EFFECT OF DIFFERENT PRETREATMENT METHODS OF CORNCOB ON BIOETHANOL PRODUCTION AND ENZYME RECOVERY

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Efficient pretreatment and reuse of cellulase are effective methods to promote the cellulosic bioethanol commercialization. Corncob was used as substrate in this study, and the effects of pretreatment methods, including dilute acid, sodium hydroxide, aqueous ammonia soaking and acid-base coupling (dilute sulfuric acid-aqueous ammonia) on glucose and ethanol concentration were analyzed during pre-hydrolysis and the simultaneous saccharification and fermentation (SSF) process. The influence of each pretreatment on the adsorption and desorption of cellulase and on the recycling effect of cellulase after re-adsorption by the fresh substrate were also discussed. The results showed that acid-base coupling pretreatment was much better than a single acid or alkali pretreatment, i.e. the cellulose content of the corncob substrate reached 73.84% after pretreatment; the desorption percent and recycling percent of cellulase after 96 h of SSF and re-adsorption in the first round was 57.7% and 62.4% respectively; ethanol concentration amounted to 62.0% of the first time in the second round of SSF after the enzyme re-adsorption.

Keywords: corncob, pretreatment, cellulase, enzyme recycling, adsorption, simultaneous saccharification and fermentation

INTRODUCTION

With the continuous fossil fuel consumption, and various kinds of environmental problems caused by the use of nonrenewable resources, people have come to realize the importance of looking for renewable energy.[1] Lignocellulose, the most abundant renewable biomass produced by photosynthesis, has the potential to serve as a sustainable supply of fuels and chemicals.[2] Corncob has higher bulk density and can be easy to collect and transport, which is considered as one of the most potential lignocellulosic feedstocks. The world's largest ethanol producer POET Company and many global energy giants have made corncob the main raw material for fuel ethanol production.[3] In China, nearly 20 million tons of corncob is produced annually from agricultural lands, which provides good raw material for bioethanol production.[4]

Pretreatment is extremely significant in the process of bioconversion of lignocellulose to bioethanol, aiming to remove lignin and hemicelluloses, to disrupt the crystalline structure of cellulose, to increase the porosity of the materials, so as to make the raw materials more accessible to the enzyme attack.[5] Various processes are being pursued globally for lignocellulose fractionation, including physical (ball-mill, hydrothermolysis), chemical (acid, alkali, ozone) and biological (fungi) technologies.[6,7] Depending on the specific circumstances, many combined pretreatments may offer better effects, such as a combination of steam explosion followed by

alkaline peroxide pretreatment,[8] hot-water followed by aqueous ammonia pretreatment,[9] and H_2O_2 followed by biological pretreatment.[10]

The high cost of the enzymes required for cellulose conversion to fermentable sugar is a major limitation hindering the commercialization of lignocellulose bioconversion to bioethanol.[11] During the past years, many researchers and enzyme companies have focused on the reduction of enzymes manufacturing cost.[12] Meanwhile, enzyme recovery and reuse is a potential way to reduce enzyme usage cost. Several main methods include immobilization, ultrafiltration and re-adsorption.[13,14,15] During enzymatic hydrolysis, cellulase can either remain adsorbed to solid residues or be free in the liquid phase, depending on its adsorption and desorption behavior. It has been proven that cellulase has relatively high stability and a natural affinity to cellulose,[16] which enable it be recovered via re-adsorption of a fresh substrate. By using such a method, the free cellulase in the liquid phase has been recovered after enzymatic hydrolysis of steam exploded and ethanol pretreated lodgepole pine,[17] which has a good effect on enzyme recycling. However, the separate hydrolysis and fermentation (SHF) process causes the accumulation of certain hydrolysis products, like glucose and especially cellobiose, which inhibits cellulase adsorption and catalysis.[18]

In this work, corncob was chosen as the substrate. Pretreatment methods, such as dilute acid, sodium hydroxide, aqueous ammonia soaking and acid-base coupling (dilute sulfuric acid-aqueous ammonia) were carried out. The effects of the pretreatment methods were analyzed during the simultaneous saccharification and fermentation (SSF) process. Furthermore, the influence of each pretreatment on the adsorption and desorption of cellulase and on the recycling effect of cellulase after the re-adsorption by the fresh substrate were also discussed. The research can provide beneficial reference for the cellulosic bioethanol commercialization.

EXPERIMENTAL

Materials

Corncob was collected from a local farm (Tianjin, China). It was pre-milled and screened, and the fraction between 20 and 80 meshes was used for experiments. The screened corncob sample was stored in hermetically closed plastic containers at -20 ºC. Cellulase (GC220, 166.5 FPU/mL) was provided by Genencor International (Palo Alto, CA, USA). Cellobiase (Novozyme 188, 926 CBU/mL) was purchased from Sigma (St. Louis, MO, USA). Commercial ethanol instant active dry yeast (*S. cerevisiae*) was obtained from Angel Yeast Co., Ltd., Wuhan, China. All other chemicals used were of analytical grades.

Pretreatment of corncob

Corncob was pretreated by the different methods described in Figure 1. A total of four different samples were prepared by adding oven-dried corncob in four identical screw-capped laboratory bottles (Pyrex glass), which were numbered from 1 to 4. Furthermore, 2 wt% H_2SO_4 was added into bottles No. 1 and 2, 2 wt% NaOH and 15 wt% aqueous ammonia into bottles No. 3 and 4, respectively, to reach a ratio of 1 g solid per 6 mL liquid. The solid/liquid slurries were heated at 121 ºC for 1 h in bottles No. 1 and 2; while they were incubated in a water bath at 80 ºC for 6 h and at 60 ºC for 12 h in bottles No. 3 and 4, respectively, with no agitation. After treatment, the solids were separated by filtering, and washed with tap water until neutral. Then the solid residue was dried in a forced-air oven at 105 ºC and weighed. Furthermore, the oven-dried solid residue of bottle No. 1 was treated with 15 wt% aqueous ammonia at 60 ºC for 12 h with a ratio of 1 g solid per 6 mL liquid in a same screw-capped laboratory bottle, and then the solid residue was separated by filtering, washed with tap water until neutral, dried in a forced-air oven at 105 ºC and weighed.

Pre-hydrolysis and SSF experiments

The pre-hydrolysis and SSF experiments were performed in 100 mL of sodium citrate buffer (50 mM, pH 4.8) containing nutrients of $(NH_4)_2HPO_4$ 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.025 g/L, yeast extract 1 g/L and antibiotic (tetracycline hydrochloride) 0.04 g/L with 1 g solid per 10 mL liquid, and the solids were obtained from four different treatments described above. The cellulase loading was 30 FPU/g cellulose, and the pre-hydrolysis reaction was incubated at 50 ºC with shaking at 120 rpm for 12 h. Samples were collected after -12, -11, -7 and 0 h (-12 ~ 0 h represented pre-hydrolysis). Then the temperature was reduced to 30 ºC and the dry yeast was added (2 g/L), converting the saccharification process into a SSF, with shaking at 100 rpm for 96 h. SSF began at 0 h and lasted for 96 h. The time of yeast addition was referred to as time 0. Samples were collected after 24, 48, 72, and 96 h. After SSF, the solid residue was washed, dried, and weighed. The chemical components were also analyzed.

Cellulase recycling experiments

A schematic diagram of the cellulase recycling process is illustrated in Figure 2. After SSF, samples were filtered using a glass microfiber membrane (Whatman GF/A). The filter cake was rinsed with an additional 10 mL of citrate buffer (pH 4.8). Fresh substrate (the same amount as in the initial SSF reaction) was added into the filtrate to reabsorb the free cellulase at 15 ºC for 90 min. The free cellulase adsorbed onto the fresh substrate was

recovered by filtration and resuspened in fresh sodium citrate buffer, which was same as above. Fresh Novozyme 188 was added with a β-glucosidase activity of 20 CBU/g cellulose. A second round of pre-hydrolysis and SSF was performed subsequently. Samples were collected after each round of re-adsorption and SSF.

Analysis

The components of raw corncob and pretreated corncob were determined according to the National Renewable Energy Laboratory (NREL, Golden, CO, USA) procedure for determination of structural carbohydrates and lignin in biomass.[19] The glucose, xylose and ethanol concentration were determined by high performance liquid chromatography (HPLC), using the Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 65 ℃ with 0.005 mol/L H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min.

The protein content of the enzyme was measured by the Bradford[20] assay, using bovine serum albumin (BSA) as the protein standard. The desorption percent and recycling percent of cellulase was defined and calculated according to the following equations:

$$\text{Desorption Percent} = \frac{\text{Protein}_S}{\text{Protein}_0} \times 100 \quad (1)$$

$$\text{Recycling Percent} = \frac{\text{Protein}_S - \text{Protein}_a}{\text{Protein}_S} \times 100 \quad (2)$$

where Protein_s is the amount of protein in the solution after SSF; Protein_0 is the amount of protein in the initial solution; Protein_a is the amount of protein in the solution after re-adsorption.

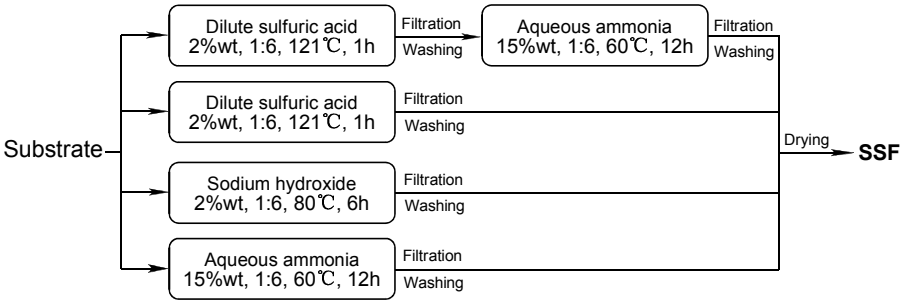

Figure 1: Procedures of different pretreatment methods

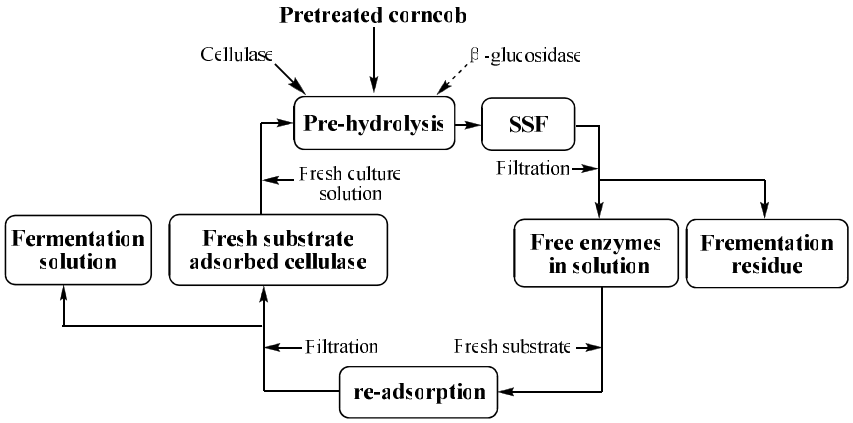

Figure 2: Schematic diagram of a cellulase recycling process

RESULTS AND DISCUSSION

Chemical composition of pretreated corncob

The results of the chemical components analysis of the raw and pretreated corncob are shown in Table 1. After dilute sulfuric acid treatment, the relative content of hemicelluloses was significantly reduced; while the relative content of lignin was obviously reduced after NaOH or aqueous ammonia treatments. The reason is that the acid solution dissolves hemicelluloses, and the alkali solution dissolves lignin. The acid-base coupling pretreatment resulted in the substrates with 73.84% cellulose, and the contents of hemicelluloses and lignin decreased to 13.03% and 9.71%, respectively. Meanwhile, cellulose recovery was up to 85.40% with 78.53% lignin reduction and 83.46%

hemicelluloses reduction. Therefore the combination of acid and alkali pretreatment was effective for the removal of hemicelluloses and lignin, resulting in high cellulose content in the substrate, which facilitated the subsequent SSF operating at high cellulose content to obtain high ethanol concentration.

Table 1
Chemical components analysis of corncob pretreated by different methods

Pretreatment	Component content			Recovery and reduction rate			
	Cellulose, %	Hemicellulose, %	Lignin, %	Solid recovery,[a] %	Cellulose recovery,[b] %	Lignin reduction,[c] %	Hemicellulose reduction,[d] %
Untreated	35.90	32.70	18.78	None	None	None	None
$NH_3 \cdot H_2O$	41.83	39.52	14.49	74.21	86.47	42.74	10.31
NaOH	42.63	41.83	13.77	72.81	86.46	46.61	6.86
H_2SO_4	54.78	13.30	25.99	59.89	91.39	17.12	75.64
H_2SO_4-$NH_3 \cdot H_2O$	73.84	13.03	9.71	41.52	85.40	78.53	83.46

[a]Solid recovery = weight of corncob after pretreatment (g)/initial quantity of corncob (g) × 100%;
[b]Cellulose recovery = weight of cellulose in corncob after pretreatment (g)/initial weight of cellulose in corncob (g) × 100%;
[c]Lignin reduction = (initial weight of lignin in corncob (g) – weight of lignin in corncob after pretreatment (g))/initial weight of lignin in corncob (g) × 100%;
[d]Hemicellulose reduction = (initial weight of hemicellulose in corncob (g) – weight of hemicellulose in corncob after pretreatment (g))/initial weight of hemicellulose in corncob (g) × 100%

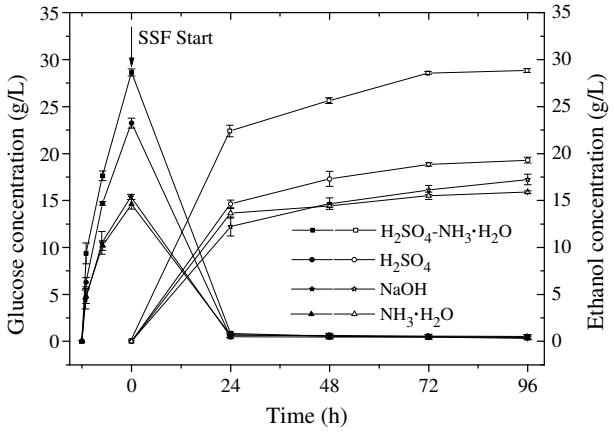

Figure 3: Comparison of glucose and ethanol concentration during pre-hydrolysis and SSF experiments of corncob with different pretreatments (□○☆△ – Ethanol concentration; ■●★▲ – Glucose concentration)

Effect of different pretreatments on ethanol production from corncob

The comparisons of glucose and ethanol concentration during pre-hydrolysis and SSF experiment of corncob with different pretreatments are presented in Fig. 3. On the time axis -12 ~ 0 h represents pre-hydrolysis, while SSF began at 0 h and lasted for 96 h.

HPLC analysis showed a similar trend of glucose formation and consumption during pre-hydrolysis and SSF for different pretreatment methods. The concentration of glucose increased rapidly and it was positively related to the cellulose content of the pretreated substrate for pre-hydrolysis. The substrate pretreated with the acid-base coupling method yielded the highest concentration of glucose (28.6 g/L after 12 h pre-hydrolysis). For all pretreatment methods, the concentration of glucose in corncob hydrolysate decreased to about 0.5 g/L within 24 h of SSF and remained constant in the following 72 h.

The trends of ethanol formation during SSF

were similar for different pretreatment methods. During the first 24 h of SSF, ethanol content obviously increased, and then the rate of ethanol production turned slow in the following 48 h. Among the four pretreatments, the concentration of ethanol after SSF from high to low was listed as follows: acid-base coupling (28.8 g/L after 96 h of SSF) > dilute acid > aqueous ammonia soaking > sodium hydroxide. Cellulose conversion using different pretreatments after SSF is shown in Table 3, in which the acid-base coupling method had a higher cellulose conversion (84.8%) than the others.

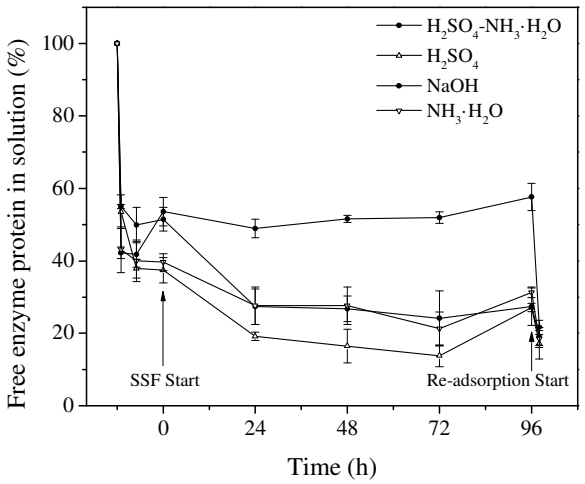

Figure 4: Free enzyme protein in solution during pre-hydrolysis, SSF and re-adsorption process for corncobs after different pretreatments

During the reaction of pre-hydrolysis and SSF, the trends of glucose and ethanol concentration for different pretreatments were both positively related to the cellulose content of the substrate, and the effect of the acid-base coupling method was better than the others. One reason for this phenomenon is that the structure of the substrate has been thoroughly damaged by the acid-base pretreatment, resulting in an easier hydrolysis. Another explanation is that most of the non-cellulosic materials have been removed, which reduced the invalid adsorption of cellulase and eliminated the inhibition for the enzymatic hydrolysis reaction, so as to make the cellulose substrate more accessible to the enzymes.

Effect of different pretreatments on cellulase recycling

In order to evaluate the feasibility of the re-adsorption strategy, the cellulase distribution between the solid and liquid phase during the pre-hydrolysis, SSF and re-adsorption was determined. By calculating "the proportion of free enzyme protein in solution to total amount of added enzyme protein" and "the proportion of enzyme absorbed on substrates to total amount of protein in the solution after re-adsorption", i.e. "desorption percent" and "recycling percent", we compared the different absorption and desorption characteristics of cellulase with regard to different pretreatment methods.[22]

Figure 4 shows the content of free enzyme protein in solution during the pre-hydrolysis, SSF and after re-adsorption for different pretreatments. On the time axis, -12 ~ 0 h represents pre-hydrolysis; while SSF began at 0 h and lasted for 96 h; subsequently re-adsorption began at 96 h. As shown in Figure 4, the enzyme protein content during the initial stage of pre-hydrolysis decreased rapidly for different pretreatment methods, which indicated that a mass of cellulase was absorbed onto the corncob substrate. With prolonging the pre-hydrolysis time to 5 h, the free enzyme protein content in solution decreased to a constant value for the corncob substrate pretreated by dilute sulfuric acid, sodium hydroxide, and aqueous ammonia. However, the free enzyme protein content increased in the hydrolysates of corncob pretreated by the acid-base coupling method after pre-hydrolysis of 5 h, which suggested that the cellulase desorbed into the solution as a form of free enzyme protein along pre-hydrolysis, and it was beneficial to cellulase recycling.

After adding yeast, the amount of free cellulase

in the fermentation liquid further decreased obviously during SSF for the corncob substrates pretreated by dilute sulfuric acid, sodium hydroxide, and aqueous ammonia. However, the acid-base coupling pretreatment method had higher cellulase content in solution than the other three methods during the SSF process. As shown in Table 2, this corresponded to a 57.7% desorption percent of cellulase after 96 h of SSF, which was about 100% higher than for the other pretreatment methods. We simultaneously analyzed the sample after fresh substrate re-adsorption in the first round. The results showed that the cellulase recycling percent for the acid-base coupling pretreatment method was 62.4% (Table 2), which was also higher than for the other pretreatment methods.

Table 2
Desorption and recycling percent for different pretreatment methods

Pretreatment	Desorption percent,[a] %	Recycling percent,[b] %
$NH_3 \cdot H_2O$	31.3	41.2
NaOH	27.4	30.4
H_2SO_4	27.1	36.5
H_2SO_4-$NH_3 \cdot H_2O$	57.7	62.4

[a]Sampled after 96 h of SSF;
[b]Sampled after 90 min of re-adsorption in the first round

Table 3
Cellulose conversion and ethanol concentration of two consecutive SSF rounds for different pretreatment methods

Pretreatment	Cellulose conversion[a] for round 0, %	Ethanol concentration for round 0, $g \cdot L^{-1}$	Cellulose conversion[a] for round 1, %	Ethanol concentration for round 1, $g \cdot L^{-1}$	Ethanol production recovery,[b] %
$NH_3 \cdot H_2O$	81.6	15.9	33.1	5.5	34.4
NaOH	83.8	17.2	22.6	2.8	16.4
H_2SO_4	78.1	19.3	12.4	2.6	13.5
H_2SO_4-$NH_3 \cdot H_2O$	84.8	28.8	57.7	17.9	62.0

[a]Cellulose conversion = (initial weight of cellulose in corncob (g) – weight of cellulose in the residue after SSF (g))/initial weight of cellulose in corncob (g) × 100%;
[b]Ethanol production recovery = ethanol concentration in the second SSF round with recycled cellulase ($g \cdot L^{-1}$) /ethanol concentration in the first SSF round ($g \cdot L^{-1}$) × 100%

For dilute sulfuric acid pretreatment, the acid mainly dissolved hemicellulose, and the remaining lignin could restrict the desorption of cellulase.[23] Therefore, the excessive cellulase absorbed onto the acid-treated corncob could not be desorbed into solution. For sodium hydroxide and aqueous ammonia pretreatment, the alkali could dissolve lignin efficiently. But as shown in Figure 4, there was not an obvious increase in the content of free enzyme protein, which indicated that the cellulase still could not be efficiently desorbed into solution during SSF. Meanwhile, for the acid-base coupling method, 57.7% of cellulase could be desorbed into solution after hydrolysis during the SSF process. This is helpful for fresh substrate adsorption, and could greatly reduce the cellulase consumption.

Furthermore, after re-adsorption of the enzyme, the fresh substrates were submitted to a new round of pre-hydrolysis and SSF process. Cellulose conversion and ethanol concentration of two consecutive SSF rounds for different pretreatment methods are shown in Table 3. Comparing the results, the acid-base coupling pretreatment method was better than the others, since it had the highest cellulose conversion (57.9%) and ethanol production recovery (62.0%). These values were in agreement with the absorption and desorption behaviors of the enzyme proteins in Figure 4 and Table 2. The results also demonstrated that corncob substrate pretreated by the acid-base coupling method could adsorb more cellulase, which led to the better recovery and to the recycling effect of the enzymes.

CONCLUSION

In this study, corncob was chosen as the

substrate. The effects of the pretreatment methods, such as dilute acid, sodium hydroxide, aqueous ammonia soaking and acid-base coupling (dilute sulfuric acid – aqueous ammonia), on the ethanol production and cellulase recycling were analyzed. The following conclusions could be drawn:

(1) Dilute sulfuric acid pretreatment mainly dissolved hemicelluloses; aqueous ammonia and NaOH pretreatment mainly dissolved lignin; acid-base coupling pretreatment could effectively remove hemicelluloses (83.46%) and lignin (78.53%) from the lignocellulose, providing a final cellulose content of 73.84% and a cellulose recovery of 85.40%.

(2) Corncob substrate pretreated by the acid-base coupling method had the highest cellulose conversion of 84.8%, and ethanol concentration of 28.8 g/L, of all the pretreatment methods.

(3) Compared to the other methods, only the acid-base coupling pretreatment had the feasibility of cellulase recovery and recycling. The desorption percent and recycling percent of cellulase after 96 h of SSF and re-adsorption in the first round were 57.7% and 62.4%, respectively. Meanwhile, the ethanol concentration after the second round of SSF amounted to 62.0% of that in the first round.

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