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Pretreatment refining leads to constant particle size distribution of lignocellulose biomass in enzymatic hydrolysis

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HIGHLIGHTS

- Constant particle size distribution of pretreated lignocellulose biomass in enzymatic hydrolysis was discovered.
- Lignin framework supports the structural integrity of vascular fibers of lignocellulose in both pretreatment and hydrolysis.
- A structural model of lignocellulose biomass in pretreatment and enzymatic hydrolysis is proposed.

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ABSTRACT

Pretreatment and enzymatic hydrolysis are the two central steps to convert lignocellulosic biomass into fermentable sugars. Here we report a discovery of constant particle size distribution of corn stover and wheat straw in enzymatic hydrolysis after pretreatment. The average particle size of the extensively pretreated corn stover and wheat straw quickly decreases to 20–30 μm in enzymatic hydrolysis and maintains constant without further size shrinking, although the hydrolysis continues to release glucose. The microstructural properties of the lignocellulose biomass pretreated by different processes and severity during the enzymatic hydrolysis were characterized by the particle size analysis, scanning electron microscopy (SEM) and the polarized light microscopy (PLM). The microstructural analysis reveals that the parenchyma tissue of the pretreated corn stover and wheat straw is quickly hydrolyzed, then the vascular bundle tissue is hydrolyzed to short vascular fibers. The fibers maintain a stable structure by the support of residual lignin framework during enzymatic hydrolysis. A structural model of lignocellulose biomass in pretreatment and enzymatic hydrolysis is proposed based on the pretreatment and hydrolysis experiments, as well as the microstructural analysis. This study takes the first insight into the uniform change of lignocellulose structure during pretreatment and enzymatic hydrolysis with the potentials on elevating hydrolysis yield and reconstruction of cell wall structure.

1. Introduction

Structural changes of lignocellulosic biomass caused by pretreatment has a great impact on the enzymatic hydrolysis, especially, the particle size of lignocellulosic biomass is the crucial impact on reaction rate, mass transfer, and rheological property of hydrolysis and consequent fermentation processes [1–4]. We observed an interesting phenomenon in our previous studies and the relevant works that the particle size distribution of the pretreated lignocellulosic biomass tends to reach a relatively constant size in enzymatic hydrolysis, although the hydrolysis of the residual cellulose continues and releases glucose [2,5]. This phenomenon is not consistent with the general chemical reaction engineering theory that solid reactant particles shrink gradually until completely disappear at the end of the reaction [6].

The particle size distribution of pretreated lignocellulose biomass in enzymatic hydrolysis had been reported in the previous publications and the rapid “cutting” model of long fibers into short fragments and the slow “peeling/excavating” model on particles surface during the enzymatic hydrolysis was proposed [5,7,8]. These models focus on the effect of the enzyme action pattern on the particle size changes, but did not consider the effect of pretreatment condition and inherent structural property of lignocellulosic biomass.

Pretreatment is the critical step of biorefining chain to render the recalcitrant structure of lignocellulose to enzymatic hydrolysis by hemicellulose removal, partial hydrolysis of acid-soluble lignin, and disrupt the crystalline cellulose structure [9]. With the development of

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visualization analysis technologies, structure change of the pretreated lignocellulose in enzymatic hydrolysis has been investigated for revealing the mechanism of enzymatic hydrolysis [5,10,11]. The architecture of microfibrils has an effect on the binding of cellulase enzymes has been observed by real time visualization method in the nanometer-scale [12]. The polarized light microscopy and confocal laser scanning microscope images revealed that the dislocated regions in the lignocellulosic fiber are easily bound by cellulase enzymes during the initial phase of hydrolysis [13,14]. However, the effects of pretreatment on the supramolecular architecture of different parts of cell walls such as vascular bundles, sclerenchyma and parenchyma cells, as well as on the structural change of lignocellulosic biomass in enzymatic hydrolysis are not clearly understood.

In this study, the constant particle size distribution of the pretreated corn stover and wheat straw during the enzymatic hydrolysis was firstly proposed. The effect of pretreatment severity and inherent cell wall structural property (such as parenchyma tissues and vascular fibers) of these lignocellulosic biomass on the phenomenon was investigated. The results reveal that the residual lignin framework in the vascular fibers provides the frame supporting for the pretreated lignocellulose in enzymatic hydrolysis. We also found that the specific pretreatment severity determines the constant particle size of vascular fibers in enzymatic hydrolysis. This study takes the first insight into the uniform change of lignocellulose structure during pretreatment and enzymatic hydrolysis with the potentials on elevating hydrolysis yield and reconstruction of cell wall structure.

2. Materials and methods

2.1. Raw materials

Wheat straw and corn stover harvested in fall 2013 (Dancheng, Henan, China) were cut coarsely and washed, then dried until constant weight with moisture of 6.5% and 5.7%, respectively. The dry biomass was crushed by a beater pulverizer with sieve pore size of 10 mm in diameter and stored in sealed plastic bags before use. The composition was measured according to the protocols by the National Renewable Energy Laboratory (NREL) [15] as shown in Table 1.

Commercial cellulase enzyme Youtell #6 was provided by Hunan Youtell Biochemical Co. (Yueyang, Hunan, China) with the filter paper activity of 135 FPU/g measured according to the NREL protocol [16] and the cellobiase activity of 344 IU/g measured according to a typical method of cellulase activity analysis [17]. All other chemicals and reagents were analytical pure and obtained from Lingfeng Chemical Reagent Co., Shanghai, China.

2.2. Dry acid pretreatment

The dry dilute acid pretreatment method was applied to pretreat corn stover and wheat straw [18]. Briefly, the sulfuric acid dosage was 25 mg per gram of dry biomass and prepared into the dilute sulfuric acid solution. The dry biomass was thoroughly mixed with the diluted sulfuric acid solution at the solid to liquid ratio of 2:1 (w/w), and stored in the plastic bag at ambient temperature for 12 h. In each batch of pretreatment, 1800 g of the mixed and presoaked lignocellulosic materials were added into a 20 L pretreatment reactor and pretreated with mild helically stirring [19]. The pretreatment conditions and the corresponding composition of pretreated materials were shown in Table 1. The target temperature with error of ± 2 °C was controlled by slightly adjust the steam flow rate entrancing into the reactor.

2.3. Enzymatic hydrolysis of lignocellulosic biomass

The enzymatic hydrolysis of corn stover or wheat straw (including raw biomass, dilute acid pretreated biomass as well as the pretreated and deliginified biomass. The raw biomass was passed the standard sieve with pore size of 250 μm) was carried out at the dry biomass loading of 2.5% (w/w) and cellulase dosage of 20 FPU per gram of dry biomass at pH 4.8, 50 °C for 72 h. 5 mL of hydrolysate samples was taken periodically and centrifuged for solid-liquid separation. The supernatant liquid was used for the analysis of soluble sugars by HPLC analysis [20]. The solid was washed with deionized water for analysis of particle size and morphology. All the results were duplicated and listed with standard deviations.

2.4. Delignification of the pretreated lignocellulosic biomass

Delignification of the pretreated lignocellulosic biomass was carried out according to the method by Ding et al. [12] to remove the lignin component from the pretreated biomass. The pretreated wheat straw or corn stover was washed with deionized water to remove the soluble substance and then dried at 80 °C till to constant weight. Then, the dry biomass was added into the solution of 0.1 M HCl contained 10% (w/w) of $NaClO_2$ with the solid to liquid ratio of 1:10 (w/v), and harshly incubated at ambient temperature for 12 h. Finally the samples were centrifuged at 11,167 × g for 5 min, and the solids were thoroughly washed with deionized water till neutral and stored in sealed plastic bags before use.

2.5. Morphology analysis of lignocellulosic biomass

The microstructural morphology of biomass was characterized by the scanning electron microscopy (SEM) and the polarized light microscopy (PLM). The lignocellulosic particles periodically taken from enzymatic hydrolysis were washed with deionized water, lyophilized, and sputtered coat with 1–2 nm gold, then observed using Hitachi S3400 N SEM (Hitachi, Kyoto, Japan) with the accelerating voltage of 15 kV. The PLM images of pretreated lignocellulosic biomass was observed using a Carl Zeiss Axio Scope A1 pol PLM (Carl Zeiss MicroImaging, Goettingen, Germany) equipped with two polarisers, a rotating stage and a Pixelink digital camera.

Table 1
Pretreatment conditions and composition of the lignocellulosic biomass.

Biomass	Pretreatment conditions	CSP[§]	Glucan[*] (%)	Xylan[*] (%)	Acid insoluble lignin[*] (%)	Ash[*] (%)	Glucan recovery[#] (%)
Corn stover	Without pretreatment	0	35.4	27.8	11.4	4.9	–
	175 °C, 2.5% H_2SO_4, 5 min	1.78	40.4	3.4	17.4	6.1	90.7
Wheat straw	Without pretreatment	0	38.7	25.9	14.9	5.2	–
	150 °C, 2.5% H_2SO_4, 1 min	0.37	39.3	7.0	14.3	5.3	99.6
	175 °C, 2.5% H_2SO_4, 1 min	1.07	41.2	3.4	19.2	5.5	100
	175 °C, 2.5% H_2SO_4, 5 min	1.78	41.6	4.3	20.6	6.3	88.8

§ CSP indicates the combined severity parameter as shown in Eq. (1).
* The composition of glucan, xylan, acid insoluble lignin and ash was calculated based on dry matter of the pretreated biomass (w/w).
# Glucan recovery presents as percentage of glucan content in the pretreated biomass divided by the initial glucan content in the raw lignocellulose feedstock.

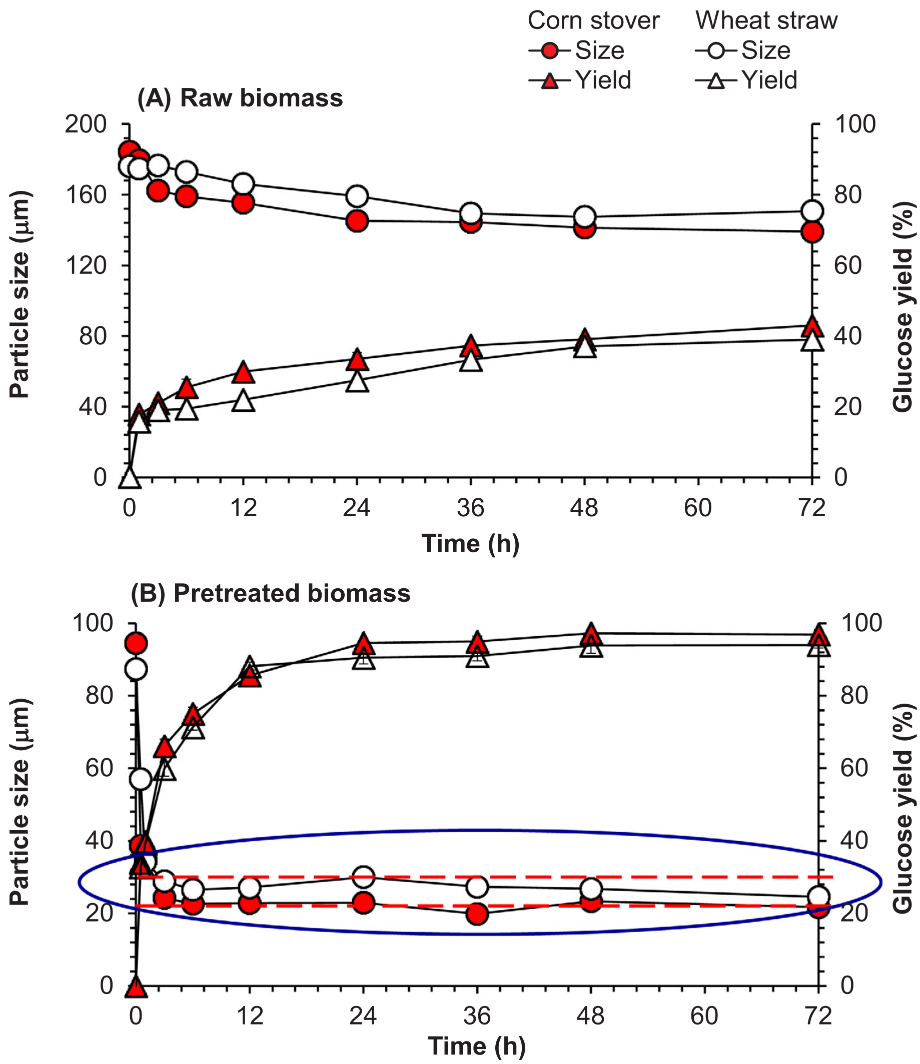

Fig. 1. Particle size and glucose yield of lignocellulosic biomass in enzymatic hydrolysis. The hydrolysis of (A) raw biomass; (B) dilute acid pretreated biomass. Pretreatment conditions: the solid to liquid ratio between the dry biomass and the dilute sulfuric acid solution was 2:1 (w/w), H_2SO_4 dosage at 25 mg per gram of the dry biomass, 175 °C for 5 min. Enzymatic hydrolysis conditions: the dry biomass loading at 2.5% (w/v), cellulase dosage at 20 FPU per gram of dry biomass at 50 °C, pH 4.8 for 72 h. Particle size is indicated by the mean diameter of equivalent sphere over the volume distribution D(4,3).

2.6. HPLC analysis

Glucose and xylose were analyzed using Shimadzu LC-20AD HPLC (Shimadzu, Kyoto, Japan) fitted with a RID-10A refractive index detector and a Bio-Rad Aminex HPX-87H column. The column oven was set at 65 °C and the mobile phase was 5 mM H_2SO_4 at 0.6 mL/min [20]. All samples were centrifuged at 11,167 × g for 5 min and the supernatant filtered through a 0.22 mm filter before analysis.

2.7. Calculation for pretreatment severity

The pretreatment severity of dry diluted acid pretreatment was calculated according to the equation of the combined severity parameter (CSP) [21,22]:

$$CSP = \mathrm{Log}R_0 - \mathrm{pH} = \mathrm{Log}\left[t\cdot\exp\left(\frac{T-100}{14.75}\right)\right] - \mathrm{pH} \quad (1)$$

where T is pretreatment temperature (°C), t is pretreatment time (min), R_0 is the severity parameter of pretreatment. The dry dilute acid pretreatment was conducted at high solids content (70% at the beginning and 50% at the end) of lignocellulose biomass, and no free liquid was generated before and after the pretreatment, therefore the pH values was calculated according to the methods by Zhang et al. [23].

2.8. Particle size distribution analysis

The particle size of lignocellulosic biomass in enzymatic hydrolysis was measured by Mastersizer 2000 laser diffraction particle size analyzer (Malvern Instruments, Worcestershire, UK) with a detection range of 0.02–2000 μm. The refractive index and absorption coefficient were taken as 1.5 and 1.0, respectively, for the correction of lignocellulosic particles. The particle size distribution of lignocellulosic biomass was represented by the volume weighted mean diameters of equivalent sphere, D [4,3], based on the volume distribution of lignocellulosic particle. All the results were the mean of repeated triplicate measurements with the residual below 1.0%.

3. Results and discussions

3.1. Particle size change of lignocellulosic biomass in enzymatic hydrolysis

Corn stover and wheat straw as typical Poaceae lignocellulosic biomass from agriculture present the similar composition and structure, which are widely used in biorefinery industry for cellulosic ethanol

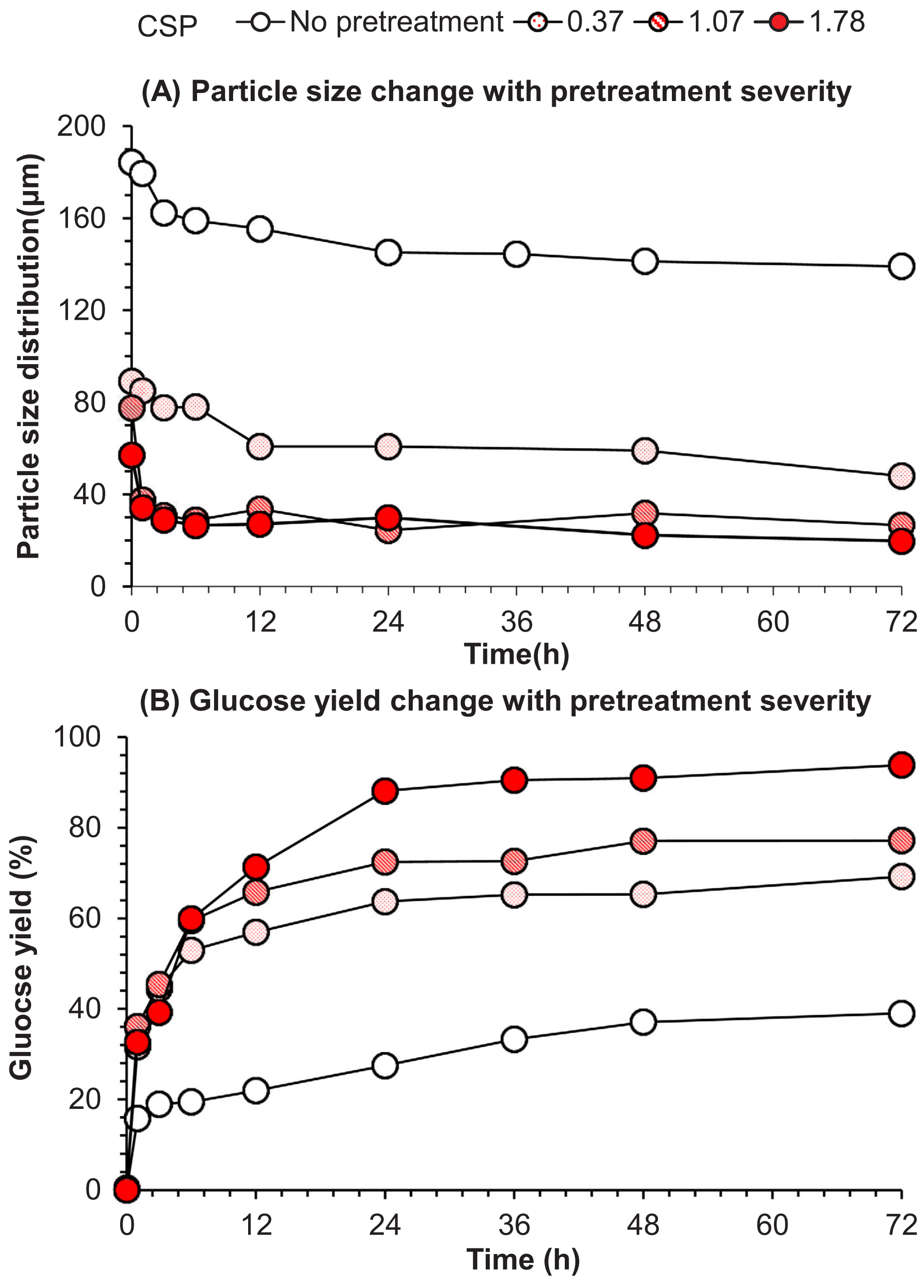

Fig. 2. Particle size and glucose yield of lignocellulosic biomass with different pretreatment severity in enzymatic hydrolysis. (A) Particle size change; (B) glucose yield; (C) correlation of constant particle size to pretreatment severity. Pretreatment conditions: the solid to liquid ratio between the dry biomass and the dilute sulfuric acid solution was 2:1 (w/w), H_2SO_4 dosage at 25 mg per gram of the dry biomass, then the pretreatment was carried at different severity: without pretreatment, combined severity parameter of 0.37, 1.07 and 1.78, respectively. Enzymatic hydrolysis conditions: the dry biomass loading at 2.5% (w/v), cellulase dosage at 20 FPU per gram of dry biomass at 50 °C, pH 4.8 for 72 h. Particle size is indicated by the mean diameter of equivalent sphere over the volume distribution D(4,3).

production [24]. Thus, they were selected as the model lignocellulosic materials for investigating particle size change of lignocellulose during the pretreatment and enzymatic hydrolysis process. The raw corn stover and wheat straw contained 35.4% and 38.7% of cellulose, 24.6% and 25.9% of xylan, 16.1% and 14.9% of lignin, 3.5% and 5.2% of ash, respectively (Table 1). For the raw biomass without pretreatment, the initial particle size distribution was 180 μm in average and then gradually decreased to 140 μm during the hydrolysis (Fig. 1a). For the pretreated biomass, the particle size rapidly decreased from 90–100 μm to 20–30 μm in the initial 3 h of the hydrolysis and then kept approximately constant till the end of the 72 hours' hydrolysis, although the hydrolysis reaction still proceeded with continuous glucose generation (Fig. 1b). Corn stover and wheat straw presented the similar phenomenon of particle size change during enzymatic hydrolysis.

We further examined the pretreatment severity on the particle size distribution in enzymatic hydrolysis. Wheat straw was pretreated at different temperature and maintained for different time to generate varied pretreatment severity (Table 1). Glucan content of the pretreated wheat straw increased with increasing pretreatment severity due to the acid hydrolyzing removal of xylan (hemicellulose), while the glucan recovery yield decreased due to partial hydrolysis of cellulose. Fig. 2 reveals that the wheat straw without pretreatment or pretreated at low severity at the combined severity parameter (CSP) of 0.37 did not show the clear tendency to constant particle size to 20–30 μm. When the CSP values increased to 1.07, the particle size started to approach to 20–30 μm. When CSP further increased to 1.78, the particle size still

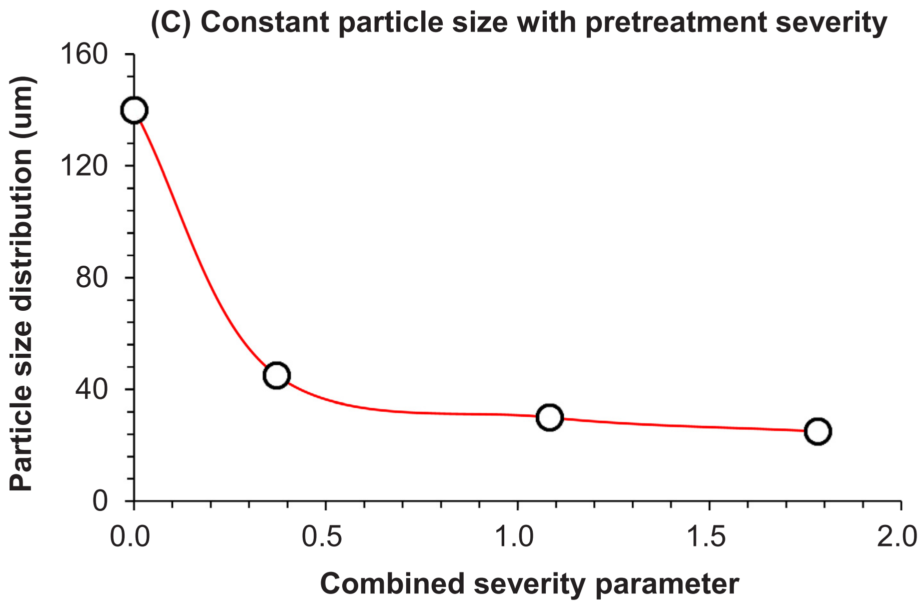

Fig. 2. (*continued*)

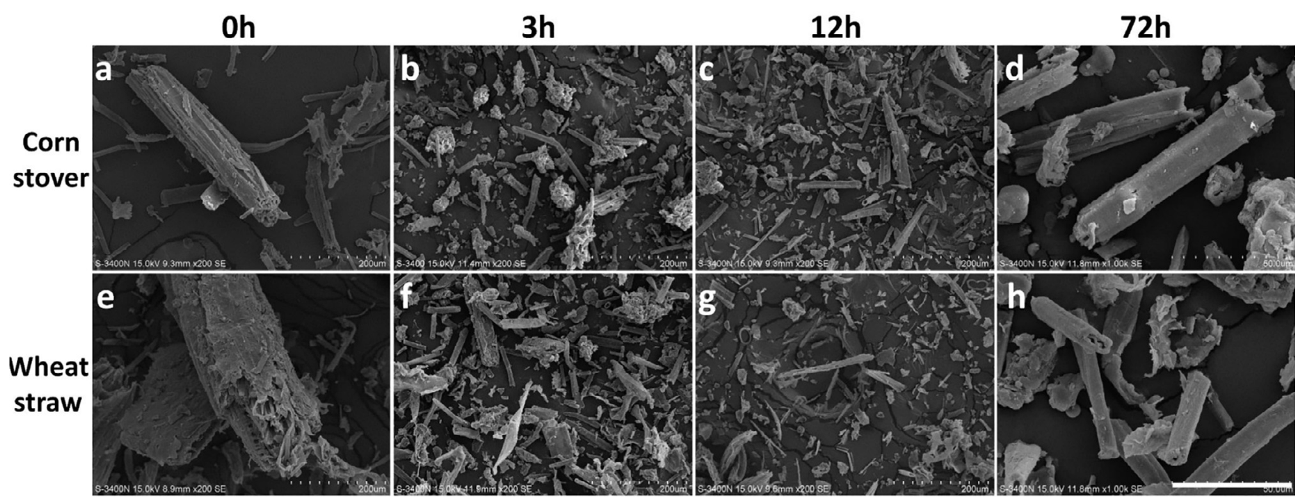

Fig. 3. SEM images of pretreated biomass in enzymatic hydrolysis. The pretreated corn stover (a, b, c and d) and wheat straw (e, f, g and h) at 0, 3, 12 and 72 h of enzymatic hydrolysis, respectively. Pretreatment conditions: the solid to liquid ratio between the dry biomass and the dilute sulfuric acid solution was 2:1 (w/w), H_2SO_4 dosage at 25 mg per gram of the dry biomass, 175 °C for 5 min. Enzymatic hydrolysis conditions: the dry biomass loading at 2.5% (w/v), cellulase dosage at 20 FPU per gram of dry biomass at 50 °C, pH 4.8 for 72 h. Bar: 200 μm for images of a, b, c, e, f, g and 50 μm for images of d, h.

approached to 20–30 μm. The highest glucose yield of wheat straw pretreated with different severity increased with the CSP increasing (Fig. 2B). Although the glucose yield increased when the CPS increased from 1.07 to 1.78, the particle size showed a similar distribution closed to 20–30 μm. This is because glucose yield depends on the pretreatment severity, which is indicated by CPS [25], while the particle size change is a combined outcome of pretreatment, enzymatic hydrolysis and inherent structure of substrate. There was no direct relation between glucose yield and particle size change during the enzymatic hydrolysis. The result suggests that the pretreatment at the extensive severity is the determent factor on the constant size distribution in enzymatic hydrolysis of the pretreated lignocellulosic biomass.

3.2. Cell wall structure on particle size in enzymatic hydrolysis

The morphological changes of the pretreated corn stover and wheat straw fibers in enzymatic hydrolysis were observed by scanning electron microscopy (SEM) (Fig. 3). The slow decrease in particle size and subtle change in surface structure of raw corn stover during enzymatic hydrolysis have been observed in our previous study [26]. The constant size distribution phenomenon was observed only for the pretreated lignocellulose biomass in enzymatic hydrolysis. Therefore, the time course of the morphological change was illustrated on the pretreated corn stover and wheat straw with the control of freshly pretreated biomass (without hydrolysis). Before the enzymatic hydrolysis, the vascular bundle tissue surrounded by parenchyma tissue cell wall was observed in the pretreated biomass (Fig. 3a and e for corn stover and wheat straw, respectively). When the enzymatic hydrolysis started, the integrated vascular fibers were broken into the smaller fiber fragments and gradually tended into the size of 10–20 μm in width and 50–100 μm in length during the initial 3 h (Fig. 3b, f). Afterwards the length decreased to 30–50 μm and the width kept approximately constant after 12 h (Fig. 3c, g). The small vascular fiber fragments maintained the similar size of 10–20 μm in width and 30–50 μm in length until the end of the 72 h' hydrolysis (Fig. 3d, h). The parenchyma tissues was observed in the initial 3 h, but disappeared after the 12 hours' hydrolysis (Fig. 3). The result suggests that the parenchyma tissues in the pretreated lignocellulose were quickly and completely hydrolyzed into

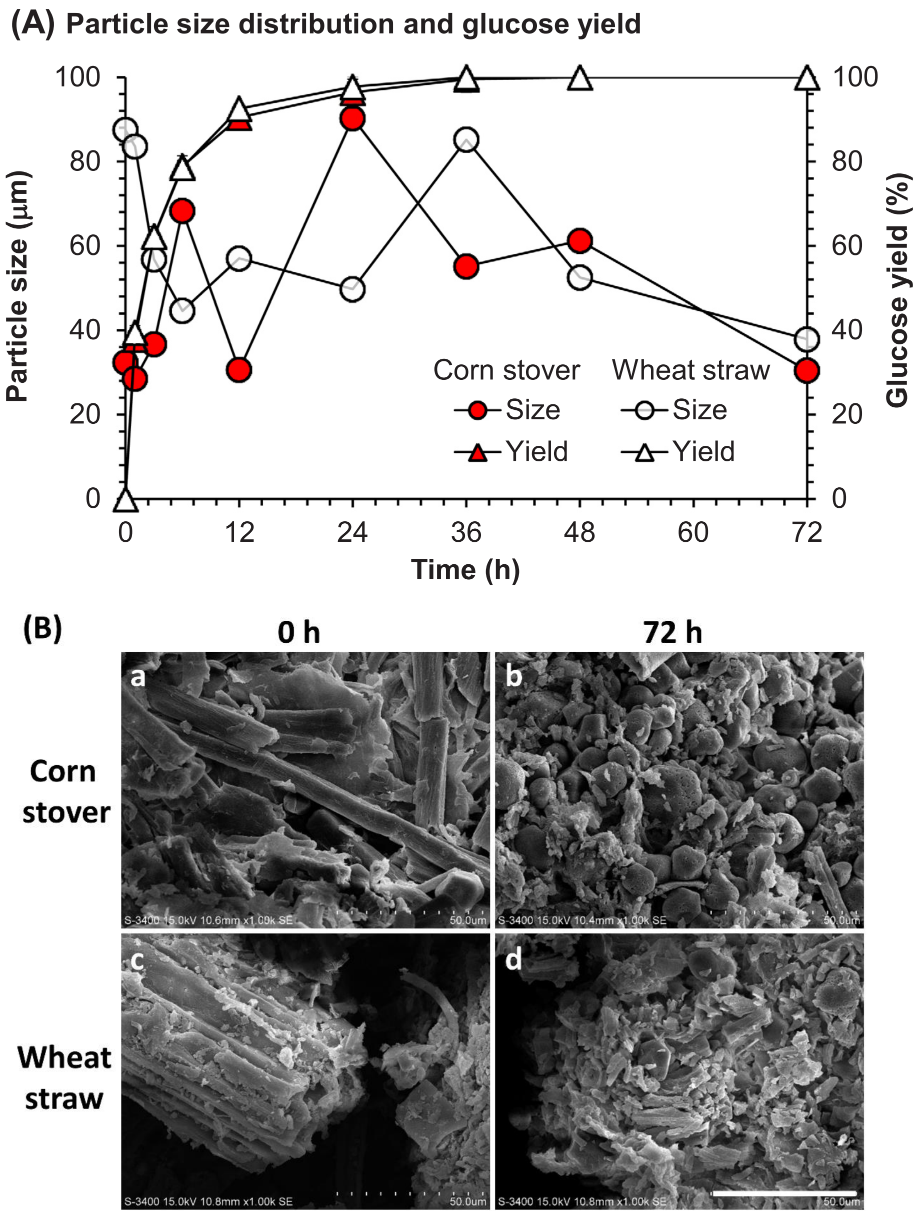

Fig. 4. Enzymatic hydrolysis of the pretreated and delignified corn stover and wheat straw. (A) Particle size distribution and glucose yield; (B) SEM images of the pretreated and delignified corn stover (a, b) and wheat straw (c, d) at the start and the 72 h of enzymatic hydrolysis. Pretreatment: the ratio of the dry biomass to the dilute sulfuric acid solution at 2:1 (w/w), H_2SO_4 dosage at 25 mg per gram of dry biomass, 175 °C for 5 min. Delignification: the dry biomass loading at 10% (w/v) in 0.1 M HCl solution containing 10% (w/w) $NaClO_2$, at ambient temperature, harsh shaking for 12 h. Enzymatic hydrolysis: the dry biomass loading at 2.5% (w/v), cellulase dosage at 20 FPU per gram of dry biomass at 50 °C, pH 4.8 for 72 h. Bar: 50 μm.

soluble sugars in the initial period, but the vascular bundles were only hydrolyzed into small fragments and kept their tubular structure till to the end of hydrolysis.

The parenchyma tissues of corn stover or wheat straw present high content of cellulose with the thin wall structure (~0.1 μm) and non-directional arrangement of cellulose microfibers, which have been demonstrated that it apt to be hydrolyzed during enzymatic hydrolysis. On the other hand, the vascular bundles present as the densely lignified thick-wall (2–5 μm) structure with tight and directional arrangement of cellulose microfibers [12,27,28]. Dilute acid pretreatment hydrolyzes the most of hemicellulose and dissolves the acid-soluble lignin, however, the acid insoluble lignin component still existed in the vascular fiber and kept its rigid and integral structure after dilute acid pretreatment [29,30]. Therefore, the parenchyma tissues of the plant cell walls were quickly hydrolyzed but the vascular fibers supported by the residual acid-insoluble lignin framework maintained the structural integrity.

To verify the function of lignin framework in the vascular fibers, the pretreated corn stover and wheat straw were delignified (complete removal of lignin) and the particle size distribution in enzymatic hydrolysis was examined. As expected, the particle size distribution of the pretreated and delignified corn stover and wheat straw no longer maintained its unique constant size. Instead, an irregular fluctuation of size distribution was presented in the enzymatic hydrolysis (Fig. 4A). This is probably because delignification caused the pretreated lignocellulose particles losing the bonding and supporting function of lignin. These particles could suddenly collapse into fragments during the hydrolysis rather than gradually decreased as the pretreated particles with lignin. Therefore, the constant size distribution of delignified lignocellulose particles disappeared. The particle size distribution fluctuated greatly with the general tendency of size decrease as shown in Fig. 4A. The SEM images show that the vascular fibers of the pretreated and delignified corn stover and wheat straw (Fig. 4B-a, 4B-c) almost completely disappeared after 72 hours' enzymatic hydrolysis (Fig. 4B-b, B-d). The glucose yield and hydrolysis rate of the delignified lignocellulose biomass in enzymatic hydrolysis were greater than those of the only pretreated biomass, apparently due to reduced lignin barrier to cellulase access (Fig. 4A). These results indicate that the complete lignin removal (delignification) caused the structure collapse of the vascular fibers and the irregular particle size distribution, and further revealed the importance of lignin for the constant particle size distribution in enzymatic hydrolysis.

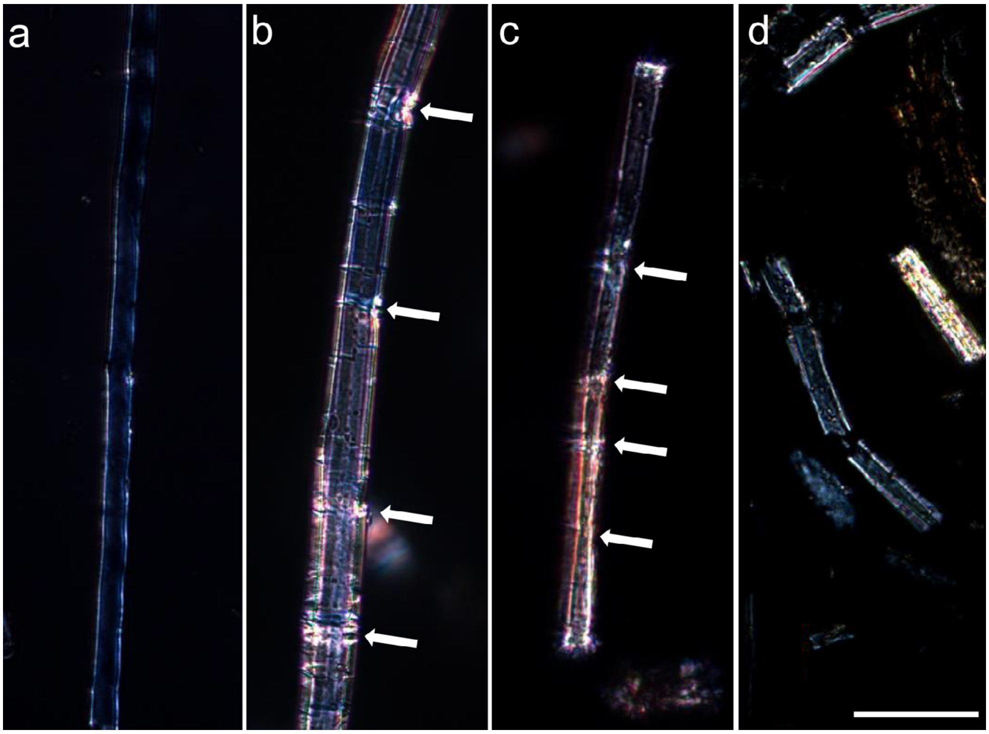

Fig. 5. Polarized light microscopy images of wheat straw fibers at various pretreatment severity. Pretreatment conditions: the solid to liquid ratio between the dry biomass and the dilute sulfuric acid solution was 2:1 (w/w), H_2SO_4 dosage at 25 mg per gram of the dry biomass, then the pretreatment carried at different severity: (a) without pretreatment, (b) 150 °C for 1 min, (c) 175 °C for 1 min, and (d) 175 °C for 5 min, respectively. Bar: 50 μm.

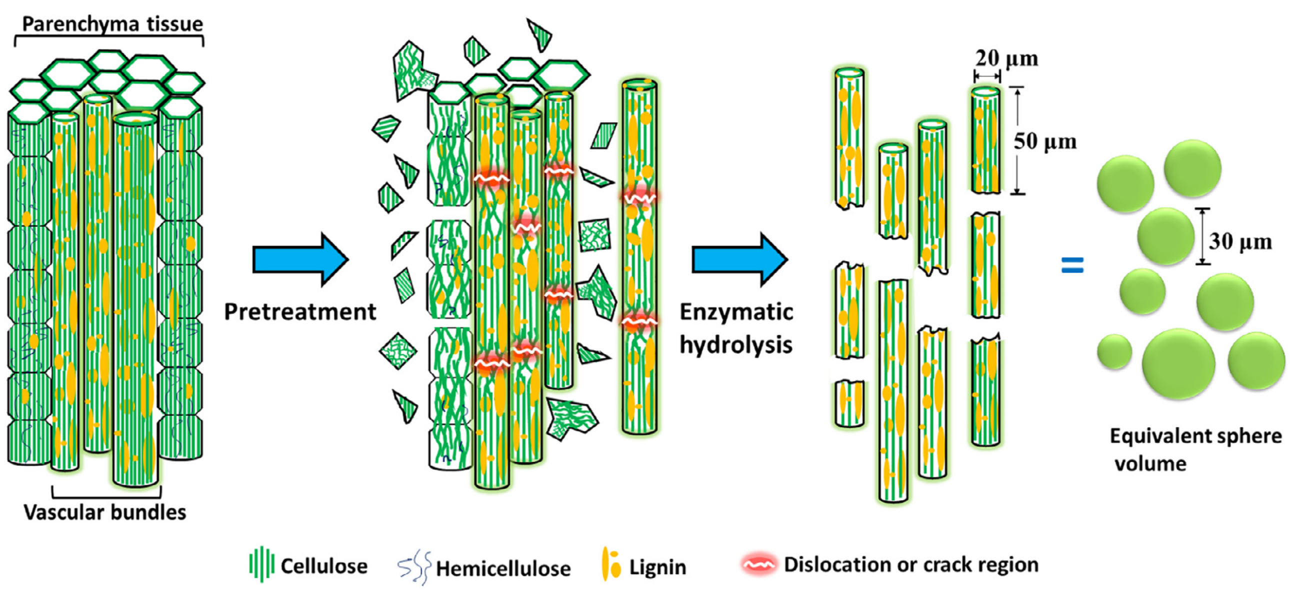

Fig. 6. Schematic illustration of pretreatment on the particle size of lignocellulosic biomass in enzymatic hydrolysis.

3.3. Microstructural analysis and structural model of enzymatic hydrolysis of lignocellulose

The crystal structure of the pretreated wheat straw with different pretreatment severity was observed using polarized light microscopy (PLM). The regions with change in the crystal structure, such as dislocations or cracks or delamination of the fibers presented a bright band across the fibers (dark region) under the polarized light [8,11]. The PLM image shows that specific regions with "scars" or "cracks" were created during the pretreated vascular fibers, and the number of the crack regions increased with increasing pretreatment severity. The average distance between the two adjacent crack regions in the vascular fibers at the pretreatment severity of 1.78 was approximately 50 μm (Fig. 5). These cracked pieces with the regular size distribution might be produced by the changes in arrangement orientation of cellulose microfibrils in the vascular fibers after pretreatment. The cellulase enzymes prefer to bind at these crack or "wounded" regions and then hydrolyze the long fibers into the short vascular fibers [31–33]. Furthermore, the short vascular fibers kept the size and vascular structure in the enzymatic hydrolysis due to the residual lignin in the pretreated vascular fiber still providing the function of support and structural integrity.

A structural model of enzymatic hydrolysis of pretreated lignocellulose biomass is proposed based on the pretreatment and hydrolysis experiments, as well as the microstructural analysis (Fig. 6). In the pretreatment step, the parenchyma tissue cell walls are disrupted and the regular crack regions with the average distance of 30–50 μm between the two adjacent crack regions in vascular fibers are created. In the enzymatic hydrolysis, the pretreated parenchyma tissue cell walls are quickly hydrolyzed and the vascular fibers at the crack regions are hydrolyzed to generate small fibers with the average length of 30–50 μm and the width of 17–22 μm. The cellulose inside the small fibers continues to release soluble glucose under the cellulase enzymes catalysis. The vascular fibers supported by the lignin framework still maintain its structural integrity of lignocellulose in the hydrolysis and the vascular fiber particle size maintains relatively. The equivalent spherical diameter of the small vascular fibers exactly equaled to the constant particle size distribution of 20–30 μm.

4. Conclusions

This study explained the interesting phenomenon of the particle size change in enzymatic hydrolysis of the dilute acid pretreated biomass by analyzing the effect of pretreatment on the inherent supramolecular structure of tissue cell walls in the lignocellulosic biomass. The pretreatment on the parenchyma and vascular bundle tissue cell walls with different structure and composition caused the particle size distribution in enzymatic hydrolysis presented a two-steps change involving the initial rapid decline followed by keeping constant size. The phenomenon of constant particle size was influenced by the combined action of pretreatment severity and vascular fiber property. The results provide the important perspectives on improving the industrial biorefinery technologies, such as the promising pretreatment operation towards the proper biomass size, cellulase enzyme cocktail for the substrates with specific size distribution, and the general rheology property prediction method for hydrolysate slurries containing regular particle size.

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