et al. 2014).

Both single NaOH treatment and single Fenton reaction can leave a large quantity of holes on the surface of the sample (Fig. 4c and Fig. 4e). NaOH treatment also aims to break up the structure of the substrate, thus causes swelling, leading to an increase in internal surface area of the sample (Liu et al. 2009b; Zhou et al. 2014), as shown in Fig. 4c. That is why the NaOH pretreatment effect was superior to the Fenton pretreatment after pretreatment (Fig. 4d and 4f). After the combined pretreatment, only erosion troughs can be seen on the surface of poplar (Fig. 4g). Consistent with this finding, the image corresponding to the highest cellulose recovery yield of 74%, after enzymatic hydrolysis, is Fig. 4h.
CONCLUSIONS

1. Approximately 74% cellulose recovery yield was obtained with combined dilute NaOH extraction and Fenton pretreatment, which showed a particularly strong enhancement of the digestion and enzymatic hydrolysis of cellulose in this study.

2. The optimum pretreatment conditions were as follows: dilute NaOH (2%) extraction at 75 °C for 3 h, and subsequent Fenton reaction with 20 mmol/g of H₂O₂ (30%) and 0.2 mmol/g of FeSO₄·7H₂O.

3. Microscopic observations of the changes in the surface structure of the pretreated residue confirmed the enhancement of the enzymatic hydrolysis of cellulose.

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