

Sequential hydrolysis of hemicellulose and lignin in lignocellulosic biomass by two-stage percolation process using dilute sulfuric acid and ammonium hydroxide

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Abstract—To obtain the total fractionation and pretreatment of the corn stover, two-stage percolation process was investigated. This process consists of two steps: use of 0.07 wt% sulfuric acid for hemicellulose recovery in first stage and ARP (ammonia recycled percolation) in the following stage for lignin recovery. Among tested conditions, the best conditions of two-stage process were as follows: 1st stage; 170 °C, 2.5 ml/min, 30 minutes using 0.07 wt% sulfuric acid and 2nd stage; 170 °C, 5.0 ml/min, 60 minutes using 15 wt% ammonium hydroxide. At above two-stage treatment conditions, the hemicellulose in corn stover was easily hydrolyzed (95%) and recovered with high yields (86%) and the extent of the lignin removal was 81%. After two-stage process, the treated biomass contained nearly pure glucan (85%). Two-stage treatment brought about enzymatic digestibility of 90% and 89% with 60 and 15 FPU/g glucan cellulase enzyme loadings, respectively.

Key words: Corn Stover, Enzymatic Hydrolysis, Lignin, Xylooligosaccharides, Bioethanol

INTRODUCTION

For the last several decades, biorefining, the utilization of lignocellulosic for production of biofuels and bio-based product has been on the rise as a promising technology for sustainable economy in the future. Although biofuels and bio-based products can make no net contribution to global warming, i.e., the carbon dioxide produced by the combustion of ethanol is consumed by the growing raw material (balance of carbon cycle), the use of fossil resources has contributed to the buildup of carbon dioxide in the atmosphere. In particular, the ethanol industry continues its rapid expansion annually by 10% since 1999 [1]. In producing biofuels and bio-based products from lignocellulosic biomass, it can typically be divided into three steps that consist in pretreatment, fermentation and separation. To achieve complete and effective utilization of biomass, two kinds of aspects should be considered in terms of pretreatment as well as fractionation of biomass. First, pretreatment of biomass is essential for efficient enzymatic hydrolysis of cellulose because of various physical and chemical barriers that inhibit the accessibility of cellulose substrate to the enzyme [2]. Practically, biomass is quite resistant to enzymatic attack as its original form (10-20% conversion by enzyme in corn stover, <10% in bagasse). Lignin has been considered as a major impeding factor in the cellulose hydrolysis by enzyme [3-8]. Lignin formula consists of many phenylpropane units and they are joined together with C-O-C (ether) and C-C linkages. The ether linkages dominate (approximately 60% of β -O-4, 10% of α -O-4, and 5% of 4-O-5), and the rest are carbon-to-carbon type (approximately 10% of 5-5', 5% of β - β). In addition, strong covalent bonds between lignin and carbohydrates have been studied [9-11]. Now, it is generally accepted that chemical bonds

called lignin-carbohydrate complex (LCC) must exist. There are three linkages (ether, ester, and glycosidic linkage) between lignin and carbohydrate. The most common and stable linkages are ester linkages followed by ether linkages and few glycosidic linkages. Early removal of lignin eliminates the interactions between lignin with cellulase enzyme, making the enzymatic hydrolysis process more efficient [5,12-14]. Furthermore, it also simplifies the overall bioconversion processing. Furthermore, hemicellulose [8], acetyl group [3,15,16], crystallinity [4,8,11,17-19], surface area [6,20], and degree of polymerization [21] have accounted for these physical and chemical barriers in the lignocellulosic biomass. Secondly, fractionation of biomass can significantly improve the overall biomass conversion technology as well as each of the biomass constituents (i.e., hemicellulose and lignin) can be utilized with high efficiency if they are recovered in relatively pure form. For instance, hemicellulose extracted from the biomass can be used to obtain chemical product or food application and lignin can replace a number of products previously made from petrochemicals.

Sulfuric acid and ammonium hydroxide have been studied for effective hemicellulose hydrolysis and lignin removal, respectively [22,23]. In our laboratory, ammonia-based pretreatment (ARP: ammonia recycle percolation) and fractionation methods have been developed [24,25,27,28]. We utilized aqueous ammonia as the pretreatment reagent and showed high degree of delignification while keeping glucan content intact. Despite the fact that ARP treatment is a pretreatment method in order to remove lignin only, the big quantity of xylan loss (40-60%) was inevitable during ARP pretreatment [24,25,29-31]. To retrieve xylan loss, we have investigated a two-stage process in which an extremely low acid percolation process and the ARP were operated in series. This process was intended to recover hemicellulose in the first stage using sulfuric acid and lignin in the second stage using ammonium hydroxide. This process resulted in pure cellulose fiber production. Sulfuric acid is a well known

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agent for hemicellulose removal. The effect of reaction conditions such as the temperature, the flow rate, and reaction time were explored in experiments using flow-through reactor. The impact of these pretreatment for enhancing the enzymatic hydrolysis was tested with pretreated and fractionated samples.

MATERIAL AND METHODS

1. Material

Corn stover was supplied by National Renewable Energy Laboratory (NREL, Golden, CO). The corn stover was screened to the nominal size of 9-35 mesh. The initial compositions of corn stover were 37.5 wt% glucan, 20.8 wt% xylan, 2.7 wt% arabinan, 0.8 wt% mannan, 1.6 wt% galactan, 17.6 wt% lignin, 6.7 wt% ash, 2.2 wt% acetyl group, 2.9 wt% protein, 3.6 wt% uronic acid and 3.6 wt% unaccounted for. α -Cellulose was purchased from Sigma (Cat# C-8200, Lot No. 11K0246). Cellulase enzyme (Spezyme CP, Lot# 301-00348-257) was provided from Genencor International Inc. (Paulo Alto, CA). An average activity of the enzyme, as determined by the NREL, is 31.2 filter paper unit (FPU)/ml. The β -glucosidase enzyme (Sigma Cat# G-0395) was purchased from Sigma-Aldrich (St. Louis, MO). Activity β -glucosidase was 12.0 IU/g.

2. Experimental Setup and Operation

A schematic diagram of the reactor setup is shown in Fig. 1. The system consists of a stock solution reservoir, pump, temperature-programmable gas chromatography oven, flow-through (percolation) reactor, and sample collecting tank #1, #2, which also served

as a backpressure vessel. Sulfuric acid or aqueous ammonia was pumped by HPLC (high-performance liquid chromatography) pump to a packed-bed-flow-through reactor. The flow rates of reagents were monitored by burette. The flow-through reactor was constructed out of SS316 tubing to the dimensions of 24.5 cm length of 2.3 cm internal diameter (101.9 cm³ of internal volume). The reactor temperature was controlled in a temperature-programmable oven. Two SS304 cylinders with 1.0 liter of internal volume were used as liquid collecting tanks coming from first or second stage treatment. The 2.5 MPa of N₂ backpressure was applied to the reactor system to maintain the system pressure above the saturated pressure of chemical solutions. Ten grams of dry biomass sample were packed into the reactor, soaked with chemical solution and left during overnight. To carry out the reaction, oven operation was set the preheating time within 15 minutes. During preheating time, #1 and #2 collecting tanks were pressurized and equalized by N₂ gas at 2.5 MPa.

2-1. Two-stage Operation

After completion of the first stage, there was a 10 minute temperature shift stage. During the temperature shift stage, sulfuric acid was pumped to the reactor with no additional washing or neutralization step. In the second series of runs, the biomass feedstocks were subjected to sequential two-stage treatment without intermittent sample taking. At the beginning of the second stage, the output effluent was switched into the second liquid collecting tanks which were switched by a 3-way valve. At the completion of a run, the reactor was pumped with water to remove the residual sugar and ammonia trapped in the treated biomass. Reaction conditions of

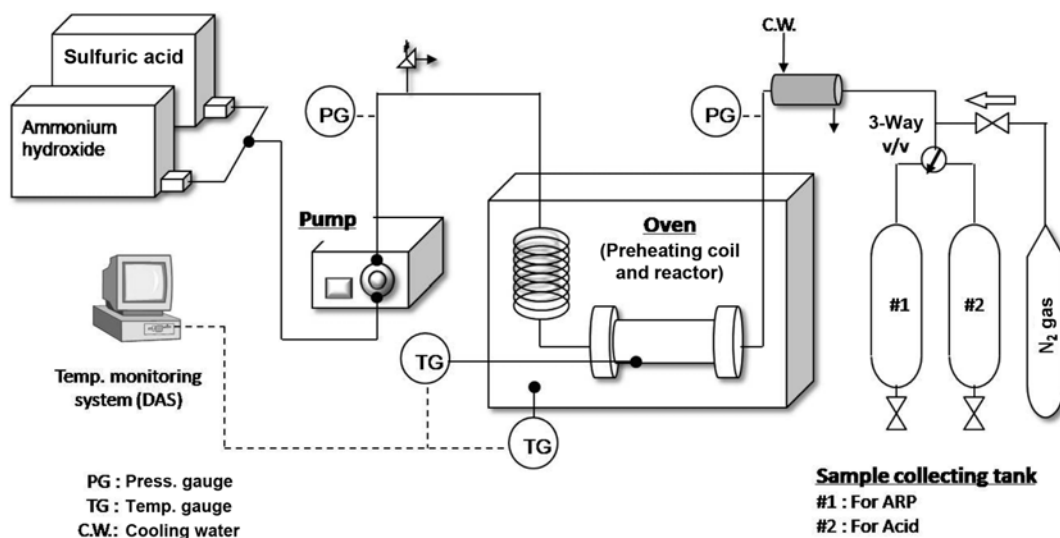


Fig. 1. Experimental set-up of two-stage percolation.

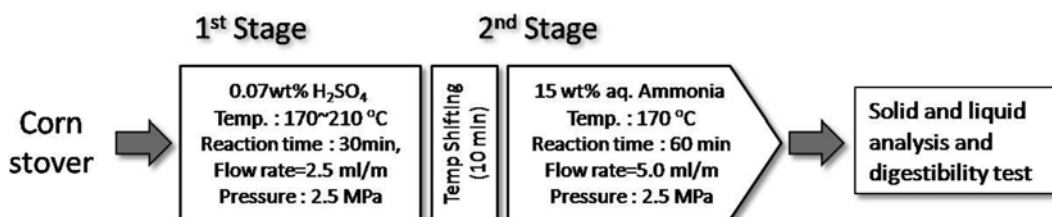


Fig. 2. Experimental conditions. Cellulase enzyme (Spezyme CP, Lot 301-00348-257, activity: 31.2 FPU), 60 or 15 FPU/g glucan, β -glucosidase supplement (Sigma, Cat No.G-0395, 30 IU/g glucan), pH 4.8, 50 °C, 150 rpm.

two-stage processing are summarized in Fig. 2. The wet solids discharged from the reactor were separated into two portions. One was dried by oven or by moisture analyzer for measurement of weight loss and further subjected to composition analysis. The other was used in the enzymatic digestibility test.

3. Digestibility Test

The enzymatic digestibilities of corn stover and α -cellulose were determined in duplicate according to the NREL Chemical Analysis and Testing Standard Procedure (CAT) [32]. The conditions of the enzymatic digestibility tests were pH 4.8 (0.05 M sodium citrate buffer) on a shaker bath agitated at 150 rpm at 50 °C. Enzyme loading of 15 and 60 FPU of Spezyme CP/g of glucan supplemented with 37 IU of β -glucosidase was used. The initial glucan concentration was 1% (w/v) based in 100 ml of total liquid and solid. The 250 ml screw-capped Erlenmeyer flasks containing the enzyme hydrolysis preparations were placed in an incubator shaker (Model Innova 4080, New Brunswick Scientific, Edison, NJ). Samples were taken periodically at appropriate sampling times (6, 12, 24, 48, 72, and 96 h) and analyzed for glucose and cellobiose content using HPLC. Total glucose content after 72 h of hydrolysis was taken to calculate of the enzymatic digestibility. α -Cellulose, substrate, and enzyme blanks were run in parallel as controls. Hydrolysis of α -cellulose was expected to be in the range of 92.0-95.0% as verification standard.

Enzyme digestibility was defined and calculated as follows:

$$\text{Enzyme digestibility [\%]} = \frac{\text{Total released glucose} \times 0.9}{\text{Initial glucan loading}} \times 100$$

0.9 is the conversion factors of glucose to equivalent glucan.

4. Analytical Methods

Solid analyses of carbohydrates and lignin were performed following the procedures of NREL Chemical Analysis and Testing Standard Procedures [32]. Each sample was analyzed in duplicate. The moisture contents of solid samples were measured by infrared moisture balance (Denver Instrument, IR-30). The sugars in the liquid samples were determined after secondary acid hydrolysis to account for the oligomer content. The conditions of the secondary hydrolysis

were 4 wt% sulfuric acid and 121 °C for one hour. For the lignin analysis, the hydrolysis solution was vacuum filtered, and the filtered hydrolyzed solid sample was dried and weighed. The dried samples were then combusted in a furnace at 575±25 °C for 16 h to determine the ash content. The difference of the two weights was taken as the acid insoluble lignin. The absorbance of the hydrolysis liquor in the aliquot obtained from the vacuum filter sample at 320 nm on a UV-Visible spectrophotometer measured the acid soluble lignin. Sugars in the hydrolysates were determined by HPLC using a Bio-Rad Aminex HPX-87P column coupled with a refractive index detector.

5. Crystallinity Index

Crystallinity of the corn stover samples was determined by X-ray diffraction using Rigaku DMAX diffractometer operated at 40 kV and 200 mA. Spectra were taken using θ - 2θ method. Duplicate samples were scanned at 1°/min from $2\theta=10$ - 30° with a step size of 0.01°. The water content retained in the sample is characterized by its scattering which has a maximum at $2\theta=28^\circ$. The crystallinity index (*CrI*) was calculated with the diffraction intensities, I_{002} at 002 peak position ($2\theta \approx 22.5^\circ$) and I_{18} at $2\theta=18^\circ$ (amorphous), as a ratio of $[(I_{002} - I_{18})/I_{002}] \times 100$ [33-35].

6. Statistical Analysis

A mean value and a standard error were calculated using JMP software version 5.0 (SAS Institute Inc.). Microsoft Office Excel 2007 was used to plot the results.

RESULT AND DISCUSSION

1. Acid Treatment

The effect of temperature in acid only treatment was tested keeping the reaction time at 30 minutes, the flow rate at 2.5 ml/min and the concentrations of sulfuric acid at 0.07 wt%. Compositional changes in liquids and solids, sugar recovery yields in liquid, and enzyme digestibilities of treated solid samples are summarized in Table 1. Among the tested temperatures, the reaction at 170 °C showed highest xylan yield in liquid. On the contrary, enzymatic digestibility of

Table 1. Effect of temperature on composition in acid only pretreatment^a

Temp. [°C]	Solid				Liquid		Total		Yield in liquid		Digestibility ^d	
	S.R. ^b [%]	Lignin ^c [%]	Glucan [%]	Xylan [%]	Glucan [%]	Xylan [%]	Glucan [%]	Xylan [%]	Glucan [%]	Xylan [%]	60 FPU [%]	15 FPU [%]
Untreated	100	17.6	37.5	20.8	-	-	37.5	20.8	-	-	21.2	16.1
170	56.6	14.1	35.3	1.7	2.6	18.1	37.9	19.8	7.0	87.2	86.4	80.4
180	55.6	12.6	34.9	1.6	2.7	17.3	37.6	19.0	7.2	83.3	87.2	81.8
190	54.7	12.6	34.9	0.9	3.6	15.9	38.5	16.8	9.5	76.6	85.6	84.3
200	53.5	13.3	34.0	0.7	3.3	12.3	37.4	13.0	8.8	59.4	89.9	88.1
210	49.8	12.5	31.9	0.2	4.8	8.5	36.7	8.7	12.7	40.9	87.3	87.2
220	49.2	14.7	29.6	0.1	5.3	5.6	34.9	5.7	13.9	27.0	91.3	90.3

^aData in the table based on the oven dry untreated biomass; Pretreatment conditions: Acid-0.07 wt% of sulfuric acid, 2.5 ml/min, 30 min, 2.5 MPa; ARP-15 wt% of ammonia, 5.0 ml/min, 60 min, 2.5 MPa; •All reactions are carried out in a Bed-Shrinking Flow-Through (BSFT) Reactor

^bS.R. stands for solid remaining after reaction

^cAcid insoluble lignin

^dDigestibility at 72 h, enzymatic hydrolysis conditions: 60 or 15 FPU/g glucan, pH 4.8, 50 °C, 150 rpm

The data in the table show the mean value (n=2; SE<1.0% for SR, SE<0.2% for lignin; SE<0.3% for glucan and xylan in solid and liquid, SE<2.1% for digestibilities, SE: standard error)

170 °C-treated solid yielded lower than other temperatures, which were 86% and 80% at 60 FPU/g-glucan and 15 FPU/g-glucan, respectively. As the treatment temperature increased, glucan and xylan removal from the solid increased, but higher decomposition of xylan in liquid was observed as reported in many literatures [36,37]; i.e., 220 °C of reaction temperature recovered only 27% of xylan in untreated corn stover even though nearly complete hydrolysis of xylan in solid occurred. The compositional analysis data in the Table 1 showed that acid treatments at 170-180 °C attained xylan recovery of 80-86%. More than 90% of xylan was recovered as oligomeric sugar form. At lower temperature range between 170 °C and 180 °C, the accountability of carbohydrate (sugar content in solid+that in liquid) was nearly 100% for glucan, and 91-95% for xylan showing no decomposition of glucose and about 5-9% decomposition of xylose. However, above 210 °C, about 5% of cellulose was hydrolyzed and severe decomposition of xylose (>50%) was observed. Delignification by dilute sulfuric acid treatment was in 17-29% range.

The 72-h digestibilities of solid samples treated at various temperatures are also shown in Table 1. Interesting trend was observed in the digestibilities with 60 and 15 FPU/g-glucan enzyme loadings. The difference of enzymatic digestibilities with two different enzyme loadings of solids treated at lower temperature was higher than the difference of solids treated at higher temperature. In other words, the enzyme digestibilities of solids samples treated above 200 °C with 15 FPU/g-glucan loading approached to those with 60 FPU/g-glucan loading. Acid treatment at 220 °C yielded the highest enzyme digestibility, although the lignin content in the treated solid increased from those of other acid treated solids. Unlike lignin contents, there was a linear relationship between xylan removal from the solids and reaction temperature. It collectively indicated that the lignin may not be the sole factor in determining enzyme hydrolysis yield of acid-treated solid samples. Considering lignin content and enzymatic digestibilities, they had a linear relationship except for the high temperature range; lignin content of the acid-treated solid gradually decreased as temperature increased to 190 °C, then lignin content increased again above 200 °C. Previous study also reported that the same unusual behavior was observed in hot-water treat-

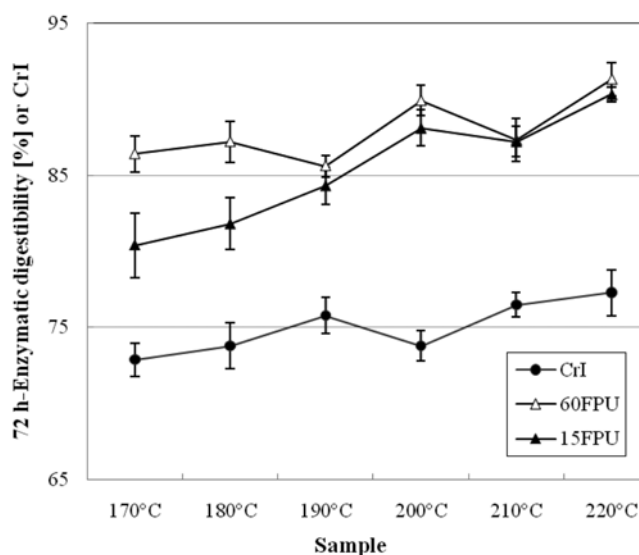


Fig. 3. Effect of temperature on enzymatic digestibility and *CrI* of acid-treated solids. Pretreatment conditions: 0.07 wt% of sulfuric acid, 2.5 ml/min, 30 min, 2.5 MPa. Digestibility is the percentage at 72 h; enzymatic hydrolysis conditions: 60 or 15 FPU/g glucan, pH 4.8, 50 °C, 150 rpm.

ment at high temperature [25-27]. We hypothesized that the lignin solubilized by sulfuric acid may undergo lignin re-polymerization reaction at high temperature; thus it increased remaining lignin in the treated solid.

The relation between *CrI* and the relevant digestibility of acid-treated solid with 60 and 15 FPU/g-glucan is summarized in Fig. 3. As the treatment temperature increased, higher *CrI* was generally calculated since *CrI* means simply a ratio of crystalline portion and amorphous portion in the biomass sample. The *CrI* of untreated corn stover was measured to be 57.7 (data not shown), which was much lower than those of acid-treated solid samples because untreated corn stover contained higher content of hemicellulose and lignin. The primary effect of acid-treatment was hemicellulose removal

Table 2. Effect of temperature on composition in acid-ARP pretreatment^a

Temp. [°C]	Solid				Liquid		Total		Yield in liquid		Digestibility ^d	
	S.R. ^b [%]	Lignin ^c [%]	Glucan [%]	Xylan [%]	Glucan [%]	Xylan [%]	Glucan [%]	Xylan [%]	Glucan [%]	Xylan [%]	60 FPU [%]	15 FPU [%]
Untreated	100	17.6	37.5	20.8	-	-	37.5	20.8	-	-	21.2	16.1
170	41.6	4.5	35.3	1.1	2.4	17.9	37.4	19.0	6.3	86.1	90.4	88.7
180	41.9	4.0	34.8	1.1	2.6	16.7	37.4	17.7	6.9	80.1	91.2	85.2
190	42.2	3.8	34.5	1.0	3.8	16.3	38.3	17.3	10.1	78.2	87.6	76.3
200	41.4	5.2	33.7	0.6	4.1	13.5	37.8	14.1	10.8	64.9	87.0	68.2
210	41.6	7.1	31.3	0.3	5.5	9.8	36.8	10.1	14.6	47.1	96.4	84.1

^aData in the table based on the oven dry untreated biomass; Pretreatment conditions: Acid-0.07 wt% of sulfuric acid, 2.5 ml/min, 30 min, 2.5 MPa; ARP-15 wt% of ammonia, 5.0 ml/min, 60 min, 2.5 MPa; •All reactions are carried out in a Bed-Shrinking Flow-Through (BSFT) Reactor

^bS.R. stands for solid remaining after reaction

^cAcid insoluble lignin

^dDigestibility at 72 hr, enzymatic hydrolysis conditions: 60 or 15 FPU/g glucan, pH 4.8, 50 °C, 150 rpm

The data in the table show the mean value (n=2; SE<1.0% for SR, SE<0.2% for lignin; SE<0.3% for glucan and xylan in solid and liquid, SE<2.2% for digestibilities, SE: standard error)

from the solid, which resulted in the increase of cellulosic portion, also meaning the increase of crystalline portion in treated solid samples. As the temperature increased above 190 °C, *CrI* decreased, and 60 FPU digestibilities slightly fluctuated corresponding to the *CrI*. Unfortunately, we found there was no direct relationship between *CrI* and digestibility.

2. Acid-ARP Treatment

The effect of temperature in two-stage treatment using dilute sulfuric acid and ammonium hydroxide (ARP) was tested keeping the reactions of ARP at 60 minutes, 170 °C, 5.0 ml/min, and 15 wt% ammonium hydroxide. In this series of runs, the corn stover was subjected to sequential two-stage treatment without intermittent sample taking. In the first stage, 0.07 wt% of sulfuric acid, 2.5 ml of flow rate, and 30 min of reaction time were applied. Five different temperatures covering 170-210 °C were applied in acid treatment stage (1st stage). Compositional changes in liquids and solids, sugar recovery yields in liquid, and enzyme digestibilities of treated solid samples are also summarized in Table 2.

The composition of two-stage treated samples showed that ARP treatment in the second stage removed mainly lignin without further decompositions of carbohydrates. Two-stage treatment in the range of 170-180 °C yielded 80-86% of xylan recovery. On the other hand, over the higher temperature range of 170-210 °C, the accountability of carbohydrate was nearly 100% for glucan, and 68-91% for xylan. No decomposition of glucose and about 9-42% decomposition of xylose were observed. Overall, glucan content in the solid samples treated below 200 °C was well preserved throughout the two-stage processing, while dilute-acid treatment above 200 °C induced noticeable decomposition of glucan and severe decomposition of xylan (9-42%) during two-stage treatment. Lignin removal after two-stage processing was in 60-78% range. As seen in acid-only treatment, an interesting behavior was also observed in the two-

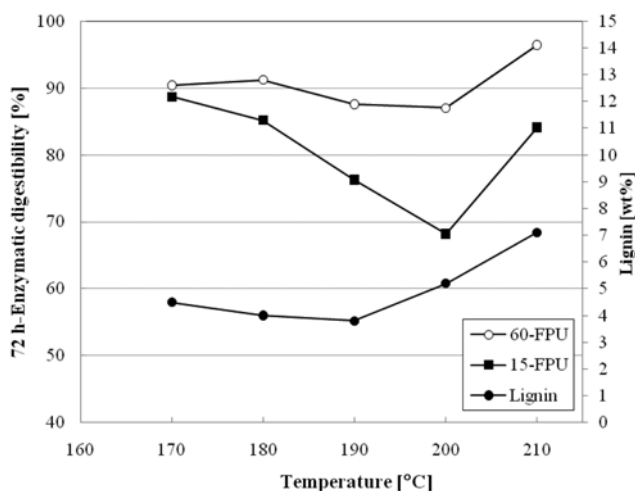


Fig. 4. Enzymatic digestibility of acid-ARP treated samples at different enzyme loading. All sugar and lignin content based on the oven-dry untreated biomass. Pretreatment conditions: Acid-0.07 wt% of acid, 2.5 ml/min of flow rate, 2.5 MPa. ARP-170 °C, 15 wt% NH₃, 5.0 ml/min of flow rate, 60 min, 2.5 MPa. Enzymatic hydrolysis conditions: 72 h, 60 or 15 FPU/g glucan, pH 4.8, 50 °C, 150 rpm. The data in the table show the mean value (n=2; SE<0.2% for lignin, SE<2.2% for digestibilities, SE: standard error).

stage treatment: lignin content in the treated solid gradually increased from 4% to 7% as temperature increased from 190 °C to 210 °C. Between 170 °C and 190 °C, lignin content decreased as temperature increased, while 210 °C treatment in the first stage drastically increased lignin content, although the same reaction conditions for ARP treatment in the second stage were used for all temperature ranges. It was concluded that high temperature dilute-acid treatment in the first stage somehow affected the extent of delignification in the following stage of ARP. We speculated that sulfuric acid hydrolyzed lignin during high temperature treatment, and then high temperature caused re-polymerization reaction or solubilized lignin,

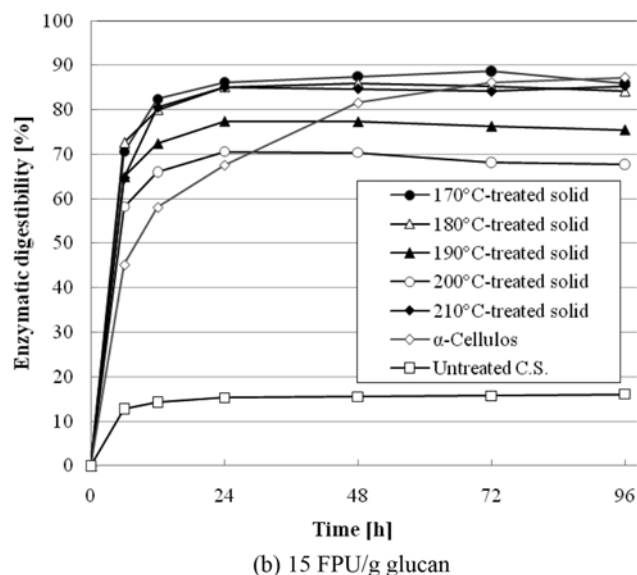
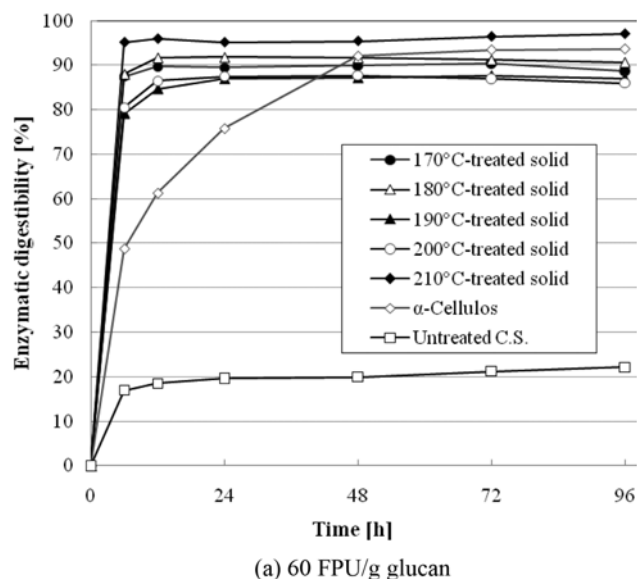


Fig. 5. Relationship between lignin content and 72-h enzymatic digestibility of two-stage treated solid samples. Pretreatment conditions: 0.07 wt% of sulfuric acid, 2.5 ml/min, 30 min, 2.5 MPa. ARP-170 °C, 15 wt% NH₃, 5.0 ml/min of flow rate, 60 min, 2.5 MPa. Digestibility is the percentage at 72 h; enzymatic hydrolysis conditions: 60 or 15 FPU/g glucan, pH 4.8, 50 °C, 150 rpm. The data points in the graph show the mean value (n=2; SE<2.2% for digestibilities, SE: standard error).

which became very resistant to alkaline extraction of lignin. The relation between lignin content in solid and relevant digestibility with 60 and 15 FPU/g-glucan for acid-ARP treatment solids is summarized in Fig. 4. In contrast to acid-only treatment, 72-h enzyme digestibilities with both 60 and 15 FPU/g-glucan enzyme loadings were reduced in the range of temperature between 170 °C and 190 °C, and a reversed trend was observed above 190 °C treatment temperature; 72-h digestibility of samples with 60 FPU/g-glucan was (1) not improved nor changed significantly between 170 °C and 180 °C, (2) slightly reduced between 180 °C and 200 °C, and then (3) enhanced significantly at 210 °C. Similar trend was observed in the case of 15 FPU/g-glucan loading, but 72-h digestibility of 210 °C-treated solid with 15 FPU/g-glucan was not as high as that with 60 FPU/g-glucan loading. According to a previous study, lignin condensation and re-polymerization at high temperature may form insoluble substrates, which could interfere with the enzymatic reaction of cellulose [9,38-40].

The digestibility test results of two-stage treated solid samples are shown in Fig. 5. The 72-h digestibilities after the two-stage treatments were 88-96% at all reaction conditions with 60 FPU/g glucan of enzyme loading. With 15 FPU/g glucan, the digestibility was 68-89%, which was substantially lower than that with 60 FPU/g glucan.

CONCLUSION

A two-stage percolation process was effective not only for fractionation but also for pretreatment of corn stover. Two-stage percolation processes fractionate corn stover into constituents such as cellulose, xylan, and lignin. The best operating temperature condition, on the basis of fractionation aspect and digestibility, was 170 °C for acid-ARP pretreatment. Under this condition, 95% of the xylan hydrolyzed, of which 86% was recovered, and 81% of lignin removal was achieved. The treated biomass contains 84.9% glucan, 2.6% xylan and 10.8% lignin. The digestibility of acid-ARP treated sample was 88.7% with 15 FPU/g glucan. We found that lignin increased unusually at certain high temperature and lignin caused an effect on cellulose hydrolysis by cellulase. This increase of remaining lignin was speculated to be due to lignin recondensation and/or soluble lignin-glucose bonding formed as these became insoluble. It was also observed that its quantity is related to the inhibition of enzymatic hydrolysis. No correlation between CrI and enzymatic digestibility was found.

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