Chinese J. Chem. Eng., **14**(6) 796—801 (2006)

Effects of Different Pretreatment Modes on the Enzymatic Digestibility of Corn Leaf and Corn Stalk*

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Abstract Corn leaf and corn stalk were pretreated with only hot water and 0.1% sulfuric acid at 160℃ or 200℃, respectively. For hot water pretreatment, the pH of corn stalk hydrolysate decreased more rapidly than that of corn leaf as the reaction time increased. On the contrary, the pH of corn leaf hydrolysate increased more than that of corn stalk with diluted acid addition. Increasing temperature enhanced the xylose dissolution rate and increased cellulose digestibility. Compared with hot water, 0.1% sulfuric acid addition improved the xylan removal and the enzymatic hydrolysis of both corn leaf and corn stalk residue. Much less xylan must be removed to achieve the same cellulose digestibility for the corn leaf as that for the corn stalk; 55% digestibility was obtained when only 32% xylan was removed from corn leaf, whereas corn stalk required removal of about 50% of the xylan to achieve the same digestibility. Overall, the descending order of enzymatic digestibility was: dilute acid hydrolysate of corn leaf > dilute acid hydrolysate of corn stalk > water-only hydrolysate of corn leaf > water-only hydrolysate of corn stalk. Finally, one separate pretreatment strategy was developed to transfer corn leaf and corn stalk to fermentable sugars for further bioenergy production.
Keywords pretreatment, corn leaf, corn stalk, xylan removal, enzymatic digestibility

1 INTRODUCTION

Enzymatic hydrolysis of the cellulose to glucose from inexpensive and abundant sources, followed by ethanol fermentation to produce alternative liquid transportation fuels is very attractive[1—4]. However, lignocellulosic biomass must first be pretreated to open up its structure, so that the high yields vital to economic success can be realized. Pretreatment is currently one of the most expensive steps in such bioconversion routes, which account for one-third of the total cost. Advanced pretreatment technologies can reduce costs, improve cellulose digestibility, simplify upstream and downstream operations, and provide the potential for additional revenues from co-products[5,6].

Two methods are available to hydrolyze lignocellulosic materials to the fermentable sugars, including concentrated acid hydrolysis and enzymatic hydrolysis[7]. Compared with acid hydrolysis, enzymatic hydrolysis is milder and more specific, but it requires pretreatment to improve the enzymatic digestibility. The pretreatment process can remove hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Among the pretreatment methods, dilute acid pretreatment has been widely studied because it is effective and inexpensive[8]. The dilute acid pretreatment can effectively solubilize hemicellulose into monomeric sugars and soluble oligomers, thus improving cellulose conversion[9]. It is especially useful for the conversion of xylan in hemicellulose to xylose that can be further fermented to ethanol by many microorganisms. However, materials that are currently used to handle dilute sulfuric acid are expensive; therefore, substantial size reduction is needed to reduce the capital investment. Furthermore, downstream neutralization will introduce some additional chemical costs and bring about processing difficulties in neutralization wastes. Apparently, the process of dilute acid pretreatment needs to be further examined to improve its economic viability[10]. Compared with dilute acid pretreatment, liquid hot water pretreatment process offers several potential advantages such as no acid supplement, no special noncorrosive construction, and much lower quantities of hydrolysate neutralization residues. Also liquid hot water appears to have the potential to generate reactive fiber, recover most of the pentosans, and produce less fermentation-inhibiting byproducts[11]. Therefore, some detailed investigations are necessary to compare the effects of the above-mentioned pretreatment modes on the enzymatic digestibility of residue.

In the present study, the investigations on the effects of liquid hot water and dilute acid pretreatment on the pH profiles of liquid hydrolysate and the yield of xylose and oligomer yield were focused. Subsequently, the enzymatic digestibility of solid residues after these different pretreatments was further compared. The study of the pretreatment of corn leaf and corn stalk provided some primary data and interesting observations; also one separate novel pretreatment strategy was developed to transform corn leaf and

Received 2006-03-10, accepted 2006-06-12.
* Supported by the National Natural Science Foundation of China (No.29976045).
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corn stalk to fermentable sugars.

2 MATERIAL AND METHODS

2.1 Sample preparation

Corn stover was obtained from the suburbs of Beijing, China. The corn leaf and corn stalk were milled to pass through a 2mm opening and then screened using a RoTap (RX-29) separator. The −420/+250μm fraction was retained in plastic Ziploc bags and kept in a freezer (−20℃) to be used in the following tests. The compositions of the corn leaf and corn stalk (Table 1) were determined through application of NREL standard procedures[12—14].

Table 1 Chemical composition of corn leaf and corn stalk

Component	Corn leaf, g·(g dry substrate)$^{-1}$	Corn stalk, g·(g dry substrate)$^{-1}$
glucan	0.342	0.365
galactan	0.025	0.024
xylan	0.221	0.216
arabinan	0.035	0.032
mannan	0.018	0.017
ash	0.084	0.052
lignin	0.164	0.174

Note: The data shown in the table are the mean value. n=2; SE<0.3% for K-lignin, SE<0.8% for ash, SE<0.3 % for glucan, galactan, xylan, arabinan and mannan, SE means standard error.

2.2 Batch reactors

The batch tube reactors were made of Hastelloy (C-276) tubing to withstand the dilute acid concentrations used in this study. Teflon plugs were inserted into either ends and then capped with stainless steel Swagelok fittings. The total working volume of these tubular reactors was 8ml. Thermocouple probes were inserted along the centerline of the tubes and were pushed 4.76mm and 50.8mm. into the reactor. Reaction temperatures of 160℃ and 200℃ were applied at 0.1% sulfuric acid with the solids concentration held at 5% (by mass).

Because temperature transients can impact biomass hydrolysis in reactor tubes, a three-bath procedure was developed to minimize these effects based on a modeling approach described later. The sequence began by preheating each tube in boiling water for 2min followed by immediately transferring them to a sand bath set at a temperature, T_1, selected through the proposed modeling approach to minimize heat-up times. After the tube was held for a specified time in this sand bath, it was transferred to a second sand bath set at the target reaction temperature T_2. The time the batch tube was placed in the second sand bath was arbitrarily set as zero reaction time. After being subjected to the target temperature for a specified reaction time, the reactants were quickly transferred to an ice-cold water bath to quench the reaction. Next, the tubes were removed from the ice-cold water bath and dried, and the end caps and Teflon plugs were removed. The contents were then pushed out and separated into liquid and solid fractions by filtration for analysis[15].

2.3 Analytical procedures

2.3.1 *pH*

A pH meter was used to measure the pH value of the liquid hydrolyzate samples when it cooled to room temperature.

2.3.2 *Sugar measurement*

The sugar concentrations were determined using a Model 2695 HPLC system (Waters, Milford, MA) equipped with a Model 2410 Differential Refractometer (Waters, Milford, MA). The column was equilibrated with de-ionized water at a flow rate of 0.6ml·min^{-1}. An Aminex column (Model HPX-87P, Bio-Rad, Sunnyvale, CA) was used for sugar separation.

2.3.3 *Determination of sugar oligomers*

Liquid hydrolyzate samples were autoclaved in 4% sulfuric acid for 1h at 121℃ to breakdown oligomers into monomeric sugars using the National Renewable Energy Laboratory (NREL) methods[13]. Sugar standards containing known sugar concentrations were also autoclaved under the same condition to estimate the hydrolysis loss factors. The amount of oligomers in the liquid sample was then calculated as:

The mass of total monomers in the posthydrolyzate liquid corrected for degradation minus monomers in the liquid before hydrolysis is the mass of oligomers.

This quantity was then divided by the sugar content of the initial biomass material to calculate the yield of oligomers.

2.3.4 *Analysis of compositions of corn leaf and corn stalk*

The cellulose, hemicellulose, and lignin contents were determined as described[16]. Moisture and ash contents were determined according to standard methods[14,17].

2.3.5 *Digestibility test*

FPU (filter paper units) and β-glucosidase were determined according to the procedures described by Zhou *et al*.[18]. One unit of enzyme activity is the amount of enzyme that liberates 1μmol of reducing sugars per minute under standard assay condition. The solid residues recovered after water or 0.1% sulfuric acid pretreatment at a solid loading of 5%, were hydrolyzed by cellulases and β-glucosidase at 50℃ for 48h in a water bath shaker, the stirring speed being 100r·min^{-1}. β-Glucosidase was used to supplement the insufficient β-glucosidase activity in the cellulases from *Trichoderma reesei*[19]. Sodium citrate buffer was used to maintain the pH at 4.8, whereas sodium azide (mass concentration 0.3%) was added to inhibit the microbial infections. Cellulases (E.C.3.2.1.4) from *T.reesei* and Novozyme 188 purchased from Sigma were the enzymes used. Enzyme activity was 1.08FPU·mg^{-1} for cellulases and 321.7CBU·ml^{-1} for

Novozyme 188. The enzyme loading concentrations were excessive: cellulases, 25FPU·(g dry biomass) $^{-1}$; β-glucosidase, 75CBU·(g dry biomass) $^{-1}$. Reducing sugars were determined using 3,5-dinitrosalicylic acid (DNS) with glucose as a standard after enzymatic hydrolysis for 48h. The enzymatic digestibility is defined as total amount of glucose released times 0.9 pertotal glucan. A dehydration factor of 0.9 is used to convert the glucose to glucan[20].

3 RESULTS AND DISCUSSION

3.1 Composition of corn leaf and corn stalk

The main components of corn leaf and corn stalk are hemicellulose, cellulose, lignin, and ash (Table 1). The cellulose and lignin content of corn stalk was higher than that of corn leaf; however, the hemicellulose and ash content of corn stalk were lower than that of corn leaf. The ash contents of corn leaf and corn stalk were considerably higher than that of wood and certain other biomasses.

3.2 Effect of two different pretreatment modes on pH of liquid hydrolyzate

Hot water pretreatment is believed to be uncatalyzed hydrolysis and is postulated to be catalyzed only by the acetyl groups released from acetic acid[21]. Although, uncatalyzed pretreatment has the advantage of minimizing the cost of chemicals and noncorrosive materials of construction, it suffers due to low sugar yields in batch systems. As the reaction progressed, the pH of the samples recorded at room temperature decreased among all the runs (Fig.1). Higher temperature would lead to the decrease of the pH more obviously. This suggested that the release of acetyl groups and/or other organic acids was seriously affected by the temperature and reaction time. An interesting phenomenon was that the pH of corn leaf hydrolysate was higher than that of corn stalk hydrolystate. This might be contributed by the different neutralization capacity of corn stalk and corn leaf hydrolysates[22]. Subsequently, the effects of dilute acid pretreatment on the pH of corn stover hydrolysate were investigated. As shown in Fig.2, higher temperature treatment increased the pH of corn stover hydrolysate. It is quite different from the effects of temperature on pH using only hot water treatment. This suggested that higher temperature might reduce the alkaline quantity to neutralize the low pH after dilute acid pretreatment. In accordance with only hot water pretreatment, the pH value of corn leaf hydrolysate was higher than that of corn stalk hydrolysate. Apparently, dilute acid pretreatment would lead to considerable decrease in pH in comparison with that in only hot water pretreatment process.

3.3 Effects of two different pretreatment modes on the yields of monomeric xylose and oligomeric xylose

The effects of temperature on xylose yield were

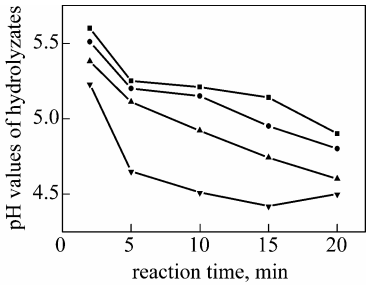

Figure 1 The pH values of hydrolyzates measured at room temperature following 160℃ and 200℃ runs at various time for uncatalyzed hydrolysis in a batch tube

(The data points shown in the graph are the mean value, SE<2.5% for pH, n=2, SE means standard error)

■ 160℃ corn leave; ● 200℃ corn leave;
▲ 160℃ corn stalk; ▼ 200℃ corn stalk

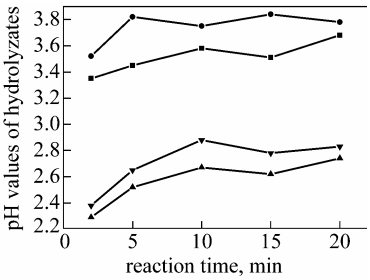

Figure 2 The pH values of hydrolyzates measured at room temperature following 0.1% sulfuric acid for 160℃ and 200℃ runs at various time for catalyzed hydrolysis in batch tubes

(The data points shown in the graph are the mean value, SE＜2.5% for pH, n=2, SE means standard error)

■ 160℃ corn leaf; ● 200℃ corn leaf;
▲ 160℃ corn stalk; ▼ 200℃ corn stalk

investigated by adding only hot water and 5% solids in the batch tubes. The results (Fig.3) showed that the temperature played an important role on the production of monomeric xylose. For corn stalk, when the temperature increased from 160℃ to 200℃, the yield of xylose in solution increased from about 4.8% to around 9.4% in the first 20min, and for corn leaf, from about 2.5% to around 3.8%. The yield of xylose from corn stalk was higher than that for corn leaf. For corn stalk and corn leaf, the highest yields of monomeric xylose were observed after 15min of reaction at 200℃ among the various conditions that were tested. The production of xylose by hot water pretreatment process was attributed to the dissociation of hemicellulose by low-pH hot water and the breakage of ether linkages in biomass by the released acid under hot water pretreatment conditions[23]. As shown in Fig.4, the effect of temperature on the yield of oligomeric xylose was also examined by adding only hot water and 5% solids in the batch tubes. The result showed that high temperature would improve the yield of oligomeric xylose significantly, especially for corn leaf. It was also observed that the yield of oligomers from corn stalk was much higher than those from corn leaf. It is generally agreed that the active hemicellulose hydrolysis

accelerator is the hydronium ion[2,24]. The different yields of xylose from corn stalk and corn leaf might be explained by further measuring hydronium ion in these two different samples. From Figs.3 and 4, the maximum yields of xylose and oligomers were obtained at 200℃, and the reaction time needed to be increased to obtain a relatively high yield at 160℃.

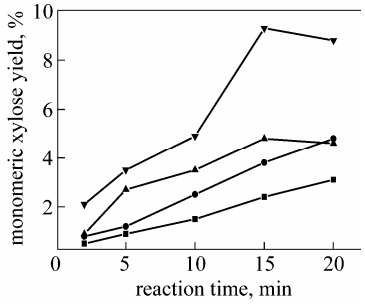

Figure 3 The monomeric xylose yield obtained under batch uncatalyzed hydrolysis as reaction time increased at 160℃ and 200℃

(The data points shown in the graph are the mean value, SE<2.5% for pH, *n*=2, SE means standard error)
■ 160℃ corn leaf; ● 160℃ corn stalk;
▲ 200℃ corn leaf; ▼ 200℃ corn stalk

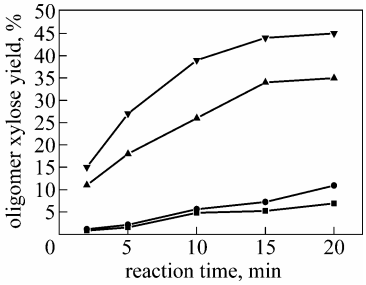

Figure 4 The oligomer xylose yield obtained under batch uncatalyzed hydrolysis as reaction time increased at 160℃ and 200℃

(The data points shown in the graph are the mean value, SE＜2.5% for pH, *n*=2, SE means standard error)
■ 160℃ corn leaf; ● 200℃ corn leaf;
▲ 160℃ corn stalk; ▼ 200℃ corn stalk

The effects of dilute acid pretreatment on the yields of monomeric xylose and xylose oligomers were further investigated. As shown in Fig.5, temperature obviously affected the removal of monomeric xylose and oligomers. For corn stalk, when the temperature was increased from 160℃ to 200℃, the yield of xylose in solution increased from about 9.8% to around 32.5% in 20min and for corn leaf, from about 13.5% to around 48%. This suggested that the removal of monomeric xylose for corn leaf was easier than that for corn stalk. Compared with the results in Fig.3, it was concluded that acid addition considerably improved the yield of xylose, although xylose degradation would be increased by the addition of dilute acid in the reaction system. As shown in Fig.6, high temperature also increased the yield of oligmers significantly. For corn leaf and corn stalk, the highest yields of oligomers were observed at 200℃ for a reaction time of 10min and 15min, respectively. Further extension of reaction time would lead to the decrease of the yield of oligomers, presumably as a result of forming xylose by a depolymerization mechanism[25]. For corn leaf, the yield of oligomers in solution was higher than that for corn stalk under the same reaction conditions.

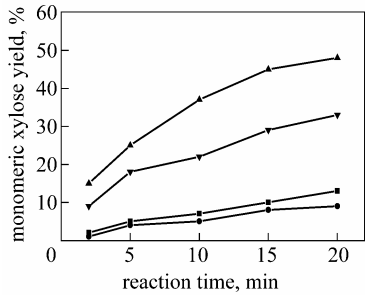

Figure 5 The monomeric xylose yield obtained under 0.1% sulfuric acid batch hydrolysis with 5% solids concentration as reaction time increased at 160℃ and 200℃

(The data points shown in the graph are the mean value, SE<2.5% for pH, *n*=2, SE means standard error)
■ 160℃ corn leaf; ● 160℃ corn stalk;
▲ 200℃ corn leaf; ▼ 200℃ corn stalk

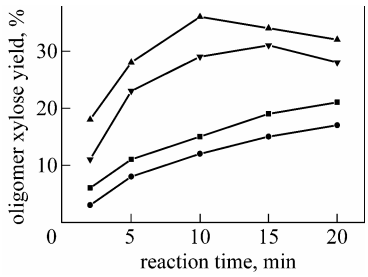

Figure 6 The oligomers xylose yield obtained under 0.1% sulfuric acid batch hydrolysis with 5% solids concentration as reaction time increased at 160℃ and 200℃

(The data points shown in the graph are the mean value, SE＜2.5% for pH, *n*=2, SE means standard error)
■ 160℃ corn leaf; ● 160℃ corn stalk;
▲ 200℃ corn leaf; ▼ 200℃ corn stalk

Based on the above experiments, it was found that two different pretreatment modes had different effects on the removal of xylan. For only hot water pretreatment, higher removal rate of xylan was obtained from corn stalk in comparison to that from corn leaf. However, in contrast, when dilute acid pretreatment was employed, higher removal rate of xylan was obtained from corn leaf in comparison to that from corn stalk. This is an interesting observation and needs to be explored in the future.

3.4 The cellulose remaining in the solid residue

Although the dissolution of cellulose was influenced by temperature, acid concentration, and reaction time, most of the cellulose still remained in the solid resides (Table 2). As for the only hot water pretreatment, the dissolution of glucan in corn stalk was

Table 2 Cellulose remaining in the solid residue at 160℃ and 200℃ runs for uncatalyzed hydrolysis and catalyzed hydrolysis in batch tubes

Heating time, min	Cellulose remaining in the solid residue, %							
	corn leaf①	corn stalk①	corn leaf②	corn stalk②	corn leaf③	corn stalk③	corn leaf④	corn stalk④
2	99.4	99.2	98.1	99.0	97.3	96.7	93.2	94.4
5	98.2	97.5	96.2	97.0	95.7	94.8	91.1	93.0
10	96.0	95.4	94.1	95.4	92.8	91.2	89.0	90.1
15	95.5	95.1	91.2	93.2	92.2	90.1	83.4	87.7
20	95.0	94.2	90.1	92.8	91.5	89.3	80.3	85.2

① Uncatalyzed hydrolysis, 160℃;
② Catalyzed hydrolysis, 160℃;
③ Uncatalyzed hydrolysis, 200℃;
④ Catalyzed hydrolysis, 200℃.
Note: The data shown in the table are the mean value. n=2; SE<0.3% for cellulose remaining, SE means standard error.

higher than that of the corn leaf at 160℃ or 200℃. However, as for dilute acid pretreatment, the dissolution of glucan in corn leaf was higher than that of corn stalk at 160℃ and 200℃.

3.5 Enzymatic hydrolyses of solid resides

The purpose of testing different pretreatment modes is to obtain efficient enzymatic hydrolyses of solid resides by celluase. As shown in Figs.7 and 8, enzymatic digestibility obviously depended on xylan removal. As for only hot water pretreatment mode, the xylan removal from corn stalk was slightly higher than that from corn leaf under the same reaction conditions. However, under the same pretreatment conditions, about 10% higher enzymatic digestibility was obtained by pre-treating corn leaf instead of corn stalk with hot water mode. As expected, 0.1% sulfuric acid addition would considerably improve the xylan removal, which resulted in the increase of enzymatic digestibility accordingly. It was also observed that much less xylan had to be removed from corn leaf to achieve the same cellulose digestibility in comparison to that from the corn stalk when 0.1% sulfuric acid was used. For example, 55% digestibility was obtained when only 32% of total xylan was removed from corn leaf, whereas, the same digestibility was achieved by removing about 50% of the xylan from corn stalk. The temperature of pretreatment also influenced the enzymatic digestibility considerably. Under the same reaction conditions, higher temperature would improve the enzymatic digestibility significantly. A highest digestibility (87%) was obtained by pretreating corn leaf for 20min with 0.1% dilute acid at 200°C. Finally, it was concluded that, under similar reaction conditions, the descending order of enzymatic digestibility after different pretreatment modes was as follows: dilute acid hydrolysate of corn leaf>dilute acid hydrolysate of corn stalk>water-only hydrolysate of corn leaf>water-only hydrolysate of corn stalk. Overall, there are some obvious differences in the enzymatic digestibility between corn leaf and corn stalk, although the quantities of main components between the two did not have considerable differences. This suggested that one separate pretreatment strategy might be integrated to develop the efficient process to utilize corn leaf and corn stalk to produce the fermentable sugar solution.

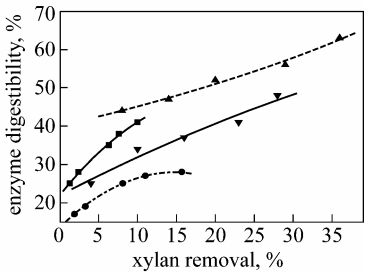

Figure 7 Effect of xylan removal on enzymatic digestility for batch pretreatment of corn leaf and corn stalk at 160℃ with water only and 0.1% sulfuric acid
(The data points shown in the graph are the mean value, SE<2.0%, n=2 for digestibility; SE means standard error)
■ water only, corn leaf; ● water only, corn stalk;
▲ 0.1% sulfuric acid, corn leaf; ▼ 0.1% sulfuric acid, corn stalk

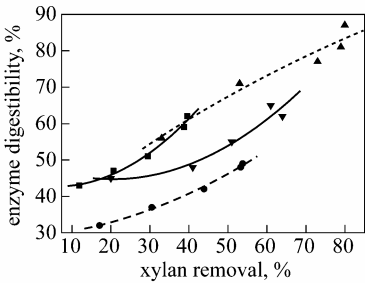

Figure 8 Effect of xylan removal on enzymatic digestility for batch pretreatment of corn leaf and corn stalk at 200℃ with only water and 0.1% sulfuric acid
(The data points shown in the graph are the mean value, SE<2.0%, n=2 for digestibility; SE means standard error)
■ water only, corn leaf; ● water only, corn stalk;
▲ 0.1% sulfuric acid, corn leaf; ▼ 0.1% sulfuric acid, corn stalk

4 CONCLUSIONS

The compositions of corn leaf and corn stalk were analyzed to provide some primary data for the pretreatment studies. Two pretreatment modes, only hot water and dilute acid pretreatments, were developed

and compared with for the purpose of achieving high enzymatic digestibility of solid resides by cellulase. It was found that the pH profiles of corn leaf hydrolysate or corn stalk hydrolysate were different with these two different modes. Also the yield of monomeric xylose and oligomeric xylose were different with these different modes under the similar pretreatment conditions. The effects of temperature and reaction time on the yields of monomeric xylose and oligomeric xylose were also investigated. Higher temperature obviously increased the xylan removal from corn leaf and corn stalk, and then improved the enzymatic digestibility of solid residues considerably.

It was also found that pH profiles of hydrolysate, xylan removal, and enzymatic digestibility were seriously affected by different types of corn stoves. Xylan removal from corn leaf was much easier than that from corn stalk under similar dilute acid conditions, and the digestibility of corn leaf resides was about 10% higher than that of corn stalk resides under the same pretreatment conditions. If corn leaf and corn stalk were pretreated together, corn leaf would counteract more acids, and the released xylose would be decreased accordingly under the rigorous conditions. Therefore, one separate pretreatment strategy would be helpful to fully utilize corn leaf and corn stalk to produce fermentable sugars. Fortunately, it is relatively easy to collect corn leaf and corn stalk separately at large scale, thus the suggested separate pretreatment strategy might be integrated to one whole process to utilize biomass to produce bioenergy efficiently.

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