

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

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

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24 **Abstract**

25 In this study, the effects and mechanism of pretreatments of three types of chemical reagents
26 combined with Fenton on poplar sawdust were studied and the optimization of enzymatic hydrolysis
27 conditions was conducted using response surface methodology. The results showed that cellulase and
28 hemicellulase had the best hydrolysis effect after NaOH-Fenton pretreatment, which were 63.73% and
29 29.29%, respectively. The optimal process of poplar substrate was to react in 1% NaOH at 100 °C for 1 h,
30 then placed in the Fenton reaction system of 0.2 mmol Fe²⁺ and 25 mmol H₂O₂ for 7 h, and finally
31 subjected to enzymatic hydrolysis for 72 h at 52 °C, with a liquid-solid ratio of 33 and 15 μL/g of
32 β-glycosidase. Under this condition, the enzymatic hydrolysis rates of cellulase and hemicellulase reached
33 86.65% and 43.9%, respectively. In conclusion, the combination of NaOH and Fenton pretreatment can
34 effectively promote the enzymatic hydrolysis of poplar sawdust, which has great potential in the
35 production of cellulosic ethanol.

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

38 **Introduction**

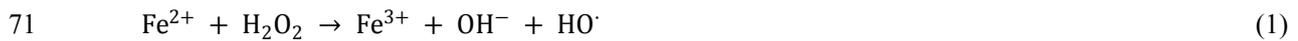
39 Growing energy demands and depletion of fossil fuels stimulated the search for biomass, thus
40 promoting the production of fuels and chemicals from lignocellulose (1, 2). Common lignocellulosic
41 biomass materials, like corn stover (3), bamboo (4), sugarcane bagasse (5), and wheat straw (6), have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

47 necessary to break the compact structure and assist in the removal of lignin, thus promoting the contact
48 between the cellulose and enzymes or microbes (10).

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

57 Fenton is an advanced oxidation reagent which used to serve for treating organic wastewater (16), and
58 the Fenton reaction could be used in lignocellulose pretreatment. After the Fenton pretreatment, cotton
59 was degraded effectively and its susceptibility to cellulase was improved (17). The enzymatic
60 saccharification effect of four different biomass feedstocks (miscanthus, switchgrass, corn stover, and
61 wheat straw) after Fenton pretreatment showed an average increase of 212% in comparison to untreated
62 control groups, which demonstrated the capacity of Fenton to improve the accessibility of cellulose to
63 enzymes (18). In the reaction mechanism, Fenton reagent generates hydroxyl radical through the reaction
64 of Fe^{2+} and H_2O_2 , as given in Eq. 1, where Fe^{2+} act as the catalyst. The consumed Fe^{2+} is regenerated by
65 the reaction of Fe^{3+} with H_2O_2 as shown in Eq. 2. These free radicals could oxidize lignocellulose
66 components (19, 20). Fenton oxidation conditions are mild, and the treatment of alkali combined with
67 Fenton has high potential in lignocellulose pretreatment. The sequential Fenton and dilute NaOH
68 extraction has been applied to pretreat corn stover which changes the cellulose structure characteristics
69 (porosity, morphology, and crystallinity) of the solid residues and enhances the enzymatic saccharification

70 (20).



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

85 In this study, poplar sawdust was treated with the combination of Fenton and dilute acid (HCl and
86 H₂SO₄), dilute alkali (ammonia and NaOH) and oxidants (H₂O₂ and NaClO), respectively, to explore the
87 differences of three chemical pretreatment methods on the removal ability of cellulose and hemicellulose
88 in poplar sawdust and subsequent enzymatic hydrolysis of cellulose. Considering the enzymatic hydrolysis
89 rates of cellulase and hemicellulase as indicators, the effects of the concentration, temperature, and time of
90 NaOH treatment as well as the H₂O₂ and Fe²⁺ additions on the enzymatic hydrolysis of sawdust were
91 investigated to obtain the optimal NaOH-Fenton pretreatment conditions. Finally, the optimal enzymatic
92 hydrolysis conditions under the optimal NaOH-Fenton pretreatment was identified using RSM. This study

93 provides a reference for maximizing the hemicellulose and cellulose in poplar sawdust into fermentable
94 sugars, reducing the cost of ethanol production, and fully improve the comprehensive utilization value of
95 poplar sawdust.

96 **Materials and Methods**

97 **Materials and Chemicals**

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

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

107 **Different Pretreatments of Poplar Sawdust**

108 **Fenton Treatment:** The tube was first loaded with 1 g of sawdust, 0.1 mmol $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ and 15
109 mmol 30% H_2O_2 , and deionized water was added to 15 mL. Sulfuric acid was used to adjust the pH to 4.
110 The tube was kept away from light for 10 h to complete the Fenton reaction. After the reaction, the mixture
111 was filtered through a 200-mesh cell sieve, and the remaining sawdust was washed with 2% oxalic acid
112 and deionized water.

113 **Acid Combined with Fenton Treatment:** Acid treatment was conducted with different concentration
114 (1%, 2%, 3%, 4%, 5%) of H_2SO_4 or HCl at 1:15 solid to liquid ratio at 80°C water for 2 h. After filtration,
115 the remaining sawdust were subjected to Fenton reaction according to the above method.

116 **Alkali Combined with Fenton Treatment:** Poplar sawdust and dilute alkali solution of different
117 concentration (NaOH was 1%, 2%, 3%, 4%, 5%, ammonia water was 5%, 10%, 15%, 20%) were reacted
118 in a 75 °C water bath for 2 h at a material-to-liquid ratio of 1:15. After solid-liquid separation, performing
119 the Fenton reaction according to the above conditions.

120 **Oxidants Combined with Fenton Treatment:** H₂O₂ treatment was to mix poplar sawdust and H₂O₂
121 solution (1.5%、2.5%、3.5%、4.5%、5.5%) at a ratio of 1:15 and incubated at 30 °C for 2h. Fenton treatment
122 is performed after filtration. NaClO treatment was conducted with NaClO solution (1.5%、2.5%、3.5%、
123 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
124 Fenton treatment.

125 **Determination of Content of Glucose and Xylose**

126 Refer to two-step acid hydrolysis, untreated poplar sawdust was used as samples. Step 1: 0.300 g
127 sample was accurately weighed and mixed with 3.0ml of 72% sulfuric acid at 30 °C for 1 h and swirled
128 every 5~10 min. Step 2: 84 mL deionized water was added to sulfuric acid to dilute the concentration to
129 4%. After reacting at 121 °C for 1 h, the filtrate obtained by suction filtration was passed through a 0.22
130 μm filter membrane. The contents of glucose and xylose in filtrate were determined by high-performance
131 liquid chromatography (HPLC, Agilent 1200, Palo Alto, CA, USA) system equipped with a refractive
132 index detector, the corresponding protection column, and a Rezex ROA. The samples were eluted with
133 0.005 M H₂SO₄ at a flow rate of 0.6 mL/min and a column temperature of 65 °C. The retention time of
134 glucose and xylose were 10.550 min and 11.000 min, respectively. Each sample was repeated three times
135 and the average was used as the calculation result.

136 **Calculation of Enzymatic Hydrolysis Conversion Rate**

137 After the pretreatment, the poplar sawdust and 25 μL of hemicellulase were mixed with 30 mL acetate
138 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

139 cycloheximide and 80 μL of tetracycline hydrochloride were added to avoid pH changes caused by
140 microbial growth. When the reaction was complete, the tube reactor was cooled, and 60 μL cellulase and
141 10 μL β -glucosidase were added to perform the hydrolysis reaction at 120 rpm and 50°C for 3 d. Finally,
142 the mixed contents were filtered through a 200-mesh cell sieve, and the enzymatic hydrolysate was
143 collected to determine the contents of glucose and xylose for the calculation of enzymatic conversion rate
144 of cellulose and hemicellulose. The calculation equations were as follows.

145 Enzymatic hydrolysis conversion rate of cellulose (%) = $\frac{C_1 \times V \times 0.90}{m \times W_1} \times 100$ (3)

146 Enzymatic hydrolysis conversion rate of hemicellulose (%) = $\frac{C_2 \times V \times 0.88}{m \times W_2} \times 100$ (4)

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

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

152 For the optimization of NaOH treatment temperature-concentration, poplar sawdust was mixed with
153 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.
154 Then, the Fenton reaction was performed under the conditions of 0.1 mmol $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ and 15 mmol
155 30% H_2O_2 for 10 h.

156 For the optimization of NaOH treatment time, poplar sawdust and NaOH solution were incubated for
157 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
158 previous experiment. Then, Fenton treatment was carried out according to the above conditions.

159 For the circulation of alkali pretreatment solution, the liquid waste was collected after NaOH
160 pretreatment, and the residual NaOH was determined by titration. According to the measured results,

161 NaOH was added to the liquid waste to reach the initial concentration. The poplar sawdust was processed,
162 and the liquid waste was collected again. The process was repeated.

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

164 The optimization of the Fenton reaction conditions was carried out under the optimal conditions of
165 NaOH treatment obtained in the above experiment. For the optimization of H₂O₂ addition amount, 1 g of
166 alkali treated sawdust was mixed with 0.1 mmol FeSO₄ · 7 H₂O and different amounts of H₂O₂ (5mmol,
167 10mmol, 15mmol, 20mmol, 25mmol, 30mmol). Deionized water was added to adjust the material-to-liquid
168 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
169 perform the Fenton reaction. For the optimization of Fe²⁺ addition amount, the sawdust was mixed with 25
170 mmol H₂O₂ and different amounts of FeSO₄ · 7 H₂O (0.05mmol, 0.1mmol, 0.2mmol, 0.3mmol, 0.4mmol),
171 and the other operations were the same as above.

172 For the optimization of Fenton reaction time, 1 g of sawdust were mixed with the optimal addition
173 amount of FeSO₄·7 H₂O and H₂O₂ obtained in the above experiment. Deionized water was added to adjust
174 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,
175 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h, respectively.

176 **Response Surface Optimization of Enzymatic Hydrolysis Conditions**

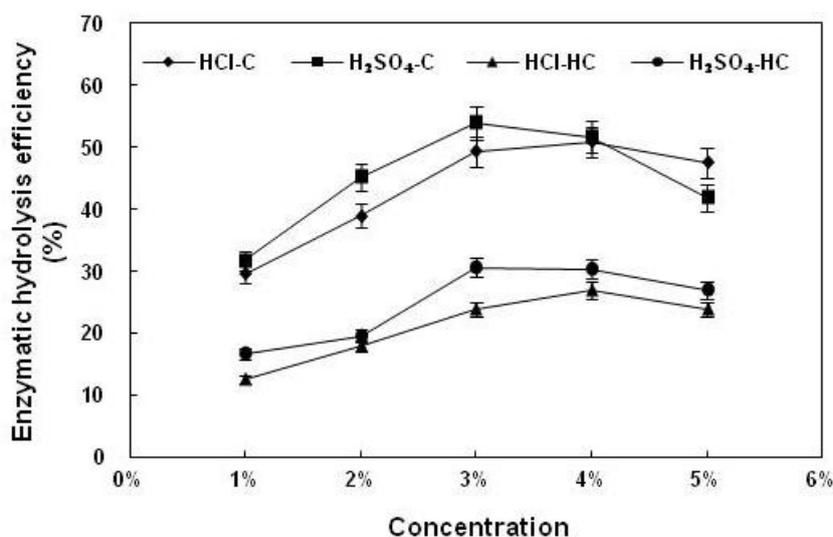
177 According to the Box-Behnken design and the preliminary test results, the enzymatic hydrolysis
178 temperature, liquid-solid ratio, enzyme concentration, and reaction time were selected, and each factor was
179 set at 3 levels to conduct response surface experiments at a total of 30 tests. The total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

182 **Result and Discussion**

183 **Comparison Analysis of Different Treatments Combined with Fenton on Lignocellulose Enzymatic**

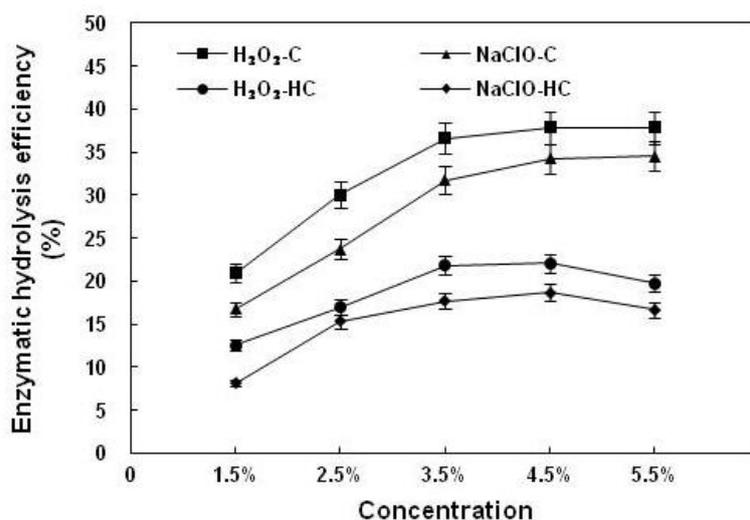
184 **Hydrolysis at Ordinary Pressure**

185 Dilute acid treatment is suitable for industrial application because of its weak corrosivity and easy
186 recovery of acid. Figure 1 depicts the effect of pretreatment with different concentrations (1% to 5%) of
187 H₂SO₄ or HCl combined with Fenton on the enzymatic hydrolysis of sawdust. The conversion rates of
188 cellulose and hemicellulose increased as the dilute acid concentration increased from 1% to 3%. When the
189 concentration exceeded 3% and 4%, the enzymatic conversion rate of cellulose and hemicellulose slightly
190 decreased. The study of Saha et al. showed that the wheat straw gave maximum sugar yield after 0.75%
191 H₂SO₄ pretreatment, and the fermentable sugar yield decreased with the increase of acid dose, which is
192 consistent with our observation. This might be because that as the concentration of acid increases, the sugars
193 gradually degraded into furfural and HMF which could influence enzymatic hydrolysis of the sawdust,
194 resulting in the decrease of the conversion of cellulose and hemicellulose (26). The enzymatic hydrolysis
195 efficiency of the dilute H₂SO₄ was higher than that of the dilute HCl. When the concentration of dilute
196 H₂SO₄ was 3%, the enzymatic hydrolysis rates of cellulose and hemicellulose were 9.5% and 28.6% higher,
197 respectively, than those of dilute HCl at the same concentration. This result shows that dilute HCl
198 pretreatment causes indistinctive delignification with subtle structural changes in the fibers (27). However,
199 dilute H₂SO₄ pretreatment can make hemicellulose more soluble, and the chemical structure in the solid
200 residue changes, making the sawdust more porous and improving the cellulose accessibility of enzymes
201 (28, 29). Therefore, the combination of 3% H₂SO₄ and Fenton is a better pretreatment condition for
202 lignocellulose, and the enzymatic hydrolysis rates of cellulose and hemicellulose reached 53.95% and
203 30.6%, respectively.



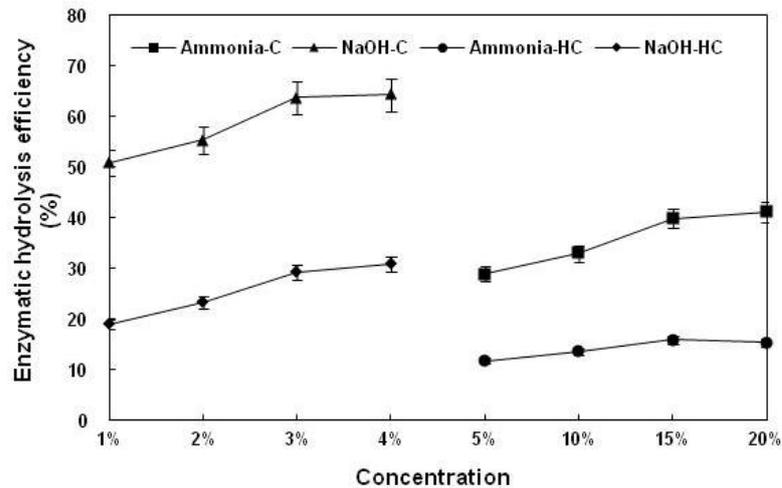
204 **Fig. 1** Effects of dilute acid concentration on enzymatic hydrolysis of pretreated sawdust (C: cellulose; HC:
 205 hemicellulose)

206 With the increase of oxidant concentration, the cellulose conversion rate gradually increased and
 207 tended to be stable at the concentration of 4.5%, while the enzymatic conversion rate of hemicellulose
 208 declined after the oxidant concentration exceeded 4.5% (Fig. 2). In addition, the enzymatic hydrolysis rates
 209 of cellulose and hemicellulose in the 4.5% H₂O₂ group were 37.88% and 22.38%, respectively, 3.86% and
 210 18.77% higher than those in the 4.5% NaClO group. Therefore, the pretreatment effect of H₂O₂ was better
 211 than NaClO, which is consistent with previous research (30). Oxidants break down the network structure
 212 of the materials through peroxidative degradation of lignin, thereby improving the enzymatic hydrolysis
 213 efficiency of cellulose and hemicellulose. As the oxidability of HClO produced by NaClO was weaker than
 214 H₂O₂, its ability to oxidize lignin was weak and it is not as effective as H₂O₂ in promoting enzymatic
 215 hydrolysis. In many cases the reaction of oxidant treatment is not selective, resulting in loss of
 216 hemicellulose and cellulose and therefore the pretreatment effect is inferior to dilute acid pretreatment (31).
 217 In conclusion, in above pretreatment experiment of poplar sawdust with oxidants combined with Fenton,
 218 the pretreatment effect of 4.5% H₂O₂-Fenton was the best.



219 **Fig. 2** Effects of oxidant concentration on enzymatic hydrolysis of pretreated sawdust (C: cellulose; HC:
 220 hemicellulose)

221 When the concentration of NaOH and ammonia was 3% to 4% and 15% to 20%, respectively, the
 222 hydrolysis rates of cellulose and hemicellulose reached the maximum (Fig. 3). The conversion rates of
 223 cellulose and hemicellulose under 15% to 20% ammonia pretreatment was much lower than that of 3% to
 224 4% NaOH. However, the enzymatic conversion rates of cellulose and hemicellulose at 4% NaOH did not
 225 increase significantly compared with those at 3% NaOH. In consequence, 3% NaOH combined with
 226 Fenton reaction was the suitable pretreatment in this experiment, and the enzymatic hydrolysis efficiencies
 227 of cellulose and hemicellulose were 63.73% and 29.29%, respectively. Although ammonia treatment
 228 promotes lignocellulose enzymatic hydrolysis less than NaOH treatment, the commonly used ammonia
 229 explosion pretreatment requires high temperature and high pressure (32). In this experiment, the combined
 230 pretreatment of ammonia and Fenton was carried out at 75°C, which was more mild and safe.



231 **Fig. 3** Effects of dilute alkali concentration on enzymatic hydrolysis of pretreated sawdust (C: cellulose;
 232 HC: hemicellulose)

233 Among the three types of chemical treatments, the cellulose conversion rate of 3% NaOH combined
 234 with Fenton pretreatment was the highest (63.73%), 68.25% and 18.11% higher than that of 4.5% H₂O₂
 235 and 3% H₂SO₄, respectively. The hemicellulose conversion rate at 3% NaOH (29.29%) was comparable to
 236 that of 3% H₂SO₄ but 32.75% higher than of 4.5% H₂O₂. The effect of some bases on lignocellulosic
 237 biomass is the basis of alkali treatments, which may be the reason why diluted NaOH is more effective
 238 than ammonia(33, 34). Solvation and saponification are the first reactions to occur in NaOH pretreatment.
 239 Solvation reaction can dissolve part of lignin into liquid components. Meanwhile, saponification of
 240 intermolecular ester bonds reduces the strength of hydrogen bonds between hemicellulose and lignin, and
 241 increases porosity and surface area, causing the expansion of the lignocellulosic biomass and makes it
 242 easier for enzymes to access (31, 35). NaOH pretreatment improved cellulose digestibility and it was more
 243 effective in the solubilization of lignin, exhibiting less dissolution of cellulose and hemicellulose than acid
 244 and oxidant processes (36). Moreover, compared with acid method, alkali treatment causes less sugar
 245 degradation, and caustic salts such as NaOH can be recovered and/or regenerated (37). NaOH extraction
 246 can also change the crystalline state of cellulose thus reducing the degree of polymerization and
 247 crystallinity (38, 39). Given the enzymatic hydrolysis efficiency after pretreatments, the combined

248 pretreatment of dilute NaOH and Fenton reaction was determined as the optimal pretreatment process in
249 this study, and the conditions were optimized in the subsequent experiments.

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

251 Figures 4a, 4b, and 4c show the enhancement of the enzymatic hydrolysis of poplar sawdust
252 pretreated by dilute NaOH (0.5% to 4.0%) at 25°C, 50°C, and 75°C for 2 h, respectively. When the NaOH
253 concentration was increased, the enzymatic hydrolysis efficiency of cellulose gradually increased at 25°C,
254 50°C, and 75°C, and reached the maximum of 36.7%, 41.5%, and 61.93% at 3% NaOH, respectively,
255 increasing by 31%, 19.6%, and 41.06% compared with 0.5% NaOH. The enzymatic conversion rate of
256 cellulose decreased when the NaOH concentration was further increased to 4%. Similar to the cellulase
257 hydrolysis rate, the enzymatic conversion rate of hemicellulase also increased with the increasing NaOH in
258 the low concentration range, reaching the highest value of 27.9%, 28.05%, and 37.4% respectively at 1%
259 to 2% NaOH, and then gradually decreased. When the alkali treatment temperature continued to rise to
260 100°C, cellulose conversion rate was higher than at 25°C, 50°C and 75°C (Fig. 4d). The enzymatic
261 hydrolysis rate of cellulose reached a maximum of 66.45% at 1.0% NaOH under 100°C for 1h and the
262 further increase of NaOH did not significantly improve the cellulase hydrolysis rate. Hemicellulase
263 hydrolysis rate also rose to the highest value of 33.7% at 1.0% NaOH. Therefore, the optimal concentration
264 of NaOH at 100°C was 1.0%.

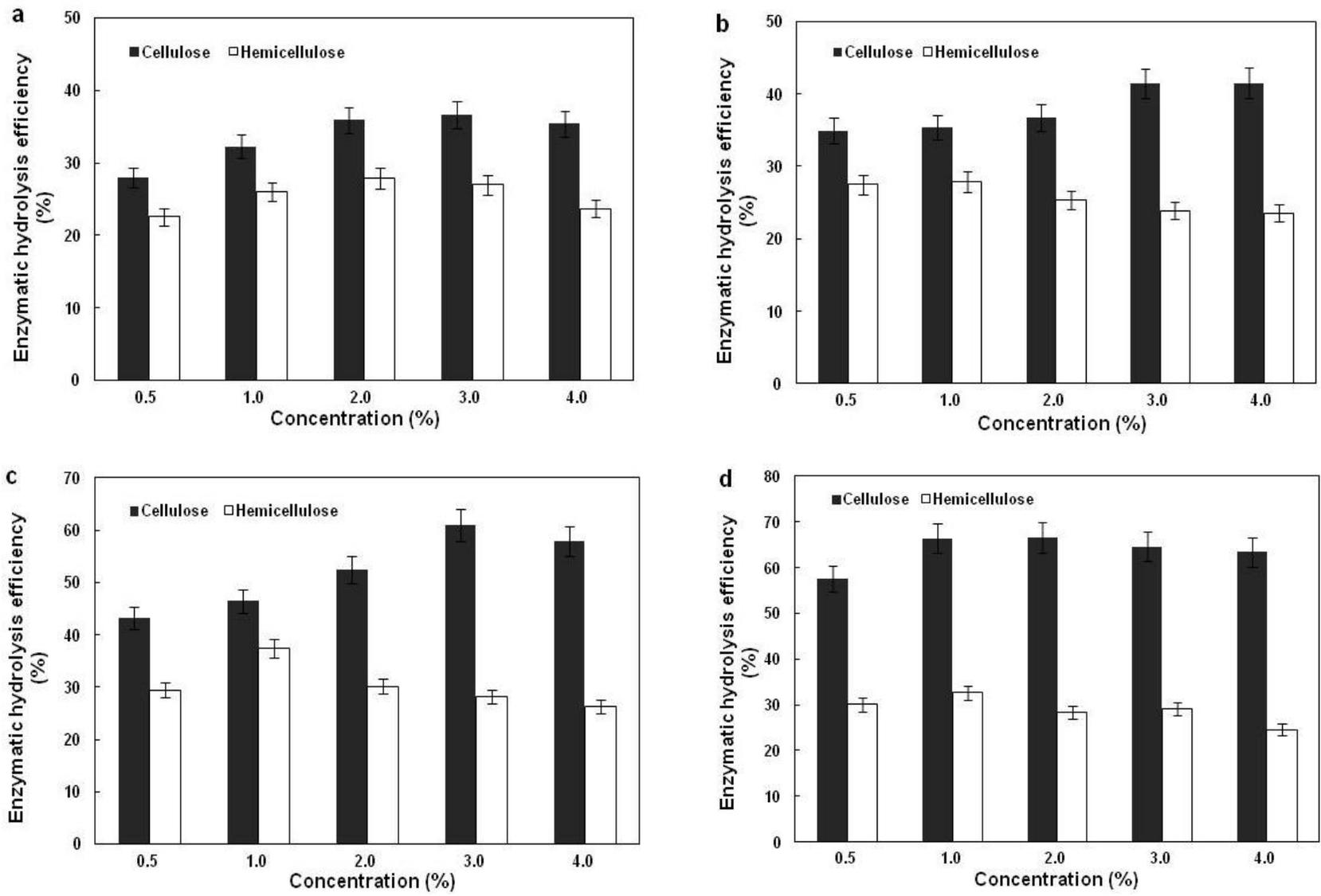
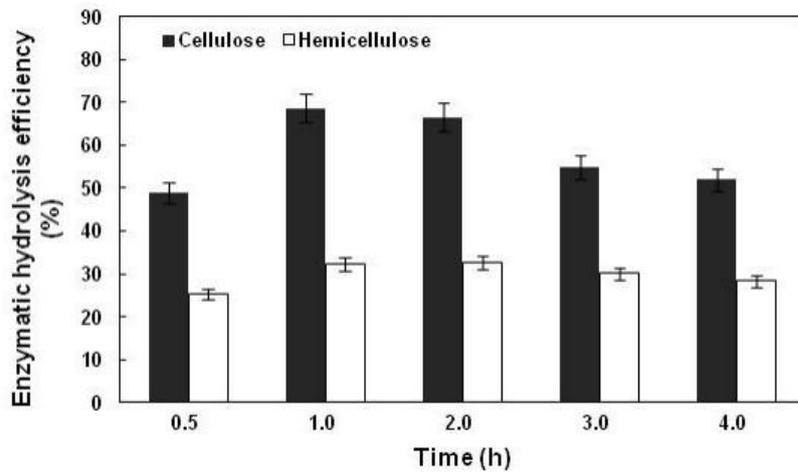


Fig. 4 Effects of dilute alkali concentration on pretreated sawdust hydrolysis at different temperatures. (a) 25°C; (b) 50°C; (c) 75°C; (d) 100°C

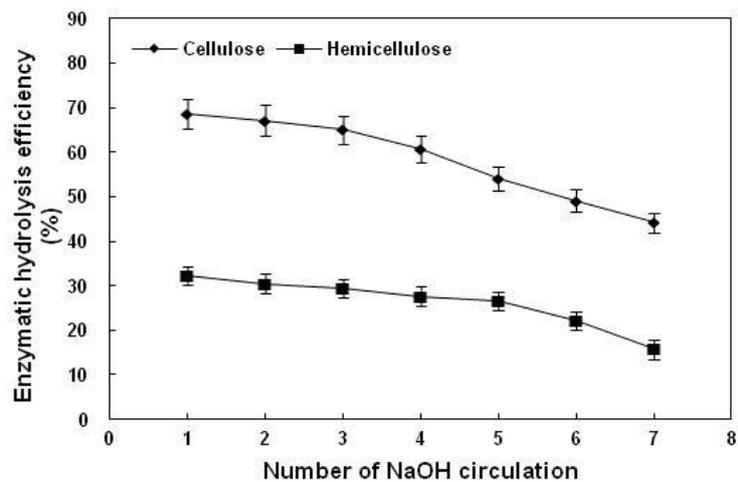
1 Alkali pretreatment removes lignin, reduces lignin molecular weight, and weakens the inhibition of
2 lignin on cellulose hydrolysis to improve hydrolysis efficiency (40). The cellulose enzymatic conversion
3 rate at 100°C was much higher than other temperatures. Moreover, the cellulose conversion rate at 1.0%
4 NaOH was 66.4%, which was 85.17%, 60.19%, and 9.05% higher than that at 25°C, 50°C, and 75°C,
5 respectively. This result can be explained by the fact that high temperature alkali pretreatment breaks the
6 fiber, leading to a loose arrangement, thus causing more serious physical structure damage to the substrate
7 (41). The 100°C NaOH pretreatment provides an efficient removal of lignin (42). In the pretreatment of
8 NaOH combined with Fenton, the optimal condition was 1% NaOH at 100°C.

9 Figure 5 shows the results of the enzymatic hydrolysis rates of different reaction time with 1.0%
10 NaOH at 100°C. The enzymatic hydrolysis rates of cellulose and hemicellulose reached a maximum of
11 68.64% and 32.6% at 1 h and 2 h, respectively. The enzymatic hydrolysis rates decreased gradually with
12 the extension of reaction time. In dilute alkali combined with Fenton treatment, the optimum condition was
13 1.0% NaOH extraction at 100°C for 1 h.



14 **Fig. 5** Effects of reaction time on pretreated sawdust hydrolysis

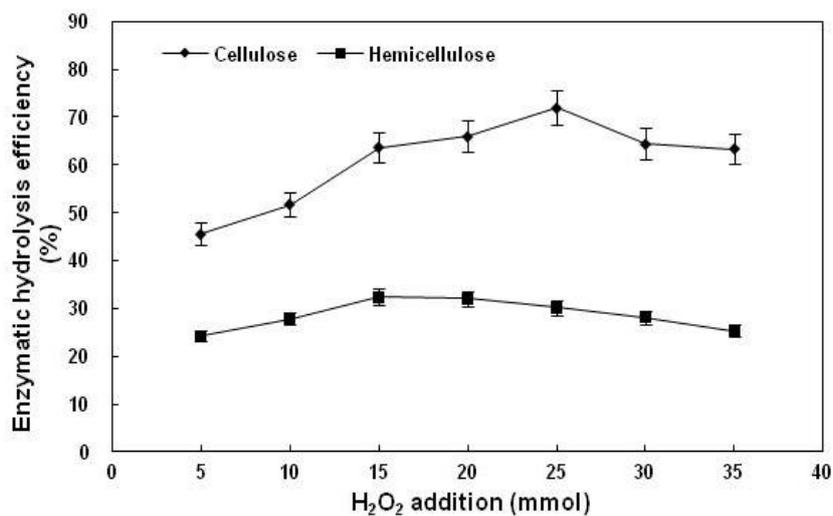
15 Alkali pretreatment solution contains phenols produced after lignin degradation and residual NaOH,
 16 which is harmful to the environment and could cause the waste of NaOH. The results of the experiment on
 17 circulation of waste liquid are shown in Fig. 6. With the increase of cycle times, the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



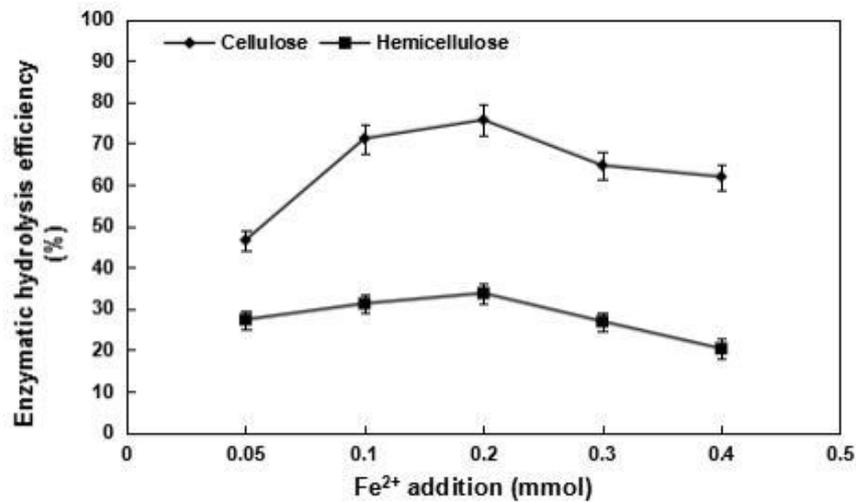
23 **Fig. 6** Effects of circulation of cycling NaOH on pretreated sawdust hydrolysis

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

25 H_2O_2 is essential in the Fenton reaction because it is converted by Fe^{2+} to generate hydroxyl radicals
26 (43). As shown in Fig. 7, at the fixed concentration of Fe^{2+} , the enzymolysis efficiency of cellulose
27 improved with the increase of H_2O_2 in the range of 5 mmol to 25 mmol, which was in accordance with the
28 previous report (44). When the H_2O_2 addition was 25 mmol, the enzymatic conversion rate of cellulose
29 reached the highest value of 72%, and as the addition amount of H_2O_2 continued to increase, it decreased
30 instead. This result was attributed to the fact that excessive H_2O_2 can oxidize Fe^{2+} to Fe^{3+} , thus inhibiting
31 the production of hydroxyl radicals and affecting the oxidation capacity of Fenton. The enzymatic
32 hydrolysis rate of hemicellulose increased slightly with the addition of H_2O_2 and reached the maximum at
33 15 mmol. The effect of H_2O_2 addition on the enzymolysis efficiency of hemicellulose was less significant
34 than that of cellulose.



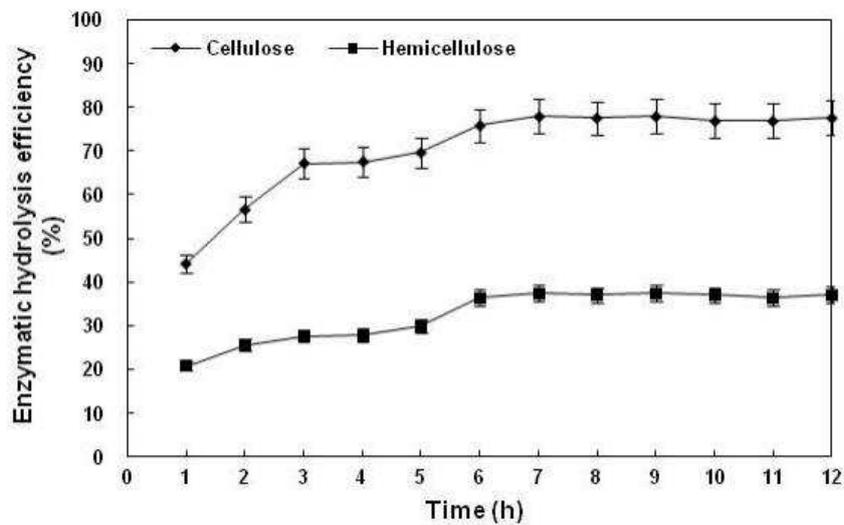
35 **Fig. 7** Effects of different H_2O_2 addition on pretreated sawdust hydrolysis



36 **Fig. 8** Effects of different Fe²⁺ addition on pretreated sawdust hydrolysis

37 Figure 8 shows the effect of Fe²⁺ addition on enzymatic hydrolysis of poplar sawdust. Specifically,
 38 with the increase of Fe²⁺, the cellulose conversion rate first rose and then decreased. When the amount of
 39 Fe²⁺ was 0.2 mmol, the enzymatic hydrolysis conversion rate of cellulose reached a maximum of 75.6%,
 40 which was 53.9% higher than that at 0.05 mmol. The hemicellulase hydrolysis conversion rate also reached
 41 a peak value of 33.7% at 0.2 mmol Fe²⁺, which was 13.4% higher than that at 0.4 mmol. Therefore, the
 42 optimized addition amount of H₂O₂ and Fe²⁺ was 25 mmol and 20 mmol, respectively.

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



49 **Fig. 9** Effects of reaction time on pretreated sawdust hydrolysis

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

52 On the basis of previous experiments, the system consisted of 29 experiments according to the
 53 Box-Behnken center combination design principle. For the investigated variables, it was assumed that the
 54 boundary conditions are enzymatic hydrolysis temperature of 45°C, 50°C, and 55°C, liquid-solid ratio of
 55 20, 30, and 40, β -glycosidase loading of 5 μ L, 10 μ L, and 15 μ L, and the enzymatic hydrolysis time of 48 h,
 56 60 h, and 72 h. The mass of the obtained reducing sugars was selected as a parameter representing the
 57 enzymatic hydrolysis efficiency. Table 1 shows the experimental design and the content of total reducing
 58 sugar obtained.

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

64 $Y = -3589.91785 + 143.82100 A - 0.62578 B - 6.74827 C + 2.39646 D + 0.16200 AB - 0.073800 AC + 0.19150$

65 $AD + 0.035250 BC - 0.061188 BD + 0.19367 CD - 1.54815 A^2 - 0.062913 B^2 - 0.062103 C^2 - 0.074289 D^2$ (5)

66 where A, B, C, and D represent enzymatic hydrolysis temperature, liquid-solid ratio, β -glycosidase content,
 67 and the enzymatic hydrolysis time, respectively. Y represents reducing sugar yield.

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

No.	A-Temperature (°C)	B-Liquid-solid Ratio	C- β -glycosidase (μ L)	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

70 The analysis of variance for the above model is shown in Table 2. The lack of fit was not significant
 71 ($P=0.1551 > 0.05$), and the model P value < 0.0001 , showing that the model selected in the experiment

72 was highly significant. In addition, the correlation coefficient (R^2) was 0.9779, indicating that the
 73 correlation between measured and predicted value was very high. The adjusted R^2 was calculated for
 74 0.9558, showing that 95.58% of the reducing sugar yield variability was explained by this statistical model,
 75 indicating that the model can predict the response. The relatively low coefficient of variation (CV) (3.28%)
 76 indicates a good repeatability of the experiments, so it can be used to predict the quality of total reducing
 77 sugar obtained by enzymatic hydrolysis.

78 **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				

79 $P \leq 0.01$ is highly significant, denoted by **; $P \leq 0.05$ is significant, denoted by *; $P > 0.05$ is not significant,
 80 denoted by NS; Correlation coefficient (R^2) = 0.9779; adjusted R^2 = 0.9558; coefficient of variation (CV) =
 81 3.28%; SS, sum of squares; DF, degree of freedom; MS, mean square.

82 In Table 2, the F test and its corresponding P values indicate the significance of the regression
 83 equation terms on reducing sugar yield. It was concluded from Table 2 that the effects of the primary term

84 A, the interaction term AD, and the quadratic term of A on reducing sugar yield are highly significant ($P <$
85 0.01). It is because the essence of cellulase is protein, which needs proper hydrolysis temperature. The
86 effects of enzymatic hydrolysis temperature (A) and time (D) on the reducing sugar yield of poplar
87 sawdust are shown in Fig. 10a. According to the shape of the response surface, the optimum temperature of
88 enzymatic hydrolysis was from 48 to 52°C, and the yield of reducing sugar obtained was lower above or
89 below this range. When the temperature was lower than 47°C, the surface dropped steeply, indicating that
90 the lower temperature had a greater effect on the recovery of reducing sugar. Figures 10a, b, and c show
91 that compared with the duration temperature of the enzymatic hydrolysis process, the trend of reducing
92 sugar yield with liquid-solid ratio, β -glycosidase content, or enzymatic hydrolysis time changed slowly.

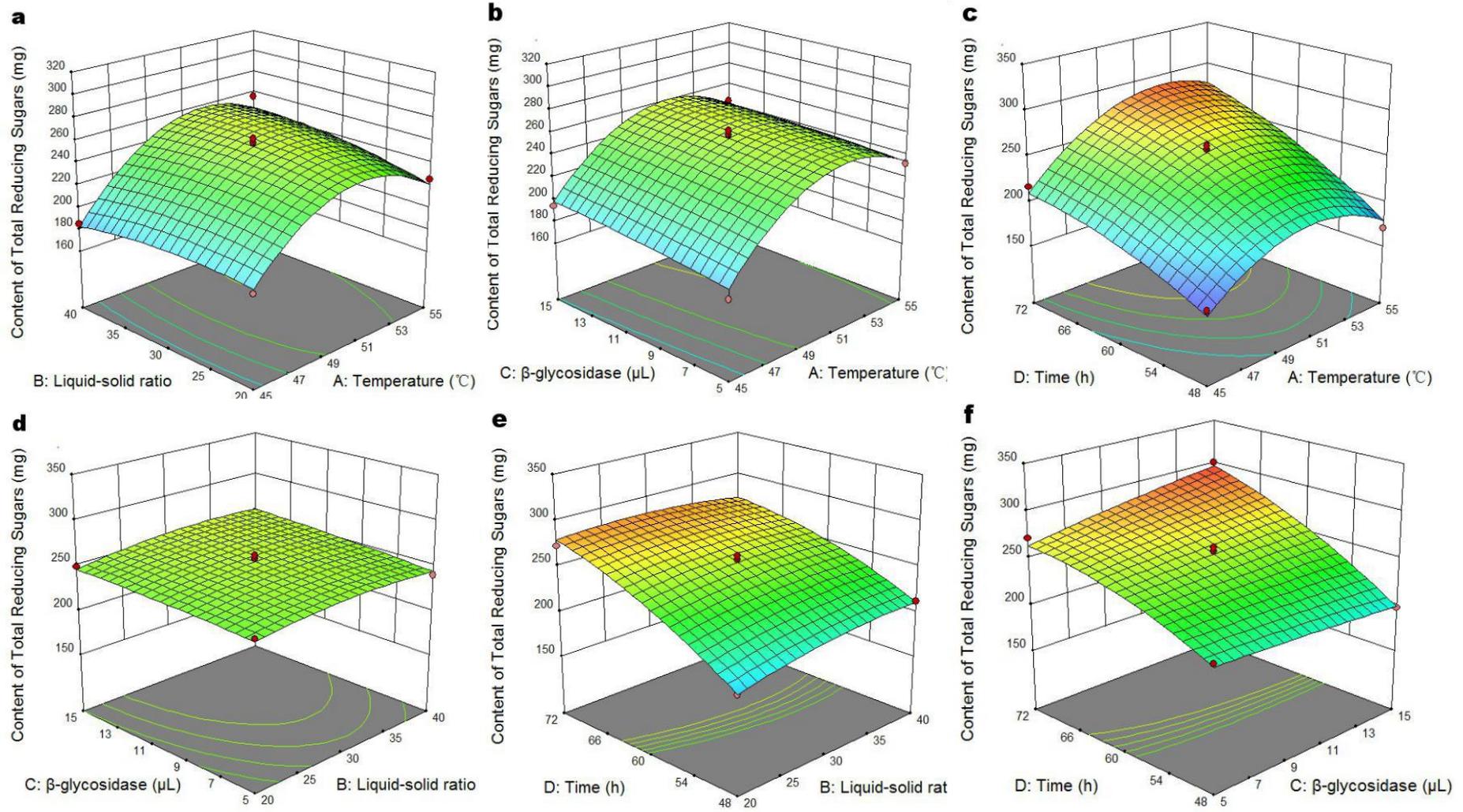


Fig. 10 Surface plot of the combined effects of temperature and reaction time on the reducing sugar yield. (a) AB; (b) AC; (c) AD; (d) BC; (e) BD; (f) CD

1 The optimum conditions of cellulase hydrolysis were further predicted within the test range. A
2 reaction temperature of 51.63 °C, liquid-solid ratio of 33.00, β -glycosidase content of 14.56 $\mu\text{L/g}$ substrates,
3 and a reaction time of 71.97 h were the optimal conditions. Considering the experimental operation, the
4 conditions were set to a reaction temperature of 52 °C, a liquid-solid ratio of 33, a β -glucosidase content of
5 15 $\mu\text{L/g}$ substrate, and a reaction time of 72 h. Under this condition, the predicted reducing sugar yield is
6 302.49 mg/g substrates after enzymatic hydrolysis. After three confirmatory tests, the total reducing sugar
7 measured was 301.47 mg/g substrates, which was very close to the predicted value. The conversion rates
8 of cellulose and hemicellulose were 86.65% and 43.9% respectively, which were higher than those before
9 optimization. Therefore, this model is effective and practical for the enzymatic hydrolysis of lignocellulose
10 after the pretreatment of NaOH combined with Fenton.

11 **Conclusion**

12 In order to realize the effective conversion of poplar sawdust to fuel ethanol, the effect and mechanism
13 of different chemical pretreatments of poplar sawdust were studied, and the pretreatment and enzymatic
14 hydrolysis conditions were optimized. The enzymatic hydrolysis effects after various pretreatments are that
15 NaOH-Fenton > H₂SO₄-Fenton > HCl-Fenton > ammonia-Fenton > H₂O₂-Fenton > NaClO-Fenton, and the
16 cellulase and hemicellulase hydrolysis rates of poplar sawdust treated with dilute NaOH-Fenton were
17 63.73% and 29.29% before optimization, respectively. The results showed that dilute alkali treatment had
18 less cellulose loss and lower degradation degree of reducing sugar compared with diluted acid treatment
19 and oxidant treatment. Moreover, the lye can be recycled, which has high environmental benefit. The
20 optimum pretreatment conditions of NaOH combined with Fenton were as follows: 1% NaOH extraction
21 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
22 optimized enzymatic hydrolysis was performed at a reaction temperature of 52 °C, a β -glycosidase content
23 of 15 $\mu\text{L/g}$ substrates, a liquid-solid ratio of 33 and a reaction time of 72 h after the NaOH-Fenton

24 pretreatment. The cellulose and hemicellulose conversion rates were 86.65% and 43.9%, respectively,
25 significantly higher than those before optimization.

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

32 **Declarations**

33 **Funding**

34 **Conflicts of interest**

35 The authors declare that they have no conflict of interest.

36 **Ethics approval** (Not applicable)

37 **Consent to participate**

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

39 **Consent for publication** (Not applicable)

40 **Availability of data and material**

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

43 **Code availability** (Not applicable)

44 **Authors' Contributions**

45 All authors contributed to the study conception and design. Material preparation, data collection and
46 analysis were completed by Fei Li, Xiaohong Lu and Yiming Li. The first draft was written by Li Fei. Ping

47 Liu contributed to the experimental design. Xiaohong Lu, Yiming Li, Xia Zhou, and Zhezhen Zhao
48 reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Figures

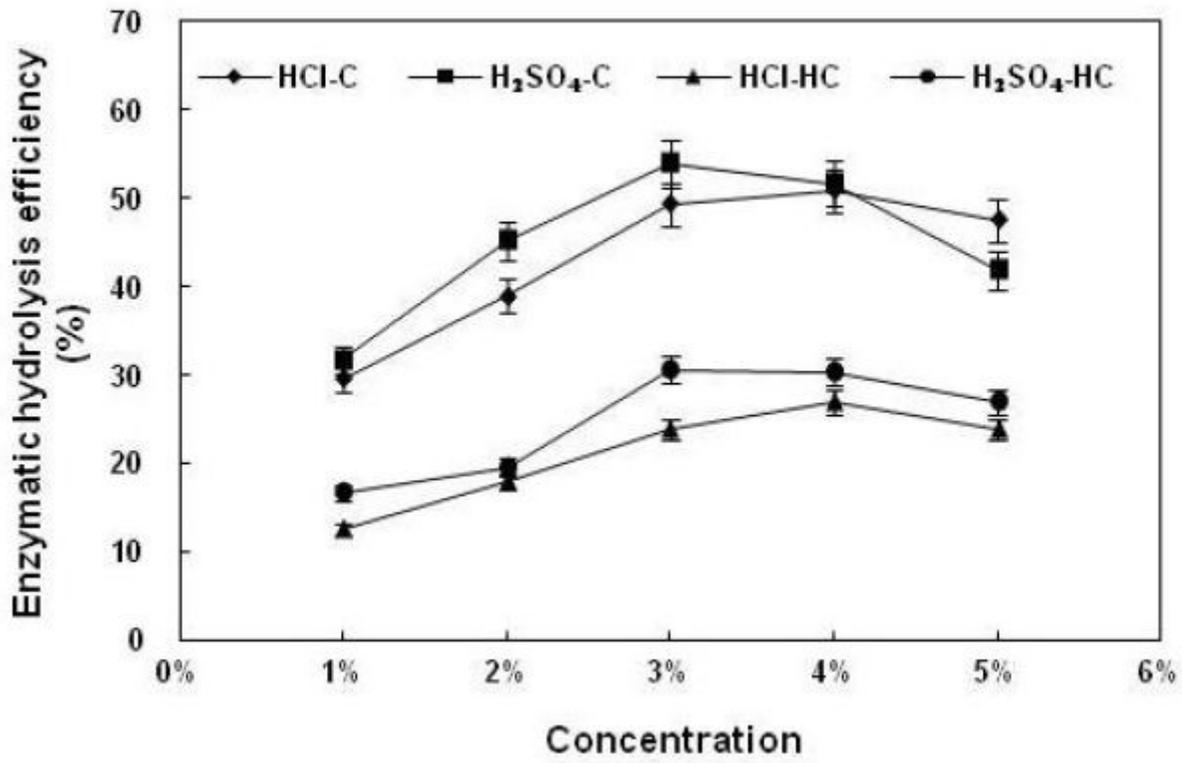


Figure 1

Effects of dilute acid concentration on enzymatic hydrolysis of pretreated sawdust (C: cellulose; HC: hemicellulose)

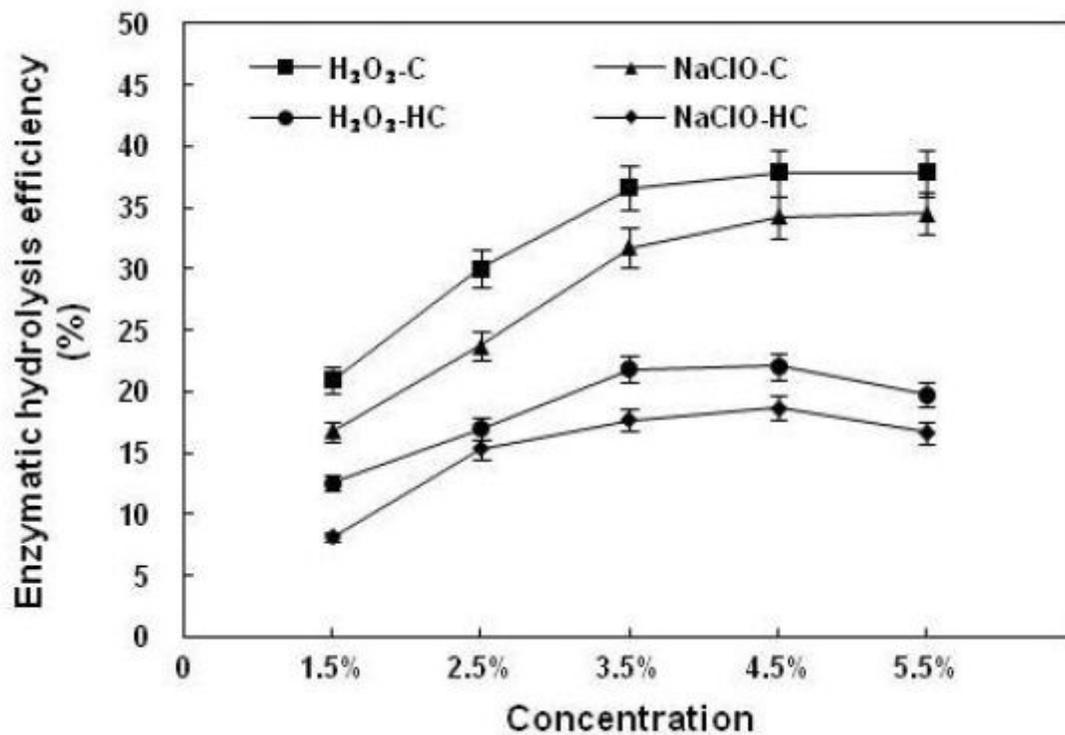


Figure 2

Effects of oxidant concentration on enzymatic hydrolysis of pretreated sawdust (C: cellulose; HC: hemicellulose)

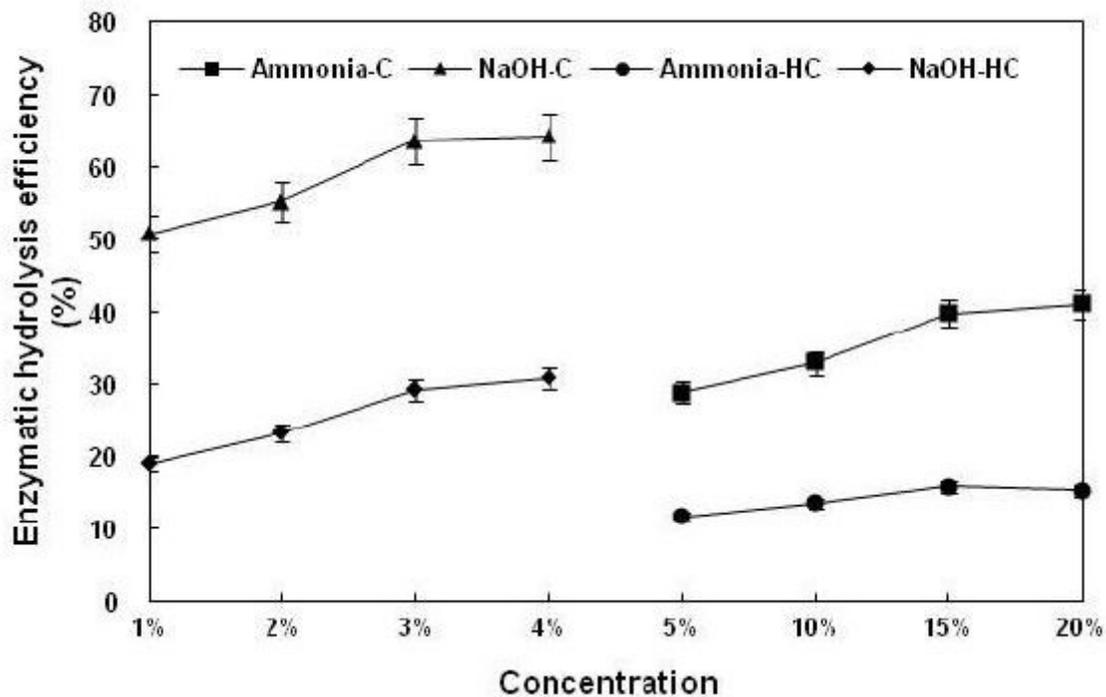


Figure 3

Effects of dilute alkali concentration on enzymatic hydrolysis of pretreated sawdust (C: cellulose; HC: hemicellulose)

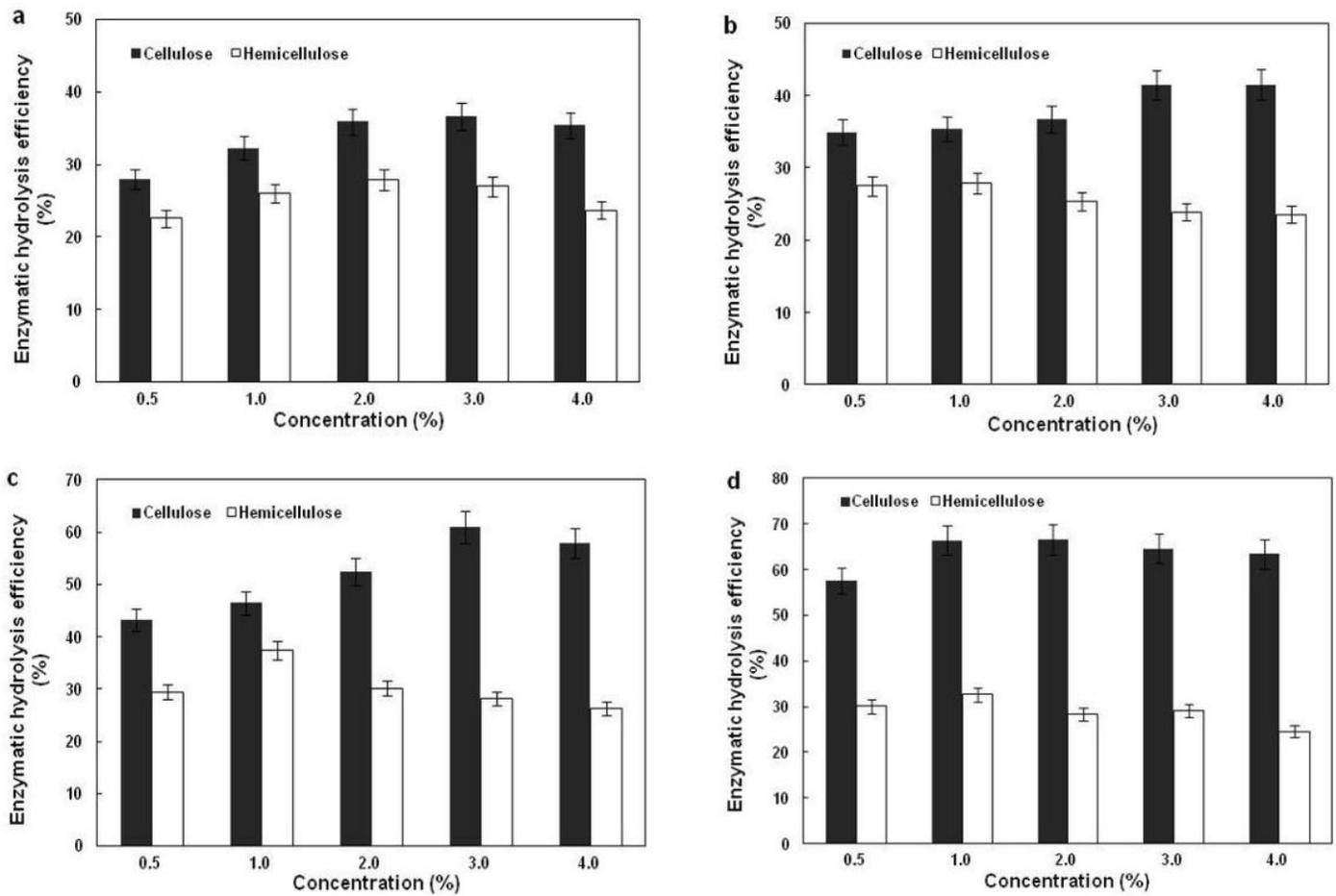


Figure 4

Effects of dilute alkali concentration on pretreated sawdust hydrolysis at different temperatures. (a) 25°C; (b) 50°C; (c) 75°C; (d) 100°C

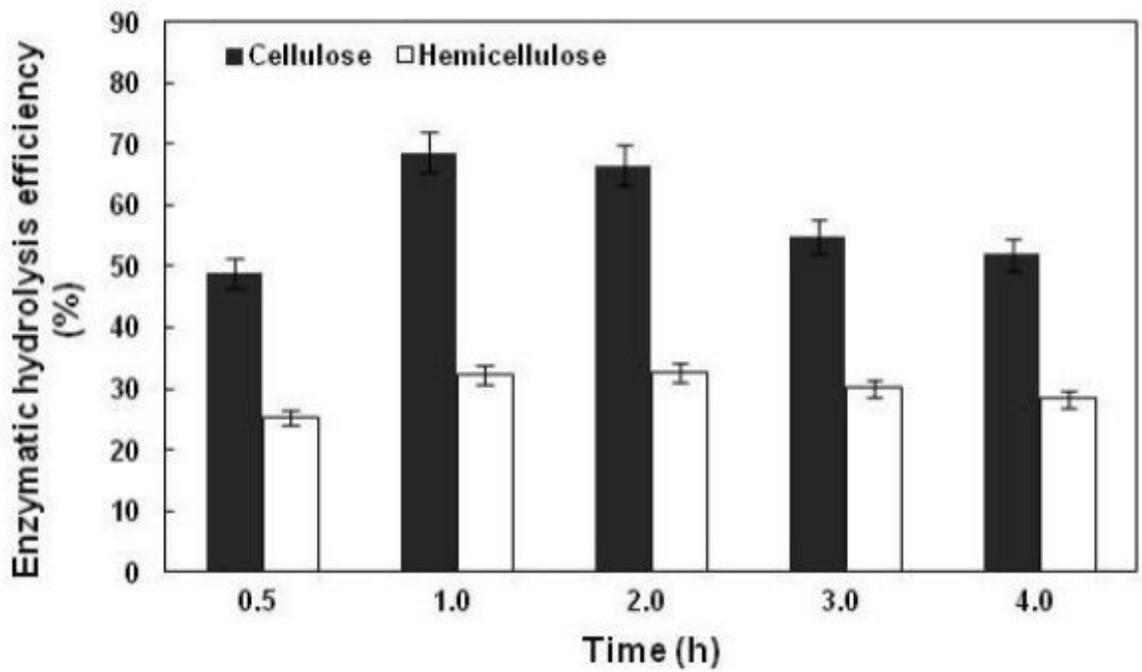


Figure 5

Effects of reaction time on pretreated sawdust hydrolysis

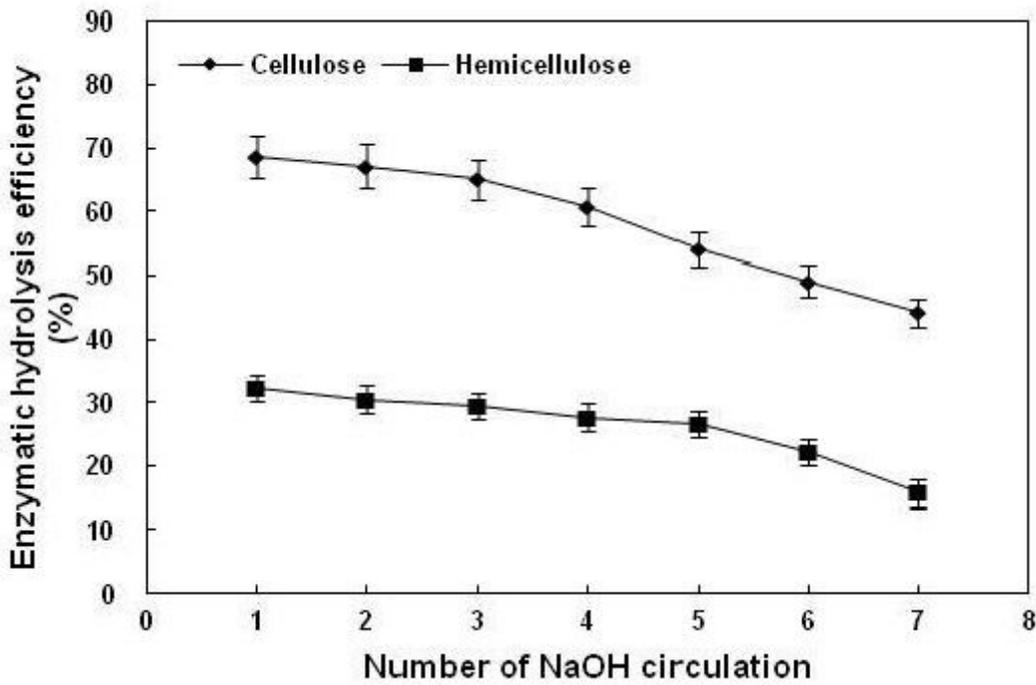


Figure 6

Effects of circulation of cycling NaOH on pretreated sawdust hydrolysis

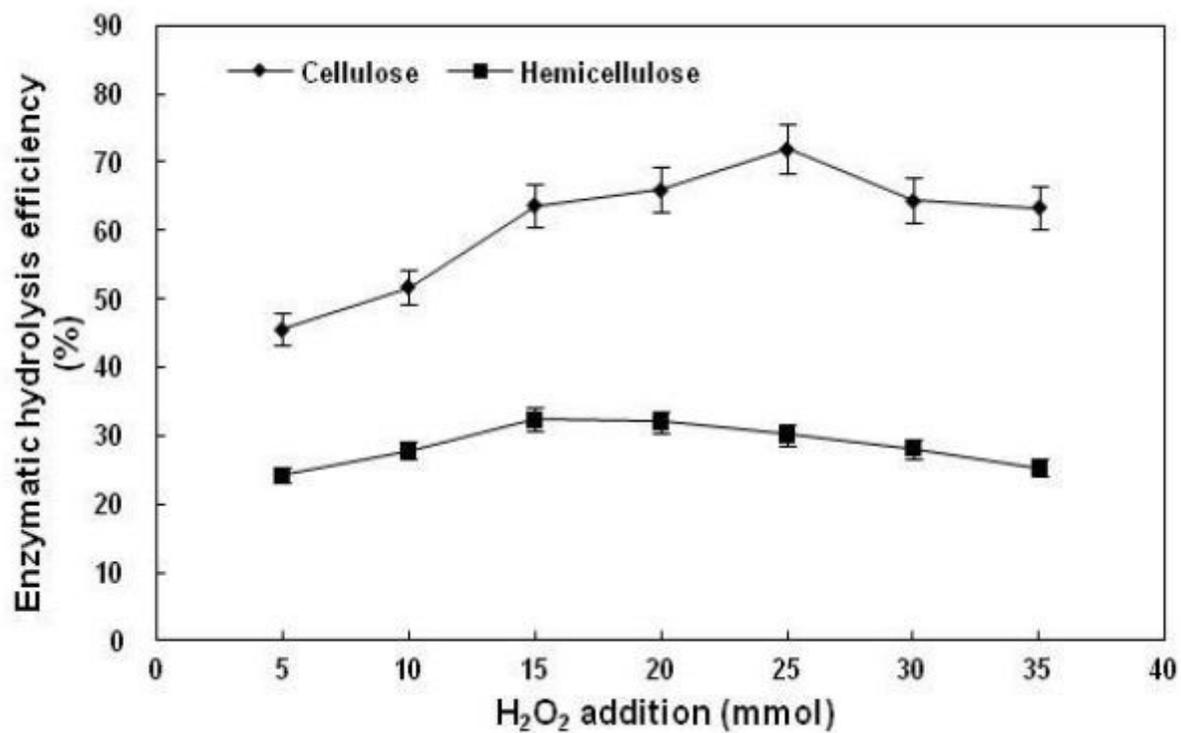


Figure 7

Effects of different H₂O₂ addition on pretreated sawdust hydrolysis

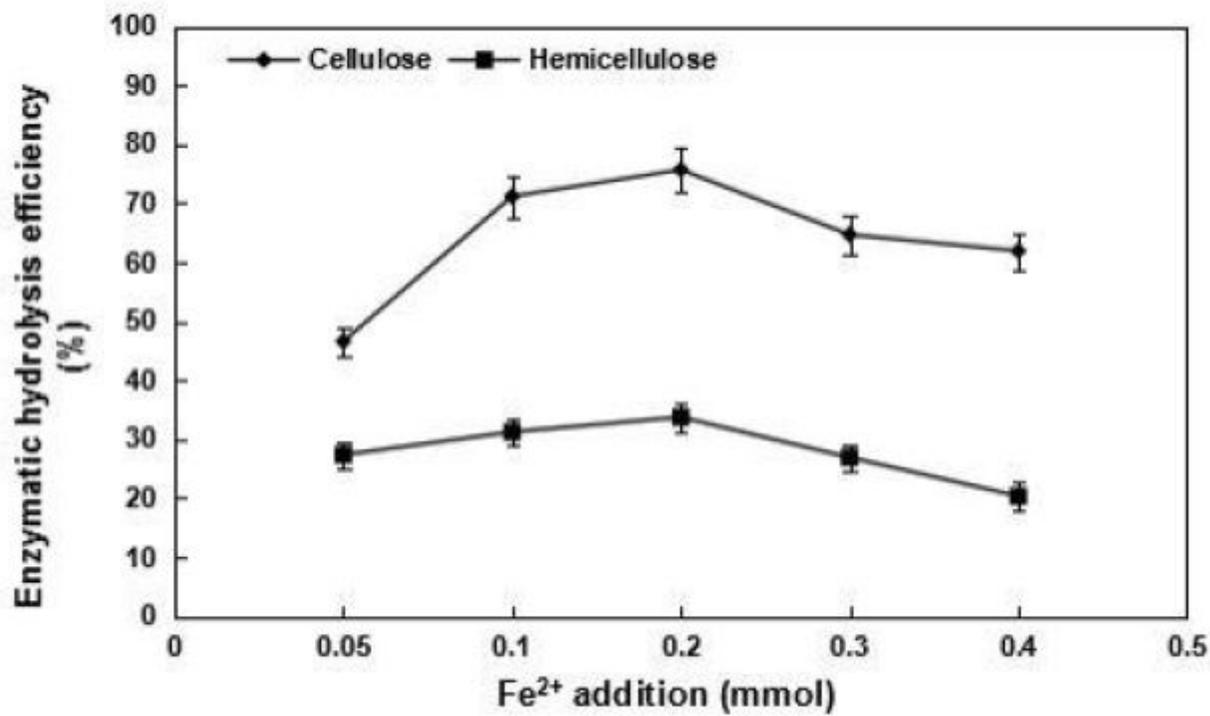


Figure 8

Effects of different Fe²⁺ addition on pretreated sawdust hydrolysis

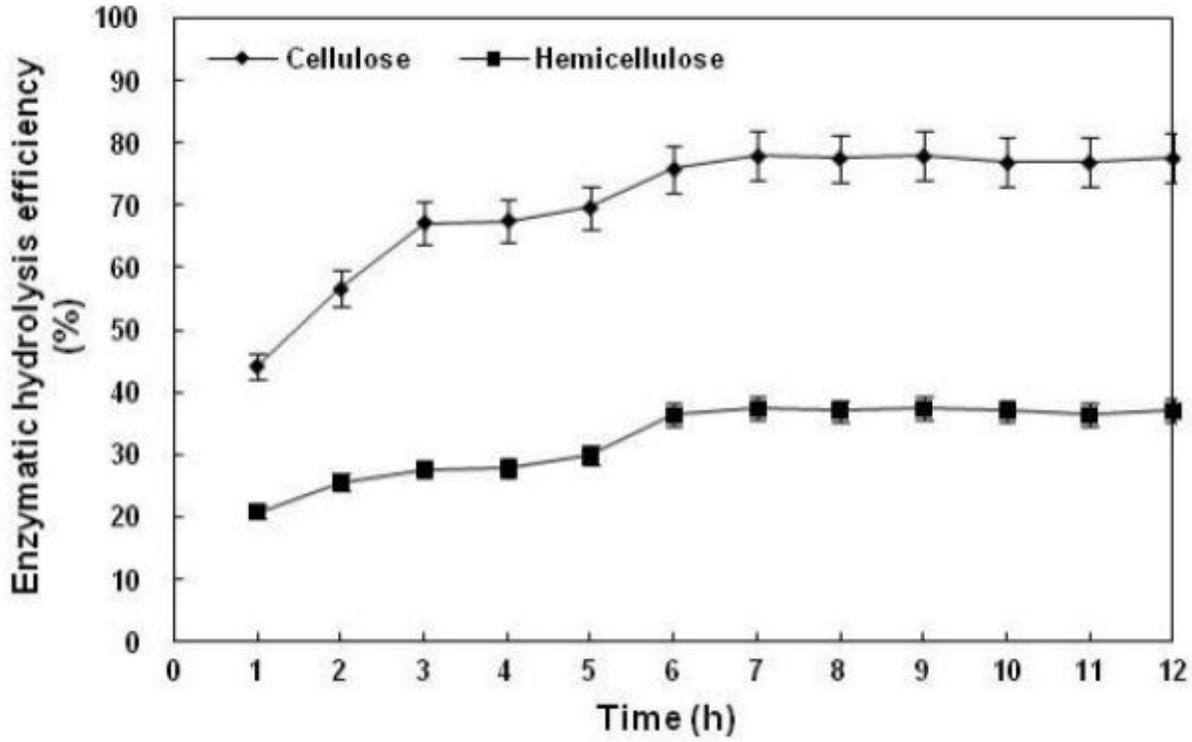


Figure 9

Effects of reaction time on pretreated sawdust hydrolysis

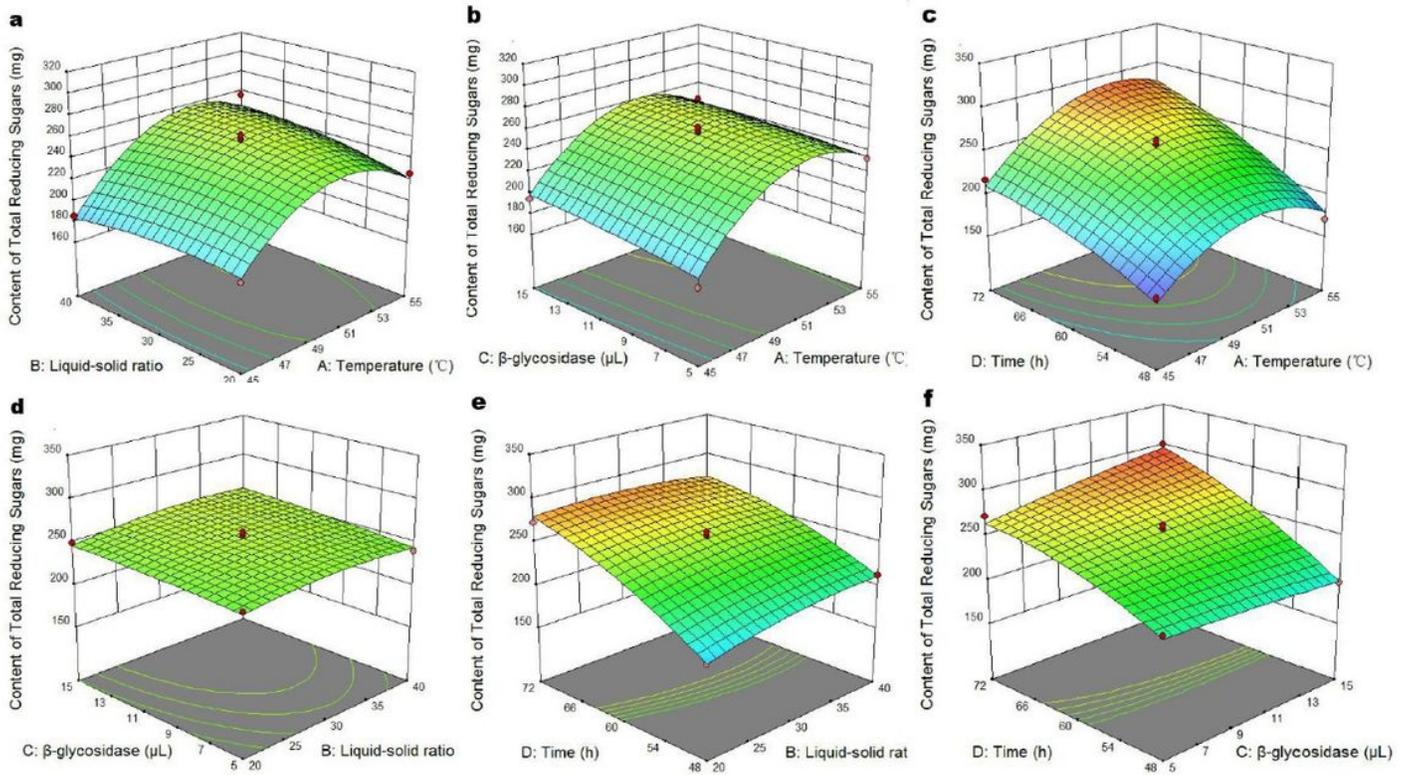


Figure 10

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