Effect and optimization of NaOH combined with Fenton pretreatment conditions on enzymatic hydrolysis of poplar sawdust

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Abstract

In this study, the effects and mechanism of pretreatments of three types of chemical reagents combined with Fenton on poplar sawdust were studied and the optimization of enzymatic hydrolysis conditions was conducted using response surface methodology. The results showed that cellulase and hemicellulase had the best hydrolysis effect after NaOH-Fenton pretreatment, which were 63.73% and 29.29%, respectively. The optimal process of poplar substrate was to react in 1% NaOH at 100 °C for 1 h, then placed in the Fenton reaction system of 0.2 mmol Fe^{2+} and 25 mmol H_{2}O_{2} for 7 h, and finally subjected to enzymatic hydrolysis for 72 h at 52 °C, with a liquid-solid ratio of 33 and 15 μL/g of β-glycosidase. Under this condition, the enzymatic hydrolysis rates of cellulase and hemicellulase reached 86.65% and 43.9%, respectively. In conclusion, the combination of NaOH and Fenton pretreatment can effectively promote the enzymatic hydrolysis of poplar sawdust, which has great potential in the production of cellulosic ethanol.

Keywords Poplar sawdust; Pretreatment of NaOH combined with Fenton; Enzymatic hydrolysis; Optimization; Response surface methodology

Introduction

Growing energy demands and depletion of fossil fuels stimulated the search for biomass, thus promoting the production of fuels and chemicals from lignocellulose (1, 2). Common lignocellulosic biomass materials, like corn stover (3), bamboo (4), sugarcane bagasse (5), and wheat straw (6), have been extensively studied. Lignocellulose mainly consists of cellulose, hemicellulose, and lignin, in which cellulose and hemicellulose can be hydrolyzed into reducing sugars, then converted into biofuels by enzymes or microbes (7). However, lignocellulose is difficult to degrade due to its complex structure and the presence of lignin (8), which affects enzyme activity, fluid permeability, and enzyme accessibility (9), hindering the utilization of lignocellulosic materials. Therefore, pretreatment of the lignocellulose is
necessary to break the compact structure and assist in the removal of lignin, thus promoting the contact between the cellulose and enzymes or microbes (10).

Pretreatment methods are generally divided into four kinds: chemical, physical, physical-chemical, biological pretreatments are widely used (11). Among the various chemical pretreatments, alkali pretreatment is the most common due to its prominent delignification ability (12). The NaOH pretreatment could reduce the lignin and hemicellulose contents and significantly improve the enzyme digestibility of the agave stalk (13). Du et al. (14) pretreated cotton straw with high-pressure assisted-NaOH, resulting in a reducing sugar yield of 293 g/g and cellulose conversion of 45.82%. However, single NaOH pretreatment has its disadvantage: the effect of enzymatic hydrolysis became worse with the increase of lignin amount except for the low lignin content (15).

Fenton is an advanced oxidation reagent which used to serve for treating organic wastewater (16), and the Fenton reaction could been used in lignocellulose pretreatment. After the Fenton pretreatment, cotton was degraded effectively and its susceptibility to cellulase was improved (17). The enzymatic saccharification effect of four different biomass feedstocks (miscanthus, switchgrass, corn stover, and wheat straw) after Fenton pretreatment showed an average increase of 212% in comparison to untreated control groups, which demonstrated the capacity of Fenton to improves the accessibility of cellulose to enzymes (18). In the reaction mechanism, Fenton reagent generates hydroxyl radical through the reaction of Fe^{2+} and H_{2}O_{2}, as given in Eq. 1, where Fe^{2+} act as the catalyst. The consumed Fe^{2+} is regenerated by the reaction of Fe^{3+} with H_{2}O_{2} as shown in Eq. 2. These free radicals could oxidize lignocellulose components (19, 20). Fenton oxidation conditions are mild, and the treatment of alkali combined with Fenton has high potential in lignocellulose pretreatment. The sequential Fenton and dilute NaOH extraction has been applied to pretreat corn stover which changes the cellulose structure characteristics (porosity, morphology, and crystallinity) of the solid residues and enhances the enzymatic saccharification
Fe$^{2+}$ + H$_2$O$_2$ → Fe$^{3+}$ + OH$^-$ + H$^+$ (1)

Fe$^{3+}$ + H$_2$O$_2$ → Fe$^{2+}$ + H$^+$ + HO$_2^-$ (2)

Cellulose can be hydrolyzed into glucose and other fermentable sugars after pretreatment. In general, common hydrolysis processes include acid hydrolysis and enzyme hydrolysis, and the latter has been widely used because of high hydrolysis efficiency and low environmental pollution (21). The existing studies on the enzymatic hydrolysis conditions of pretreated biomass materials are mainly focused on the optimization of enzyme addition amount, process time, temperature, and pH. The optimal conditions for two-step alkaline-enzymatic hydrolysis are an NaOH concentration of 9%, enzyme ratio of 0.95, and time of 16 h (22). In addition, lignocellulose digestibility is affected by liquid-solid ratio, buffer concentration, degree of crystallization, and porosity (23). When considering the effects of many factors, response surface methodology (RSM) is an effective tool for optimizing the process (24). It can reduce the number of experiments needed to evaluate multiple parameters, and the experimental methodology forms a mathematical model to describe the whole process, in addition to analyzing the influence of independent variables. At present, RSM has been successfully used to optimize the enzymatic reaction conditions (25).

In this study, poplar sawdust was treated with the combination of Fenton and dilute acid (HCl and H$_2$SO$_4$), dilute alkali (ammonia and NaOH) and oxidants (H$_2$O$_2$ and NaClO), respectively, to explore the differences of three chemical pretreatment methods on the removal ability of cellulose and hemicellulose in poplar sawdust and subsequent enzymatic hydrolysis of cellulose. Considering the enzymatic hydrolysis rates of cellulase and hemicellulase as indicators, the effects of the concentration, temperature, and time of NaOH treatment as well as the H$_2$O$_2$ and Fe$^{2+}$ additions on the enzymatic hydrolysis of sawdust were investigated to obtain the optimal NaOH-Fenton pretreatment conditions. Finally, the optimal enzymatic hydrolysis conditions under the optimal NaOH-Fenton pretreatment was identified using RSM. This study
provides a reference for maximizing the hemicellulose and cellulose in poplar sawdust into fermentable sugars, reducing the cost of ethanol production, and fully improve the comprehensive utilization value of poplar sawdust.

Materials and Methods

Materials and Chemicals

Poplar sawdust was obtained from a farm in the outskirts of Beijing. The raw material was smashed and sifted through a 20-mesh sieve, then dried to a constant weight. Through two-step acid hydrolysis, the contents of cellulose, hemicellulose, and lignin were determined to be 32.57%, 13.88%, and 32.11%, respectively.

The cellulase, β-glucosidase, hemicellulase and cycloheximide were purchased from Sigma (St. Louis, MO, USA). Tetracycline hydrochloride was obtained from Amresco (Solon, OH, USA). The glucose standard, xylose standard, and cellobiose are chromatographically pure, and all supplied from Exin Biotechnology Co., Ltd. (Shanghai, China). The other reagents used in the experiment were of analytical purity and were purchased from Beijing Chemical Works (Beijing, China).

Different Pretreatments of Poplar Sawdust

Fenton Treatment: The tube was first loaded with 1 g of sawdust, 0.1 mmol FeSO₄·7 H₂O and 15 mmol 30% H₂O₂, and deionized water was added to 15 mL. Sulfuric acid was used to adjust the pH to 4. The tube was kept away from light for 10 h to complete the Fenton reaction. After the reaction, the mixture was filtered through a 200-mesh cell sieve, and the remaining sawdust was washed with 2% oxalic acid and deionized water.

Acid Combined with Fenton Treatment: Acid treatment was conducted with different concentration (1%, 2%, 3%, 4%, 5%) of H₂SO₄ or HCl at 1:15 solid to liquid ratio at 80°C water for 2 h. After filtration, the remaining sawdust were subjected to Fenton reaction according to the above method.
**Alkali Combined with Fenton Treatment:** Poplar sawdust and dilute alkali solution of different concentration (NaOH was 1%, 2%, 3%, 4%, 5%, ammonia water was 5%, 10%, 15%, 20%) were reacted in a 75°C water bath for 2 h at a material-to-liquid ratio of 1:15. After solid-liquid separation, performing the Fenton reaction according to the above conditions.

**Oxidants Combined with Fenton Treatment:** H$_2$O$_2$ treatment was to mix poplar sawdust and H$_2$O$_2$ solution (1.5%, 2.5%, 3.5%, 4.5%, 5.5%) at a ratio of 1:15 and incubated at 30°C for 2 h. Fenton treatment is performed after filtration. NaClO treatment was conducted with NaClO solution (1.5%, 2.5%, 3.5%, 4.5%, 5.5%) at 1:15 solid to liquid ratio at 80°C water for 2 h. After filtration, the sawdust was subjected to Fenton treatment.

**Determination of Content of Glucose and Xylose**

Refer to two-step acid hydrolysis, untreated poplar sawdust was used as samples. Step 1: 0.300 g sample was accurately weighed and mixed with 3.0 ml of 72% sulfuric acid at 30 °C for 1 h and swirled every 5~10 min. Step 2: 84 mL deionized water was added to sulfuric acid to dilute the concentration to 4%. After reacting at 121 °C for 1 h, the filtrate obtained by suction filtration was passed through a 0.22 μm filter membrane. The contents of glucose and xylose in filtrate were determined by high-performance liquid chromatography (HPLC, Agilent 1200, Palo Alto, CA, USA) system equipped with a refractive index detector, the corresponding protection column, and a Rezex ROA. The samples were eluted with 0.005 M H$_2$SO$_4$ at a flow rate of 0.6 mL/min and a column temperature of 65 °C. The retention time of glucose and xylose were 10.550 min and 11.000 min, respectively. Each sample was repeated three times and the average was used as the calculation result.

**Calculation of Enzymatic Hydrolysis Conversion Rate**

After the pretreatment, the poplar sawdust and 25 μL of hemicellulase were mixed with 30 mL acetate buffer (pH 4.8) in a 50-mL centrifugal tube and placed in a 70°C water bath for 1 d. Also, 60 μL of
cycloheximide and 80 μL of tetracycline hydrochloride were added to avoid pH changes caused by microbial growth. When the reaction was complete, the tube reactor was cooled, and 60 μL cellulase and 10 μL β-glucosidase were added to perform the hydrolysis reaction at 120 rpm and 50 °C for 3 d. Finally, the mixed contents were filtered through a 200-mesh cell sieve, and the enzymatic hydrolysate was collected to determine the contents of glucose and xylose for the calculation of enzymatic conversion rate of cellulose and hemicellulose. The calculation equations were as follows.

\[
\text{Enzymatic hydrolysis conversion rate of cellulose (\%) = } \frac{C_1 \times V \times 0.90}{m \times W_1} \times 100
\]  \hspace{1cm} (3)

\[
\text{Enzymatic hydrolysis conversion rate of hemicellulose (\%) = } \frac{C_2 \times V \times 0.88}{m \times W_2} \times 100
\]  \hspace{1cm} (4)

where \( C_1/C_2 \) was the concentration of glucose/xylose in enzymatic hydrolysate (mg/mL), \( V \) was the total volume (mL), 0.90 was the conversion factor for glucose to equivalent cellulose, 0.88 was the conversion factor for xylose to equivalent hemicellulose, \( m \) was the quality of poplar sawdust (mg), and \( W_1/W_2 \) is the content of glucose/xylose in two-step acid hydrolysis.

**Optimization of NaOH Treatment under Fixed Fenton Conditions**

For the optimization of NaOH treatment temperature-concentration, poplar sawdust was mixed with dilute NaOH (1%, 2%, 3%, 4%, 5%) at 1:15 solid to liquid ratio at 25 °C, 50 °C, 75 °C, and 100 °C for 2 h. Then, the Fenton reaction was performed under the conditions of 0.1 mmol FeSO\(_4\) \( \cdot \) 7 H\(_2\)O and 15 mmol 30% H\(_2\)O\(_2\) for 10 h.

For the optimization of NaOH treatment time, poplar sawdust and NaOH solution were incubated for 0.5 h, 1.0 h, 2.0 h, 3.0 h and 4.0 h at the optimal NaOH concentration and temperature obtained in the previous experiment. Then, Fenton treatment was carried out according to the above conditions.

For the circulation of alkali pretreatment solution, the liquid waste was collected after NaOH pretreatment, and the residual NaOH was determined by titration. According to the measured results,
NaOH was added to the liquid waste to reach the initial concentration. The poplar sawdust was processed, and the liquid waste was collected again. The process was repeated.

Optimization of Fenton Treatment under Fixed NaOH Treatment Conditions

The optimization of the Fenton reaction conditions was carried out under the optimal conditions of NaOH treatment obtained in the above experiment. For the optimization of H₂O₂ addition amount, 1 g of alkali treated sawdust was mixed with 0.1 mmol FeSO₄ • 7 H₂O and different amounts of H₂O₂ (5mmol, 10mmol, 15mmol, 20mmol, 25mmol, 30mmol). Deionized water was added to adjust the material-to-liquid ratio to 1:15, and sulfuric acid was used to adjust the pH to 4. The tube was kept out of the light for 10 h to perform the Fenton reaction. For the optimization of Fe²⁺ addition amount, the sawdust was mixed with 25 mmol H₂O₂ and different amounts of FeSO₄ • 7 H₂O (0.05mmol, 0.1mmol, 0.2mmol, 0.3mmol, 0.4mmol), and the other operations were the same as above.

For the optimization of Fenton reaction time, 1 g of sawdust were mixed with the optimal addition amount of FeSO₄•7 H₂O and H₂O₂ obtained in the above experiment. Deionized water was added to adjust the material-to-liquid ratio to 1:15. Then, the mixture reacted away from the light for 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, respectively.

Response Surface Optimization of Enzymatic Hydrolysis Conditions

According to the Box-Behnken design and the preliminary test results, the enzymatic hydrolysis temperature, liquid-solid ratio, enzyme concentration, and reaction time were selected, and each factor was set at 3 levels to conduct response surface experiments at a total of 30 tests. The total reducing sugar obtained by enzymatic hydrolysis was taken as the response value to optimize the enzymatic hydrolysis of pretreated poplar sawdust. The reducing sugar content of the sample was determined by DNS method.

Result and Discussion

Comparison Analysis of Different Treatments Combined with Fenton on Lignocellulose Enzymatic
Hydrolysis at Ordinary Pressure

Dilute acid treatment is suitable for industrial application because of its weak corrosivity and easy recovery of acid. Figure 1 depicts the effect of pretreatment with different concentrations (1% to 5%) of H₂SO₄ or HCl combined with Fenton on the enzymatic hydrolysis of sawdust. The conversion rates of cellulose and hemicellulose increased as the dilute acid concentration increased from 1% to 3%. When the concentration exceeded 3% and 4%, the enzymatic conversion rate of cellulose and hemicellulose slightly decreased. The study of Saha et al. showed that the wheat straw gave maximum sugar yield after 0.75% H₂SO₄ pretreatment, and the fermentable sugar yield decreased with the increase of acid dose, which is consistent with our observation. This might because that as the concentration of acid increases, the sugars gradually degraded into furfural and HMF which could influence enzymatic hydrolysis of the sawdust, resulting in the decrease of the conversion of cellulose and hemicellulose (26). The enzymatic hydrolysis efficiency of the dilute H₂SO₄ was higher than that of the dilute HCl. When the concentration of dilute H₂SO₄ was 3%, the enzymatic hydrolysis rates of cellulose and hemicellulose were 9.5% and 28.6% higher, respectively, than those of dilute HCl at the same concentration. This result shows that dilute HCl pretreatment causes indistinctive delignification with subtle structural changes in the fibers (27). However, dilute H₂SO₄ pretreatment can make hemicellulose more soluble, and the chemical structure in the solid residue changes, making the sawdust more porous and improving the cellulose accessibility of enzymes (28, 29). Therefore, the combination of 3% H₂SO₄ and Fenton is a better pretreatment condition for lignocellulose, and the enzymatic hydrolysis rates of cellulose and hemicellulose reached 53.95% and 30.6%, respectively.
Fig. 1 Effects of dilute acid concentration on enzymatic hydrolysis of pretreated sawdust (C: cellulose; HC: hemicellulose)

With the increase of oxidant concentration, the cellulose conversion rate gradually increased and tended to be stable at the concentration of 4.5%, while the enzymatic conversion rate of hemicellulose declined after the oxidant concentration exceeded 4.5% (Fig. 2). In addition, the enzymatic hydrolysis rates of cellulose and hemicellulose in the 4.5% H$_2$O$_2$ group were 37.88% and 22.38%, respectively, 3.86% and 18.77% higher than those in the 4.5% NaClO group. Therefore, the pretreatment effect of H$_2$O$_2$ was better than NaClO, which is consistent with previous research (30). Oxidants break down the network structure of the materials through peroxidative degradation of lignin, thereby improving the enzymatic hydrolysis efficiency of cellulose and hemicellulose. As the oxidability of HClO produced by NaClO was weaker than H$_2$O$_2$, its ability to oxidize lignin was weak and it is not as effective as H$_2$O$_2$ in promoting enzymatic hydrolysis. In many cases the reaction of oxidant treatment is not selective, resulting in loss of hemicellulose and cellulose and therefore the pretreatment effect is inferior to dilute acid pretreatment (31). In conclusion, in above pretreatment experiment of poplar sawdust with oxidants combined with Fenton, the pretreatment effect of 4.5% H$_2$O$_2$-Fenton was the best.
When the concentration of NaOH and ammonia was 3% to 4% and 15% to 20%, respectively, the hydrolysis rates of cellulose and hemicellulose reached the maximum (Fig. 3). The conversion rates of cellulose and hemicellulose under 15% to 20% ammonia pretreatment was much lower than that of 3% to 4% NaOH. However, the enzymatic conversion rates of cellulose and hemicellulose at 4% NaOH did not increase significantly compared with those at 3% NaOH. In consequence, 3% NaOH combined with Fenton reaction was the suitable pretreatment in this experiment, and the enzymatic hydrolysis efficiencies of cellulose and hemicellulose were 63.73% and 29.29%, respectively. Although ammonia treatment promotes lignocellulose enzymatic hydrolysis less than NaOH treatment, the commonly used ammonia explosion pretreatment requires high temperature and high pressure (32). In this experiment, the combined pretreatment of ammonia and Fenton was carried out at 75℃, which was more mild and safe.
Fig. 3 Effects of dilute alkali concentration on enzymatic hydrolysis of pretreated sawdust (C: cellulose; 
HC: hemicellulose)

Among the three types of chemical treatments, the cellulose conversion rate of 3% NaOH combined 
with Fenton pretreatment was the highest (63.73%), 68.25% and 18.11% higher than that of 4.5% H₂O₂ 
and 3% H₂SO₄, respectively. The hemicellulose conversion rate at 3% NaOH (29.29%) was comparable to 
that of 3% H₂SO₄ but 32.75% higher than of 4.5% H₂O₂. The effect of some bases on lignocellulosic 
biomass is the basis of alkali treatments, which may be the reason why diluted NaOH is more effective 
than ammonia (33, 34). Solvation and saponification are the first reactions to occur in NaOH pretreatment. 

Solvation reaction can dissolve part of lignin into liquid components. Meanwhile, saponification of 
intermolecular ester bonds reduces the strength of hydrogen bonds between hemicellulose and lignin, and 
increases porosity and surface area, causing the expansion of the lignocellulosic biomass and makes it 
easier for enzymes to access (31, 35). NaOH pretreatment improved cellulose digestibility and it was more 
effective in the solubilization of lignin, exhibiting less dissolution of cellulose and hemicellulose than acid 
and oxidant processes (36). Moreover, compared with acid method, alkali treatment causes less sugar 
degradation, and caustic salts such as NaOH can be recovered and/or regenerated (37). NaOH extraction 
can also change the crystalline state of cellulose thus reducing the degree of polymerization and 
crystallinity (38, 39). Given the enzymatic hydrolysis efficiency after pretreatments, the combined
pretreatment of dilute NaOH and Fenton reaction was determined as the optimal pretreatment process in this study, and the conditions were optimized in the subsequent experiments.

**Improvement of Alkali Pretreatment Conditions of Lignocellulose under Fixed Fenton Reaction**

Figures 4a, 4b, and 4c show the enhancement of the enzymatic hydrolysis of poplar sawdust pretreated by dilute NaOH (0.5% to 4.0%) at 25°C, 50°C, and 75°C for 2 h, respectively. When the NaOH concentration was increased, the enzymatic hydrolysis efficiency of cellulose gradually increased at 25°C, 50°C, and 75°C, and reached the maximum of 36.7%, 41.5%, and 61.93% at 3% NaOH, respectively, increasing by 31%, 19.6%, and 41.06% compared with 0.5% NaOH. The enzymatic conversion rate of cellulose decreased when the NaOH concentration was further increased to 4%. Similar to the cellulase hydrolysis rate, the enzymatic conversion rate of hemicellulase also increased with the increasing NaOH in the low concentration range, reaching the highest value of 27.9%, 28.05%, and 37.4% respectively at 1% to 2% NaOH, and then gradually decreased. When the alkali treatment temperature continued to rise to 100°C, cellulose conversion rate was higher than at 25°C, 50°C and 75°C (Fig. 4d). The enzymatic hydrolysis rate of cellulose reached a maximum of 66.45% at 1.0% NaOH under 100°C for 1h and the further increase of NaOH did not significantly improve the cellulase hydrolysis rate. Hemicellulase hydrolysis rate also rose to the highest value of 33.7% at 1.0% NaOH. Therefore, the optimal concentration of NaOH at 100°C was 1.0%.
Fig. 4 Effects of dilute alkali concentration on pretreated sawdust hydrolysis at different temperatures. (a) 25°C; (b) 50°C; (c) 75°C; (d) 100°C.
Alkali pretreatment removes lignin, reduces lignin molecular weight, and weakens the inhibition of lignin on cellulose hydrolysis to improve hydrolysis efficiency (40). The cellulose enzymatic conversion rate at 100°C was much higher than other temperatures. Moreover, the cellulose conversion rate at 1.0% NaOH was 66.4%, which was 85.17%, 60.19%, and 9.05% higher than that at 25°C, 50°C, and 75°C, respectively. This result can be explained by the fact that high temperature alkali pretreatment breaks the fiber, leading to a loose arrangement, thus causing more serious physical structure damage to the substrate (41). The 100°C NaOH pretreatment provides an efficient removal of lignin (42). In the pretreatment of NaOH combined with Fenton, the optimal condition was 1% NaOH at 100°C.

Figure 5 shows the results of the enzymatic hydrolysis rates of different reaction time with 1.0% NaOH at 100°C. The enzymatic hydrolysis rates of cellulose and hemicellulose reached a maximum of 68.64% and 32.6% at 1 h and 2 h, respectively. The enzymatic hydrolysis rates decreased gradually with the extension of reaction time. In dilute alkali combined with Fenton treatment, the optimum condition was 1.0% NaOH extraction at 100°C for 1 h.
Fig. 5 Effects of reaction time on pretreated sawdust hydrolysis

Alkali pretreatment solution contains phenols produced after lignin degradation and residual NaOH, which is harmful to the environment and could cause the waste of NaOH. The results of the experiment on circulation of waste liquid are shown in Fig. 6. With the increase of cycle times, the enzymatic hydrolysis conversion rates decreased gradually. In the fourth cycle, the enzymatic hydrolysis rates of cellulose and hemicelluloses were 60.59% and 27.61%, respectively, which were 11.73% and 16.6% lower than the first cycle. As the cycle continued, the enzymatic hydrolysis efficiency dropped. Taking production efficiency and cost into consideration, the waste liquid can be recycled for four times to reduce about 31% NaOH consumption.

Fig. 6 Effects of circulation of cycling NaOH on pretreated sawdust hydrolysis
Improvement of Fenton Reaction Conditions of Lignocellulose under Fixed Alkali Pretreatment

H₂O₂ is essential in the Fenton reaction because it is converted by Fe²⁺ to generate hydroxyl radicals (43). As shown in Fig. 7, at the fixed concentration of Fe²⁺, the enzymolysis efficiency of cellulose improved with the increase of H₂O₂ in the range of 5 mmol to 25 mmol, which was in accordance with the previous report (44). When the H₂O₂ addition was 25 mmol, the enzymatic conversion rate of cellulose reached the highest value of 72%, and as the addition amount of H₂O₂ continued to increase, it decreased instead. This result was attributed to the fact that excessive H₂O₂ can oxidize Fe²⁺ to Fe³⁺, thus inhibiting the production of hydroxyl radicals and affecting the oxidation capacity of Fenton. The enzymatic hydrolysis rate of hemicellulose increased slightly with the addition of H₂O₂ and reached the maximum at 15 mmol. The effect of H₂O₂ addition on the enzymolysis efficiency of hemicellulose was less significant than that of cellulose.

Fig. 7 Effects of different H₂O₂ addition on pretreated sawdust hydrolysis
Fig. 8 Effects of different Fe$^{2+}$ addition on pretreated sawdust hydrolysis

Figure 8 shows the effect of Fe$^{2+}$ addition on enzymatic hydrolysis of poplar sawdust. Specifically, with the increase of Fe$^{2+}$, the cellulose conversion rate first rose and then decreased. When the amount of Fe$^{2+}$ was 0.2 mmol, the enzymatic hydrolysis conversion rate of cellulose reached a maximum of 75.6%, which was 53.9% higher than that at 0.05 mmol. The hemicellulase hydrolysis conversion rate also reached a peak value of 33.7% at 0.2 mmol Fe$^{2+}$, which was 13.4% higher than that at 0.4 mmol. Therefore, the optimized addition amount of H$_2$O$_2$ and Fe$^{2+}$ was 25 mmol and 20 mmol, respectively.

As shown in Fig. 9, the enzymatic hydrolysis conversion rates of cellulose and hemicellulose rose with the increase of reaction time in the first 7 h of Fenton, and the hydrolysis rates of cellulose and hemicellulose increased by 76.21% and 79.58% at 7 h, respectively, compared with that at 1 h. With a further extension of the reaction time after 7 h, the enzymatic hydrolysis rates did not ascend anymore and reached a plateau. This trend was consistent with a previous study (45). Therefore, the appropriate time for Fenton reaction is 7 h.
Fig. 9 Effects of reaction time on pretreated sawdust hydrolysis

Optimization of Sawdust Enzymatic Hydrolysis Conditions after Pretreatment of NaOH Combined with Fenton

On the basis of previous experiments, the system consisted of 29 experiments according to the Box-Behnken center combination design principle. For the investigated variables, it was assumed that the boundary conditions are enzymatic hydrolysis temperature of 45°C, 50°C, and 55°C, liquid-solid ratio of 20, 30, and 40, β-glycosidase loading of 5 μL, 10 μL, and 15 μL, and the enzymatic hydrolysis time of 48 h, 60 h, and 72 h. The mass of the obtained reducing sugars was selected as a parameter representing the enzymatic hydrolysis efficiency. Table 1 shows the experimental design and the content of total reducing sugar obtained.

The effects of process parameters (temperature, liquid-solid ratio, the β-glycosidase content, and the time of the enzymatic hydrolysis process) on the reducing sugars yield were examined. The relationships among four factors during the enzymatic hydrolysis process were determined by quadratic multiple regression method. After performing analysis of variance (ANOVA) on the data in Table 1, the quadratic regression equation takes the following form,

\[
Y = -3589.91785 + 143.82100 A - 0.62578 B - 6.74827 C + 2.39646 D + 0.16200 AB - 0.07380 AC + 0.19150 AD + 0.03525 BC - 0.06118 BD + 0.19367 CD - 1.54815 A^2 - 0.062913 B^2 - 0.074289 C^2 - 0.081825 D^2
\] (5)
where A, B, C, and D represent enzymatic hydrolysis temperature, liquid-solid ratio, β-glycosidase content, and the enzymatic hydrolysis time, respectively. Y represents reducing sugar yield.

Table 1. Box-Behnken Design for Enzymatic Hydrolysis of Pretreated Poplar Sawdust and the Response Values

| No. | A-Temperature (°C) | B-Liquid-solid Ratio | C-β-glycosidase (μL) | D-Time (h) | Content of Total Reducing Sugars (mg) |
|-----|--------------------|----------------------|----------------------|------------|--------------------------------------|
| 1   | 45                 | 20                   | 10                   | 60         | 187.77                               |
| 2   | 55                 | 20                   | 10                   | 60         | 225.99                               |
| 3   | 45                 | 40                   | 10                   | 60         | 185.40                               |
| 4   | 55                 | 40                   | 10                   | 60         | 256.02                               |
| 5   | 50                 | 30                   | 5                    | 48         | 215.07                               |
| 6   | 50                 | 30                   | 15                   | 48         | 197.61                               |
| 7   | 50                 | 30                   | 5                    | 72         | 272.23                               |
| 8   | 50                 | 30                   | 15                   | 72         | 301.25                               |
| 9   | 45                 | 30                   | 10                   | 48         | 162.23                               |
| 10  | 55                 | 30                   | 10                   | 48         | 170.35                               |
| 11  | 45                 | 30                   | 10                   | 72         | 217.12                               |
| 12  | 55                 | 30                   | 10                   | 72         | 271.20                               |
| 13  | 50                 | 20                   | 5                    | 60         | 243.37                               |
| 14  | 50                 | 40                   | 5                    | 60         | 240.37                               |
| 15  | 50                 | 20                   | 15                   | 60         | 249.67                               |
| 16  | 50                 | 40                   | 15                   | 60         | 253.72                               |
| 17  | 45                 | 30                   | 5                    | 60         | 176.56                               |
| 18  | 55                 | 30                   | 5                    | 60         | 233.26                               |
| 19  | 45                 | 30                   | 15                   | 60         | 194.56                               |
| 20  | 55                 | 30                   | 15                   | 60         | 243.88                               |
| 21  | 50                 | 20                   | 10                   | 48         | 189.20                               |
| 22  | 50                 | 40                   | 10                   | 48         | 212.37                               |
| 23  | 50                 | 20                   | 10                   | 72         | 272.98                               |
| 24  | 50                 | 40                   | 10                   | 72         | 266.78                               |
| 25  | 50                 | 30                   | 10                   | 60         | 251.44                               |
| 26  | 50                 | 30                   | 10                   | 60         | 258.39                               |
| 27  | 50                 | 30                   | 10                   | 60         | 250.76                               |
| 28  | 50                 | 30                   | 10                   | 60         | 253.41                               |
| 29  | 50                 | 30                   | 10                   | 60         | 262.23                               |

The analysis of variance for the above model is shown in Table 2. The lack of fit was not significant \((P=0.1551 > 0.05)\), and the model \(P\) value < 0.0001, showing that the model selected in the experiment...
was highly significant. In addition, the correlation coefficient ($R^2$) was 0.9779, indicating that the correlation between measured and predicted value was very high. The adjusted $R^2$ was calculated for 0.9558, showing that 95.58% of the reducing sugar yield variability was explained by this statistical model, indicating that the model can predict the response. The relatively low coefficient of variation (CV) (3.28%) indicates a good repeatability of the experiments, so it can be used to predict the quality of total reducing sugar obtained by enzymatic hydrolysis.

Table 2. ANOVA for the Quadratic Model

| Source   | SS      | DF | MS      | F Value | P value | Statistical significance |
|----------|---------|----|---------|---------|---------|-------------------------|
| Model    | 35744.27| 14 | 2553.16 | 44.29   | < 0.0001** |                         |
| A        | 6396.85 | 1  | 6396.85 | 110.97  | < 0.0001** |                         |
| B        | 173.89  | 1  | 173.89  | 3.02    | 0.1044 NS  |                         |
| C        | 298.30  | 1  | 298.30  | 5.17    | 0.0392 *   |                         |
| D        | 17231.61| 1  | 17231.61| 298.92  | < 0.0001** |                         |
| AB       | 262.44  | 1  | 262.44  | 4.55    | 0.0510 NS  |                         |
| AC       | 13.62   | 1  | 13.62   | 0.24    | 0.6345 NS  |                         |
| AD       | 528.08  | 1  | 528.08  | 9.16    | 0.0091**   |                         |
| BC       | 12.43   | 1  | 12.43   | 0.22    | 0.6496 NS  |                         |
| BD       | 215.65  | 1  | 215.65  | 3.74    | 0.0736 NS  |                         |
| CD       | 540.10  | 1  | 540.10  | 9.37    | 0.0085**   |                         |
| A²       | 9716.67 | 1  | 9716.67 | 168.56  | < 0.0001** |                         |
| B²       | 256.74  | 1  | 256.74  | 4.45    | 0.0533 NS  |                         |
| C²       | 15.64   | 1  | 15.64   | 0.27    | 0.6106 NS  |                         |
| D²       | 742.30  | 1  | 742.30  | 12.88   | 0.0030**   |                         |
| Residual | 807.05  | 14 | 57.65   |         |           |                         |
| Lack of fit | 710.40 | 10 | 71.04   | 2.94    | 0.1551 NS  |                         |
| Pure error | 96.64  | 4  | 24.16   |         |           |                         |
| Total    | 36551.32| 28 |         |         |           |                         |

$P \leq 0.01$ is highly significant, denoted by **; $P \leq 0.05$ is significant, denoted by *; $P > 0.05$ is not significant, denoted by NS; Correlation coefficient ($R^2$) = 0.9779; adjusted $R^2$ = 0.9558; coefficient of variation (CV) = 3.28%; SS, sum of squares; DF, degree of freedom; MS, mean square.

In Table 2, the F test and its corresponding $P$ values indicate the significance of the regression equation terms on reducing sugar yield. It was concluded from Table 2 that the effects of the primary term
A, the interaction term AD, and the quadratic term of A on reducing sugar yield are highly significant ($P < 0.01$). It is because the essence of cellulase is protein, which needs proper hydrolysis temperature. The effects of enzymatic hydrolysis temperature (A) and time (D) on the reducing sugar yield of poplar sawdust are shown in Fig. 10a. According to the shape of the response surface, the optimum temperature of enzymatic hydrolysis was from 48 to 52°C, and the yield of reducing sugar obtained was lower above or below this range. When the temperature was lower than 47°C, the surface dropped steeply, indicating that the lower temperature had a greater effect on the recovery of reducing sugar. Figures 10a, b, and c show that compared with the duration temperature of the enzymatic hydrolysis process, the trend of reducing sugar yield with liquid-solid ratio, β-glycosidase content, or enzymatic hydrolysis time changed slowly.
Fig. 10 Surface plot of the combined effects of temperature and reaction time on the reducing sugar yield. (a) AB; (b) AC; (c) AD; (d) BC; (e) BD; (f) CD
The optimum conditions of cellulase hydrolysis were further predicted within the test range. A reaction temperature of 51.63 °C, liquid-solid ratio of 33.00, \(\beta\)-glucosidase content of 14.56 \(\mu\)L/g substrates, and a reaction time of 71.97 h were the optimal conditions. Considering the experimental operation, the conditions were set to a reaction temperature of 52°C, a liquid-solid ratio of 33, a \(\beta\)-glucosidase content of 15 \(\mu\)L/g substrate, and a reaction time of 72 h. Under this condition, the predicted reducing sugar yield is 302.49 mg/g substrates after enzymatic hydrolysis. After three confirmatory tests, the total reducing sugar measured was 301.47 mg/g substrates, which was very close to the predicted value. The conversion rates of cellulose and hemicellulose were 86.65% and 43.9% respectively, which were higher than those before optimization. Therefore, this model is effective and practical for the enzymatic hydrolysis of lignocellulose after the pretreatment of NaOH combined with Fenton.

**Conclusion**

In order to realize the effective conversion of poplar sawdust to fuel ethanol, the effect an mechanism of different chemical pretreatments of poplar sawdust were studied, and the pretreatment and enzymatic hydrolysis conditions were optimized. The enzymatic hydrolysis effects after various pretreatments are that NaOH-Fenton > H₂SO₄-Fenton > HCl-Fenton > ammonia-Fenton > H₂O₂-Fenton > NaClO-Fenton, and the cellulase and hemicellulase hydrolysis rates of poplar sawdust treated with dilute NaOH-Fenton were 63.73% and 29.29% before optimization, respectively. The results showed that dilute alkali treatment had less cellulose loss and lower degradation degree of reducing sugar compared with diluted acid treatment and oxidant treatment. Moreover, the lye can be recycled, which has high environmental benefit. The optimum pretreatment conditions of NaOH combined with Fenton were as follows: 1% NaOH extraction at 100°C for 1 h and subsequent 25 mmol H₂O₂ and 0.2 mmol Fe²⁺ for a Fenton reaction period of 7 h. The optimized enzymatic hydrolysis was performed at a reaction temperature of 52°C, a \(\beta\)-glucosidase content of 15 \(\mu\)L/g substrates, a liquid-solid ratio of 33 and a reaction time of 72 h after the NaOH-Fenton
pretreatment. The cellulose and hemicellulose conversion rates were 86.65% and 43.9%, respectively, significantly higher than those before optimization.

This study explored a suitable pretreatment method for poplar sawdust to promote the degradation of lignocellulose in biomass feedstock. Optimization of the conditions of pretreatment and enzymatic hydrolysis effectively improves the utilization of lignocellulose, which lays the foundation for subsequent research on its conversion to biofuels. The structural characterization of pretreated poplar sawdust need to study to further clarify its mechanism, and the recycling of alkali black liquor will also be considered in the future.

Declarations

Funding

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethics approval (Not applicable)

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication (Not applicable)

Availability of data and material

All authors declare that all data and materials support their published claims and comply with field standards.

Code availability (Not applicable)

Authors' Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were completed by Fei Li, Xiaohong Lu and Yiming Li. The first draft was written by Li Fei. Ping
Liu contributed to the experimental design. Xiaohong Lu, Yiming Li, Xia Zhou, and Zhezhen Zhao reviewed and revised the manuscript. All authors read and approved the final manuscript.

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