# Physical and Chemical Characterizations of Corn Stover from Leading Pretreatment Methods and Effects on Enzymatic Hydrolysis

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ABSTRACT: Lignocellulosic biomass is difficult for hydrolysis due to its recalcitrance on chemical compositions and physical properties. The 3D microstructure of corn stover by X-ray tomography showed the visualized inner pore and fiber structure, indicating closed spaces and density distribution. Dilute sulfuric acid pretreatment on corn stover stems resulted in high lignin content, which led to slow hydrolysis at the beginning stage of enzymatic hydrolysis. The low crystallinity and high porosity of steam explosion pretreated stems exhibited enzymatic hydrolysis faster at the initial stage, while lower final glucan conversion was observed after steam explosion pretreatment due to the lack of lignin removal compared with sulfuric acid pretreatment. The removal of

most lignin and increased porosity in the inner biomass by aqueous ammonia pretreatment led to the fastest hydrolysis and highest glucan conversion. Leaves and husks pretreated by sulfuric acid exhibited a higher cellulose digestibility and sugar release rate than the stems due to their lower lignin content. The results suggested that lignin composition played a more important role than porosity and crystallinity during the hydrolysis of lignocellulose.

KEYWORDS: Lignocellulose, Pretreatment, Thermal gravimetric analysis, Crystallinity, Porosity, X-ray tomography
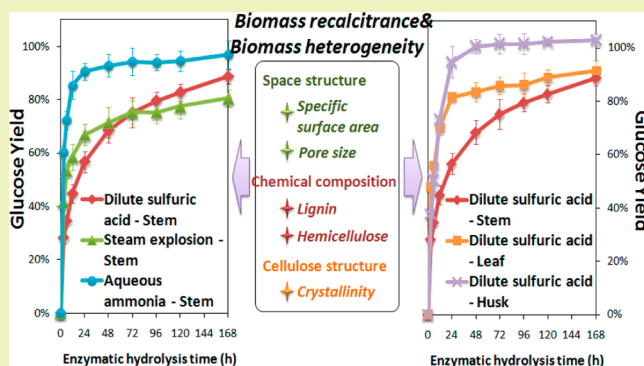
INTRODUCTION

Pretreatment is a prerequisite processing step for producing biofuels and other biobased products from lignocellulosic biomass. Various pretreatment methods including physical pretreatment, chemical pretreatment, physicochemical pretreatment, and biological pretreatment have been developed to overcome biomass recalcitrance during the last decades.[1−3] The goal of the pretreatment process is to disrupt the lignin—carbohydrate complex structure and decrease the structural and compositional impediments to improve enzymatic hydrolysis and increase fermentable sugar yields from cellulose or hemicellulose.[2,3] Various pretreatments can be put into three categories: low pH, neutral pH, and high pH.[4] Typically, low pH pretreatments (e.g., dilute acid pretreatment and steam explosion) remove most of the hemicellulose and degrade a large portion of lignin.[5,6] Near neutral pH pretreatment such as pH-controlled liquid hot water removes much of the hemicellulose but leaves most of the cellulose and lignin intact.[3] High pH pretreatments (e.g., aqueous ammonia and ammonia fiber expansion) remove a large fraction of lignin and some hemicellulose.[7,8]

Most of the pretreatment technologies could improve accessible surface area (ASA) for cellulase.[9,10] ASA is related with several factors altered by pretreatments directly, including porosity structure factors (pore size and volume and specific surface area),[11] chemical composition factors (lignin, hemi-

cellulose, and acetyl group),[12,13] and cellulose structure factor (cellulose crystallinity).[14] A previous study showed no single factor absolutely dominated ASA and glucose yields from enzymatic hydrolysis of various pretreated solids.[15] Furthermore, ASA may vary with tissue specificity (e.g., leaf and stem). The tissue specificity of corn stover affected enzyme digestibility of cellulose in both compositions and physical structures.[16,17] Thus, we are still lacking the in-depth understanding into the effects of these leading pretreatment methods on biomass features. In addition, key factor parameters for enzyme digestibility in leading pretreatments have never been systematically characterized.

Plant cell wall recalcitrance is in multiple scales, including several orders of magnitude encompassing both macroscopic and microscopic barriers.[18,19] The investigation on the microstructure by light and electron microscopy is difficult due to surface artifacts caused by the thermal—physical—chemical treatment during biomass sample preparation. Furthermore, it is hard to predict the cellulase accessibility of feedstock with simple surface characteristics because it is a segment of the integral cell wall structure. X-ray micro-computed tomography as a powerful and promising tool was

Table 1. Pretreatment Methods, Conditions, and Severity[a]

pretreatment methods	pretreatment conditions	severity, Log R_o	ref
dilute sulfuric acid (DA)	180 °C, 30 min, 1% H_2SO_4 (w/w), 10% solids	3.83	23
oxalic acid (OA)	180 °C, 30 min, 1% oxalic acid (w/w), 10% solids	3.83	23
steam explosion (SE)	200 °C, 10 min, 75% solids	3.94	24
liquid hot water (LHW)	200 °C, 30 min, 10% solids	4.42	–
aqueous ammonia (AA)	180 °C, 30 min, 20% ammonia (w/w), 10% solids	3.83	8

[a]Severity parameters included only time and temperature; Log R_o= Log $[t \times \exp((H\text{-}R)/14.75)]$, where t is time, min; H is pretreatment temperature, °C; and R is a reference temperature, 100 °C.

widely used to investigate the 3D structure of materials.[20,21] X-ray microcomputed tomography characterizes the feedstocks without destructiveness through penetrative 3D imaging of the internal microstructure at submicron resolution. In addition, fully automated X-ray microcomputed tomography in three dimensions allows virtual cuts through the sample in any direction, and so the information about the distribution and location of the fibers, fiber orientation and size, porosity structure, and other compositions distribution can be obtained using 3D image analysis.[22]

■ MATERIALS AND METHODS

Materials. Corn stover (CS) biomass used for this study was obtained from the suburb of Tianjin, China, and stems, leaves, and husks were separated. Materials were dried to the moisture content of 5−10% at room temperature and then was milled by knife mill and passed through a screen of 2 mm. The compositions of different CS fractions were determined following the Laboratory Analysis Protocol (LAP) of the National Renewable Energy Laboratory (NREL), Golden, CO, U.S.A.

Avicel (Sigma-Aldrich, Shanghai, China) consisting of 98% glucan of dry matter was used as the pure cellulose. The protein concentration and enzyme activity of the enzymes are as follows: Accellerase 1500 (Genencor, Wuxi, China, 89 mg proteins/mL, 77 FPU/mL) and Novozyme 188 (Sigma-Aldrich, 67 mg proteins/mL, 850 CBU/mL).

Pretreatment and Enzyme Digestibility of Corn Stover Biomass. Dilute sulfuric acid (DA), oxalic acid (OA), liquid hot water (LHW), and aqueous ammonia (AA) pretreatment were conducted with a tube reactor heated by an oil bath, as previous described.[23] Steam explosion (SE) pretreatment was conducted with a 15 L steam explosion reactor as previous described.[24,25] The pretreatment methods and conditions are listed in Table 1. The pretreated slurry was pressed through a filtration cloth to separate pretreated solids from free liquids. The solid fractions were washed with distilled water until the filtrate was neutral and then dried at room temperature until the moisture was less than 10% and subjected to analysis of physicochemical properties and enzymatic hydrolysis.

The pretreated solids were hydrolyzed by commercial enzymes mixtures. The cellulase mixture consisted of Accellarase 1500 (15 FPU/g cellulose, equivalent to 17.5 mg proteins/g cellulose) and Novozyme 188 (30 CBU/g cellulose, equivalent to 2.4 mg proteins/g cellulose). The cellulose loading for enzymatic hydrolysis was kept at 3.0% by weight. The reaction was carried out at pH 4.8 (5 mM citrate buffer), 50 °C, and 200 rpm agitation for 168 h. Each hydrolysis was conducted in a 250 mL Erlenmeyer flask with 100 g of total saccharification mixture. The sugars in the hydrolysate were analyzed by HPLC equipped with a Bio-Rad Aminex 87H column at 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min.

Thermal Gravimetric Analysis. The thermal gravimetric analysis (TGA) was conducted on a TA Instruments Q600 SDT thermal analyzer (U.S.A.). The samples were heated by steady 20 °C/min increase from 25 to 800 °C in a nitrogen medium.

Biomass Crystallinity Analysis. The biomass crystallinity was measured using a D8 Fucos X-ray diffractometer (Bruker AXS Co., Germany). Samples were scanned at a speed of 2°/min, range from 2θ

= 10−40°, and with a step size of 0.02° at room temperature by positioning the samples on a quartz sample holder using a Rigaku Miniflex diffractometer in conjunction with a Cu Kα radiation source (k = 0.154 nm) operated at 30 kV. Biomass crystallinity as expressed by the crystallinity index (CrI) was determined as follows

$$\text{CrI} = 100 \times [(I_{002} - I_{amorphous})/I_{002}]$$

where, I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at about 2θ = 22.5, and $I_{amorphous}$ is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at about 2θ = 18.7.

Porosity Property Analysis. The Brunauer−Emmett−Teller (BET) surface area was determined by N_2 physisorption using an Intelligent 3H-2000A automated system according to the multipoint BET method (Beishide Instrument-S&T Co., Ltd., China). Air-dried samples were oven-dried at 75 °C for 8 h to minimize structural changes prior to BET analysis. BET analysis was carried out for a relative vapor pressure of 0.04−0.2 at −196 °C. Average pore diameter and total pore volume were determined from N_2 desorption at relative vapor pressure of 0.01−0.99 following a BJH model.

3D Microstructures Analysis. The untreated and pretreated fibers (∼1 mm) were air-dried and subjected to tomography. The 3D microstructure of the biomass was conducted by a X-ray 3D microscope (Nanovoxel-2100, Sanying Precision Engineering Research Center, China). During microtomography data acquisition, the sample was set on a rotation stage. An X-ray irradiated the sample. The X-ray source has a focal spot the size <1 μm. The sample was scanned at an average tube voltage of 50 kV, a current of 40 mA, and an exposure time of 1500 ms per image. A rotation step size of 0.36° was used. These images were then gathered to virtually reconstruct the internal sample microstructure using a filter back projection algorithm. The obtained data sets represent a 3D map of the relative absorption coefficient of the sample constituents. Therefore, the different constituents (air and natural lignocellulosic fibers) were represented in the images by different gray levels, where the pores were in dark and fibers are bright. The size of the observed cylinder was 1 mm × 1 mm (diameter × height). The resolution was 0.9 μm × 0.9 μm × 0.9 μm per voxel.

■ RESULTS

Chemical Composition. The composition of each corn stover fraction is listed in Table 2. This result is similar to

Table 2. Compositions for Different Corn Stover Fractions[a]

component (%, dry weight)	stems	leaves	husks
glucan	41.2 (1.1)	35.6 (0.7)	39.1 (0.8)
xylan	18.9 (0.6)	23.5 (1.0)	26.9 (0.9)
arabinan	2.1 (0.2)	5.3 (0.1)	6.1 (0.4)
acetyl	5.1 (0.3)	3.3 (0.2)	4.4 (0.3)
acid-insoluble lignin	15.8 (0.4)	13.1 (0.5)	10.7 (0.3)
extractives	12.9 (0.7)	10.2 (0.5)	7.2 (0.5)
ash	2.6 (0.2)	5.6 (0.1)	3.4 (0.1)
total	98.6	96.6	97.8

[a]Standard deviation is representative of three replicates.

Table 3. Compositions for Different Pretreated Corn Stover[a]

fractions	pretreatments	composition (%, dry weight)			
		glucan	xylan	acid-insoluble lignin	total
stems	DA	51.5 (1.5)	−	43.3 (2.4)	94.8
	OA	59.3 (1.2)	1.3 (0.2)	33.2 (2.1)	93.8
	SE	62.3 (1.3)	7.3 (0.4)	24.4 (1.4)	94.0
	LHW	58.9 (1.5)	5.1 (0.3)	31.6 (1.8)	96.6
	AA	70.4 (2.1)	19.5 (1.0)	5.6 (1.3)	95.5
leaves	DA	58.8 (1.6)	−	40.1 (1.8)	98.9
husks	DA	53.4 (1.3)	−	36.1 (1.6)	89.5

[a]Values are means of duplicate experiments.

previous study.[16] The compositions of different fractions after pretreatment are in Table 3. DA-L had the highest glucan content and DA-S had the highest lignin content. Furthermore, the lignin recovery for most pretreatments was more than 100% due to the formation of pseudo lignin.[26,27] The results of the compositions of stems from different pretreatments were consistent with previous reports.[8,23−25]

Thermal Gravimetric Analysis. The derivative thermal gravimetry (DTG) curves of different fractions of corn stover biomass subjected to various pretreatments are shown in Figure 1. The DTG profiles of all samples exhibited initial peaks between 30 and 130 °C, which correspond to the vaporization of water. The curve for untreated corn stover biomass exhibited two decomposition steps, and the decomposition peaks temperatures were at 290 and 340 °C, which correspond to the pyrolysis of hemicellulose and cellulose, respectively.[28,29] The heights of the peaks at 290 °C were different and decreased from husk > leaves > stem, which follow the hemicellulose contents in Table 2. It is believed that pyrolysis decomposition profile is dependent on its lignocellulosic composition.[30] The changes in pyrolysis in the pretreated samples reflect removal of structural cell wall components.

Crystallinity. Crystallinity index (CrI) of untreated and pretreated corn stover is shown in Table 4. The CrI of untreated corn stover fractions increased obviously by husk < leaves < stem, indicating husk and leaves contain more proportion of hemicellulose and amorphous cellulose. The CrI of husk and leaves was significantly increased after DA, because hemicellulose and amorphous cellulose was broken down.[4] Table 4 showed that all pretreatments increased the CrI of pretreated stem except SE. SE-S exhibited the lowest CrI due to the lignin relocalization and serious degradation of the fibrillar structure by mechanical shear force effect at instantaneous decompression step.[24,25] The CrI of LHW-S increased by 15.6% compared with untreated stem, because most hemicellulose was removed and amorphous cellulose was depredated after LHW.[31] The stem pretreated by AA had the highest CrI among all pretreatments due to the removal of lignin and hemicellulose.[8]

Porosity Structures. ASA of biomass is a critical factor for cellulose digestibility, which is greatly related to porosity structure properties, such as specific surface area (SSA), average pore diameter (APD), and total pore volume (TPV).[32,33] The parameters including SSA, APD, TPV, and true density for untreated and pretreated corn stover are shown in Table 4. The SSA, APD, and TPV were relatively low for untreated corn stover fractions and increased from stem < leaves < husk. For different pretreatments, SAA, APD, and TPV were increased from AA < LHW < OA < DA < SE. Higher SSA often accompanied higher APD and TPV.

X-ray Tomography Revealing 3D Structure Changes of Pretreated Corn Stover. In order to investigate the internal microstructure changes after pretreatments, the untreated and pretreated samples were evaluated using three-dimensional microtomographic reconstructions in the present study (Figure 2). The lighter gray regions are enriched with cellulose fibers, whereas regions of the cell wall that were stained black/dark gray are porosity structures. As observed in Figure 2A, C, and E, the highly rigid and ordered plant cell structure of the untreated stem, leaves, and husk were clearly visible. The 3D image of the stem was taken from the epidermis of the stem, where vessel cells and tighter structures were observed than in the leaves and husks. A large number of loose mesophyll cells and larger intercellular space was observed in the leaves. Furthermore, the cell walls of the leaves were thicker than these of the stem and husk. The tissue structure of the husk is similar to that of the stem. After DA, major and ordered fiber structures were obviously altered and cannot be clearly observed (Figure 2B, D, and F). A large number of pores and fractures in DA-S, DA-L, and DA-H were observed. Figure 2G showed the 3D profile of AA-S. The fibers presented in most of the stem interior were separated and modified from the original structure of the biomass, and the cellulose macrofibers were also clearly observed. Furthermore, lots of meso- and macro-pores were formed during AA, which were obviously larger than those in DA-S and SE-S. This observation was opposite to the porosity parameter and certified the formation of some close pores and "bottle ink" structures, which reduced the overall nitrogen accessible porosity by the BET method, leading to underestimation of the total porosity.[19] The disrupted fibers of the stem after SE could be clearly observed in the micrometer scale (Figure 2H). It is interesting to note that many circle structures thicker than the ordinary cell wall were observed in SE-S. The diameter of these "steam explosion circles" is within 100 μm, and the height is about 50 μm. A similar structure was also observed in the hydrolyzed corn leaf and pith pretreated by liquid hot water.[17] The formation of the "steam explosion circles" may be due to the heterogeneity of the stem and instantaneous decompression during escape of steam with high pressure. Except for the "steam explosion circles", other fibers and pores of SE-S were homogeneous, and the boundaries of the fibers were irregular and fuzzy. At the instantaneous decompression step of SE, the particles were exploded into small pieces, and the crystalline structure of cellulose was disrupted. The internal space was enlarged, and the fibers were looser than those of DA-S.

Enzymatic Hydrolysis. In enzymatic hydrolysis of un-treated and pretreated corn stover, glucan conversion and glucose release rate were used to evaluate the enzyme digestibility performance in present study. The glucan
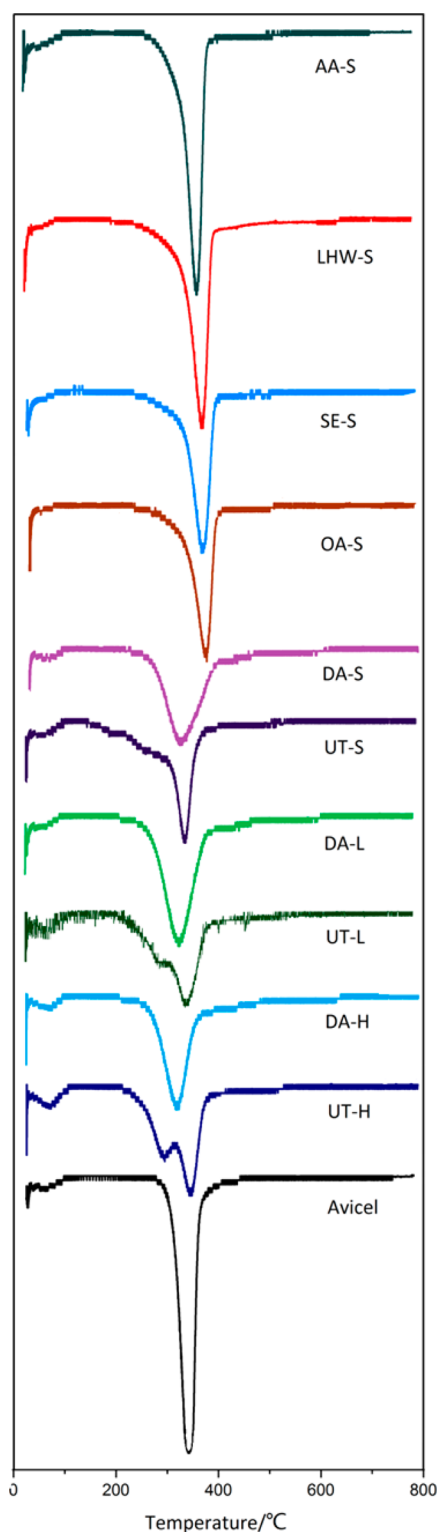
Figure 1. DTG curves of untreated and pretreated corn stover fractions.

Table 4. Crystallinity Index (CrI), Specific Surface Area (SSA), Average Pore Diameter (APD), Total Pore Volume (TPV), and True Density for Untreated and Pretreated Corn Stover[a]

	crystallinity index (%)	specific surface area (m²/g)	average pore diameter (nm)	total pore volume ($\times 10^{-2}$ mL/g)	true density (g/mL)
DA-S	49.46	4.41	36.31	3.32	1.45
OA-S	51.44	3.02	34.89	2.59	1.54
SE-S	43.80	4.73	42.47	3.52	1.46
LHW-S	53.76	2.88	35.28	2.59	1.45
AA-S	55.81	1.31	31.20	1.11	1.56
DA-L	47.46	4.79	24.14	2.89	1.54
DA-H	46.93	5.62	39.71	5.58	1.42
stems	46.52	0.78	15.10	0.29	1.45
leaves	35.72	1.01	17.04	0.43	1.24
husks	24.84	1.24	23.61	0.93	1.35

[a]Values are means of duplicate experiments (standard error <5%).

of pretreated stem increased from OA < SE < LHW < DA < AA with the digestibility time increasing from 72 to 168 h. The glucose release rate at 0−3 h of the pretreated stem increased from DA < LHW < OA < SE < AA (Table 5). It should be noticed that DA-S exhibited the lowest glucose release rate during the first 12 h of digestibility, while it exhibited the highest glucose release rate between 12 and 168 h.

The glucan conversions of untreated stem, leaves, and husk were 20.2%, 26.5%, and 28.3%, respectively. Leaves, husk, and stem were pretreated by dilute sulfuric acid and subsequently digested by cellulase (Figure 3B). DA-H exhibited the highest final glucose yield, followed by DA-L and DA-S, which showed the same trend as the previous study.[34] The glucose release rate for DA-L and DA-H was much higher than that for DA-S. The initial glucose release rate at 0−3 h increased from stem < husk < leaves after DA (Table 5).

■ DISCUSSION

Chemical composition of feedstocks is the important factor affecting enzyme digestibility. Stems exhibited higher glucan and lignin content than leaves and husks due to the abundant secondary tissues and thick wall cells. The higher acetyl and lignin content lead to low enzyme digestibility of stems compared with leaves and husks.[16,35] The enzyme digestibility of DA pretreated corn stover fractions indicated that lignin content affected glucan conversion at the beginning of enzyme digestibility. The melted lignin played a negative role at the beginning stage of hydrolysis due to absorbed cellulase reducing the active enzyme quantity. The removal of xylan did not obviously improve glucan conversion of DA-S at the beginning 48 h digestibility compared with that of OA-S. Low lignin content in SE-S and LHW-S compared with DA-S and OA-S should be one of the potential reasons for higher glucan conversion of SE-S and LHW-S at 72 h.

Cellulose crystallinity determined by X-ray diffraction is also believed to be closely related with the enzyme digestibility of biomass.[36] It is noticeable that the low CrI of SE-S led to higher glucan conversion than DA-S, OA-S, and LHW-S at the first 72 h digestibility. However, the highest glucan conversion was observed for AA-S with the highest CrI, while the relatively low CrI of DA-S resulted in the lowest glucan conversion in the first 72 h of hydrolysis. The results indicated that lignin content was
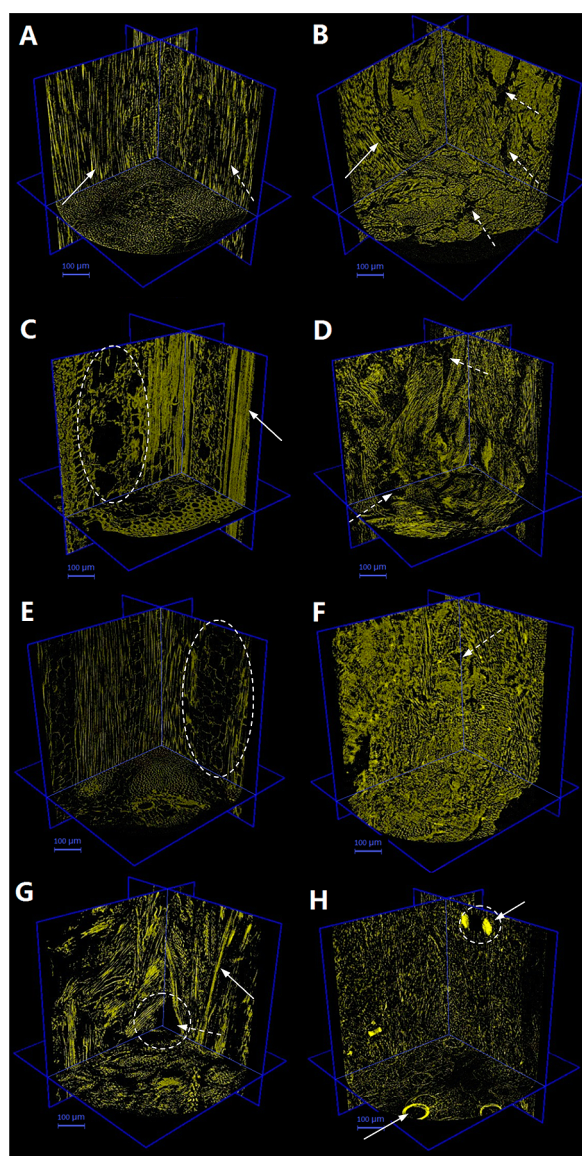
conversion of pretreated stem increased from DA < LHW < OA < SE < AA during the first 12 h in hydrolysis (Figure 3A). It is interesting to note that the glucan conversion of DA-S increased rapidly between 12 and 72 h digestibility, and it surpassed that of OA-S at 48 h as well as SE-S and LHW-S at 72 h. In addition, the glucan conversion of LHW-S surpassed that of OA-S at 12 h and SE-S at 72 h. The glucan conversion

Figure 2. 3D Microstructure imaging of different corn stover fractions. (A) untreated stem, (B) DA pretreated stem, (C) untreated leaf, (D) DA pretreated leaf, (E) untreated husk, (F) DA pretreated husk, (G) AA pretreated stem, and (H) SE pretreated stem.

Figure 3. Enzyme kinetics of corn stover pretreated by leading pretreatments. DA-S, dilute sulfuric acid pretreated stem; OA-S, oxalic acid pretreated stem; SE-S, steam explosion pretreated stem; LHW-S, liquid hot water pretreated stem; AA-S, aqueous ammonia pretreated stem; DA-L, dilute sulfuric acid pretreated leaves; and DA-H, dilute sulfuric acid pretreated husk.

the preferential factor affecting initial digestibility compared with crystallinity.

Porosity properties have been considered very important to lignocellulose digestibility. As the pretreated biomass was dried at room temperature, the loss of water led to pore collapse at the cell wall due to shrinkage of the cross-linking of microfibrils,[37] reorganization of the hydrogen bond in cellulose,[38] and fiber hornification.[39] The surface area of wet biomass determined by the methods for wet materials (e.g., solute exclusion[40] and NMR thermoporometry[41]) was several folds more than that of dry biomass.[38] Herein, pore collapse of pretreated biomass was taken into consideration to investigate its effect on enzyme digestibility. BET nitrogen adsorption is a method to determine pore parameters of dry materials. The BET results showed that leading pretreatments obviously increased SSA, APD, and TPV of pretreated corn stover fractions. However, there is no evident relationship between pore size and glucan conversion. AA led to the lowest SSA,
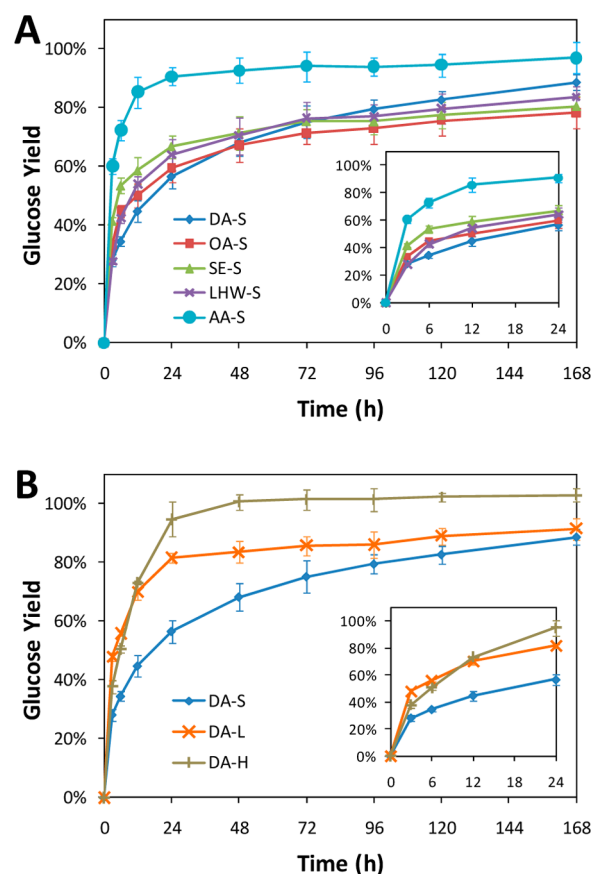
APD, and TPV but the highest enzyme digestibility among the five pretreatments. This indicates that the surface area and pore size of dry biomass, or the pore collapse, are not the main factors to enzyme digestibility. A similar result also exhibited no significant relationship between pore volumes measured by the wet method and cellulose digestibility for DA pretreatment.[41] Another defect of surface determination methods is the confusion of the surface area of cellulose and other compositions. For example, the increased SSA of DA-S may be attributed to the increasing surface of the melting lignin.

3D Tomography was used to detect the inner structure of the dry biomass without cutting, and visualization of the inner structure was revealed *in situ* without any artifacts generated during the intricate sample preparation. In addition, 3D tomography could present the inner structure more readily than a 2D electron microscope. The fibril linkage, big pores, and big spaces in AA-S and the density distribution in SE-S were determined clearly in this study. This suggests the pore collapse was carried out at part of the fibrils and formed a "bottle neck" instead of being stuffed solid in AA-S. The inner pore of AA-S may be a reason for its high cellulose digestibility. The heterogeneous density in SE-S makes the porous region easy to hydrolyze but the condensed region hard to hydrolyze.

Table 5. Glucose Release Rate in Enzymatic Hydrolysis of Pretreated Corn Stover[a]

fractions	pretreatments	glucose release rate (g L^{-1} h^{-1})								
		0−3 h	3−6 h	6−12 h	12−24 h	24−48 h	48−72 h	72−96 h	96−120 h	120−168 h
stems	DA	2.02	1.43	0.52	0.30	0.15	0.10	0.05	0.04	0.02
	OA	3.35	1.13	0.42	0.16	0.10	0.05	0.03	0.02	0.01
	SE	4.15	1.40	0.36	0.16	0.05	0.04	0.01	0.02	0.01
	LHW	2.77	1.47	0.58	0.30	0.06	0.07	0.02	0.03	0.02
	AA	6.02	1.22	0.65	0.26	0.01	0.00	0.00	0.01	0.00
leaves	DA	4.29	1.60	0.56	0.24	0.05	0.07	0.00	0.00	0.02
husks	DA	3.78	1.78	0.88	0.54	0.07	0.01	0.00	0.01	0.00

[a]Values are means of duplicate experiments (standard error <5%).

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Notes
The authors declare no competing financial interest.

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