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

Chunyan Zhang,[a] Haisheng Pei,[b] Shanshan Wang,[a] Zhongyi Cui,[a] and Ping Liu [a,]*

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 H_2O_2 (30%) and 0.2 mmol/g of $FeSO_4·7H_2O$ 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

*Contact information: a: College of Food Science and Nutritional Engineering, China Agricultural University, P.O. Box 398, 17 Qinghua Donglu, Haidian District, Beijing 100083, China; b: Chinese Academy of Agricultural Engineering, 41 Maizidian street, Chaoyang District, Beijing 100125, China; * Corresponding author: liuping@cau.edu.cn*

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 H_2O_2) 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 (Bhange *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 H_2O_2 itself is a very powerful oxidant that proceeds through a radical mechanism, and Fe^{2+} ions present in the pretreatment solution serve as a catalyst. The Fenton reaction involves the oxidation of Fe^{2+} to Fe^{3+} by H_2O_2 and then the reduction of Fe^{3+} to Fe^{2+}, as given in Eqs. 1 and 2 (Arantes *et al.* 2012), simultaneously bringing about two kinds of oxygen radicals, $HO^{\bullet}$ and $HOO^{\bullet}$ (Arantes *et al.* 2011; He *et al.* 2015). These oxygen radicals initiate chain reactions for oxidizing lignocellulosic components (He *et al.* 2015).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + HO^{\bullet} \tag{1}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + H^{+} + HOO^{\bullet} \tag{2}$$

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 1.0×6.0 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 H_2O_2 (30%) and $FeSO_4 \cdot 7H_2O$ 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,

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

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

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 (Bhange *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., $FeSO_4 \cdot 7H_2O$ and H_2O_2, 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

Pretreatment Process	Solid Recovery (%)	Cellulose (%)	Hemicellulose (%)
Control group	100	33.4	35.0
H_2O	92.3	35.8	32.4
CaO	88.9	36.4	32.6
NaOH	82.1	36.3	31.7
Fenton	86.5	35.1	28.2
NaOH + Fenton	66.2	41.5	31.9

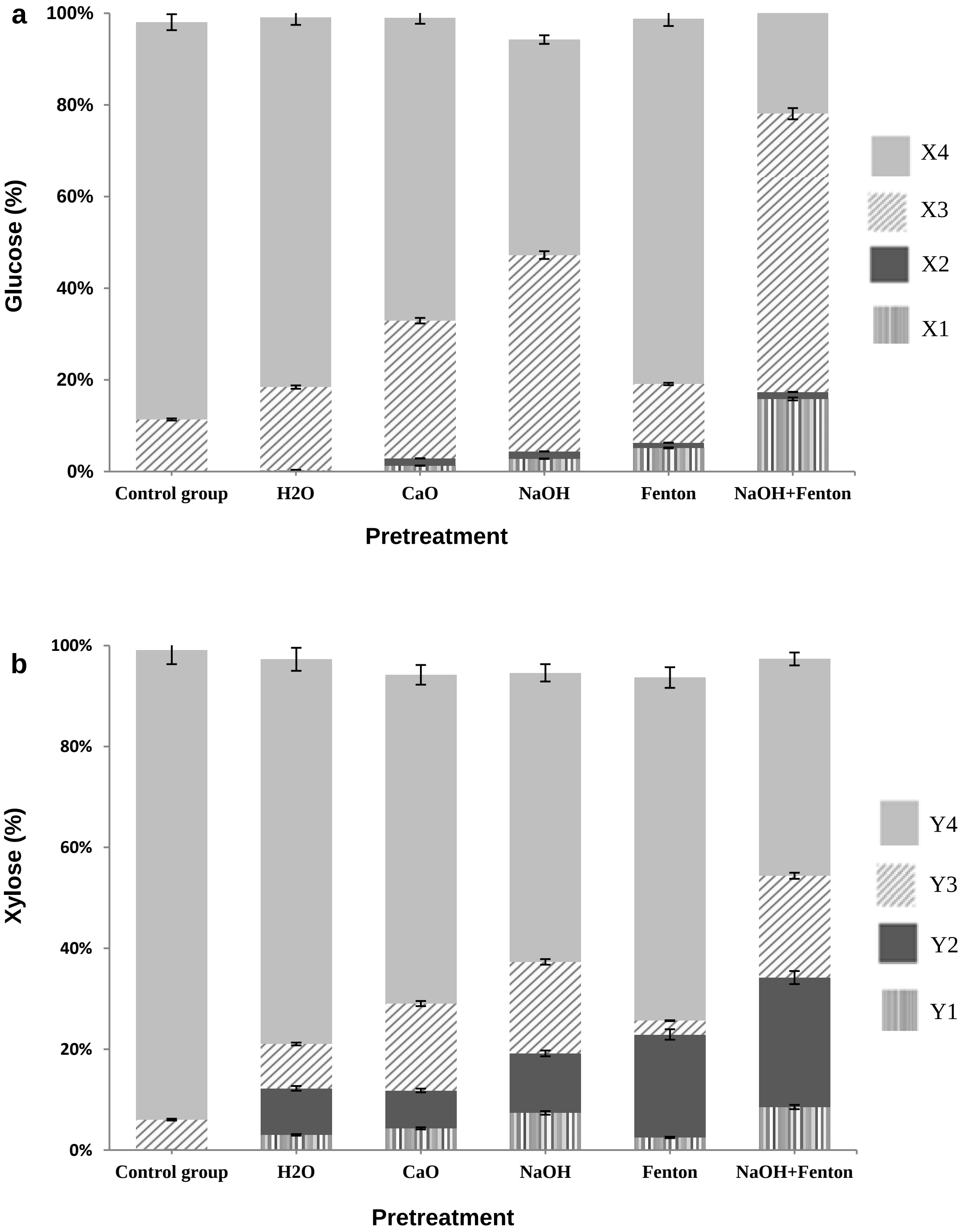


Fig. 1. Enzymatic hydrolysis of (a) cellulose and (b) hemicellulose with various pretreatment processes.

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 (H_2O_2 1 mL/g, $FeSO_4 \cdot 7H_2O$ 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.

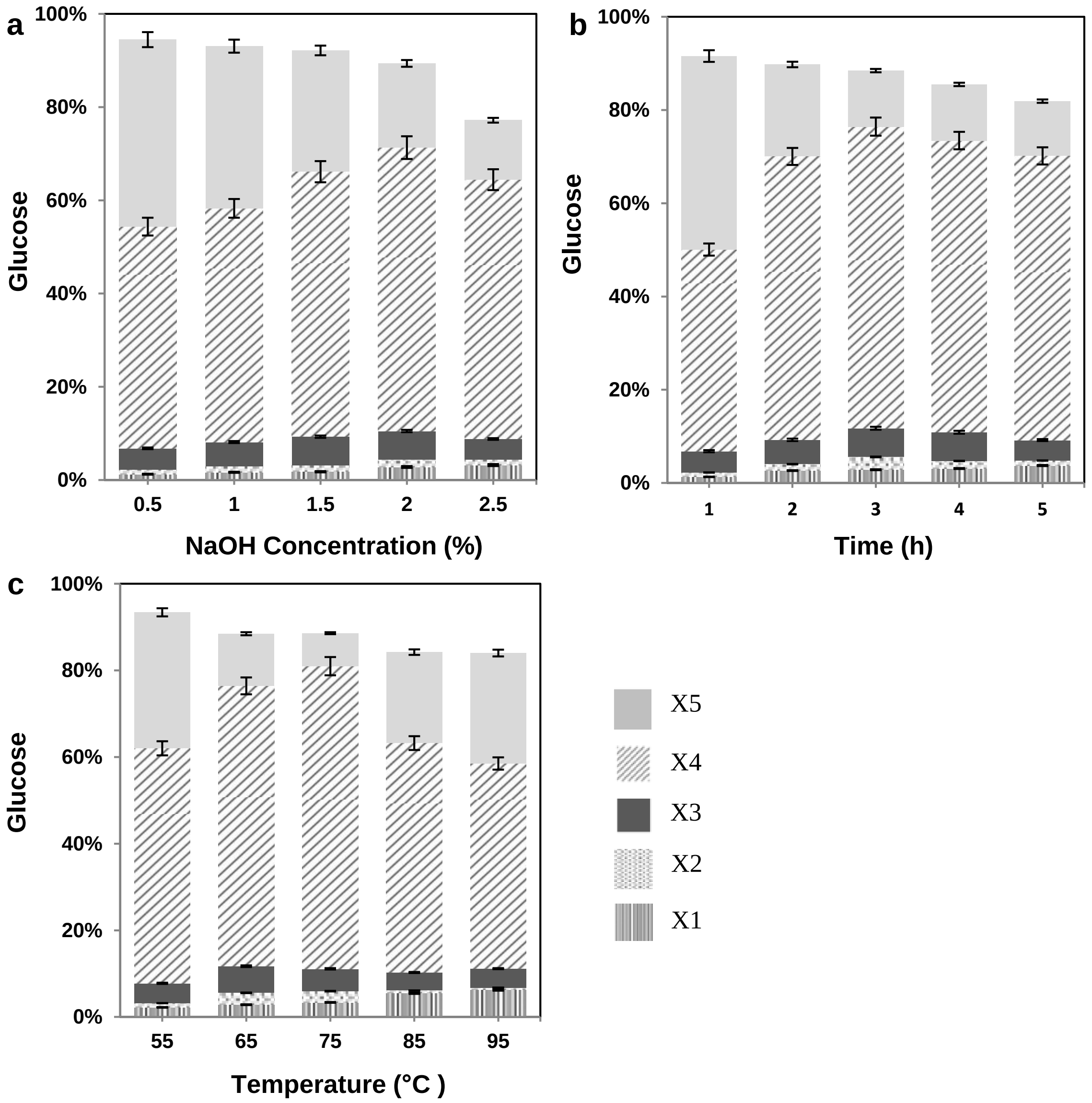


Fig. 2. Effects of NaOH treatment conditions on enzymatic hydrolysis of pretreated poplar, where enzymatic hydrolysis was carried out under the following conditions: (a) pretreatment by dilute NaOH (0.5% to 2.5%) at 65 °C for 2 h and the subsequent Fenton reaction for 12 h; (b) poplar pretreated by dilute 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

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

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_2O_2 and $FeSO_4{\cdot}7H_2O$ involved in the Fenton's reagent, 5 to 30 mmol/g H_2O_2 and 0.05 to 0.4 mmol/g $FeSO_4{\cdot}7H_2O$ 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_2O_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).

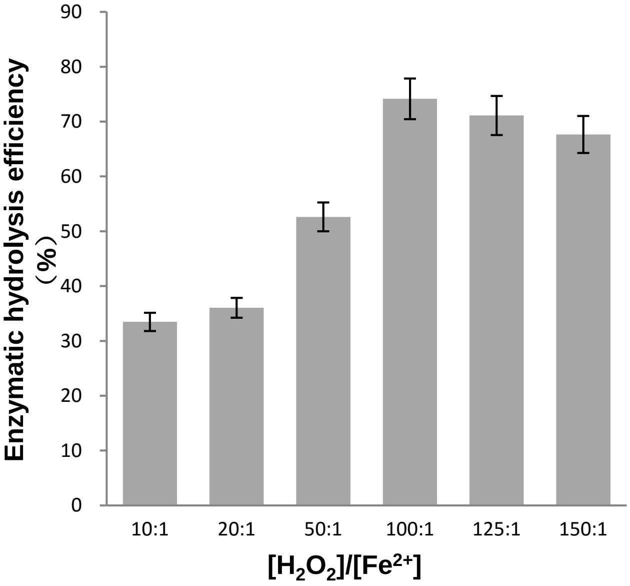


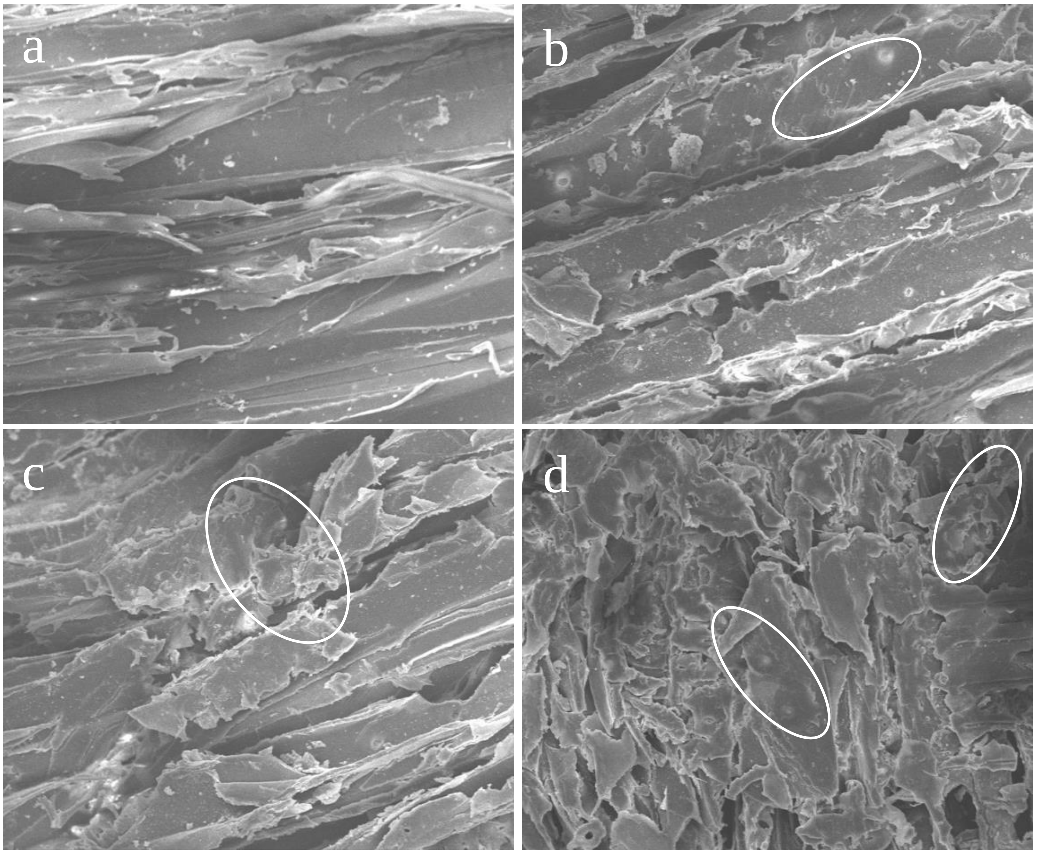
Fig. 3. Effects of different [H_2O_2]/[Fe^{2+}] ratios on enzymatic hydrolysis of cellulose

Figure 3 also indicates that an increase in H_2O_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_2O_2 and 0.2 mmol/g of $FeSO_4{\cdot}7H_2O$ seems to work the best for a high enzymatic hydrolysis efficiency of cellulose.

Thus, the Fenton reagent media containing 20 mmol/g of H_2O_2 (30%) and 0.2 mmol/g of $FeSO_4{\cdot}7H_2O$ 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.



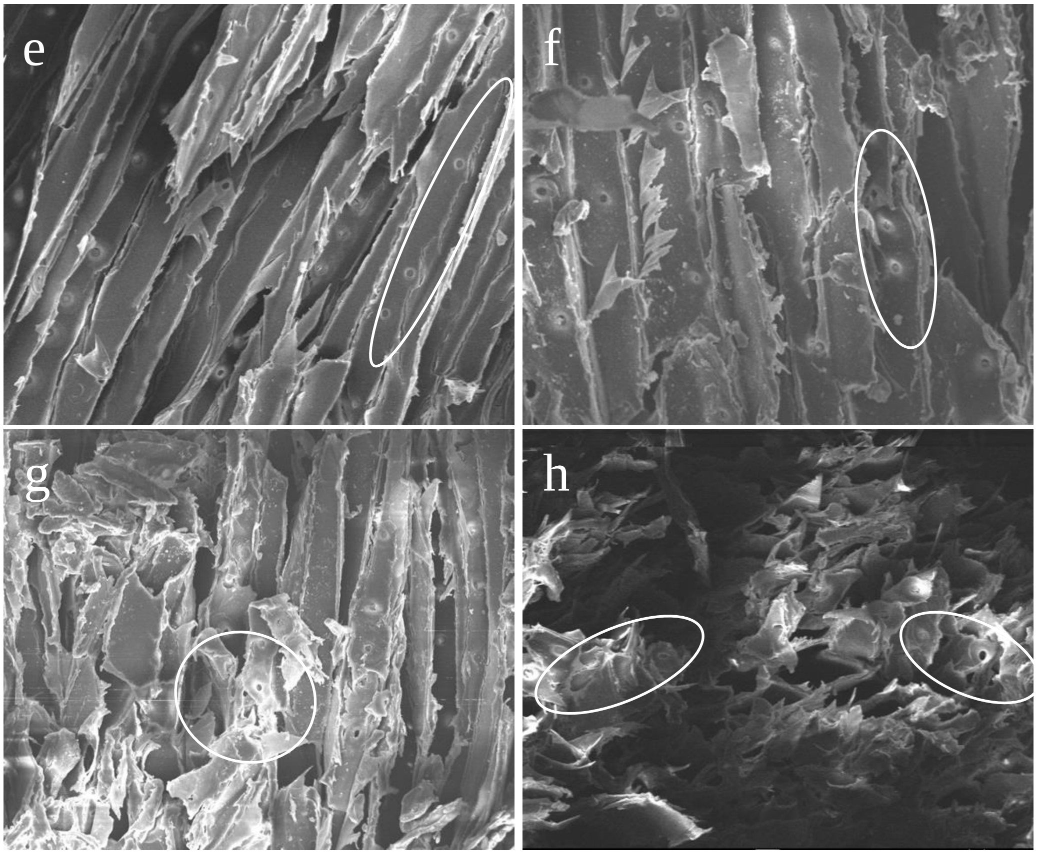


Fig. 4. SEM images of (a) untreated poplar (500x); (b) untreated poplar after enzymatic hydrolysis (500x); (c) NaOH pretreated poplar (500x); (d) NaOH pretreated poplar after enzymatic hydrolysis (500x); (e) Fenton pretreated poplar (500x); (f) Fenton pretreated poplar after enzymatic hydrolysis (500x); (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 H_2O_2 (30%) and 0.2 mmol/g of $FeSO_4 \cdot 7H_2O$.
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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REFERENCES CITED

Arantes, V., Milagres, A. M., Filley, T. R., and Goodell, B. (2011). "Lignocellulosic polysaccharides and lignin degradation by wood decay fungi: The relevance of nonenzymatic Fenton-based reactions," *Journal of Industrial Microbiology and Biotechnology* 38(4), 541-555. DOI: 10.1007/s10295-010-0798-2

Arantes, V., Jellison, J., and Goodell, B. (2012). "Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass," *Applied Microbiology and Biotechnology* 94(2), 323-338. DOI: 10.1007/s00253-012-3954-y

Banerjee, G., Car, S., Scott-Craig, J. S., Hodge, D. B., and Walton, J. D. (2001). "Saccharification of lignocellulosic materials by the cellulases of *Bacillus subtilis*," *International Journal of Agriculture and Biology* 3(2), 199-202. DOI: 1560-8530/2001/03-2

Bhange, V. P., William, S. P. M. P., Sharma, A., Gabhane, J., Vaidya, A. N., and Wate, S. R. (2015). "Pretreatment of garden biomass using Fenton's reagent: Influence of Fe^{2+} and H_2O_2 concentrations on lignocellulose degradation," *Journal of Environmental Health Science and Engineering* 13, 12. DOI: 10.1186/s40201-015-0167-1

Batool, S., Khalid, A., Chowdury, A. J. K., Sarfraz, M., Balkhair, K. S., and Ashraf, M. A. (2015). "Impacts of azo dye on ammonium oxidation process and ammonia oxidizing soil bacteria," *RSC Advances* 5, 34812-34820. DOI: 10.1039/C5RA03768A

Cao, W., Sun, C., Liu, R., Yin, R., and Wu, X. (2012). "Comparison of the effects of five pretreatment methods on enhancing the enzymatic digestibility and ethanol production from sweet sorghum bagasse," *Bioresource Technology* 111, 215-221. DOI: 10.1016/j.biortech.2012.02.034

Coimbra, M. C., Duque, A., Saéz, F., Manzanares, P., Garcia-Cruz, C. H., and Ballesteros, M. (2016). "Sugar production from wheat straw biomass by alkaline extrusion and enzymatic hydrolysis," *Renewable Energy* 86(4), 1060-1068. DOI: 10.1016/j.renene.2015.09.026

Ding, S. Y., Liu, Y. S., Zeng, Y. N., Himmel, M. E., Baker, J. O., and Bayer, E. A. (2012). "How does plant cell wall nanoscale architecture correlate with enzymatic digestibility," *Science* 338(3), 1055-1060. DOI: 10.1126/science.1227491

He, Y. C., Ding, Y., Xue, Y. F., Yang, B., Liu, F., Wang, C., Zhu, Z. Z., Qing, Q., Wu, H., Zhu, C., *et al*. (2015). "Enhancement of enzymatic saccharification of corn stover with sequential Fenton pretreatment and dilute NaOH extraction," *Bioresource Technology* 193(6), 324-330. DOI: 10.1016/j.biortech.2015.06.088

Jain, P., and Vigneshwaran, N. (2012). "Effect of Fenton's pretreatment on cotton cellulosic substrates to enhance its enzymatic hydrolysis response," *Bioresource Technology* 103(1), 219-226. DOI: 10.1016/j.biortech.2011.09.110

Ju, X., Engelhard, M., and Zhang, X. (2013). "An advanced understanding of the specific effects of xylan and surface lignin contents on enzymatic hydrolysis of lignocellulosic biomass," *Bioresource Technology* 132, 137-145. DOI: 10.1016/j.biortech.2013.01.049

Jung, Y. H., Kim, H. K., Park, H. M., Park, Y. C., Park, K., Seo, J. H., and Kim, K. H. (2015). "Mimicking the Fenton reaction-induced wood decay by fungi for pretreatment of lignocellulose," *Bioresource Technology* 179, 467-472. DOI: 10.1016/j.biortech.2014.12.069

Jung, Y. H., Kim, I. J., Kim, H. K., and Kim, K. H. (2013). "Dilute acid pretreatment of lignocellulose for whole slurry ethanol fermentation," *Bioresource Technology* 132, 109-114. DOI: 10.1016/j.biortech.2012.12.151

Jung, Y. H., Kim, I. J., Kim, H. K., and Kim, K. H. (2014). "Whole slurry fermentation of maleic acid-pretreated oil palm empty fruit bunches for ethanol production not necessitating a detoxification process," *Bioprocess and Biosystems Engineering* 37(4), 659-665. DOI: 10.1007/s00449-013-1035-y

Kato, D. M., Elia, N., Flythe, M., and Lynn, B. C. (2014). "Pretreatment of lignocellulosic biomass using Fenton chemistry," *Bioresource Technology* 162, 273-278. DOI: 10.1016/j.biortech.2014.03.151

Kim, T. H., and Lee, Y. Y. (2005). "Pretreatment and fractionation of corn stover by ammonia recycle percolation process," *Bioresource Technology* 96(18), 2007-2013. DOI: 10.1016/j.biortech.2005.01.015

Kohlmann, Westgate, P., Velayudhan, A., Weil, J., Sarikaya, A., Brewer, M. A., Hendrickson, R. L., and Ladisch, M. R. (1996). "Enzyme conversion of lignocellulosic plant materials for resource recovery in a controlled ecological life support system," *Advances in Space Research* 18(1-2), 251-265. DOI: 10.1016/0273-1177(95)00815-V

Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H. V., Auer, M., Vogel, K. P., Simmons, B. A., and Singh, S. (2010). "Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification, and enzymatic saccharification," *Bioresource Technology* 101(13), 4900-4906. DOI: 10.1016/j.biortech.2009.10.066

Li, J., Zhou, P., Lv, X., Xiao, W., Gong, Y., Lin, J., and Liu, Z. (2016). "Use of sugarcane bagasse with different paricle sizes to determine the relationship between physical properties and enzymatic hydrolysis," *BioResources* 11(2), 4745-4757. DOI:10.15376/biores.11.2.4745-4757

Liu, L., Sun, J., Cai, C., Wang, S., Pei, H., and Zhang, J. (2009a). "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, L., Sun, J., Li, M., Wang, S., Pei, H., and Zhang, J. (2009b). "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

Ma, L. J., Cui, Y. Z., Cai, R., Liu, X. Q., Zhang, C. Y., and Xiao, D. G. (2015). "Optimization and evaluation of alkaline potassium permanganate pretreatment of corncob," *Bioresource Technology* 180(2), 1-6. DOI:10.1016/j.biortech.2014.12.078

Mesquita, J. F., Ferraz, A., and Aguiar, A. (2016). "Alkaline-sulfite pretreatment and use of surfactants during enzymatic hydrolysis to enhance ethanol production from sugarcane bagasse," *Bioprocess and Biosystems Engineering* 39(3), 441-448. DOI:10.1007/s00449-015-1527-z

Michalska, K., Miazek, K., Krzystek, L., and Ledakowicz, S. (2012). "Influence of pretreatment with Fenton's reagent on biogas production and methane yield from lignocellulosic biomass," *Bioresource Technology* 119, 72-78. DOI: 10.1016/j.biortech.2012.05.105

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., and Ladisch, M. R. (2005). "Optimization of pH controlled liquid hot water pretreatment of corn stover," *Bioresource Technology* 96(18), 1986-1993. DOI: 10.1016/j.biortech.2005.01.013

Negro, M. J., Duque, A., Manzanares, P., Sáez, F., Oliva, J. M., Ballesteros, I., and Ballesteros, M. (2015). "Alkaline twin-screw extrusion fractionation of olive-tree pruning biomass," *Industrial Crops and Products* 74, 336-341. DOI: 10.1016/j.indcrop.2015.05.018

Qing, Q., Hu, R., He, Y., Zhang, Y., and Wang, L. (2014). "Investigation of a novel acid-catalyzed ionic liquid pretreatment method to improve biomass enzymatic hydrolysis conversion," *Applied Microbiology and Biotechnology* 98(6), 5275-5286. DOI: 10.1111/j.1365-2621.2006.01372.x

Qiu, W., and Chen, H. (2012). "Enhanced the enzymatic hydrolysis efficiency of wheat straw after combined steam explosion and laccase pretreatment," *Bioresource Technology* 118, 8-12. DOI: 10.1016/j.biortech.2012.05.033

Sun, F., and Chen, H. (2008). "Enhanced enzymatic hydrolysis of wheat straw by aqueous glycerol pretreatment," *Bioresource Technology* 99(14), 6156-6161. DOI: 10.1016/j.biortech.2007.12.027

Wan, C., and Li, Y. (2011). "Effectiveness of microbial pretreatment by *Ceriporiopsis subvermispora* on different biomass feedstocks," *Bioresource Technology* 102(16), 7507-7512. DOI: 10.1016/j.biortech.2011.05.026

Wang, K., Yang, H., Chen, Q., and Sun, R.-C. (2013). "Influence of delignification efficiency with alkaline peroxide on the digestibility of furfural residues for bioethanol production," *Bioresource Technology* 146, 208-214. DOI: 10.1016/j.biortech.2013.07.008

Wang, Q. Q., Wei, W., Li, X., Sun, J. Z., He, J., and He, M. X. (2016). "Comparative study of alkali and acidic cellulose solvent pretreatment of corn stover for fermentable sugar production," *Bioresources* 11(1), 482-491. DOI:10.15376/biores.11.1.482-491

Yamashita, Y., Shono, M., Sasaki, C., and Nakamura, Y. (2010). "Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo," *Carbohydrate Polymers* 79(4), 914-920. DOI: 10.1016/j.carbpol.2009.10.017

Yang, M., Kuittinen, S., Zhang, J., Keinanen, M., and Pappinen, A. (2013). "Effect of dilute acid pretreatment on the conversion of barley straw with grains to fermentable sugars," *Bioresource Technology* 146, 444-450. DOI: 10.1016/j.biortech.2013.07.107

Zeng, M., Mosier, N. S., Huang, C. P., Sherman, D. M., and Ladisch, M. R. (2007). "Microscopic examination of changes of plant cell structure in corn stover due to hot water pretreatment and enzymatic hydrolysis," *Biotechnology and Bioengineering* 97(2), 265-278. DOI: 10.1002/bit.21298

Zhang, Y., Li, Q., Su, J., Lin, Y., Huang, Z., Lu, Y., Sun, G., Yang, M., Huang, A., Hu, H., *et al*. (2015). "A green and efficient technology for the degradation of cellulosic materials: Structure changes and enhanced enzymatic hydrolysis of natural cellulose pretreated by synergistic interaction of mechanical activation and metal salt," *Bioresource Technology* 177, 176-181. DOI: 10.1016/j.biortech.2014.11.085

Zhou, W., Yang, M., Wang, C., Liu, J., and Xing, J. (2014). "Changes in plant cell-wall structure of corn stover due to hot compressed water pretreatment and enhanced enzymatic hydrolysis," *World Journal of Microbiology and Biotechnology* 30(8), 2325-2333. DOI: 10.1007/s11274-014-1651-y

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