Ammonia-Ethanol-Water Pretreatment of Wheat Straw for Facilitating Enzymatic Saccharification Integrated with the Preparation of Submicron Lignin Spheres

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Ammonia-ethanol-water (AEW) pretreatment was adopted to treat wheat straw for ameliorating saccharification with the integrated preparation of submicron lignin spheres (SLS). Results showed that AEW pretreatment could remove 77% of lignin and 90% of extractives, thus increasing specific surface area and porosity of the substrate and finally enhancing the release of fermentable sugars in saccharification. Under the optimal pretreatment conditions (170 °C for 2 h, ethanol concentration 55% (v/v), ammonia concentration 7.5 wt%), the final total sugar yield reached 81.7% after pretreatment and saccharification, which was 2.25 times higher compared to the conventional ethanol organosolv pretreatment. Moreover, washing could be excluded for AEW-pretreated wheat straw before saccharification, and both ethanol and ammonia could be readily recovered and reused, making the AEW pretreatment clean and sustainable. In addition, SLS with hollow structure and average diameter of 161.2 ± 53.6 nm were fabricated using the fractionated lignin, which could offset the overall cost of AEW pretreatment.

Keywords: Ammonia-ethanol-water pretreatment; Enzymatic saccharification; Wheat straw; Submicron lignin spheres; Biorefinery

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INTRODUCTION

The excessive use of fossil fuels is leading to a severe energy and environmental crisis around the world (Ding *et al.* 2012). Thus, the utilization of lignocelluloses has attracted increased attention, as plants can fix CO_2 by photosynthesis and produce biomass (Xu *et al.* 2016). The annual production of lignocelluloses is estimated to be over 200 billion tons on earth (Hu and Ragauskas 2012). Hence, it is of crucial importance to make better use of the abundant lignocelluloses, particularly in the case of agricultural wastes (*e.g.*, wheat straw), to protect the environment, avoid waste of cellulosic materials, and promote the sustainable development of human society.

The cell wall of lignocellulose is mainly composed of cellulose, hemicellulose, and lignin. Cellulose (glucan) as a linear homo-polymer is present as semicrystalline elementary fibrils, while hemicelluloses are hetero-polysaccharides, consisting of main chains (*e.g.*, xylan) and branched side groups. Both cellulose and hemicelluloses are the sources of fermentable sugars. Lignin is an amorphous biopolymer of phenylpropanoids closely associated with hemicelluloses (Sixta 2006). In plant cell walls, cellulose fibrils that play a role of skeleton support are tightly covered by hemicellulose-lignin matrix, and this well evolved structure functions just like a strong reinforced concrete structure (Zhu *et al.* 2016), which provides a robust natural recalcitrance against outside attack. Thus, in conversion of lignocelluloses to cellulosic ethanol or chemicals, pretreatment is an indispensable step to break the natural resistance of lignocelluloses as a means of facilitating sugar release in downstream saccharification (Sun *et al.* 2016; Li *et al.* 2017).

To obtain a high yield of fermentable sugars, lignin removal is usually a priority strategy because it can rupture the physical barrier of lignin to cellulose and reduce the nonproductive absorption of lignin to enzymes (Mosier *et al.* 2005). As reported, many pretreatments can selectively remove lignin, such as alkali-based pretreatment, biological methods, sulfite pretreatment, deep eutectic solvents pretreatment, and organosolv pretreatment (Agbor *et al.* 2011; Liu *et al.* 2019). Among these methods, organosolv pretreatment using ethanol is considered to be a very promising pretreatment due to its inherent advantages, such as good lignin solubility, high retention of available carbohydrates, coproduction of high-purity lignin, low cost of solvent, lack of toxicity, good miscibility of ethanol with water, and the easy recovery and reuse of the solvent (Zhou *et al.* 2018). In ethanol organosolv pretreatment, lignin dissolution is catalyzed by acetic acid (generated from the hydrolysis of acetyl groups on hemicelluloses) mainly through the cleavage of β-O-4 linkage in lignins (Wen *et al.* 2013), and simultaneously, part of hemicellulose is degraded (Guo *et al.* 2015). Ethanol is beneficial to the penetration of reagent to plant tissue, and also the dissolution of degraded lignin fragments (Zhou *et al.* 2018). Yet, the lignin removal of ethanol organosolv pretreatment is typically less than 70% (Hallac *et al.* 2010). Hence, acid, inorganic salts, or alkali agents can be added in ethanol organosolv pretreatment to enhance delignification and reduce pretreatment severity. Either inorganic (*e.g.*, sulfuric acid) (Sidiras and Koukios 2004) or organic acids (*e.g.*, formic acid) (Li *et al.* 2016) can be used, but acids can cause severe degradation of hemicelluloses, generation of inhibitors, lignin condensation and precipitation, equipment corrosion, and generation of acid wastewater (Tarasov *et al.* 2018). Replacing acid with inorganic salts (*e.g.*, $FeCl_3$, $MgCl_2$) can cause less corrosion and less degradation of hemicellulose in ethanol pretreatment, and these salts could be easily recycled (Huijgen *et al.* 2011). However, the use of metal salts might complicate process control and affect the purity of separated lignin. As for alkali agents, both inorganic (*e.g.*, NaOH) and organic base (*e.g.*, diethylamine, ethylenediamine) can be used to promote lignin removal in ethanol pretreatment because alkali can efficiently break the linkages of lignin subunits and the crosslinking bonds between carbohydrates and lignin (Xu *et al.* 2016), and hence the delignification can reach up to over 80% (Mesa *et al.* 2011; Tang *et al.* 2017). In contrast to other bases, ammonia is a promising agent, because it is inexpensive, easily available, non-corrosive, non-polluting, miscible with water and ethanol, and easier to be recovered and reused (Hashemi *et al.* 2019). Additionally, the residual ammonia in pretreated substrate as the N source is beneficial to downstream fermentation (Kim *et al.* 2003). Thus, ammonia is expected be a suitable alkaline reagent which could be used to enhance lignin removal in ethanol organosolv pretreatment. However, ammonia-ethanol-water (AEW, ammonia-assisted ethanol) pretreatment of lignocellulose for improving enzymatic hydrolysis is very little reported. Only Park and Phillips (1988) found that the enzymatic susceptibility of poplar was increased approximately 6-fold after ammonia-assisted ethanol organosolv pretreatment in comparison with the untreated poplar.

On the other hand, lignin valorization is of vital importance to increase the economic feasibility of the entire process for lignocellulose conversion (Luo *et al.* 2020;

Mei *et al.* 2020). Lignin nano-/micron-spheres are relatively new biomaterials that can be potentially used as fillers in composites (Nair *et al.* 2014), carriers for controlled release of agricultural actives or drugs (Sameni *et al.* 2018), functional building blocks, *etc.*, and they can be fabricated using different methods (*e.g.*, self-assembly, homogenization) through simple chemical or mechanical mechanisms with different lignin sources (*e.g.*, alkali lignin, organosolv lignin) (Zhao *et al.* 2016). However, the preparation of submicron lignin spheres (SLS) derived from AEW pretreatment has not been reported.

Therefore, in this work, AEW pretreatment was used to treat wheat straw, and the impact of pretreatment conditions on fermentable sugar release of saccharification and feature changes of the pretreated wheat straw were comprehensively investigated. It is expected that the addition of ammonia in ethanol organosolv pretreatment could efficiently enhance the pretreatment effectiveness of wheat straw. Also, both ethanol and ammonia could be readily recovered and reused, which could make the pretreatment process clean. Thus, the recovery of ethanol and ammonia were evaluated in this work. Most importantly, compared to other alkali reagents, ammonia is relatively safe to environment, and the use of ammonia will not introduce salt ions to pretreatment system, and the separated lignin would have a high purity. In addition, the preparation and characterization of SLS using the fractionated lignin from AEW pretreatment were conducted for the first time. The fabrication of SLS can valorize lignin, thus offsetting the overall process cost of AEW pretreatment.

EXPERIMENTAL

Materials

Wheat straw was collected in the summer of 2018 from Weifang of Shandong province, China. As tested following the National Renewable Energy Laboratory (NREL) analytical procedures (Sluiter *et al.* 2008), the native wheat straw contained 30.3 ± 0.2% of glucan, 13.5 ± 0.1% of xylan, 1.0 ± 0.1% of arabinan, 0.7 ± 0.1% of acetyls, 25.1 ± 0.3% of lignin (acid soluble lignin + acid insoluble lignin), 14.4 ± 0.7% of extractives (hot water extractives + ethanol extractives), and 13.6 ± 0.1% of ash. Before pretreatment, the wheat straw with water content of 66.5% was pulverized by a pilot twin screw extrusion machine (manufactured by Tianzheng Screening Pulping Equipment Co., Ltd., Hebei, China) with the maximum throughput of 200 kg/h (Liu *et al.* 2013). The extrusion duration of wheat straw was only about 30 seconds, and just one pass was conducted to avoid over damage of wheat straw. The pulverized wheat straw with the particle size less than 2 cm in length was stored in a sealed plastic bag in a cold room at 4 °C.

All analytical-grade chemicals, including aqueous ammonia (25 wt%), ethanol (99.5 wt%), and others, were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and used as received. The cellulase enzyme used for enzymatic hydrolysis was supplied by Qingdao Vland Biotech Inc. (Qingdao, China, a local supplier) and the activity of cellulase was 86 FPU/mL (80 mg protein/mL) as measured by the standard method (Ghose 1987).

Methods

The AEW pretreatment

The AEW pretreatment of wheat straw was performed in a 15-L stainless steel reactor (PL1-00; Xianyang TEST Equipment Co., Ltd., Xianyang, China) equipped with four small cooking bombs inside, and the volume of each bomb was 1 L. In this case, four parallel pretreatment experiments took place simultaneously, and the rotational speed of the reactor was 1 rpm. For each pretreatment, 50 g of wheat straw (based on the dry weight) was used, and the pretreatment was implemented under the certain experiment conditions with the liquid-to-solid ratio of 8 (w/w). After pretreatment, the cooking bombs were immediately cooled with cold running water. Then, the spent liquor was squeezed out using a 500-mesh washing bag and collected in a cold trap apparatus in a fume hood. Subsequently, the spent liquor was concentrated after recovery of ammonia and ethanol with rotary evaporation, and then sulfuric acid was added to the concentrated spent liquor until the pH was adjusted to 3 to get the precipitated lignin (named as P-lignin), which was further purified by washing with deionized water. For comparison, the lignin sample (referred as D-lignin) was also collected by direct drying of the concentrated spent liquor and then followed by washing with deionized water. The solid fraction after AEW pretreatment was first washed once with ethanol (55%, v/v) at 50 °C and then washed with deionized water at 50 °C to neutral pH. For comparison, the unwashed solid samples after pretreatment and phase separation were also collected. Finally, both the washed and unwashed samples were stored at 4 °C for further tests.

In pretreatment experiments, key factors of ethanol concentration (0 to 78%, v/v), ammonia dosage (0 to 100 wt%, NH_3 dosage was based on the dry weight of wheat straw), and energy input were varied to optimize the process and check the effectiveness of pretreatment. When other pretreatment conditions were fixed, energy input can be represented as H-factor, which combines pretreatment time and temperature into a unique variable. This single variable is traditionally used in the pulping industry to indicate the extent of cooking reaction, and it can also be used to evaluate pretreatment (Vergara *et al.* 2018). The H-factor is calculated by the Eq. 1 (Vroom 1957),

$$H = \int_0^t e^{\left(43.2 - \frac{16115}{T}\right)} dt \tag{1}$$

where T is the pretreatment temperature (K), and t is the pretreatment time (h) at T. The designed energy input and the calculated values of corresponding H-factors are listed in Table 1.

Table 1. Energy Input and the Corresponding H-factor for AEW Pretreatment

Pretreatment Temperature (°C)	Pretreatment Time (h)	H-factor
150	2	329
170	1	920
170	2	1840
170	3	2760
190	2	8852

Enzymatic saccharification

In this work, enzymatic saccharification was used to evaluate the effectiveness of pretreatment, according to the NREL standard procedure (Selig *et al.* 2008). The saccharification of pretreated wheat straw was conducted in a 50-mL bottle with a substrate solid loading of 2 wt% in an incubator shaker (50 °C, pH 4.8, for 48 h). Approximately 0.02 wt% sodium azide was added to prevent microbial contamination, and the cellulase dosage was 25 FPU/g-glucan (23 mg protein/g-glucan). A high loading of enzyme was used due to the relatively low activity of cellulase. After saccharification, the samples of hydrolysate were taken out and immediately cooled to 4 °C for monosaccharide analysis with high performance liquid chromatography (HPLC).

Preparation of submicron lignin spheres (SLS)

The SLS particles were prepared *via* the self-assembly method using the acetylated lignin, as reported previously (Qian *et al.* 2014). Firstly, the purified lignin was acetylated in the mixed solution of acetic acid and acetyl bromide (volume ratio of acetic acid to acetyl bromide was 95 : 5) at 55 °C for 3 h, and the reaction was carried out in a single port flask (500 mL) in water bath with magnetic stirring (200 rpm). Upon completion, the solvent was recovered by rotary evaporation under vacuum at 55 °C, and then the solids were completely dried at 105 °C for 2 h in a drier (DGH-9076A, Shanghai, China). After that, the resulting acetylated lignin was immediately dissolved in tetrahydrofuran (THF). Finally, the SLS particles were obtained through self-assembly when the deionized water was slowly and gradually added to the acetylated lignin-THF solution. In the resultant dispersion of SLS, the end volume ratio of water to THF was 67 : 33.

Chemical composition analysis

The chemical compositions of raw and pretreated wheat straw were tested based on the two-step sulfuric acid hydrolysis following the NREL procedure (Sluiter *et al.* 2008). After being filtered with 0.22-μm membrane, the acidic and enzymatic hydrolyzates were analyzed by a HPLC system (Model 1200; Agilent, Santa Clara, CA, USA). A Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm) and refractive index detector were equipped in the HPLC system, and the column was operated at 55 °C with the mobile phase of 0.005 M sulfuric acid solution at a flow rate of 0.6 mL/min. All samples were measured at least three times based on the calibration curve established with the standard sugars with the known and gradient concentrations. The sugar losses during acid hydrolysis were corrected by subjecting known amounts of standard sugars throughout the same test procedures. Equations 2 through 8 are as follows,

$$R_{\text{solid}}\,(\%) = \frac{M_{\text{pretreated biomiss}}}{M_{\text{orignal biomiss}}} \times 100 \tag{2}$$

$$R_{\text{glucan}}\,(\%) = \frac{M_{\text{glucan in pretreated biomiss}} \times R_{\text{solid}}}{M_{\text{glucan in orignal biomiss}}} \times 100 \tag{3}$$

$$R_{\text{xylan}}\,(\%) = \frac{M_{\text{xylan in pretreated biomiss}} \times R_{\text{solid}}}{M_{\text{xylan in orignal biomiss}}} \times 100 \tag{4}$$

$$D_{\text{lignin}}\,(\%) = \left(1 - \frac{M_{\text{lignin in pretreated biomiss}} \times R_{\text{solid}}}{M_{\text{lignin in orignal biomiss}}}\right) \times 100 \tag{5}$$

$$C_{\text{glucose}}\,(\%) = \frac{M_{\text{glucose in hydrolyzate}} \times 0.9}{M_{\text{glucan in pretreated biomiss}}} \times 100 \tag{6}$$

$$C_{\text{xylose}}\,(\%) = \frac{M_{\text{xylose in hydrolyzate}} \times 0.88}{M_{\text{xylan in pretreated biomiss}}} \times 100 \tag{7}$$

$$Y_{\text{total sugar}}\,(\%) = \frac{M_{\text{glucose in hydrolyzate}} \times 0.9 + M_{\text{xylose in hydrolyzate}} \times 0.88}{M_{\text{glucan in orignal biomiss}} + M_{\text{xylan in orignal biomiss}}} \times 100 \tag{8}$$

where, M is the mass of the corresponding substance (g).

The calculations for solid recovery (R_{solid}), the recovery efficiencies of glucan (R_{glucan})/xylan (R_{xylan}), and the delignification efficiency (D_{lignin}) after pretreatment, as well as the enzymatic conversion extents of glucan (C_{glucan})/xylan (C_{xylan}) and the final total sugar yields (glucose + xylose) ($Y_{\text{total sugar}}$) after saccharification were carried out following the previous work (Xie *et al.* 2018), and the detailed calculation equations are given in Eqs. 2 to 8. In brief, R_{glucan} and R_{xylan} indicate how much glucan and xylan remained in the pretreated wheat straw, respectively. The C_{glucan} and C_{xylan} are defined as the percentages of the released glucose and xylose in enzymatic hydrolyzate after saccharification divided by the corresponding glucan and xylan in the pretreated wheat straw accordingly. The $Y_{\text{total sugar}}$ is defined as the percent of the released glucose plus xylose in hydrolysate after saccharification divided by the corresponding total sugars in original wheat straw.

In this work, all experiments of pretreatment, enzymatic saccharification, and chemical composition were conducted at least three times, and the average value with standard deviation are reported accordingly. The statistical analyses were performed using Microsoft Excel 2013.

Characterizations

The surface morphologies of wheat straw and SLS were observed with a scanning electron microscope (SEM, S-4800; Hitachi, Tokyo, Japan) with the accelerating voltages from 3.0 to 8.0 kV, and the freeze-dried samples were coated with platinum (E-1045; Hitachi, Tokyo, Japan) before observation.

Fourier transform infrared spectroscopy (FTIR) spectra of wheat straw before and after pretreatment were recorded on a spectrometer (Nicolet 6700; Thermo Fisher, Waltham, MA, USA) in the wavenumber range of 4000 to 500 cm^{-1} at resolution 4 cm^{-1}. Samples were prepared using the standard KBr pellet method, and 32 scans were collected for each sample. The absorption peaks at 2900, 1430, 1372, and 897 cm^{-1} were used for the calculation of cellulose crystalline index. The total crystallinity index (*TCI*) of cellulose was computed as the ratio of absorption peak at 1372 to 2900 cm^{-1} (Nelson and O'Connor 1964), and the lateral order index (*LOI*) of cellulose was calculated as the ratio of absorption peak at 1430 to 897 cm^{-1} (Hurtubise and Krassig 1960).

The crystallinity index (*CrI*) of the raw and pretreated wheat straw was determined using an X-ray diffraction (XRD) instrument (D8 ADVANCE; Bruker Co., Karlsruhe, Germany) with the Ni-filtered Cu Kα radiation of 80 mA and 40 kV. The scanning speed was 0.5°/min in the scattering angle (2θ) range of 5 to 45°, and the *CrI* was calculated in terms of the empirical method reported by Segal *et al.* (1959).

The specific surface area and pore volume of raw and pretreated wheat straw samples were measured with a Brunauer-Emmet-Teller (BET) surface integral analyzer (Autosorb-iQ; Quantachrome, Boynton, FL, USA) at N_2 environment with the test models of BET theory and Barrett-Joyner-Halenda (BJH) method, respectively. Before testing, samples were lyophilized for 24 h and degassed under vacuum at 80 °C for 8 h.

The organic elements of C, H, O, N, and S in the raw and pretreated wheat straw were analyzed on an elemental analyzer (Vario EL cube; Elementar Co., Hanau, Germany) after the samples were burned with oxygen at temperature of 1200 °C for 70 s.

The molecular weight of acetylated lignin was tested by gel permeation chromatography (GPC, HELEOS System; Wyatt Co., Santa Barbara, CA, USA) with a refractive index detector (G1362A). The mobile phase was THF and the flow rate was 1 mL/min. Polystyrene was used as the standard material for calibration.

The morphologies of the as-prepared SLS were characterized using a transmission electron microscope (TEM, Hitachi H-7600; Hitachi, Tokyo, Japan) with an accelerating voltage of 120 kV and atomic force microscope (AFM, Agilent 5400, Palo Alto, CA, USA) in the tapping mode, respectively.

RESULTS AND DISCUSSION

It is well known that biomass pretreatment is essential for the facilitation of sugar release in enzymatic saccharification (Li *et al.* 2017; Xie *et al.* 2018). On the basis of structure features of lignocelluloses and the requirements of industrial application, a good pretreatment method should meet the requirements of high recovery of polysaccharides, limited formation of inhibitors, suitable fractionation and utilization of lignin, low chemical consumption or efficient chemical recovery, low energy consumption and reduced wastewater, and most importantly, high compatibility with enzymatic saccharification (Mosier *et al.* 2005; Liu *et al.* 2020).

Impact of Key Process Variables on the Effectiveness of AEW Pretreatment

In this work, AEW pretreatment was applied to treat wheat straw. The impact of key variables of ethanol concentration, ammonia dosage, and energy input on the effectiveness of AEW pretreatment was evaluated in terms of R_{solid}, R_{glucan}, R_{xylan}, D_{lignin}, and the standard enzymatic hydrolysis.

In ethanol organosolv pretreatment, ethanol concentration is of vital importance for pretreatment efficiency. Shown in Fig. 1a and 1b are the impact of ethanol concentration on the effect of AEW pretreatment of wheat straw. The ethanol concentration ranged from 0 to 78% (v/v) with the fixed ammonia dosage of 5 wt% at 170 °C for 2 h. As shown in Fig. 1a, after AEW pretreatment, both R_{glucan} and R_{xylan} increased with the increase of ethanol concentration. For instance, the R_{glucan} and R_{xylan} increased 15.3% and 58.9%, respectively, when ethanol concentration increased from 0 to 55% (v/v). These results indicated that higher ethanol concentration could reduce the degradation of carbohydrates (particularly for xylan) in AEW pretreatment, which was probably because both cellulose and xylan are insoluble in ethanol. Figure 1a also exhibits that the lignin removal ascended approximately 50% when ethanol concentration increased from 0 to 37% (v/v), which was because ethanol could promote the impregnation of plant tissue by transferring reagents to lignin, and simultaneously transport the soluble lignin fragments from the inside tissue to the bulk solution (Zhou *et al.* 2018). However, the D_{lignin} descended when ethanol concentration was higher than 55% (v/v). This phenomenon was because higher concentration of ethanol could reduce the reaction between NH_3 and H_2O, generating less OH^-, which was in line with the decreased pH in a higher ethanol concentration (Fig. 1a). A weaker alkalinity reduced the lignin degradation. Moreover, the AEW fractionated lignin *via* ammonolysis might have a lower solubility in a higher ethanol concentration, because different lignin has distinct hydrogen-bonding capacity in different solvents (Ni and Hu 1995). Thus, the increase of the preservation of carbohydrates and the decrease of D_{lignin} led to a rise of R_{solid} when the ethanol concentration was over 55% (v/v). Correspondingly, as displayed in Fig. 1b, the C_{glucan}, C_{xylan}, and $Y_{\text{total sugar}}$ had similar trends with D_{lignin}. This phenomenon was because lignin content in pretreated substrate is one of main hindrances to enzymatic hydrolysis (Xie *et al.* 2018). In fact, based on all data obtained in this work, it was found that lignin content had a linearly negative impact on enzymatic conversion and $Y_{\text{total sugar}}$ (Fig. 2). Similar correlations were also reported previously for alkali-based pretreatments (Yang *et al.* 2013; Xu *et al.* 2015). With the ethanol concentration of 55% (v/v) and ammonia dosage of 5 wt%, D_{lignin} after AEW pretreatment could only reach 61% (Fig. 1a) and the corresponding $Y_{\text{total sugar}}$ after saccharification was only approximately 64% (Fig. 1b). Hence, to further boost lignin removal and $Y_{\text{total sugar}}$, ammonia dosage can be properly increased.

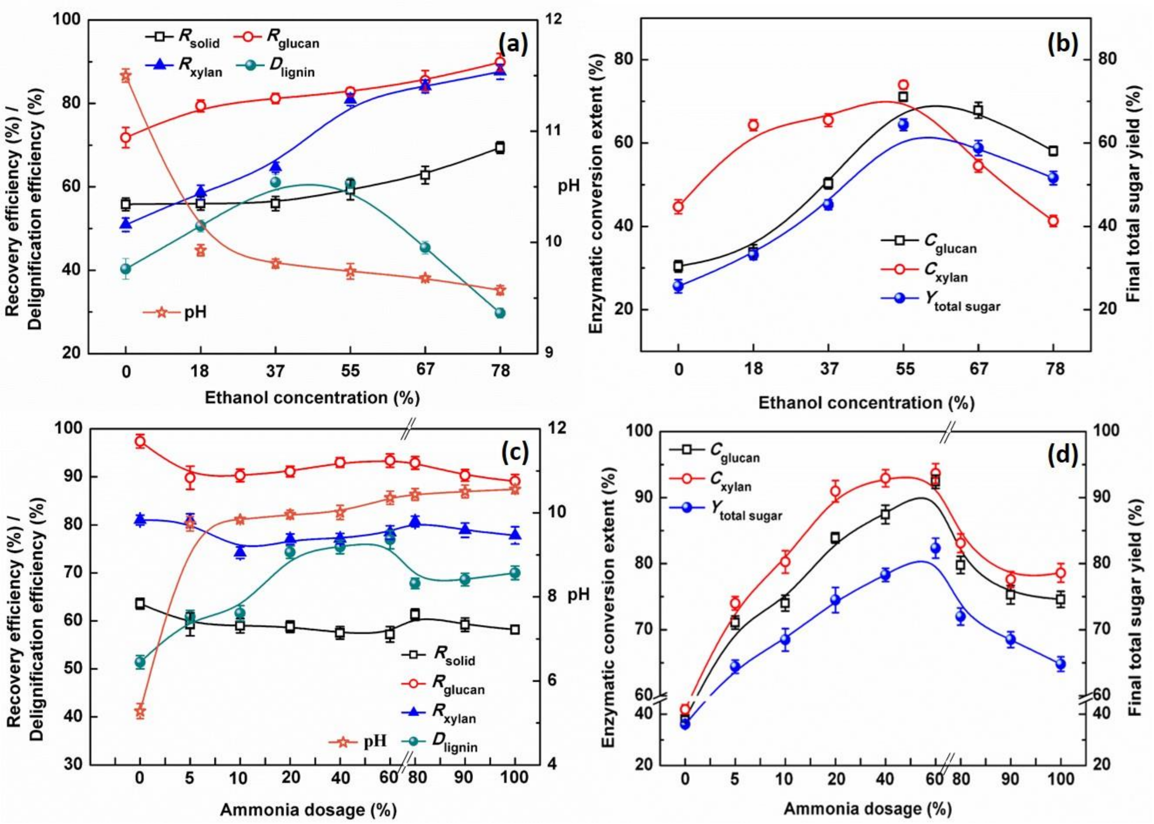

Fig. 1. Effect of ethanol concentration and ammonia dosage on the effectiveness of AEW pretreatment (a: impact of ethanol concentration on recovery and delignification, ammonia dosage 5 wt%, 170 °C for 2 h; b: impact of ethanol concentration on enzymatic conversion and final total sugar yield, ammonia dosage 5 wt%, 170 °C for 2 h; c: impact of ammonia dosage on recovery and delignification, ethanol concentration 55% (v/v), 170 °C for 2 h; d: impact of ammonia dosage on enzymatic conversion and final total sugar yield, ethanol concentration 55% (v/v), 170 °C for 2 h)

Figures 1c and 1d present the impact of ammonia dosage on the effect of AEW pretreatment with the fixed ethanol concentration of 55% (v/v) at 170 ℃ for 2 h. It can be observed from Fig. 1c that the pH value kept increasing with the increment of ammonia dosage. This is because NH_3 can react with H_2O, releasing the nucleophilic reagent OH^- ions, which have a higher activity to attack lignin. As known, aqueous ammonia can swell lignocelluloses and enhance lignin removal by breaking aryl-ether bonds in lignin (mainly the dearylation of non-phenolic β-aryl ether bonds) as well as ester and ether bonds in the lignin-carbohydrate complex under the attack of OH^- and NH_3 (Kim *et al.* 2003; Hashemi *et al.* 2019). Thus, with the increase of ammonia dosage from 0 to 60 wt%, D_{lignin} kept increasing from 51.4 to 76.9%, and that was an approximate 50% increase of lignin removal. Additionally, to achieve a higher ammonia dosage over 60 wt%, the raw wheat straw was completely dried for tuning of the ratio of ethanol to aqueous ammonia without adding of additional water before pretreatment. However, as shown in Fig. 1c, when ammonia dosage increased to 80 to 100 wt%, the corresponding D_{lignin} (67 to 70%) was clearly lower compared to the one (approximate 77%) with the ammonia dosage of 60 wt%. This result was due to the more compact structure of wheat straw induced by water loss after complete drying (Luo *et al.* 2011), which could reduce mass transfer in pretreatment. Therefore, the corresponding enzymatic conversion and $Y_{\text{total sugar}}$ were all decreased greatly (Fig. 1d) because of the less removal of lignin (thus less accessibility of substrate to cellulase) along with some degradation (approximately 3% more) of carbohydrates. In addition, Fig. 1c also displays that the increase of ammonia dosage from 5 to 100 wt% did not cause severe degradation of carbohydrates, and the R_{glucan} and R_{xylan} varied in the ranges of 89 to 93% and 75 to 81%, respectively. Again, lignin content of pretreated wheat straw (*i.e.*, lignin removal) was closely related to the enzymatic conversion and $Y_{\text{total sugar}}$ (Figs. 1 and 2), and the highest $Y_{\text{total sugar}}$ of 81.4% could be achieved with the D_{lignin} of 77%. To achieve this point, the suitable ammonia dosage was 60 wt% for AEW pretreatment.

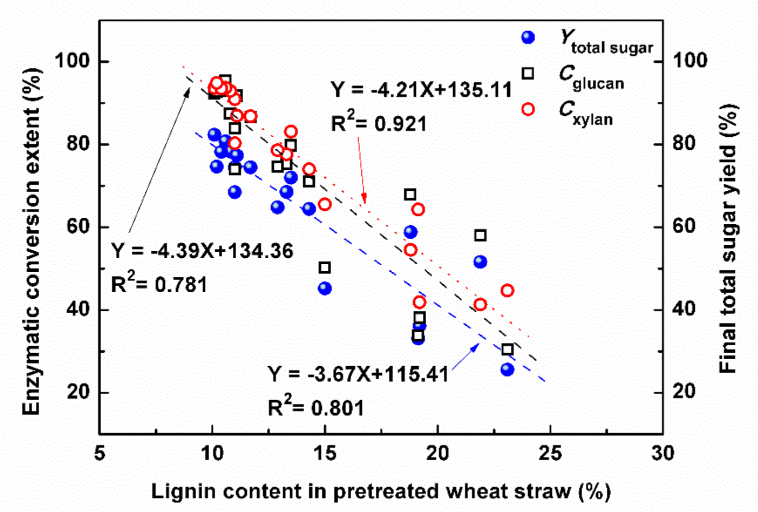

Fig. 2. Correlations between the lignin content of pretreated wheat straw and enzymatic conversion / final total sugar yields

Energy input (presented as H-factor) in pretreatment also plays an important role on pretreatment efficiency and process cost. The impact of H-factor on the effectiveness of AEW pretreatment is shown in Fig. 3. In the range of H-factor from 329 to 8852, the ethanol concentration and ammonia dosage were fixed at 55% (v/v) and 60 wt%, respectively. As expected, R_{solid} decreased with the increase of H-factor, because more energy input could promote chemical reactions in pretreatment (Sixta 2006), leading to more lignin removal and more degradation of carbohydrates (Fig. 3a). Additionally, when H-factor ascended from 329 (150 °C, 2 h) to 1840 (170 °C, 2 h), the increased lignin removal was closely related to the increase of C_{glucan}, C_{xylan}, and $Y_{\text{total sugar}}$. For example, when H-factor increased from 920 to 1840, D_{lignin} increased approximately 5%, and the corresponding C_{glucan}, C_{xylan}, and $Y_{\text{total sugar}}$ improved approximately 4, 9, and 4.5%, respectively. However, when H-factor was higher than 1840, severe degradation of glucan and xylan took place, resulting in the decrease of $Y_{\text{total sugar}}$, although both the corresponding C_{glucan} and C_{xylan} were basically steady over 90% (Fig. 3b). Therefore, to obtain a higher

$Y_{\text{total sugar}}$, excessive increase of H-factor is not needed, because severe degradation of carbohydrates should be avoided.

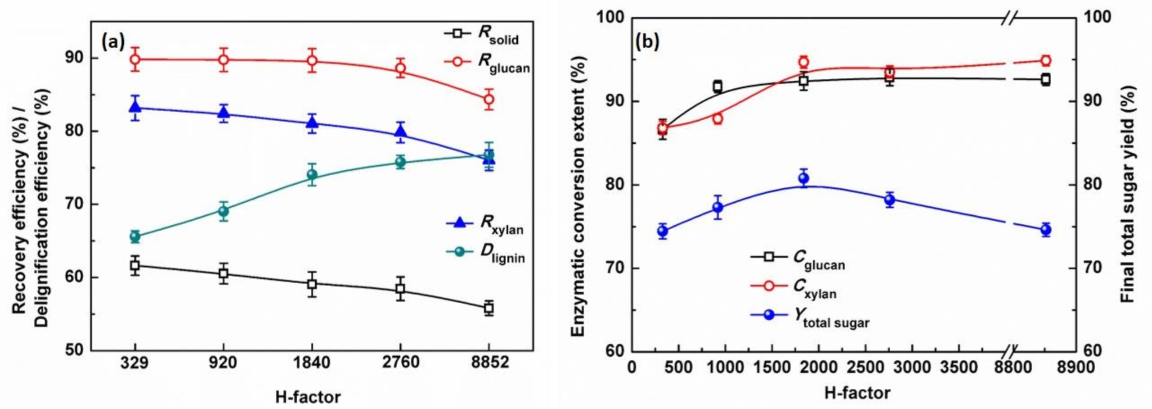

Fig. 3. Effect of H-factor on the effectiveness of AEW pretreatment (a: for recovery and delignification; b: for enzymatic conversion and final total sugar yield)

Based on the results obtained, the suitable H-factor was 1840, corresponding to the energy input at 170 °C for 2 h, and the suitable ethanol concentration and ammonia dosage were 55% (v/v) and 60 wt%, respectively. Under such conditions, the corresponding C_{glucan}, C_{xylan}, and $Y_{\text{total sugar}}$ could reach up to 92.4 ± 1.1, 94.1 ± 0.7, and 81.7 ± 1.0%, respectively. This $Y_{\text{total sugar}}$ was 2.25 times higher compared to the ethanol organosolv pretreatment without any addition of ammonia (only 36.8 ± 1.2%, Fig. 1d).

Furthermore, the C_{glucan}, C_{xylan}, and $Y_{\text{total sugar}}$ as the function of enzymatic hydrolysis time for the wheat straw pretreated under the optimal conditions are exhibited in Fig. 4.

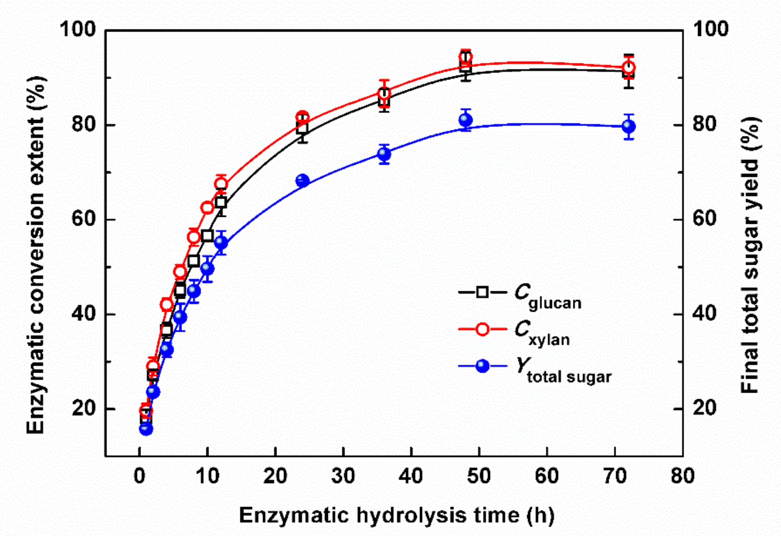

Fig. 4 Enzymatic conversion extent and final total sugar yield as the function of enzymatic hydrolysis time (wheat straw pretreated at 170 °C for 2 h with ethanol concentration of 55% (v/v) and ammonia dosage of 60 wt%)

As shown, the C_{glucan}, C_{xylan}, and $Y_{\text{total sugar}}$ increased rapidly in the first 24 h (especially in the first 12 h), indicating a high accessibility of the pretreated wheat straw after sufficient removal of lignin and extractives. As shown in Tables 2 through 4, the remaining extractives of the AEW-pretreated wheat straw were in the range of 2.1 to 5.6%, which was obviously lower than the extractives content (14.4%) of raw wheat straw and the alkali pretreated wheat straw (7.4 to 12.4%) (Xie *et al.* 2018). The highest removal efficiency of extractives could reach 90% after AEW pretreatment, which was probably because wheat straw contains over 50% of lipophilic extractives and these lipophilic extractives (such as fatty acids, waxes, steryl esters) could be easily extracted by ethanol (Sun and Suan 2001). However, AEW pretreatment could not efficiently remove ash (Tables 2 through 4). Both extractives and ash had a barrier effect on saccharification as well (Yuan *et al.* 2018).

Table 2. Effect of Ethanol Concentration on the Chemical Composition of AEW-pretreated Wheat Straw

Ethanol Concentration (%)	R_{solid} (%)	Glucan (%)	Xylan (%)	Lignin (%)	Extractives (%)	Ash (%)
0	55.8 ± 0.1	41.7 ± 0.1	13.5 ± 0.1	23.1 ± 0.1	4.9 ± 0.4	9.3 ± 0.5
18	56.0 ± 0.1	45.9 ± 0.3	15.5 ± 0.3	19.1 ± 0.4	4.8 ± 0.2	10.7 ± 0.9
37	56.0 ± 0.2	47.0 ± 0.3	17.1 ± 0.2	15.0 ± 0.5	4.3 ± 0.5	11.8 ± 0.1
55	59.3 ± 0.1	45.2 ± 0.4	20.2 ± 0.2	14.3 ± 0.4	4.2 ± 0.3	9.6 ± 0.4
67	62.8 ± 0.4	44.1 ± 0.4	19.8 ± 0.1	18.8 ± 0.5	3.3 ± 0.2	10.2 ± 0.3
78	69.4 ± 0.3	42.0 ± 0.2	18.7 ± 0.2	21.9 ± 0.4	4.5 ± 0.4	9.7 ± 1.2

Composition of raw wheat straw: glucan 30.3 ± 0.2%, xylan 13.5 ± 0.1%, lignin 25.1 ± 0.3%, extractives 14.4 ± 0.7%, and ash 13.6 ± 0.1%

Table 3. Effect of Ammonia Dosage on the Chemical Composition of AEW-pretreated Wheat Straw

Ammonia Dosage (%)	R_{solid} (%)	Glucan (%)	Xylan (%)	Lignin (%)	Extractives (%)	Ash (%)
0	63.6 ± 0.4	46.4 ± 0.5	17.2 ± 0.4	19.2 ± 0.4	5.6 ± 0.3	12.0 ± 0.1
5	59.3 ± 0.1	45.2 ± 0.4	20.2 ± 0.2	14.3 ± 0.4	4.2 ± 0.2	10.7 ± 0.4
10	59.0 ± 0.3	50.0 ± 0.1	19.1 ± 0.0	11.0 ± 0.2	3.7 ± 0.4	9.6 ± 0.1
20	58.8 ± 0.7	47.0 ± 0.6	17.7 ± 0.5	11.0 ± 0.6	3.2 ± 0.2	10.1 ± 0.5
40	57.5 ± 0.4	49.0 ± 0.6	18.1 ± 0.3	10.8 ± 0.2	2.9 ± 0.5	9.2 ± 0.3
60	57.2 ± 0.3	49.5 ± 0.3	18.5 ± 0.1	10.1 ± 0.3	2.1 ± 0.3	10.7 ± 0.7
80	61.3 ± 0.3	45.9 ± 0.2	17.8 ± 0.2	13.5 ± 0.3	5.1 ± 0.4	10.9 ± 0.2
90	59.2 ± 0.2	48.1 ± 0.5	18.6 ± 0.4	13.3 ± 0.2	3.7 ± 0.2	9.5 ± 1.3
100	58.2 ± 0.4	46.4 ± 0.4	18.0 ± 0.2	12.9 ± 0.4	5.0 ± 0.4	9.8 ± 1.6

Composition of raw wheat straw: glucan 30.3 ± 0.2%, xylan 13.5 ± 0.1%, lignin 25.1 ± 0.3%, extractives 14.4 ± 0.7%, and ash 13.6 ± 0.1%

Figure 4 also shows that after 24 h hydrolysis, the increase of conversion (C_{glucan} and C_{xylan}) and final sugar yield gradually became slow, which was due to the inhibition effect of the increased sugar concentration (Jing *et al.* 2009). After 48 h hydrolysis, the

C_{glucan}, C_{xylan}, and $Y_{total\ sugar}$ basically kept steady, indicating the end of enzymatic saccharification. Interestingly, C_{xylan} was higher than C_{glucan} (Fig. 4), which was probably because most extracted xylan was likely re-adsorbed on the surface of AEW-pretreated wheat straw as xylan was not soluble in ethanol, leading to a higher exposure of xylan to enzymes (the enzyme used in this work contained some xylanase), and thus a higher C_{xylan} could be obtained.

Table 4. Effect of H-factor on the Chemical Composition of AEW-pretreated Wheat Straw

Energy Input	H-factor	R_{solid} (%)	Glucan (%)	Xylan (%)	Lignin (%)	Extractives (%)	Ash (%)
150 °C 2 h	329	61.6 ± 0.5	47.2 ± 0.5	20.1 ± 0.3	11.7 ± 0.6	5.4 ± 0.2	11.6 ± 0.3
170 °C 1 h	920	60.5 ± 0.4	48.1 ± 0.4	20.2 ± 0.3	11.1 ± 0.5	4.4 ± 0.4	10.9 ± 0.1
170 °C 2 h	1840	59.1 ± 0.7	50.2 ± 0.8	20.3 ± 0.7	10.6 ± 1.1	2.6 ± 0.7	9.8 ± 1.2
170 °C 3 h	2760	58.5 ± 0.2	49.1 ± 0.3	20.2 ± 0.4	10.4 ± 0.3	3.4 ± 1.3	10.1 ± 0.7
190 °C 2 h	8852	55.8 ± 0.4	49.0 ± 0.3	20.2 ± 0.3	10.2 ± 0.4	3.7 ± 0.5	9.7 ± 1.4

Composition of raw wheat straw: glucan 32.4 ± 0.2%, xylan 14.8 ± 0.3%, lignin 21.6 ± 0.4%, extractives 14.5 ± 0.9%, and ash 14.5 ± 0.4%

In addition, the effect of washing after AEW pretreatment on enzymatic conversion and final sugar yields is demonstrated in Table 5, which depicts that sufficient washing could wash out the extracted lignin, extractives, and degraded carbohydrates, leading to a lower R_{solid}. More removal of lignin and extractives yielded higher C_{glucan} and C_{xylan}, but comparable $Y_{total\ sugar}$ could be obtained after AEW pretreatment with or without washing. Based on variance analysis, for the samples with ammonia dosage of 40 or 60 wt% (Table 5), the effect of washing after pretreatment on $Y_{total\ sugar}$ was statistically different ($0.01 <$ P-value < 0.05), but the difference of $Y_{total\ sugar}$ (with or without washing) was still comparable (< 1.5%). For the samples with ammonia dosage of 80 or 90 wt% (Table 5), the impact of washing on $Y_{total\ sugar}$ was not statistically different (with the P-value > 0.05). The comparable $Y_{total\ sugar}$ with or without washing was because parts of degraded carbohydrates remained in the pretreated wheat straw after phase separation but without washing, hence offsetting the sugar loss of carbohydrates degradation in pretreatment with washing. Please note that, under the alkaline conditions, 5-hydroxymethylfurfural (5-HMF) and furfural were not detectable in the spent liquor of AEW pretreatment, and this was also beneficial to the followed enzymatic hydrolysis of the pretreated wheat straw without washing. Therefore, the washing step could be omitted after AEW pretreatment before saccharification, which will reduce the cost for the production of fermentable sugars.

Table 5. Effect of Washing on the Effectiveness of AEW Pretreatment

NH_3 Dosage (wt%)	Washing (Yes/No)	R_{solid} (%)	R_{glucan} (%)	R_{xylan} (%)	D_{lignin} (%)	Extractives Content (%)	C_{glucan} (%)	C_{xylan} (%)	$Y_{total\ sugar}$ (%)
40	Yes	57.5 ± 0.4	93.0 ± 0.7	77.1 ± 0.4	75.3 ± 0.8	2.9 ± 0.3	91.1 ± 0.2	93.4 ± 0.1	75.1 ± 0.3
	No	68.6 ± 0.3	93.8 ± 0.1	78.4 ± 0.6	68.6 ± 1.1	16.9 ± 0.7	86.9 ± 0.5	84.7 ± 0.9	73.8 ± 0.6
60	Yes	57.2 ± 0.7	92.6 ± 0.3	78.4 ± 0.2	77.0 ± 0.3	2.1 ± 0.6	93.2 ± 0.7	94.9 ± 0.3	81.7 ± 0.2
	No	67.8 ± 1.0	93.4 ± 0.8	86.5 ± 1.3	70.3 ± 0.7	14.6 ± 1.2	91.8 ± 0.1	85.7 ± 1.4	80.2 ± 0.6
80	Yes	61.3 ± 0.2	92.1± 0.3	80.7 ± 0.9	69.5 ± 1.4	5.2 ± 0.1	79.1 ± 0.3	82.6 ± 0.1	71.9 ± 0.4
	No	75.0 ± 0.6	92.9 ± 0.4	92.2 ± 0.7	59.7 ± 0.2	16.0 ± 0.5	77.4 ± 0.5	78.1 ± 1.2	70.3 ± 0.9
90	Yes	59.2 ± 0.1	89.4 ± 0.3	78.9 ± 0.1	68.6 ± 0.5	3.7 ± 0.4	78.5 ± 0.1	81.1 ± 0.6	70.5 ± 1.1
	No	72.0 ± 0.3	90.3 ± 0.6	89.5 ± 0.9	61.7 ± 1.1	15.0 ± 0.7	77.8 ± 0.6	79.7 ± 0.8	71.2 ± 0.5

Feature Changes of Wheat Straw Before and After Pretreatment

Feature changes of wheat straw before and after AEW pretreatment were comprehensively characterized, and further revealed why AEW pretreatment could clearly facilitate the enzymatic saccharification of wheat straw. Shown in Fig. 5 are the SEM images of wheat straw.

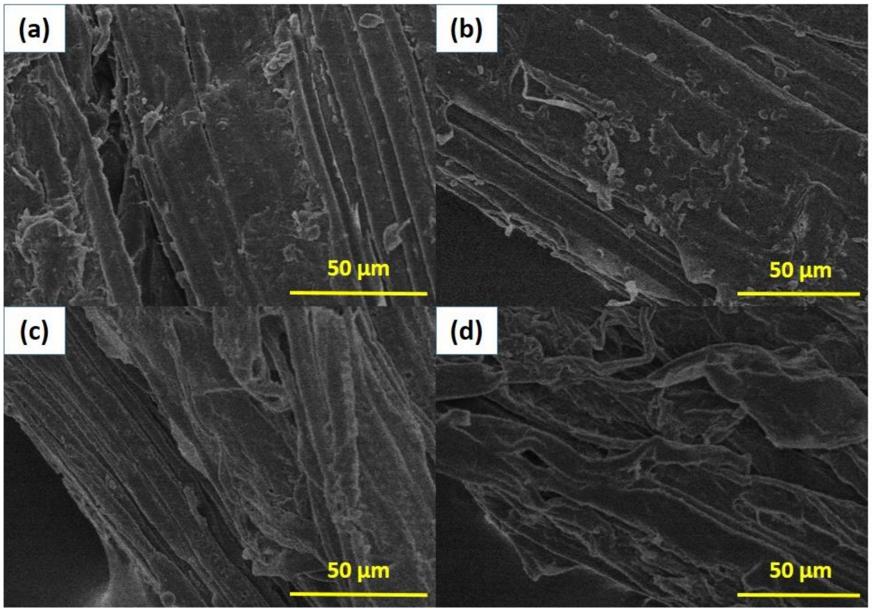

Fig. 5. SEM images of wheat straw before and after AEW pretreatment with a magnification of 800 times (a: raw materials; b: wheat straw pretreated at 150 °C for 2 h with ethanol concentration of 55% (v/v) and ammonia dosage of 60 wt%; c: wheat straw pretreated at 170 °C for 2 h with ethanol concentration of 18% (v/v) and ammonia dosage of 60 wt%; d: wheat straw pretreated at 170 °C for 2 h with ethanol concentration of 55% (v/v) and ammonia dosage of 60 wt%)

As shown, raw wheat straw (smashed by extrusion) had a relatively smooth surface, and the fiber bundles were tightly compacted (Fig. 5a), indicating that pulverization by extrusion did not cause clear fiber delamination and fibrillation in this work. After AEW pretreatment, the fiber surface became rougher and the compact structure became looser under the relatively mild pretreatment conditions (Fig. 5b). With the increase of energy input, the fiber bundles were fragmented and the open structures could be clearly observed (Fig. 1c). Through further increasing ammonia dosage to 60 wt% in AEW pretreatment, the fiber bundles were remarkably loosened, a large number of long fibers were broken, and microfibers were clearly exposed (Fig. 5d), leading to the increments of specific surface area and porosity. In fact, based on BET analysis, the specific surface area and pore volume of wheat straw after AEW pretreatment under optimal conditions increased approximately 20% and 67%, respectively, compared to the raw material. These phenomena were because AEW pretreatment could efficiently remove lignin and extractives (Fig. 1 and Tables 2 to 4), leading to the improvement of enzyme accessibility to pretreated substrates. Thus, the enzymatic conversion efficiencies of wheat straw were clearly promoted after AEW pretreatment (Figs. 1 and 3).

In addition, the removal of lignin and extractives after AEW pretreatment of wheat straw can also be verified by FTIR and XRD analyses. As displayed in Fig. 6, the relatively decreased intensities of the characteristic peaks of hemicelluloses (at 1740 cm^{-1}) and lignin (at 1510 and 1423 cm^{-1}), as well as the relatively increased intensity of the feature peak of cellulose (at 897 cm^{-1}) indicated some degradation of hemicellulose (removal of acetyl groups and carboxylic acids) and delignification after pretreatment (Xie *et al.* 2018; Chen *et al.* 2019). Furthermore, crystallinity of lignocellulose is also an indicator of enzyme accessibility (Xu *et al.* 2019).

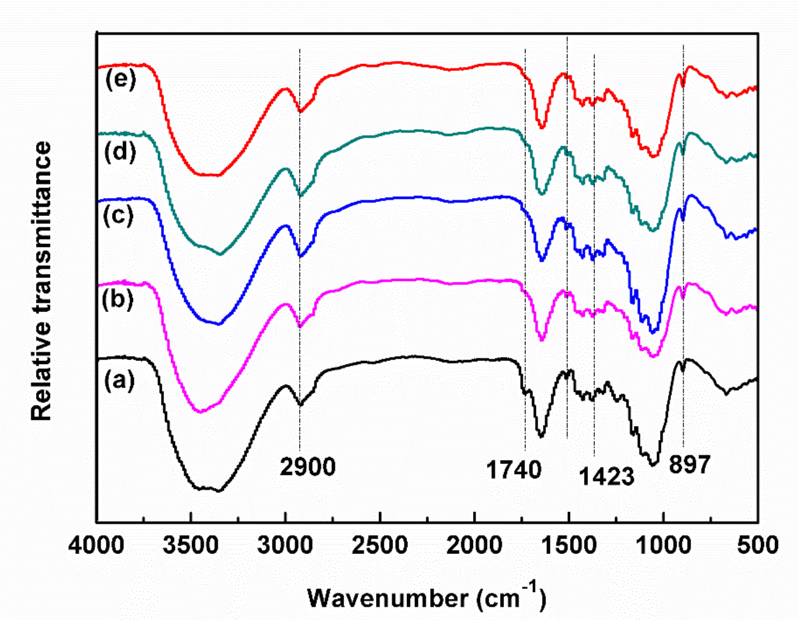

Fig. 6. FTIR spectra of wheat straw before and after AEW pretreatment (a: raw material; b: wheat straw pretreated at 150 °C for 2 h with ethanol concentration of 55% (v/v) and ammonia dosage of 60 wt%; c: wheat straw pretreated at 170 °C for 2 h with ethanol concentration of 18% (v/v) and ammonia dosage of 60 wt%; d: wheat straw pretreated at 170 °C for 2 h with ethanol concentration of 55% (v/v) and ammonia dosage of 20 wt%; e: wheat straw pretreated at 170 °C for 2 h with ethanol concentration of 55% (v/v) and ammonia dosage of 60 wt%)

Table 6 lists the crystallinity values of wheat straw samples evaluated by FTIR and XRD. Both *TCI* and *LOI* were calculated based on FTIR analysis, reflecting the crystallinity changes of cellulose in wheat straw samples, while the *CrI* values according to XRD measurement indicated the crystallinity modification of the entire wheat straw sample. Table 7 shows that both *TCI* and *LOI* of cellulose slightly increased, indicating some degradation of non-crystalline regions of cellulose after pretreatment (Li *et al.* 2015). However, the *CrI* of pretreated wheat straw gradually increased with the increase of the severity of AEW pretreatment. For instance, the *CrI* (55.8%) of wheat straw pretreated at the optimal conditions was 25.4% higher compared to the raw material (44.5%). The increments of *CrI* were associated with the removal of amorphous components (such as lignin, extractives) in pretreated wheat straw (Xu *et al.* 2015), which were in good agreement with the obtained results shown in Fig. 1 and 3, as well as Tables 2 to 4.

Table 6. Crystallinity of Wheat Straw Samples Evaluated by FTIR and XRD

Samples	Energy Input	Ethanol Concentration (%, v/v)	Ammonia Dosage (wt%)	*TCI*	*LOI*	*CrI* (%)
a	Raw wheat straw			0.902	0.71	44.5
b	150 °C, 2 h	55	60	0.934	0.75	47.0
c	170 °C, 2 h	18	60	0.991	0.81	50.6
d	170 °C, 2 h	55	20	1.080	0.83	53.2
e	170 °C, 2 h	55	60	1.110	0.86	55.8

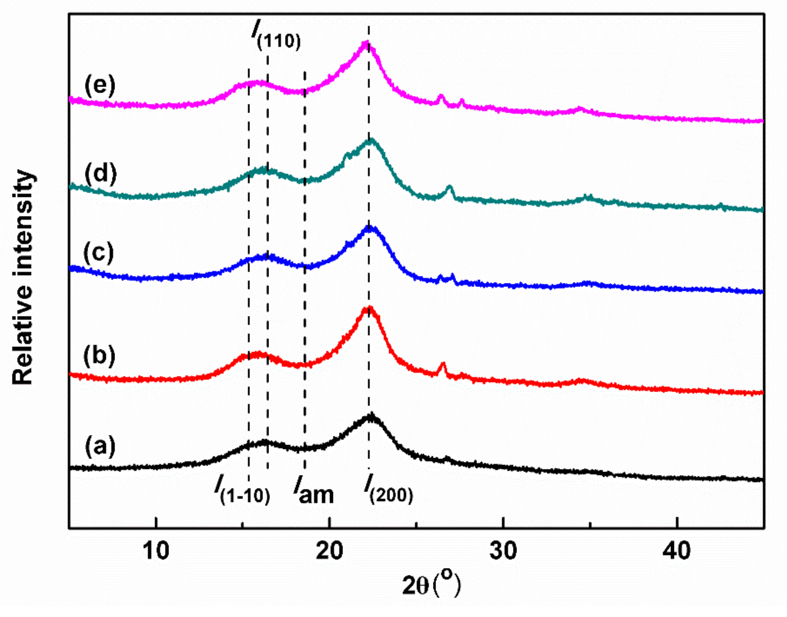

Fig. 7. XRD patterns of wheat straw before and after AEW pretreatment (a: raw material; b: wheat straw pretreated at 150 °C for 2 h with ethanol concentration of 55% (v/v) and ammonia dosage of 60 wt%; c: wheat straw pretreated at 170 °C for 2 h with ethanol concentration of 18% (v/v) and ammonia dosage of 60 wt%; d: wheat straw pretreated at 170 °C for 2 h with ethanol concentration of 55% (v/v) and ammonia dosage of 20 wt%; e: wheat straw pretreated at 170 °C for 2 h with ethanol concentration of 55% (v/v) and ammonia dosage of 60 wt%)

Characterization of SLS

The fractionated and purified lignin from AEW pretreatment with optimal conditions was first acetylated and then used to prepare SLS. Before acetylation, lignin samples of P-lignin and D-lignin were obtained by acid precipitation and direct drying of spent liquor, respectively, after recovery of ethanol and ammonia. As tested by GPC, the molecular weights (M_w) of P-lignin and D-lignin were 4000 and 29290, respectively, and the corresponding polydispersity index values were 1.13 and 1.17, respectively. The higher M_w of D-lignin indicated that more re-polymerization and condensation of lignin occurred in direct drying of the concentrated spent liquor.

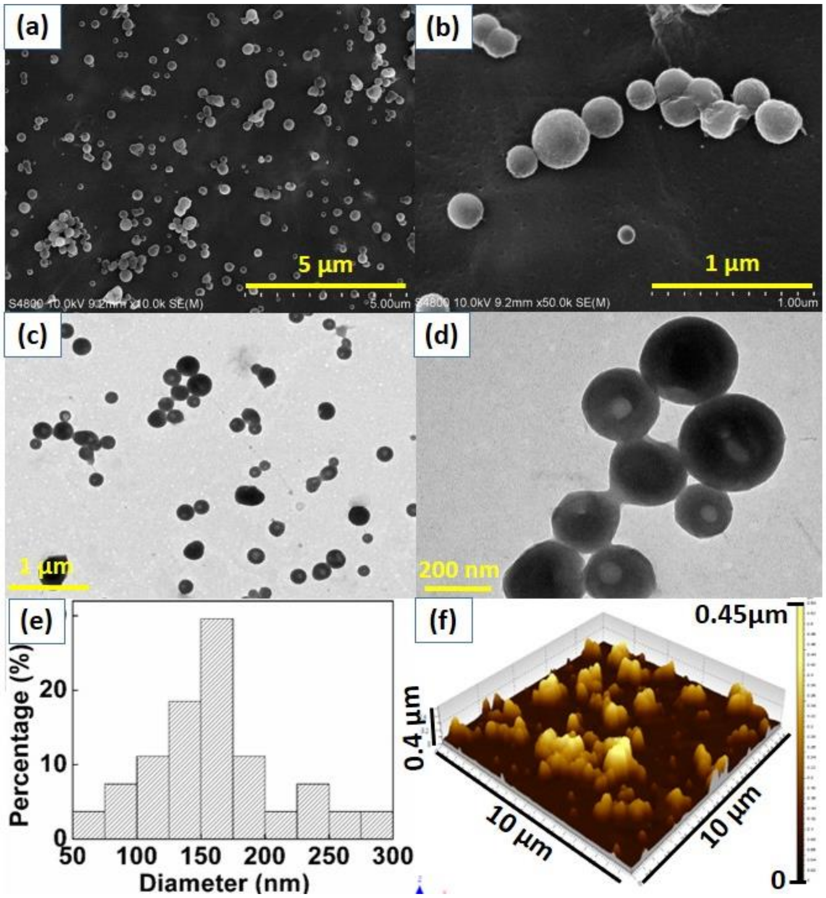

Fig. 8. Morphologies of submicron lignin spheres prepared using the lignin obtained by acid precipitation from the spent liquor derived from AEW pretreatment of wheat straw (a and b: SEM images with magnifications of 10k and 50k respectively; c and d: TEM images; e: Diameter distribution based on TEM analysis; f: AFM image)

The morphologies of SLS prepared using P-lignin are given in Fig. 8. As shown in Figs. 8a and c, the resulting SLS particles had good dispersibility in the mixed THF-water solution. The SLS particles showed clear boundaries, smooth surface, and spherical morphologies (Fig. 8b and c). The diameters of the SLS particles ranged from 50 to 300 nm and the corresponding average diameter was 161.2 ± 53.6 nm, based on the statistics of TEM analysis. Each SLS particle clearly exhibited a hollow structure, as displayed in Fig. 8c. But the conditions of AEW pretreatment had no clear impact on the morphologies and size of the obtained SLS by the same preparation method in this work. This was likely because the acid precipitation and acetylation of lignin reduced the difference of the obtained lignin samples, thus minimizing the effects of pretreatment conditions on the properties of the final SLS. More work on the structure characterization of lignin samples should be done in future. The as-prepared SLS particles with the unique structure have many potential applications, such as the carrier for the controlled release of drugs or agricultural activities (Sameni *et al.* 2018), the fillers in composites (Zhang *et al.* 2019).

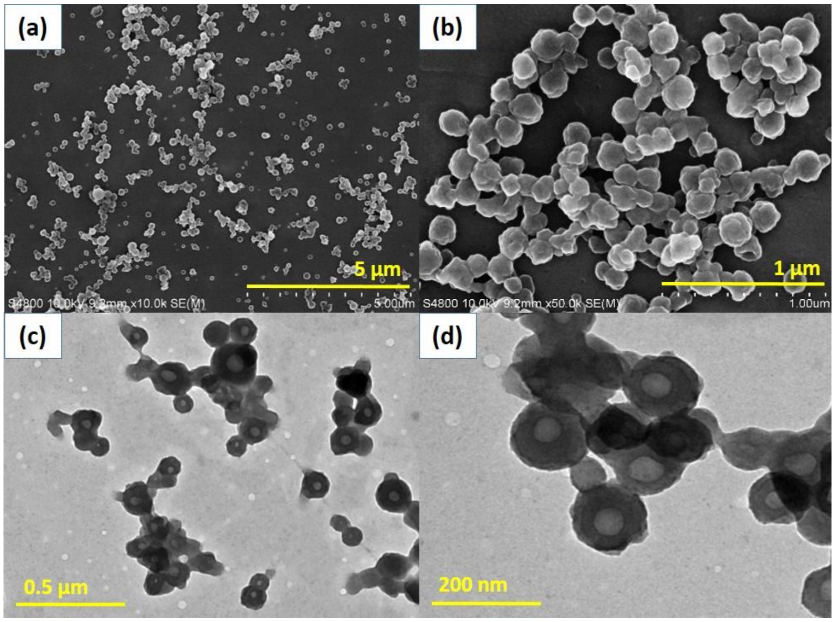

Fig. 9. Morphologies of submicron lignin spheres prepared using the lignin obtained after direct dry of spent liquor derived from AEW pretreatment of wheat straw (a and b: SEM images with magnifications of 10k and 50k respectively; c and d: TEM images)

For comparison, the SLS particles were also prepared using D-lignin. The resulting SLS particles had more uniform diameters in the range of 100 to 200 nm with the smaller average diameter of 134.1 ± 43.6 nm, but also exhibited a hollow structure (Fig. 9). However, the obtained SLS particles from D-lignin showed rougher surface and more aggregation compared to the one fabricated with P-lignin. The morphology difference of SLS is largely dependent on the nature of the obtained lignin itself (such as molecular weight, purity, and functional groups) (Zhao *et al.* 2016). The nonuse of acid in the fabrication of SLS is a cleaner and more sustainable process.

Mass Balance

The mass balance of AEW pretreatment under the optimal conditions and enzymatic saccharification was evaluated, as shown in Fig. 10. Pulverized wheat straw (1 kg, oven-dried basis) was pretreated at 170 °C for 2 h with an ethanol concentration of 55% (v/v) and an ammonia dosage of 60 wt% (corresponding ammonia concentration 7.5 wt%). Upon completion of AEW pretreatment, solid and liquid phases were separated, and the cellulosic solid residue (with a yield of 57.2%) was washed to remove the extracted substances (*e.g.*, degraded lignin, extractives) in pretreatment. The washed solid with 283 g glucan, 106 g xylan, 58 g lignin, and 12 g extractives was subjected to enzymatic hydrolysis. After saccharification, 404 g fermentable sugars (291 g glucose + 113 g xylose) were obtained in the hydrolyzate, and the corresponding $Y_{\text{total sugar}}$ was 81.7 ± 0.2%, which was comparable with the modified alkali pretreatment (80%) with 11 wt% NaOH, 2 wt% sodium lignosulfonate, and 0.1 wt% anthraquinone (Xu *et al.* 2015). Additionally, comparable $Y_{\text{total sugar}}$ (80.2 ± 0.6%) was obtained for AEW pretreatment without washing before saccharification (Fig. 10b), although purification of hydrolyzate might be needed for the downstream process. However, AEW pretreatment does not need the high capital cost for NaOH recovery, because both ethanol and ammonia can be easily recovered and reused. According to the authors' experimental results, the recovery efficiencies of ethanol and ammonia were 93.7% and 85.7%, respectively.

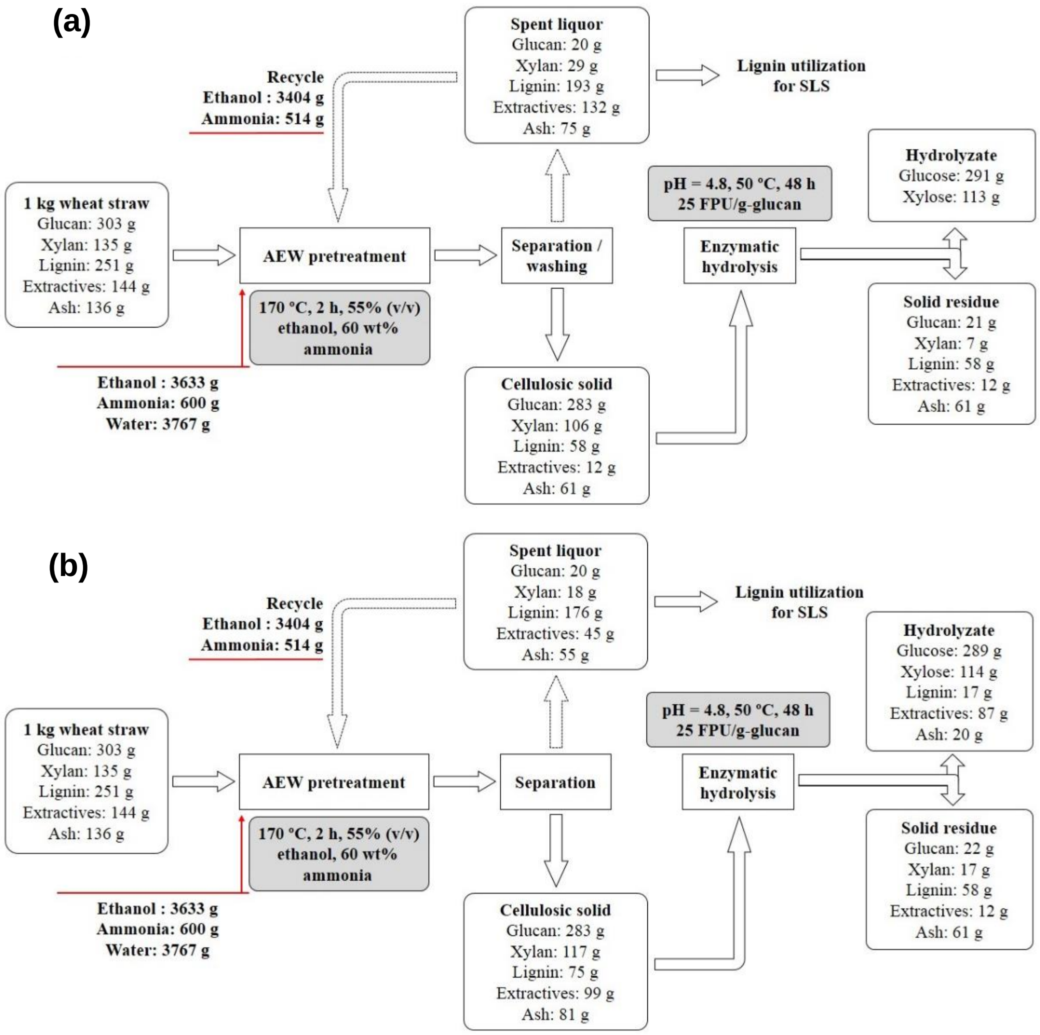

Fig. 10. Mass balance of AEW pretreatment and saccharification (a: with washing; b: without washing)

Based on the organic element analysis (Table 7), nitrogen content in the fractionated lignin was 5.2%, which was obviously higher compared to raw wheat straw (approximately 0.6%). This was because ammonia could react with lignin, causing lignin degradation *via* ammonolysis (Kim *et al.* 2003). The additional loss of approximately 9% ammonia and 6% ethanol could be further lowered in large-scale application with good sealing of equipment and efficient operation. Therefore, AEW pretreatment was a more economical and sustainable process compared to alkali-based pretreatment, particularly for a relatively small plant for the treatment of agricultural waste with a limited collection radius. In addition, with the concept of integrated biorefinery (Van Heiningen 2006; Jahan and He 2018), the fractionated lignin can be used to prepare the high value SLS to further increase the economic feasibility of the overall process of AEW pretreatment. As for the preparation of SLS in THF, the solvent of THF can also be readily recovered by evaporation because the boiling point of THF is 66 °C. As tested in this work, the recovery of THF could be above 98%.

Table 7. Organic Element Analysis of Wheat Straw Before and After AEW Pretreatment

Samples	N (%)	O (%)	C (%)	H (%)	S (%)
Raw Materials	0.58	51.09	42.62	4.90	0.81
Pretreated Wheat Straw	0.32	54.43	39.43	5.00	0.82
Fractionated Lignin [a]	5.20	33.86	55.53	4.60	0.81

[a] Purified lignin obtained by acid precipitation of spent liquor and the followed washing with deionized water

CONCLUSIONS

1. Ammonia-ethanol-water pretreatment of wheat straw was conducted and evaluated by enzymatic saccharification. Results exhibited that ammonia-ethanol-water (AEW) pretreatment is a promising approach with many advantages, such as high recovery of available carbohydrates after pretreatment as well as high lignin removal (77%) and high extractives removal (90%).
2. The AEW pretreatment can achieve easy recovery and reuse of chemicals and solvent (clean process), reduced water use (without washing of substrate before saccharification), and the high value utilization of the fractionated lignin.
3. The preparation of submicron lignin spheres with unique structure and average diameter of 161.2 ± 53.6 nm could valorize lignin, thus further increasing the economic viability of the entire process of AEW pretreatment.

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