Response Surface Optimization of Ammonium Sulfite Pretreatment for Fermentable Sugar Production from Wheat Straw

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The development of a clean and sustainable pretreatment is of great importance for the production of fermentable sugars. In this study, an ammonium sulfite (AS) pretreatment of wheat straw was optimized based on response surface methodology with a three-level, three-factor Box-Behnken design. The investigated factors were AS dosage, pretreatment time, and pretreatment temperature. The effectiveness of the AS pretreatment was evaluated using the standard enzymatic hydrolysis procedure. A second-order polynomial fit was performed to fit the experimental data, and the model analysis showed that the effect of the AS dosage on the final total sugar yields was much more significant than that of the other two factors. Under the optimum pretreatment conditions (27% of the AS dosage (based on the dry wheat straw) at 160 ºC for 63 min), the final total sugar yield achieved was 74.4% after saccharification, which was in agreement with the predicted value (76.5%). Furthermore, it was found that pre-impregnation with acetic acid before AS pretreatment or the post-mechanical refining after AS pretreatment could further increase the fermentable sugar yields to approximately 77%. In addition, the spent liquor containing nitrogen could be used for the production of lignin-based fertilizer, thus making the whole process clean and sustainable.

Keywords: Wheat straw; Ammonium sulfite pretreatment; Response surface methodology; Fermentable sugars; Biorefinery

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INTRODUCTION

Due to the increasing consumption of fossil fuels, the development of environmentally friendly and renewable energy has received special attention (Rastogi and Shrivastava 2017). Lignocellulose is the most abundant renewable material with an annual global production of approximately 200 billion metric tons (Juhász *et al.* 2005), and its utilization is not in competition with food. Thus, converting lignocellulosic biomass into liquid fuel, valuable chemicals, and materials has been the research hotspot in recent years (Moreira *et al.* 2011; Kumar *et al.* 2014).

Wheat straw, as one of the most abundant renewable lignocellulose resources (the global annual output of wheat straw was over 600 million tons next to rice straw and corn stover), usually contains 33% to 40% cellulose and 20% to 25% hemicellulose (Prasad *et*

al. 2007), which can be converted to fermentable sugars (*i.e.*, sugar platform) for further conversion. However, most of wheat straw has been burned directly in the field in China for a long time, causing serious environmental issues such as the increase of fine particulate matter (particulate matter (PM) 2.5, particles smaller than 2.5 μm) in the air (Li *et al.* 2018). Currently, the burning of agricultural waste is strictly prohibited by the Chinese government. Therefore, to make better use of agricultural wastes (*e.g.*, wheat straw), the production of fermentable sugars for the manufacturing of bioethanol has become an urgent concern both for environmental protection and the development of alternative energy sources for vehicles and other forms of transportation (Xie *et al.* 2018).

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin, which are strongly interacted and connected by non-covalent forces, covalent cross-linkages, or hydrogen bonding, resulting in a strong resistance to degradation against outside forces (Ding *et al.* 2012). Therefore, pretreatment is required to loosen the recalcitrant structure of natural lignocelluloses for saccharification and fermentation for biofuel production (Xu *et al.* 2016).

Over the past decades, extensive efforts have been made and various pretreatment approaches have been employed, such as biological (*e.g.*, white-rot fungi), physical (*e.g.*, grinding, irradiation), chemical (*e.g.*, alkali, acids, solvents, or supercritical fluids), and a combination of these methods (Galbe and Zacchi 2007; Lee *et al.* 2009; Fatriasari *et al.* 2014, 2015). However, different pretreatments have different advantages and disadvantages, such as low efficiency, strict operating conditions, or serious pollution risk, which may dramatically increase the process cost and hinder the feasibility of commercialization (Li *et al.* 2017a,b).

Furthermore, a high lignin content of lignocellulosic biomass could restrict the enzymatic hydrolysis, because of the nonproductive binding between lignin and enzyme, causing the inactivation of cellulase (Paixão *et al.* 2016). Therefore, the efficient removal of lignin from lignocelluloses is the most commonly used strategy for biomass pretreatment (Xu *et al.* 2016). Up to now, several kinds of pretreatment approaches have been developed for lignin removal with varied successes, including alkali-based pretreatments (Li *et al.* 2017b), organosolv pretreatment (Mou *et al.* 2014), deep eutectic solvents pretreatment (Satlewal *et al.* 2018), and sulfite pretreatment (Zhu *et al.* 2009; Yang *et al.* 2013). However, the process' cost or capital cost for chemical or solvent recovery is quite high, particularly for the small-scale mills using agricultural waste (*e.g.*, wheat straw) as the feedstock, due to the limitation of the material collection radius (Xie *et al.* 2018). Thus, the development of an economical and eco-friendly pretreatment process for efficient delignification of lignocellulose is of crucial importance for the production of fermentable sugars *via* enzymatic saccharification, particularly for industrial applications (Wu *et al.* 2018).

In the case of sulfite pretreatment, the degradation and sulfonation of lignin promotes lignin removal, thus increasing the digestibility of the pretreated substrate (Li *et al.* 2014; Qi *et al.*, 2018). Additionally, the hydrophilicity of the residual lignin could be increased due to the sulfonation, leading to the reduction of non-productive hydrophobic adsorption of enzymes by lignin (Gong *et al.* 2015). Usually, sodium sulfite is used for sulfite pretreatment (Zhu *et al.* 2009; Yang *et al.* 2013), and the chemical recovery has to be well handled (Jin *et al.* 2015). In the authors' previous work, ammonium sulfite (AS) pretreatment was used to treat tobacco stalk (Wang *et al.* 2018) and *Eulaliopsis binata* (Wu *et al.* 2018) for efficient delignification, and the spent liquor of AS pretreatment can be used to produce fertilizer due to the high nitrogen content. Qi *et al.* (2018) studied the

impact of AS pretreatment on the improvement of enzymatic hydrolysis of wheat straw by single factor experiments, and verified that lignin could be efficiently removed by sulfonation and ammonolysis during AS pretreatment. But they didn't investigate the combined effect of the AS pretreatment factors, and the AS pretreatment process was not fully optimized. In addition, the safety of the production of fertilizer using the spent liquor from AS pretreatment was not evaluated.

Therefore, in the present work, AS pretreatment was used to treat wheat straw for the production of fermentable sugars, and the effectiveness of AS pretreatment was evaluated through enzymatic hydrolysis following the standard National Renewable Energy Laboratory (NREL) procedure (Sluiter *et al.* 2008). Based on the authors' previous study (single factor experiment), the response surface methodology (RSM) with a three-level, three-factor Box-Behnken design was adopted for the optimization of AS pretreatment and the investigation of the combined impact of the key factors of AS pretreatment on the downstream saccharification. Three key variables of AS dosage, pretreatment temperature, and pretreatment time were investigated for the RSM experiments, and the final total sugar yield after AS pretreatment and saccharification was selected as the response variable. In addition, the mass balance under the optimized conditions was discussed, and the wheat straw before and after the AS pretreatment was comprehensively characterized using elemental analysis, scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy, and X-ray diffraction (XRD) to investigate the impact of pretreatment on the property changes of wheat straw. Most importantly, the safety of the spent liquor derived from AS pretreatment for the production of lignin-based fertilizer was evaluated. The manufacture of lignin-based fertilizer using spent liquor could make the whole process of AS pretreatment clean and sustainable. In a word, the clean and effective production of fermentable sugars is of crucial importance for the sustainable conversion of lignocelluloses to bioenergy or chemicals (*e.g.*, furfural, glycol).

EXPERIMENTAL

Materials

Wheat straw was collected from Dingyuan, Anhui province, China. The air-dried straw was ground and milled with a twin-screw extrusion (self-designed; Manufactured by Tianzheng Screening Pulping Equipment Co., Ltd., Hebei, China) system (the maximum throughput was 200 kg/h) before the experiments (Liu *et al.* 2013), and the milled wheat straw (with the size of about 1 cm) was stored in sealed plastic bags at 4 ºC for moisture balance before pretreatment. According to the analysis based on the NREL procedure, the wheat straw contained 34.6% ± 0.3% glucan, 16.2% ± 0.1% xylan, 1.0% ± 0.0% arabinan, 0.7% ± 0.0% acetyl groups, 22.6% ± 0.2% lignin, 14.4% ± 0.2% extractives, and 10.6% ± 0.0% ash. All analytical-grade chemical reagents, including ammonium sulfite (AS), sodium citrate, citric acid, ethanol, acetic acid, and sulfuric acid, were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and directly used without further purification. The cellulase (from *Trichoderma reesei*) used for enzymatic hydrolysis was gifted by Qingdao Vland Biotech Inc. (Qingdao, China), and the activity of cellulase was 85 FPU/mL, which was determined by the standard procedure (Ghose 1987).

Pretreatment of wheat straw

The AS pretreatments were completed in a Parr Hastelloy reactor (Series 4560; Moline, IL, USA) that consisted of a heater, stirrer, stainless steel vessel (300 mL), and controllers for the adjustment of stirring speed and pretreatment temperature. For each pretreatment, 10 g of wheat straw (dry weight basis) was used with a certain AS dosage and a solid/liquid ratio of 1:8 (w/w). The acquired amount of AS was dissolved in water to make AS solution before use, and then wheat straw was added in AS solution. After sufficient mixing, the mixtures were heated from room temperature to the designated temperature (150, 160, and 170 °C, respectively) with a stirring speed of 200 rpm (optimized). The key factors of pretreatment time, pretreatment temperature, and AS dosage were investigated for the optimization of AS pretreatment. After pretreatment, the heater was turned off and the cooking pot was rapidly cooled for 10 min with cool water. Then, the pretreated material was removed and separated into solid (pretreated wheat straw) and liquid (spent liquor) using a filtration with non-woven fabric bag (self-made) with 500 mesh. The pretreated stock was washed with de-ionized water until neutrality. The washed stock and spent liquor samples were stored at 4 °C for further tests and analysis. All of the experiments were conducted in triplicate in this study and the average value was reported.

In addition, the scale-up experiment of AS pretreatment under the optimized conditions was also conducted in a cooking digester (VRD-42SD-A; China Pulp and Paper Research Institute, Beijing, China) with a total sample amount of 200 g (dry weight straw).

Additionally, a higher content of extractives and ash in the substrate could hinder the downstream enzymatic hydrolysis (Yuan *et al.* 2018), and post mechanical refining could increase the specific surface area of substrate to facilitate the enzymatic hydrolysis (Xu *et al.* 2014). Hence, a pre-impregnation stage (for the removal of extractives and ash) before AS pretreatment and post-mechanical refining (for the increase of the specific surface of substrate) after AS pretreatment was employed to further improve the enzymatic digestibility of the pretreated wheat straw. According to the authors' previous work, pre-impregnation was performed at 40 °C for 3 h with 3 wt% of acetic acid (AA) dosage (based on the dry weight of straw) and a solid/liquid ratio of 1:10 (w/w). Upon completion of pre-impregnation, the solid stock was washed with de-ionized water until neutrality, and then the washed stock was stored at 4 °C for further treatment and analysis.

As for post-mechanical refining, the pretreated and washed wheat straw was refined by a pulp refining instrument (PFI) mill (PL11-00; Xianyang TEST Equipment Co., Ltd., Xianyang, China), which was conducted with 10 wt% of solid consistency, 4000 revolution numbers, and refining clearance of 0.24 mm, based on the authors' previous work (Xu *et al.* 2014). Afterwards, the refined pulp was collected and stored at 4 °C for the further evaluation of enzymatic hydrolysis.

Experimental Design

To examine the individual and combined effects of the key pretreatment variables on the response factor, the Box-Behnken design with three key variables was employed to optimize the AS pretreatment conditions. The selected independent variables were pretreatment temperature (°C), AS dosage (wt%), and pretreatment time (min). The total sugar yield (%) was selected as a response for analysis. The levels of factors were chosen based on preliminary trials to ensure a proper range. A set of 17 trials that ran with five replicates at central points were designed using Design-Expert 8.0.6 software (Stat-Ease Inc., Minneapolis, MN, USA). Each of the trial points was replicated three times.

The multifactor experiment was designed by the interaction between the pretreatment temperature, pretreatment time, and AS dosage, and the response of the total sugar yield to the varied conditions were modeled *via* RSM to obtain the optimal conditions. The ranges of factors were chosen based on preliminary trials to describe the reaction space. The independent variables were transformed to the range between -1 and 1 for the appraisals of factors and the corresponding levels for the three variables are summarized in Table 1. The data were fitted with a regression model that was described using a quadratic polynomial equation, and the fit of the models were evaluated through comparing R^2 and the adjusted R^2. The validations under the optimized conditions and critical conditions were performed, and the statistical analysis of the data was performed using an analysis of variance (ANOVA).

Table 1. Ranges of Each Independent Variable in the Box-Behnken Design

Coded Levels of Factors	AS Dosage (wt%)	Time (min)	Temperature (°C)
Low level (-1)	10	40	150
Central level (0)	20	60	160
High level (+1)	30	80	170

Methods

Enzymatic hydrolysis

The enzymatic hydrolysis of pretreated wheat straw was performed in 50-mL bottles with the addition of 0.4 g pretreated biomass (dry weight) and cellulase (25 FPU/g-substrate, or 30 FPU/g-glucan). The pH was adjusted to 4.8 with a sodium citrate buffer, and 0.02% sodium azide was added to prevent microbial contamination, resulting in a final substrate consistency of 2% (w/v). The mixture was then incubated in an incubator shaker at 50 °C with 120 rpm for 48 h. At the end of the process, the mixture was immediately cooled to 4 °C to stop saccharification, and then the supernatants were filtered through a membrane (pore size of 0.22 μm) and analyzed for monomeric sugars using high performance liquid chromatography (HPLC).

Composition analysis

The compositional analysis of the native and pretreated wheat straw was determined using the two-step sulfuric acid hydrolysis method following the NREL analytical procedure (Sluiter *et al.* 2008). The amount of monomeric sugars in the liquid fractions were measured directly by an HPLC system (Model 1200; Agilent Technologies, Santa Clara, CA, USA) after neutralization and filtration through 0.22-μm nylon filters. The HPLC system was equipped with a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Waters, USA) at 55 °C and a refractive index detector (Chengdu GELAI Co., Ltd., Chengdu, China). The analysis was completed using sulfuric acid (0.005 M) as the mobile phase at a flow rate of 0.6 mL/min for 20 min. The evaluation of AS pretreatment and enzymatic hydrolysis was calculated using the following equations,

$$R_{\text{solid}}\ (\%) = \frac{M_{\text{pretreated biomass}}}{M_{\text{orignal biomass}}} \times 100 \quad (1)$$

$$R_{\text{glucan}}\ (\%) = \frac{R_{\text{solid}} \times C_{\text{glucan in pretreated biomass}}}{C_{\text{glucan in original biomass}}} \times 100 \quad (2)$$

$$R_{\text{xylan}}\ (\%) = \frac{R_{\text{solid}} \times C_{\text{xylan in pretreated biomass}}}{C_{\text{xylan in original biomass}}} \times 100 \quad (3)$$

$$D_{\text{lignin}}\ (\%) = 1 - \frac{R_{\text{solid}} \times C_{\text{lignin in pretreated biomass}}}{C_{\text{lignin in original biomass}}} \times 100 \quad (4)$$

$$Y_{\text{total sugar}}\ (\%) = \frac{M_{\text{glucose in hydrolyzate}} \times 0.9 + M_{\text{xylose in hydrolyzate}} \times 0.88}{M_{\text{glucan in original biomass}} + M_{\text{xylan in original biomass}}} \times 100 \quad (5)$$

where M refers to the mass of the corresponding substance (g), C refers to the content of the corresponding component in biomass (wt%), and R_{solid} (%) is the percentage of solid recovery after pretreatment. R_{glucan} (%) and R_{xylan} (%) are the recovery rates of glucan and xylan, respectively, and D_{lignin} (%) is the delignification rate. The total sugar yield ($Y_{\text{total sugar}}$, %) was calculated as the percentage of the total sugar (glucose + xylose) in enzymatic hydrolysate divided by the total sugar in the corresponding raw material. All of the experiments were performed in triplicate, and the average data were reported.

Characterization

The FTIR analysis was conducted to detect the changes in the functional groups of the raw and pretreated wheat straw. Sample tablets were prepared by mixing each sample with potassium bromide (the ratio of sample to KBr was 1:100) before analysis. The spectra were recorded within the frequency range of 400 cm^{-1} to 4000 cm^{-1} using a FTIR spectrometer (Nicolet 6700; Thermo Fisher Scientific Inc., Waltham, MA, USA) with a detector at 4 cm^{-1} resolution and 32 scans per sample.

The surface morphologies and characteristics of the untreated and pretreated wheat straw were analyzed using SEM (S4800; Hitachi, Tokyo, Japan) and energy dispersive spectroscopy (EDS) (D8 Advance; Bruker, Karlsruhe, Germany) with the accelerating voltage of 5.0 kV and the backscatter electron detector. The images were taken with the magnification of 2000, and the working distance was 8.1-8.6 mm for different images. The freeze-dried samples were sputter coated (vacuum spraying for 90 s) with a thin gold layer of 2.5-2.8 nm in thickness (Hitachi, Tokyo, Japan) prior to analysis.

Inductively coupled plasma mass spectrometry (ICP-MS) (Agilent ICP-OES 730; Agilent Technologies, Santa Clara, CA, USA) as a highly sensitive mass spectrometry was used on the spent liquor samples to assess the presence of several trace elements.

The crystallinities of the lyophilized samples were measured using an X-ray diffractometer (D8 ADVANCE; Bruker Co., Karlsruhe, Germany). The scattering angle (2θ) ranged from 10º to 50º with a scanning speed of 0.5º per min and the Ni-filtered Cu Kα radiation was generated at 80 mA and 40 kV. The crystallinity index (*CrI*) was calculated according to the empirical method (Segal *et al.* 1959),

$$CrI\ (\%) = (I_{002} - I_{\text{am}}) / I_{002} \times 100 \quad (6)$$

where I_{002} is the maximum of the diffraction intensity (the 002 plane) and I_{am} is the minimum diffraction intensity (between 101 peaks and 002 peaks).

RESULTS AND DISCUSSION

Model Fitting and Statistical Analysis

A summary of the experimental design is listed in Table 2. The final total sugar yields ($Y_{\text{total sugar}}$) for the 17 trials were in the range of 42.4% to 75.8%, while the corresponding delignification rate ranged from 44.7% to 78.0%. Severe pretreatment conditions could lead to a higher lignin removal. For instance, for Trial 1 with 10 wt% AS

dosage at 160 °C for 40 min, the D_{lignin} was 57.6%, which was lower compared to Trial 2 (D_{lignin} of 66.2%) that had a higher AS dosage (30 wt%). However, excessive reaction severity could cause the over-degradation of carbohydrates. To obtain a high final total sugar yield, a high delignification rate was needed for the improvement of cellulase accessibility, and simultaneously, a higher sugar recovery was also required after pretreatment (Mathew *et al*. 2011; Kim and Han 2012). Therefore, Trial 14 with the D_{lignin} of 67% and solid recovery of 61% had the highest $Y_{total\ sugar}$ (75.8%), but Trial 8 with a relatively higher D_{lignin} of 78% had a lower $Y_{total\ sugar}$ (66.6%) because the corresponding R_{solid} was only 47%, which indicated a severe sugar loss during pretreatment.

Table 2. Summary of the Box-Behnken Design Used for RSM Analysis and the Corresponding Responses

Trial	Variables			Responses						
	AS Dosage (wt%)	Time (min)	Temp. (°C)	R_{solid} (%)	R_{glucan} (%)	R_{xylan} (%)	D_{lignin} (%)	Actual $Y_{total\ sugar}$ (%)	Predicted $Y_{total\ sugar}$ (%)	Residual
1	10	40	160	59.4±1.50	93.0±2.26	75.0±2.53	57.6±0.61	59.3±0.80	56.7	2.61
2	30	40	160	62.6±1.33	93.6±2.61	81.5±1.00	66.2±1.44	72.4±0.42	71.6	0.80
3	10	80	160	54.6±0.68	72.5±0.36	68.6±0.16	65.2±1.86	57.5±0.14	58.3	-0.80
4	30	80	160	53.4±0.75	77.6±0.97	65.9±0.55	75.1±1.51	70.7±0.08	73.3	-2.61
5	10	60	150	66.4±1.11	79.0±0.55	89.6±0.42	44.7±1.35	42.4±0.67	43.5	-1.06
6	30	60	150	60.0±1.47	93.4±1.43	84.3±1.15	61.4±0.47	70.8±0.46	70.0	0.75
7	10	60	170	51.7±0.81	74.1±0.07	59.0±0.20	65.0±0.10	61.5±0.56	62.3	-0.75
8	30	60	170	47.0±0.89	84.3±0.61	51.8±0.15	78.0±2.00	66.6±0.79	65.6	1.06
9	20	40	150	66.8±0.55	93.8±2.20	93.5±3.49	49.6±2.11	54.3±0.20	55.8	-1.55
10	20	80	150	64.8±0.16	90.1±0.22	87.1±0.06	55.0±2.55	64.6±0.14	62.8	1.85
11	20	40	170	56.6±0.77	86.8±0.30	66.0±0.15	66.9±0.65	66.5±1.42	68.3	-1.85
12	20	80	170	48.2±0.61	87.4±0.29	52.0±0.39	77.2±0.89	66.2±0.31	64.6	1.55
13	20	60	160	59.8±1.51	90.1±2.40	67.0±2.22	66.1±0.04	72.9±1.06	74.0	-1.08
14	20	60	160	61.0±1.13	89.3±2.01	70.5±2.11	67.0±0.48	75.8±0.59	74.0	1.84
15	20	60	160	59.5±2.01	91.5±1.29	66.2±0.84	65.6±0.08	75.3±1.20	74.0	1.32
16	20	60	160	62.2±1.15	88.8±1.13	68.1±1.45	65.2±0.15	73.1±0.47	74.0	-0.91
17	20	60	160	58.7±0.79	90.8±0.96	63.2±1.06	67.6±0.32	72.8±0.66	74.0	-1.18

According to the regression analysis of each response, quadratic models were selected, as verified by the software based on the effect of the combined independent variables on the responses. The $Y_{total\ sugar}$ for the trials was set as the responsive variable for modelling to reach the highest final total sugar yield. Thus, the final quadratic polynomial model in terms of actual factors is shown by Eq. 7,

$$Y_{total\ sugar}\ (\%) = -2382.3755 + 12.3636X_1 + 3.1409X_2 + 27.5134X_3 - 2.5 \times 10^{-5}X_1X_2 - 0.0582X_1X_3 - 0.0133X_2X_3 - 0.0579X_1^2 - 8.0819 \times 10^{-3}X_2^2 - 0.0787X_3^2 \quad (7)$$

where, X_1, X_2, and X_3 are the AS dosage (wt%), pretreatment time (min), and pretreatment temperature (°C), respectively.

In the above equation, positive signs of the regression coefficients showed a synergistic effect, while negative signs indicated an antagonistic effect. In addition, statistical analysis of the full quadratic models was performed using an analysis of variance (ANOVA) to evaluate the effects of the variables on $Y_{total\ sugar}$ and the possible interaction between the key variables. The fit of the models were evaluated by comparing the R^2 and the adjusted R^2. The statistical significance was determined by an F-test and P-value. It has been suggested that the R^2 value should be no less than 0.80, and the P-value should be

less than 0.05, for a good fit of the developed model (Ruangmee and Sangwichien 2013; Auxenfans *et al.* 2014).

The ANOVA results of the developed model are presented in Table 3. The model fit the data with an R^2 of 0.9692 for $Y_{\text{total sugar}}$, which implied that only 3.08% of the total variation could not be explained by the model. This regression model was significant at the 99.98% confidence level indicating that the model equation adequately described the response. The low P-value (0.0002) of the model also confirmed that the fit was good and the predictive power of the adjusted model was rather high. Additionally, the lack of fit (LOF) value represented the misfit probability of the predicted and adjusted coefficient, and no significant LOF indicated a good fitting of the data (Sindhu *et al.* 2011). The F-value of 4.68 implied the LOF was not significant relative to the pure error, and there was only an 8.50% chance that this large (F) could occur because of noise.

Table 3. ANOVA for Response Surface Quadratic Model for Total Sugar Yield

Source	Sum of Squares	DF	Mean Square	F Value	P-value Prob. > F	Significance
Model	1209.31	9	134.37	24.49	0.0002	**
A	446.41	1	446.41	81.37	< 0.0001	**
B	5.53	1	5.53	1.01	0.3489	
C	103.03	1	103.03	18.78	0.0034	**
AB	1.000E-004	1	1.000E-004	1.823E-005	0.9967	
AC	135.26	1	135.26	24.65	0.0016	**
BC	28.36	1	28.36	5.17	0.0572	
A^2	140.92	1	140.92	25.69	0.0015	**
B^2	44.00	1	44.00	8.02	0.0253	*
C^2	260.97	1	260.97	47.57	0.0002	**
Residual	38.40	7	5.49			
Lack of Fit	29.89	3	9.96	4.68	0.0850	
Pure Error	8.51	4	2.13			
Corrected Total	1247.71	16				
R^2	0.9692					

DF: degree of freedom; A: AS dosage; B: pretreatment time; C: pretreatment temperature
**denotes very significance difference at P< 0.01; *denotes significance difference at P< 0.05

Moreover, as shown in Table 3, the AS dosage (X_1) and pretreatment temperature (X_3) ($P < 0.05$) were significant in affecting the total sugar yield, while the impact of pretreatment time (X_2) was not significant ($P > 0.05$). According to the P-value, the impact of independent variables followed the order $X_1 > X_3 > X_2$, and the quadratic terms of X_1^2, X_2^2, and X_3^2 were all significant with a P-value less than 0.05. Additionally, the interactions of X_1X_3 were significant, while the interactions of X_1X_2 and X_2X_3 were not significant. All statistical data showed that the developed model could be used to analyze and predict the relationship between the pretreatment variables and $Y_{\text{total sugar}}$.

Impact of Variables on Response

Response surfaces for the impacts of variables and their mutual effects on $Y_{\text{total sugar}}$ are shown in Fig. 1 (A to C), and the corresponding contour plots are presented in Fig. 1 (a to c), respectively. Two variables were varied in the certain ranges, while the other one was fixed at the central value. The slope of response surface indicated the extent of the response

with pretreatment conditions. For example, the steep curved surface meant that the response value was very sensitive to the pretreatment conditions. At the same time, the shape of the contour plot can reflect the intensity of the interaction effects. In general, the oval reveals a strong interaction of the two factors, which was opposite to the circle (Lu *et al.* 2017).

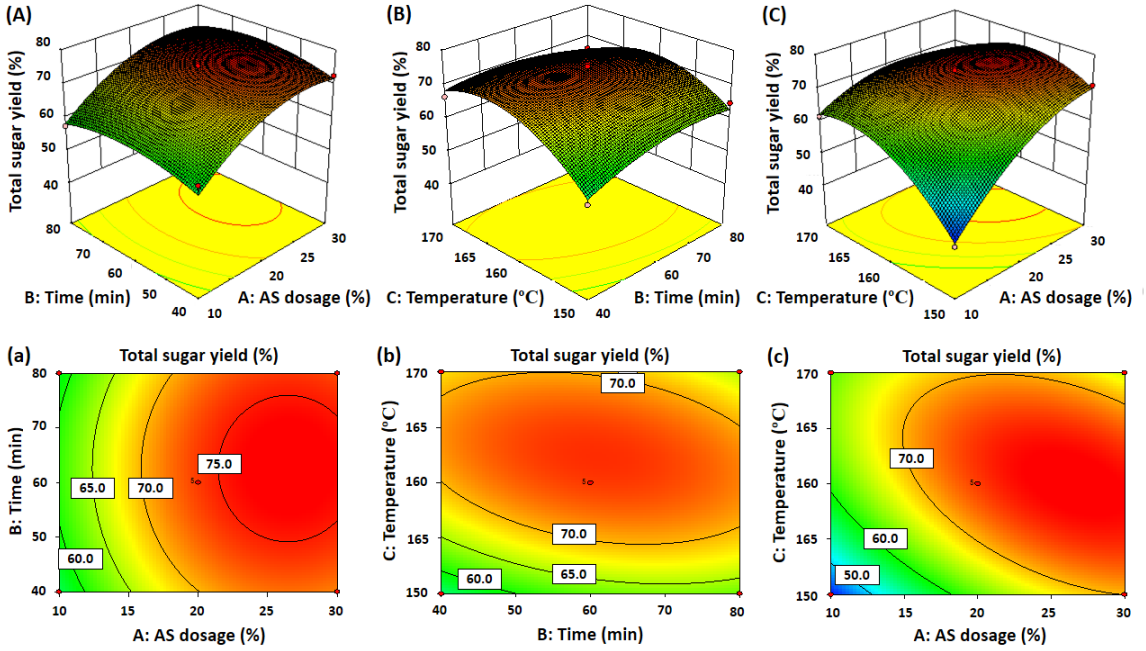

Fig. 1. Response surfaces and contour plots for the impacts of variables and their mutual effects on total sugar yield: (A, a) AS dosage and time (temperature = 160 ºC); (B, b) time and temperature (AS dosage = 20%); and (C, c) AS dosage and temperature (time = 60 min)

Figure 1A shows the effects of pretreatment time and AS dosage on the final total sugar yield. The $Y_{\text{total sugar}}$ increased with increased pretreatment time when the AS dosage was in the range of 10 wt% to 30 wt%, but when the AS dosage was fixed, the impact of the pretreatment time on $Y_{\text{total sugar}}$ was relatively weak. As shown in Fig. 1B, with the prolonging of pretreatment time, the $Y_{\text{total sugar}}$ first increased and then gradually decreased, which indicated that the excessive pretreatment time could lead to the severe degradation of carbohydrates (Li *et al.* 2014), reducing the sugar recovery after pretreatment. Therefore, to obtain a higher $Y_{\text{total sugar}}$, the pretreatment time should be properly shortened. In addition, the combined effect of pretreatment temperature and AS dosage on $Y_{\text{total sugar}}$ is displayed in Fig. 1C, which exhibits that when the pretreatment time was fixed at 60 min, both the increase of AS dosage and pretreatment temperature were beneficial for the improvement of $Y_{\text{total sugar}}$; however, excessively severe pretreatment conditions were not needed. An excessively high AS dosage (*e.g.*, 30 wt%) and pretreatment temperature (170 ºC) could increase the process' cost and cause over-degradation of carbohydrates, resulting in a lower final total sugar yield. Glucan was more resistant to harsh conditions when compared with xylan (Xu *et al.* 2016), but excessively high pretreatment temperatures could lead to a degradation of sugars, thus gaining a lower solid recovery after pretreatment (Table 2).

In addition, Fig. 1a and Fig. 1b clearly show that the change of $Y_{\text{total sugar}}$ was not obvious with the increase of pretreatment time under the constant AS dosage and pretreatment temperature, indicating the interactions of the AS dosage *vs.* pretreatment

time and pretreatment temperature *vs.* pretreatment time were not significant. However, the interaction between the AS dosage and pretreatment temperature was strong, as presented in Fig. 1c. The results were in line with the ANOVA analysis (Table 3).

Validation of the Model

Based on the above model analysis, the optimized AS pretreatment conditions were an AS dosage of 27 wt%, a pretreatment time of 63 min, and a pretreatment temperature of 160 ºC. Under these optimized conditions, the predicted $Y_{\text{total sugar}}$ was 76.5%. To validate the adequacy and appropriateness of the model Eq. 5, additional AS pretreatment experiments under the optimum conditions were repeatedly conducted, and the experiment result was 75.8% ± 0.6%, which was in good agreement with the predicted value. The authors also did a scale-up of the AS pretreatment experiment with a larger sample size of 200 g (dry wheat straw), and it verified that the scale-up result (74.4% ± 0.5%) also mostly matched the predicted value (76.5%) by the developed model with the accuracy rate of 97.3%. The relatively lower result of the scale-up experiment was probably due to the different reactors with different mixing approaches (*i.e.*, small size reactor with mechanical stirring, while large size reactor with the blending of the rotational reactor (Chi *et al.* 2019)). The mixing technique could affect the mass transfer during pretreatment, thus influencing the result. The substrate obtained from the scale-up experiment was used for saccharification and the evaluation of mass balance. In addition, the accuracy rate of the predicted value under the critical conditions was larger than 95% as well.

Enzymatic Hydrolysis of the Pretreated Wheat Straw

After AS pretreatment under the optimum conditions, the pretreated wheat straw was subjected to enzymatic hydrolysis. The sugar conversion and the sugar yields as the function of saccharification time are displayed in Figs. 2a and 2b, respectively. The sugar conversion was calculated as the percent of the glucose/xylose in hydrolysate divided by the corresponding glucose/xylose in the substrates (*i.e.*, pretreated wheat straw). As shown, the enzymolysis ran rapidly in the first 4 h and then gradually slowed down. This trend was attributed to the increased concentration of fermentable sugars in the hydrolysate, which inhibited the enzymatic hydrolysis of the substrate (Xu *et al.* 2015). Similar results were also reported in previous studies (Liu *et al.* 2013; Gong *et al.* 2015). Figure 2a exhibits that the glucan conversion was up to 99%, indicating that almost all glucan in the pretreated wheat straw was hydrolyzed to glucose. In other words, the wheat straw had a very good digestibility of glucan after the AS pretreatment under the optimum conditions. Yet, approximately 89% of xylan conversion was obtained, which was probably because the xylanase content in the enzyme was not high enough. Furthermore, Fig. 2b presents that the glucose yield and xylose yield were approximately 80% and 60%, respectively, although the corresponding sugar conversion was relatively high in saccharification. These results were due to the degradation of carbohydrates during AS pretreatment, particularly for xylan. To reduce the sugar loss in pretreatment, the addition of suitable carbohydrate stabilizers (*e.g.*, anthraquinone or magnesium salts) could be a possible solution (Xu *et al.* 2015). In addition, the $Y_{\text{total sugar}}$ was 74.4% after 48 h saccharification, which was 3.8 times higher in comparison with the raw wheat straw (19.6%).

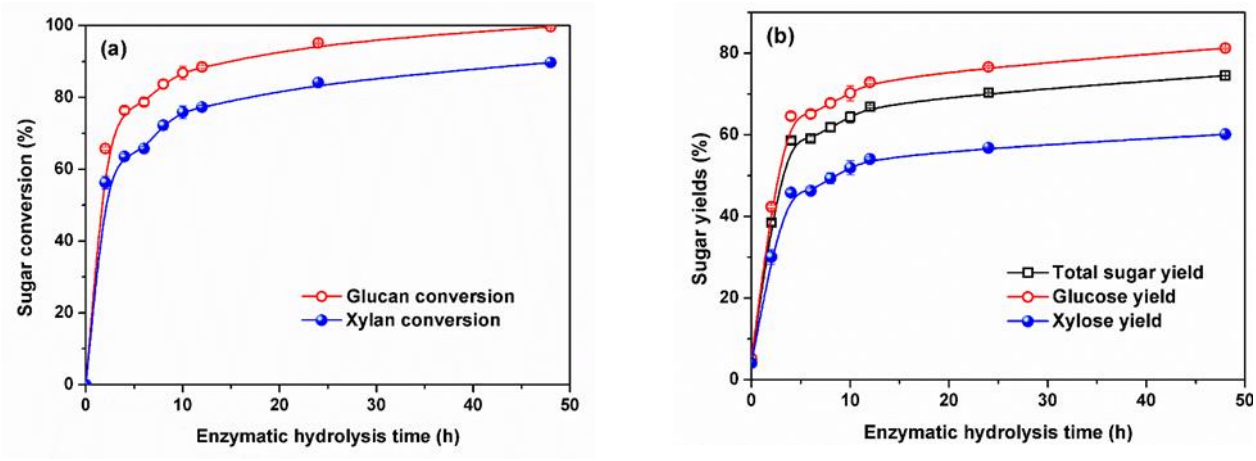

Fig. 2. Sugar conversion (a) and sugar yields (b) as the function of the enzymatic hydrolysis time

Effect of Pre-impregnation and Mechanical Refining on Enzymatic Hydrolysis

It has been demonstrated that the high content of extractive and ash in a substrate can impede the enzymatic hydrolysis (Mou *et al.* 2013; Yuan *et al.* 2018), and post-mechanical refining after chemical pretreatment can increase the porosity of the substrate and decrease the crystallinity of cellulose, thus improving enzymatic saccharification (Xu *et al.* 2014). It was reported that about 10 to 20% improvement of biomass digestibility could be achieved by post-mechanical refining (Chen *et al.* 2013), but the improvement was highly dependent upon the severity of chemical treatment (Jones *et al.* 2014; Xu *et al.* 2016). Therefore, to further ameliorate the final total sugar yield, pre-impregnation before AS pretreatment and post-mechanical refining after AS pretreatment were completed. Figure 3 shows the constituent changes of wheat straw before and after different treatments. In Fig. 3, both glucan and xylan contents in wheat straw increased after pre-impregnation, which was mainly due to the removal of extractives and ash (just 24% of lignin was removed during pre-impregnation). The removal rates of extractives and ash were 57% and 43%, respectively, after pre-impregnation. Compared to the raw material, the glucan content increased from 34.6% to 48.9% with the AS pretreatment alone. This phenomenon was due to the efficient delignification (74%), as well as the removal of extractives and ash, hence leading to high sugar conversion in saccharification (Fig. 2a). In addition, the glucan and xylan contents were further increased after pre-impregnation combined with AS pretreatment (Fig. 3), and a larger removal of extractives and ash could lead to a higher accessibility of enzymes to polysaccharides (Yuan *et al.* 2018), thus further raising the final total sugar yield from 74.4% ± 0.5% (without pre-impregnation) to 77% ± 0.6%.

In contrast, as expected, post-mechanical refining after AS pretreatment increased the $Y_{\text{total sugar}}$ to 76.9% ± 1.4%. These results indicated that we could either do pre-impregnation or do post mechanical refining to further improve enzymatic hydrolysis. Additionally, by combining pre-impregnation, AS pretreatment, and post-mechanical refining, the $Y_{\text{total sugar}}$ could reach to 78% ± 0.8%, which was comparable with the reported alkali-based pretreatments (Gong *et al.* 2015; Xu *et al.* 2015). However, the full combination of pre-impregnation and post mechanical refining for AS pretreatment was not feasible with only about 1% increase of $Y_{\text{total sugar}}$, due to the increase of process cost.

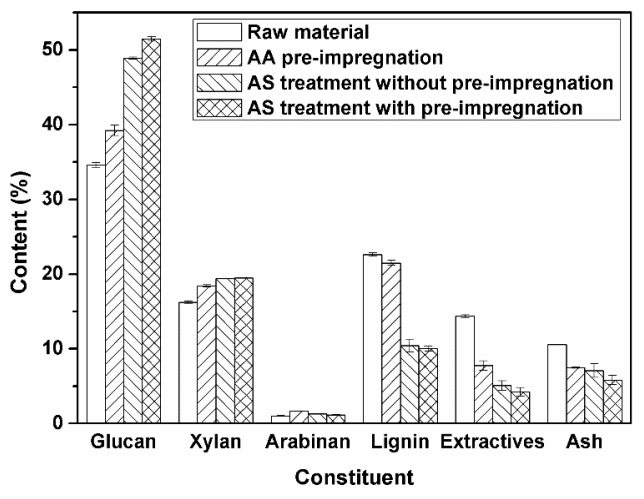

Fig. 3. Constituent of raw material and the samples after different treatments (AS pretreatment was run under the optimum conditions)

Physicochemical Properties of the Pretreated Wheat Straw

To further evaluate the effectiveness of AS pretreatment, the changes of the physicochemical properties of the pretreated wheat straw were comprehensively characterized. Figure 4 presents the morphology changes of wheat straw before and after pretreatment. As shown in Fig. 4a, the untreated wheat straw appeared as a smooth, tight, and rigid surface with a highly ordered surface structure, which hindered the accessibility of cellulase to cellulose.

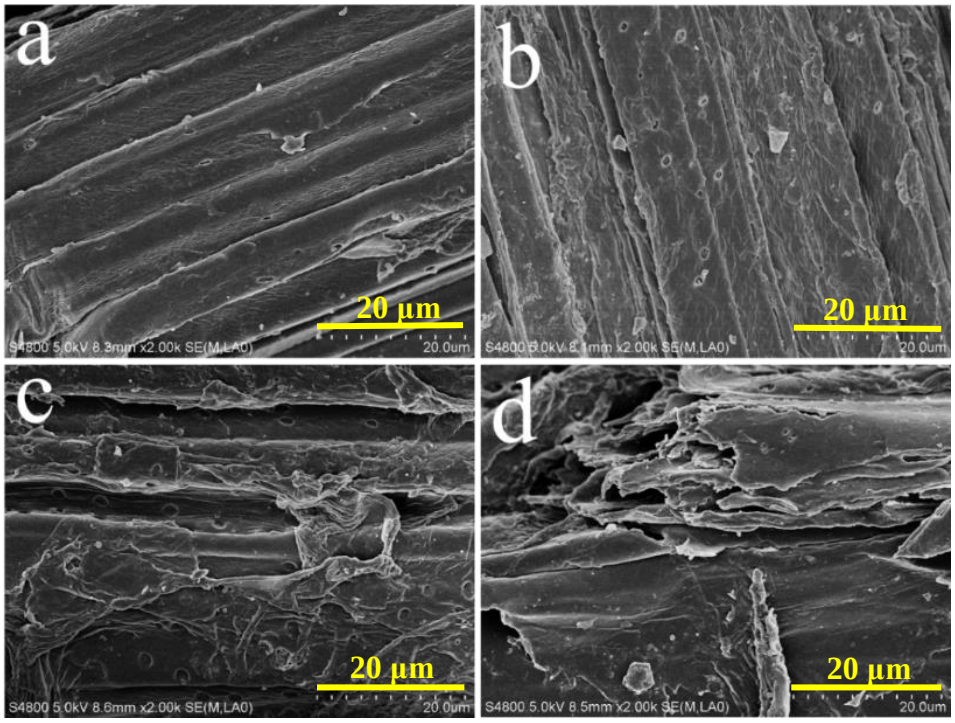

Fig. 4. (a) SEM images of untreated wheat straw, (b) treated wheat straw by pre-impregnation, (c) the AS pretreated wheat straw after pre-impregnation, and (d) the PFI refined wheat straw after AS pretreatment (AS pretreatment was run under the optimum conditions); the scale in the lower right hand corner for each image indicates a length of 20 µm.

During pre-impregnation, acetic acid first destroyed the dense structure formed by hemicellulose, cellulose, and lignin, and then lots of pores were generated (Fig. 4b) due to the partial removal of extractives and ash (Fig. 3); these changes were more conducive to the subsequent chemical penetration as well as the exposure of cellulose surface (Yuan *et al.* 2018). As presented in Fig. 3 and Table 2, AS pretreatment could efficiently remove lignin, hence leading to a coarser and looser surface with more exposure of internal structures and fibers (Fig. 4c). In addition, deeper delamination and fibrillation occurred during post-mechanical PFI refining, and these actions could further increase the external surface area and porosity of the substrate (Fig. 4d), leading to a higher enzyme accessibility. Similar results were also previously reported (Xu *et al.* 2014, 2015; Fatriasari *et al.* 2018).

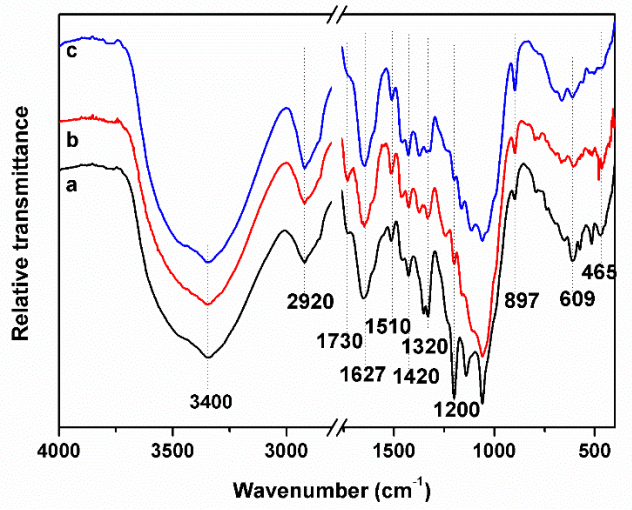

Fig. 5. (a) FTIR spectra of untreated wheat straw, (b) the AS pretreated wheat straw without pre-impregnation, and (c) the AS pretreated wheat straw with pre-impregnation (AS pretreatment was run under the optimum conditions)

The FTIR spectroscopy was used to investigate the changes of the chemical groups and chemical compositions of untreated and pretreated wheat straw (Fig. 5). The spectral differences of the samples implied that pretreatment led to significant changes of chemical groups. In Fig. 5, the broad absorption band located within the wavenumber range of 3100 cm^{-1} to 3500 cm^{-1} was due to the stretching of the hydrogen-bonded hydroxyl groups from cellulose, hemicellulose, and lignin (Chen *et al.* 2018). The obvious absorption at 2900 cm^{-1} was assigned to the stretching vibration of CH_2 groups (Li *et al.* 2013). All samples showed obvious characteristic bands of the aromatic skeletal vibrations at 1510 cm^{-1} and 1420 cm^{-1} (Mou *et al.* 2014; Chen *et al.* 2019), indicating that the benzene ring structure was not damaged significantly during pretreatment.

Additionally, the peak at 897 cm^{-1} was related to the β-glycosidic linkage of cellulose (Xu *et al.* 2015), and the relative increase of this peak intensity supported the fact that the cellulose content increased with the breakdown of lignin and hemicellulose after pretreatment (Fig. 3), while the decrease of the peak intensity at 1740 cm^{-1} represented the deacetylation and the partial degradation of hemicelluloses (Xu *et al.* 2015). The bands positioned at 1627 cm^{-1} and 1510 cm^{-1} corresponded to the aromatic ring C=C stretching in lignin, and the clear peaks at 1380 cm^{-1} and 1320 cm^{-1} corresponded to the CH stretching and C-O stretching of guaiacyl, respectively (Mou *et al.* 2014). These peaks related to

lignin were significantly decreased after pretreatment, suggesting the removal of the lignin, which agreed with the elemental analysis of O/C value (Table 4). A higher O/C value indicated a lower lignin content in biomass (Mou *et al.* 2013). In addition, it was noted that the decrease of the absorption at 465 cm^{-1} (Si-O) for the pretreated wheat straw was due to the partial removal of ash, particularly for the one with pre-impregnation (Fig. 5c). This result was also in line with the reduction of Si content by elemental analysis of pretreated wheat straw, as shown in Table 4.

Table 4. Elemental Analysis of Wheat Straw Before and After AS Pretreatment

Samples	N (%)	S (%)	Si (%)	C (%)	O (%)	O/C
Raw wheat straw	0.25	0.92	2.05	62.52	30.93	0.49
AS pretreated wheat straw	0.50	1.32	0.61	55.47	31.97	0.58

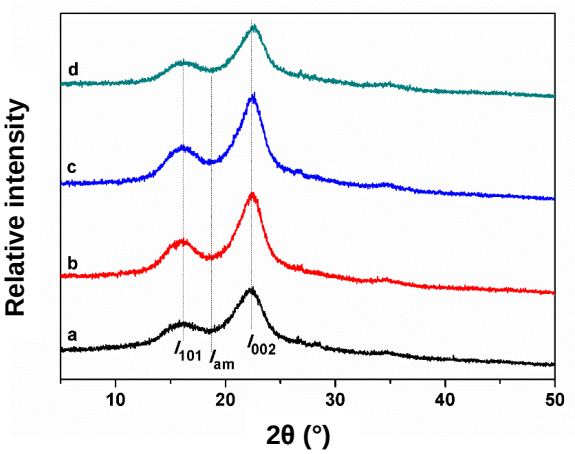

Fig. 6. (a) XRD patterns of untreated wheat straw, (b) the AS pretreated wheat straw without pre-impregnation, (c) the AS pretreated wheat straw with pre-impregnation, (d) and the PFI refined wheat straw after AS pretreatment (AS pretreatment was run under the optimum conditions).

Pretreatment affects the crystallinity of lignocellulosic biomass, which was believed as a crucial factor significantly affecting the enzymatic digestibility of lignocellulosic biomass (Xu *et al.* 2014; Qi *et al.*, 2018). The XRD patterns of the native and pretreated wheat straw samples are shown in Fig. 7, which presents that the samples showed the typical crystalline structure of cellulose I. The *CrI* of raw wheat straw was 50.7%, which was lower than that of the AS-pretreated wheat straw (58.7%). This was attributed to the partial removal of the amorphous components, such as hemicelluloses and lignin (Xu *et al.* 2015). For the sample with pre-impregnation and AS pretreatment, the *CrI* further increased to 68.7%, which was mainly due to the deeper removal of extractives (Fig. 3). In addition, the *CrI* of the PFI refined sample (Fig. 6d) was reduced to 55.2%, because of the partial damage of the crystalline region of cellulose during mechanical refining. The decrease of cellulose crystallinity could facilitate the subsequent enzymatic digestibility of cellulosic materials (Xu *et al.* 2014, 2019).

Characterization of Spent Liquor

As tested, the solid content of AS spent liquor was 8.2%, and the pH value was 7.6, which was neutral. In the solids of spent liquor, the inorganic content was 14.1%, which

was derived from the ash of straw and the remaining AS, while the organic content was 86.0%, which was the degradation products of carbohydrates, removed lignin, and extractives (Xu *et al.* 2015). To verify the safety and feasibility of the use of the AS spent liquor for the production of fertilizer, elemental analysis was conducted by ICP-MS and EDS. As shown in Table 5, the spent liquor contained an increase of nutrients needed for plant growth, such as nitrogen, trace elements (*e.g.*, zinc, magnesium), and a minute quantity of harmful elements such as arsenic and chromium, which was safe enough for the fertilizer production (Wu *et al.* 2018).

Table 5. Elemental Analysis of Ammonium Sulfite Spent Liquor and Solid Fraction

Element	Content [a] (mg/L)	Element	Content [b] (wt%)
Ca	2.36	C	27.22
Mg	1.98	H	5.48
Fe	4.91	O	49.15
Mn	0.48	N	8.41
Cu	0.55	S	9.74
Zn	0.98		
Cd	0		
Cr	0.07		
Hg	0		
As	0.08		

[a]: Based on the spent liquor of AS pretreatment;
[b]: based on the solid fraction of the spent liquor from AS pretreatment

Overall Mass Balance

The overall mass balance of AS pretreatment and enzymatic saccharification of wheat straw is presented in Fig. 7.

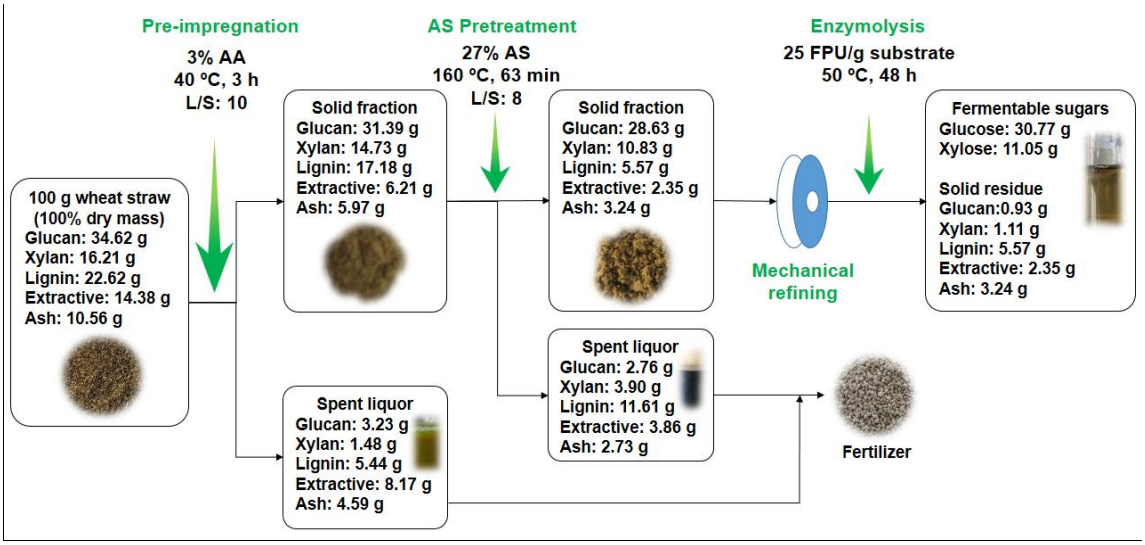

Fig. 7. Overall mass balance of AS pretreatment and enzymatic hydrolysis of wheat straw

As exhibited in Fig. 7, 100 g of dry wheat straw contained 34.6 g glucan, 16.21 g xylan, 22.6 g lignin, 14.38 g extractives, and 10.56 g ash was used as the starting material.

First, pre-impregnation was conducted, and approximately 56.9% of extractives and 43.5% of ash were extracted. Second, AS pretreatment was conducted under the optimum conditions with the AS dosage of 27 wt% at 160 ºC for 63 min. After AS pretreatment, most of lignin (75.4%) was removed, and more extractives and ash were extracted as well, as verified by FTIR (Fig. 5) and XRD (Fig. 6) analysis, leading to a more porous structure of substrate (Figs. 4b and 4c). Lastly, post-mechanical refining of the pretreated wheat straw was implemented to further increase the fibrillation and specific surface area of the substrate (Fig. 4d) and decrease the cellulose crystallinity (Fig. 6), thus enhancing the digestibility of the substrate. In this case, 99% of glucose and 89% of xylose were released from the substrate during enzymolysis (Fig. 2b), and the corresponding final total sugar yield was 78%, which was 4.8% higher compared to the one with AS pretreatment alone. In addition, based on the concept of integrated biorefinery, the spent liquor of AS pretreatment contained an increase of nutrients (Table 5) that could be used to produce lignin-based fertilizer to nourish soil (Wu *et al.* 2018). Therefore, the AS pretreatment process for the production of fermentable sugars would be more economically and environmentally viable.

CONCLUSIONS

1. Wheat straw as an abundant agricultural residue was used as the feedstock for the establishment of the sugar platform *via* AS pretreatment and saccharification. According to the response surface experiment, the AS pretreatment was systematically optimized to gain a high final total sugar yield. The results under the optimum conditions achieved a 74% final total sugar yield.
2. Proper removal of lignin, extractives, and ash, as well as the further increase of specific surface of substrate and decrease of cellulose crystallinity boosted the final total sugar yield to 78%.
3. Based on the concept of integrated biorefinery, the spent liquor derived from AS pretreatment contained nitrogen, which could be used for the production of fertilizer to put nutrients back into soil.
4. Therefore, the ammonium sulfite pretreatment will be a sustainable and effective pretreatment technology, which can be applied for the production of fermentable sugars for the further conversion of bioenergy or chemicals.

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