Combination Pretreatment of Steam Explosion and NaOH Enhances Enzymatic Saccharification of Corn Stover

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The presence of lignin sheaths and the crystalline structure of cellulose are two key barriers in the development of corn stover (CS). To remove these two barriers and improve the digestibility of carbohydrates in CS, CS (pretreated by H_2O_2, H_2SO_4, NaOH, an enzyme, steam explosion [SE], and SE combined with NaOH [SE-NaOH]) was hydrolyzed. The total reducing sugar yield (Y_{trs}), accessibility of the enzyme to the substrate ($K_{obs,0}$), and gradual loss of the enzyme activity (K_i) were compared by regression analysis of the kinetic model, scanning electron microscopy, Fourier transform infrared spectroscopy, and X-ray diffraction analysis. The pretreatment dramatically increased the Y_{trs} and $K_{obs,0}$, and remarkably decreased the K_i. The maximum increase in the Y_{trs} (106.57%) was obtained after the saccharification reaction of the CS pretreated by SE-NaOH. Physicochemical characterizations of the CS pretreated by SE-NaOH showed that the SE-NaOH pretreatment effectively reduced the lignin sheath, decreased cellulose crystallization, and created favorable conditions for enzymatic diffusion and penetration.

Keywords: Corn stover; Pretreatment; Hydrolysis; Kinetic

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INTRODUCTION

Corn stover (CS) is one of the most abundant agricultural residues in China. Bioconversion of CS to biofuel can alleviate the energy crisis caused by the depletion of fossil energy and reduce environmental pollution caused by the combustion of fossil energy (Yang *et al.* 2015; Chang *et al.* 2016). The bioconversion process of lignocellulose includes four steps: pretreatment of the CS, saccharification of the cellulose, fermentation of the saccharification products, and biorefining of the biofuel. As a critical processing step for bioethanol and bio-based material production, pretreatment results in a high sugar yield *via* the bioconversion route (Zhao *et al.* 2016). The primary objective of pretreatment is to reduce the impeding effects of the lignin and hemicellulose, destroy the cellulose crystallinity, decrease the degree of polymerization (DP) of the cellulose, and increase the accessible surface area (Sun *et al.* 2015).

Numerous approaches, including physical, chemical, physicochemical, and biological pretreatments, have been applied to pretreat lignocellulose (Alvira *et al.* 2010). Long-term research has been conducted on the enzymatic pretreatment strategy of CS and a complete microbial pretreatment system has been developed (Fan *et al.* 2019). The saccharification reaction of cellulose in pretreated CS is a dynamic reaction process. To

explore its behavioral mechanism (dynamic model mechanism), it is necessary to investigate the factors and laws affecting the efficiency of enzymatic hydrolysis.

It has been reported that the total reducing sugar yield (Y_{trs}) is closely related to the changes in the compositions and morphologies of the lignocellulose after pretreatment, which was determined through scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transform infrared (FTIR) spectroscopy (Li *et al.* 2016). The pretreatment of lignocellulose can increase the Y_{trs} by depolymerizing the lignin and hemicellulose fractions and disrupting the crystalline structure of the cellulose (Behera *et al.* 2014).

Li *et al.* (2016) has reported that sulfuric acid pretreatment can result in achieving a maximum glucose yield (94.2%), and a distinct change in the composition and crystal texture of the solid residuals was found by XRD and SEM analyses. Some studies have shown with SEM micrographs and XRD crystallinity measurements that the increase in the accessible surface area of cellulose is more important than the removal of lignin when the goal is to achieve high sugar yields (Rollin *et al.* 2011). Fourier transform infrared spectroscopy has been used to characteristically assess the structural changes in cellulose and lignin before and after pretreatment (Ma *et al.* 2015). Physical treatment is more effective than typical chemical methods at decomposing the crystal structure of CS (Chang *et al.* 2012). Chemical treatment can effectively degrade lignin. However, microbial fermentation and enzymatic hydrolysis are also more efficient at converting CS into reducing sugars and low-molecular weight carbohydrates compared with physicochemical pretreatment (Huang *et al.* 2017).

To further enhance the pretreatment efficiency, it is necessary to compare the mechanical differences of these methods using a kinetic model. This is because the hydrolysis of cellulose is done in a heterogeneous reaction system and the reaction rate constant is affected by many factors, including diffusion, mass transfer, adsorption, transfer, desorption, and enzyme denaturation of the reactant molecules, product molecules, and catalyst surface (Bansal *et al.* 2009). Therefore, the conventional Michaelis-Menten equation cannot be used to describe the hydrolysis reaction of cellulose and the model needs to be modified to describe the enzymatic reaction in heterogeneous systems (Yang and Fang 2015).

Kopelman (1988) stated that the kinetics of heterogeneous reactions do not follow the classic kinetic model. A heterogeneous reaction system includes the following characteristics: 1) the rate constant changes with time; 2) the rate constant is related to the reaction order and spectrum dimension; and 3) the kinetics of the enzymatic reaction belong to the fractal dynamic model. Väljamäe *et al.* (2003) used fractal-like kinetics to study the hydrolysis process of *Trichoderma reesei* and suggested that fractal-like kinetics are suitable for the enzymatic hydrolysis of cellulose. Huang (1975) studied the hydrolysis of substrates at various concentrations with different concentrations of insoluble enzymes, proposed a kinetic model for rapid adsorption, and discussed methods for determining the kinetic parameters. Zhang *et al*. (2017) applied the impeded Michaelis model (IMM) to fit the enzymatic hydrolysis of cellulose.

In the present study, the IMM was further used to fit the kinetic process of cellulose hydrolysis from CS pretreated by physicochemical and biological methods and to determine the mechanical differences between these methods.

EXPERIMENTAL

Materials

Corn stover was purchased from a local farm (Henan, China), dried at 105 °C to a constant weight, ground into a fine powder, and sieved through a 0.25-mm sieve. The ground CS powder consisted of 26.2% hemicellulose, 32.1% cellulose, and 15.4% lignin. A commercial *T. reesei* cellulase was supplied by Ningxia NCM Biotechnology Ltd. (Ningxia, China). The activities of the carboxymethyl-cellulase, filter paper enzyme, and β-glucosidase were 6.11×10^4 U/mL, 844 FPU/mL, and 10.9 U/mL, respectively. All of the chemicals were of standard analytical grade and used as received without further purification.

Pretreatment

Acid, H_2O_2, alkaline, and enzyme pretreatments

The CS was pretreated with sulfuric acid, H_2O_2, NaOH, and lignin peroxidase according to previously described methods (Chen *et al.* 2009; Tai and Keshwani 2014; Ramadoss and Muthukumar 2015; Zhang *et al.* 2015). After the CS was washed, the pretreated CS was dried at 80 °C to a constant weight and preserved at room temperature.

Steam explosion pretreatment

The dried CS was rapidly heated by high-pressure steam in the absence of any chemicals. The pressure inside of the digester was 1.5 MPa and the steam mixture was held for 400 s. The effects of the operating parameters during steam explosion (SE), such as the pressure (0.6 MPa, 0.8 MPa, 1.2 MPa, 1.5 MPa, and 2 MPa) and reaction time (200 s, 400 s, and 600 s), were investigated.

SE-NaOH pretreatment

The dried CS pretreated by SE under the optimum conditions was washed, dried at 80 °C, and then treated with NaOH (SE-NaOH). The pretreated biomass was washed with distilled water until a neutral pH was achieved. Subsequently, the CS was dried at 80 °C to a constant weight and preserved at room temperature.

Enzymatic Saccharification

All of the saccharification tests of the pretreated CS were conducted in 100-mL Erlenmeyer flasks. Briefly, 1 g of pretreated CS was added to 20 mL of 0.1 M acetate buffer containing 3% cellulase (pH = 4.4). After the mixture was fully blended, the initial concentration of the reducing sugar was denoted as C_0. The saccharification reaction was conducted at 47 °C in a water shaking bath (160 rpm) for 32 h. Subsequently, samples were taken at 1 h, 2 h, 4 h, 8 h, 16 h, and 32 h. Each sample was immediately cooled in an ice bath to room temperature to terminate the reaction. The Y_{trs} in each flask was determined according to the 3,5-dinitrosalicylic acid method (Miller 1959) and denoted as C_1. The Y_{trs} was calculated according to Eq. 1,

$$Y_{trs}\ (\%) = (C_1 - C_0) \times V/G \times 100\% \quad (1)$$

where Y_{trs} is the total reducing sugar yield, V is the volume of the reaction solution (mL), G is the weight of the total dry substrate (g), and C_0 and C_1 are the reducing sugar concentrations (g/mL) at 0 h and t h of reaction, respectively.

IMM

According to the IMM (Yang and Fang 2015; Zhang *et al*. 2017), the pretreatment efficiency depends upon the $K_{obs,0}$ (h^{-1}) and K_i (h^{-1}). The variable $K_{obs,0}$ expresses the comprehensive action of the initial accessibility of cellulase to the substrate and the activity of the cellulase. The variable $K_{obs,0}$ was calculated as follows,

$$K_{obs,0} = k_2(E_0) / K_m \quad (2)$$

where E_0 is the initial enzyme concentration (mol/L), k_2 is the rate constant from the enzyme-substrate complex to the product, and K_m (mol/L) is the Michaelis constant.

The correlation between the Y_{trs}, reaction time (t, h), and $K_{obs,0}$ was as follows,

$$\frac{dY_{trs}}{dt} = K_{obs,0} \frac{1}{(1+\alpha t)^2}(1 - Y_{trs}) \quad (3)$$

where α is a constant, and K_i is the coefficient of the time-dependent inactive enzyme.

The coefficient K_i can be calculated according to the constant α and the following equation:

$$K_i = \frac{2\alpha}{1+\alpha t} \quad (4)$$

Equation 3 can be solved to give:

$$-\ln(1 - Y_{trs}) = \frac{K_{obs,0} t}{1+\alpha t} \quad (5)$$

Equation 5 then can be rearranged as:

$$-\frac{t}{\ln(1 - Y_{trs})} = \frac{1}{K_{obs,0}} + \frac{\alpha}{K_{obs,0}} t \quad (6)$$

Equation 6 was applied to fit the experimental results by plotting $-t/n(1 - Y_{trs})$ *versus* t. The coefficient of the t and constant term are $\alpha/K_{obs,0}$ and $1/K_{obs,0}$, respectively. The value for K_i was calculated with Eq. 4. All of the determinations were repeated at least three times, and the results were presented as the means with the standard errors. The regressions and analysis of variance (ANOVA) were performed using SPSS 17.0 software (SPSS Company, Chicago, USA).

Physical and Chemical Characterizations of the CS

SEM analysis

Photographs of the samples with different pretreatments were acquired by SEM analysis. Before observation, the samples were passed through an 80-mesh screen sieve and then pasted on the side of the glass using a double-sided adhesive. The tissues were randomly selected for image testing at different magnifications, such as 500 and 2000 times.

FTIR analysis

To analyze the changes in the chemical components of the differently pretreated CS, the FTIR spectra of the CS were obtained by a Nicolet iS50 spectrometer (Massachusetts, USA). The samples were passed through a 100-mesh screen sieve. The FTIR spectra were generated on the FTIR spectrometer using KBr translucent disks

containing 1% sample in the absorbance mode. The data was recorded within the region of 4000 cm^{-1} to 400 cm^{-1} at a resolution of 4 cm^{-1} with 128 scans (Jiang *et al.* 2015).

XRD analysis

The crystallinity index (CrI) has been used to describe the relative amount of crystalline material in cellulose. CrI was analyzed by XRD analysis (D8 ADVANCE, Karlsruhe, Germany) with Cu Kα radiation (λ = 1.54 nm) and operated at 30 kV. The samples were passed through a 100-mesh screen sieve, and the scattering angle ranged from 10° to 40° with a scan rate of 2°/min and step size of 0.02°.

The CrI was calculated according to the method developed by Segal *et al.* (1959),

$$CrI\ (\%) = (I_{002} - I_{am}) / I_{002} \times 100\% \quad (7)$$

where I_{002} is the intensity of the crystalline portion in the biomass (cellulose), and I_{am} is the intensity of the amorphous portion (such as the cellulose, hemicellulose, and lignin).

RESULTS AND DISCUSSION

The IMM contains two parameters, $K_{obs,0}$ and K_i, which are calculated with the α. The variable $K_{obs,0}$ is considered to be the initial activity of the cellulase and accessibility of cellulase to the substrate, and K_i shows the loss and inactivation of the enzymatic activity in a heterogeneous system caused by the inhibitory reaction of inert and non-reactive substances. The IMM can clearly reflect the effects of the structural characteristics and residual lignin on saccharification of cellulase in pretreated CS. In the present study, the relationship between the Y_{trs} and structural characteristics of the pretreated CS with various methods was determined by comparing the accessibility of cellulase to the substrate and enzymatic loss in the process of cellulose hydrolysis. Therefore, IMM analysis was introduced in the present study.

Effect of the SE Pressure and Maintenance Time on the Y_{trs}

To determine the effectiveness of the SE pretreatment, the saccharification results of the CS pretreated with SE under different conditions are shown in Table 1. The Y_{trs} at the different time points showed a positive correlation with the pressure and maintenance time in the SE process. The $K_{obs,0}$, K_i, and ANOVA results were fitted by Eq. 6 (Table 1).

Table 1. Y_{trs} of the CS Pretreated with SE under Various Conditions after Saccharification

SE Conditions[1]	Y_{trs}[2](%)					
	1 h	2 h	4 h	8 h	16 h	32 h
0.6 MPa/400 s	25.087	26.946	29.664	31.952	38.007	42.679
0.8 MPa/400 s	25.325	27.518	30.570	33.764	37.912	43.156
1.2 MPa/400 s	25.278	27.709	30.284	34.050	40.629	44.348
1.5 MPa/200 s	27.568	30.605	33.060	35.806	36.805	45.167
1.5 MPa/400 s	27.859	30.855	34.724	38.136	43.046	46.957
1.5 MPa/600 s	28.359	31.021	33.809	38.802	41.298	49.703
2 MPa/200 s	27.693	30.855	32.894	38.219	42.213	49.286
2 MPa/400 s	29.149	30.73	34.225	39.301	42.962	50.285
2 MPa/600 s	27.901	30.314	31.313	36.721	41.881	51.783

[1]: Pressure/maintenance time of SE; and [2]: Y_{trs} is the total reducing sugar yield at 32 h

Table 2 shows that the R^2 values of the data for all of the conditions were higher than 0.968 and were close to the adjusted R^2 values ($p < 0.001$, and F-value > 100). These results indicated that the IMM analysis could fit the experimental results well and it possessed a high interpretation rate and reliability.

The Y_{trs} at 32 h and $K_{obs,0}$ in Table 1 increased from 426.8 mg/g to the maximum value of 453.5 mg/g and from 0.242 h^{-1} to the maximum value of 0.330 h^{-1}, respectively, and K_i was not significantly changed when the pressure increased from 0.6 MPa to 1.5 MPa. The significant change in the $K_{obs,0}$ and insignificant change in the K_i indicated that more of the internal cellulose was exposed on the surface because of the SE process, and destruction of the crystalline structure of the cellulose decreased the DP of the cellulose and increased the accessible surface area, which led to an increased enzyme accessibility. Li and Chen (2014), Zhao and Chen (2013), and Kojiro *et al.* (2010) have shown that in the SE process, the saturated steam can penetrate the pores of CS and tear all of the pores in a rapid decompression process, and the exposure of cellulose breaks the straw organization, which results in a huge hole and the exposure of most fiber bundle sheaths. These conclusions supported the findings of the current study. At the same pressure, an increase in the SE maintenance time had a minor effect on the K_i; thus, the SE pretreatment was conducted under the conditions of 1.5 MPa and 400 s.

Table 2. Regressive Analysis of the Y_{trs} Obtained from the CS Pretreated by SE under Various Conditions *versus* Time after Saccharification

SE Conditions	Y_{trs}(%)	$K_{obs,0}$	K_i	R^2	Adj-R^2	F-value	p value
0.6 MPa/400 s	42.679	0.2418	0.0581	0.990	0.987	382.311	0.000
0.8 MPa/400 s	43.156	0.2608	0.0584	0.991	0.989	441.312	0.000
1.2 MPa/400 s	44.348	0.2573	0.0581	0.993	0.991	548.904	0.000
1.5 MPa/200 s	45.167	0.2772	0.0585	0.978	0.973	181.246	0.000
1.5 MPa/400 s	46.957	0.3304	0.0588	0.995	0.994	873.246	0.000
1.5 MPa/600 s	49.703	0.2671	0.0577	0.981	0.976	206.355	0.000
2 MPa/200 s	49.286	0.2650	0.0577	0.986	0.983	282.757	0.000
2 MPa/400 s	50.285	0.2756	0.0577	0.986	0.982	278.638	0.000
2 MPa/600 s	51.783	0.2141	0.0562	0.968	0.960	120.002	0.000

Adj-R^2 – adjusted R^2

To analyze the effects of the pressure and time on the SE process, the Y_{trs} at 32 h was used as the dependent variable, the pressure (P) and time (t) were used as the independent variables, and Eq. 8 was developed,

$$Y_{trs} = \alpha e^{P} + \beta t + \gamma \quad (8)$$

where α and β are the coefficients of the e^P and t, respectively, and γ is a constant.

The model fitting F-value (80.078) and p value (0.000) in Table 3 showed that there was a correlation between the Y_{trs} and P, that t was extremely significant, and that the reliability of the analysis was extremely high. The R^2 value was 0.964, which was higher than the R^2 value of 0.80 (Biswas *et al.* 2015). The adjusted R^2 value (0.952) was close to the R^2 value. The above-mentioned results suggested that the predictability and adaptability of this model was relatively reliable. The coefficients of the e^P and t were significant ($p < 0.01$) (Table 3). Among them, the coefficients of the e^P and t were greater than 0, which revealed that the Y_{trs} had a significant positive correlation with the P and t. Therefore, the small coefficient of t showed that the effect of the SE maintenance time on the Y_{trs} was

small in the experimental scope. The e^P value (13.976) showed that the Y_{trs} and SE pressure exponentially correlated, and that the effect of the pressure on the Y_{trs} was extensive. A higher pressure resulted in a higher Y_{trs}.

Table 3. Regression Analysis of Each Item between the Y_{trs}, P, and T

Item	Coefficient	*t*-value	Significance
Constant	368.495	38.285	0.000
e^P	13.976	11.719	0.000
T	0.088	4.777	0.003

F-value = 80.078, significance: p = 0.000, R^2 = 0.964, and adj-R^2 = 0.952

Comparison of the Various Pretreatment Methods

Table 4 shows the Y_{trs} of the CS pretreated with physiochemical or biological methods after saccharification. Compared with the untreated CS, the Y_{trs} of the pretreated CS after saccharification significantly increased. The maximum Y_{trs} obtained from the CS pretreated with SE-NaOH increased by 106.57%.

Table 4. Y_{trs} of the CS Pretreated with Various Methods after Saccharification

Method	Y_{trs} (%)					
	1 h	2 h	4 h	8 h	16 h	32 h
Untreated	21.399	25.496	27.954	32.866	34.868	38.817
NaOH	37.292	41.249	49.974	56.029	65.278	74.098
H_2SO_4	27.614	28.901	37.292	38.627	41.774	50.546
H_2O_2	23.847	26.136	32.572	33.764	38.198	43.108
Enzyme	24.277	24.706	29.235	32.524	36.338	39.199
SE	25.659	29.331	31.619	36.005	41.392	45.349
SE + NaOH[1]	41.154	44.920	54.265	61.082	77.197	80.391
SE + NaOH[2]	40.534	44.682	52.691	59.700	71.380	77.959

Enzyme: lignin peroxidase; SE + NaOH[1]: the pressure of SE was 1.5 MPa; and SE + NaOH[2]: the pressure of SE was 2.0 MPa

Table 5 indicates that the $K_{obs,0}$, K_i, and ANOVA results in Table 4 were fitted by Eq. 6. The R^2 values for all of the data were higher than 0.936 and were close to the adjusted R^2 values ($p < 0.01$, F-value > 100). These results showed that the IMM analysis could fit the experimental results of the various pretreatment methods, which suggested a high interpretation rate and reliability. Moreover, there was a smaller difference in the $K_{obs,0}$ between the CS pretreated by H_2SO_4 and H_2O_2 and the untreated CS compared with that between the NaOH and SE-pretreated CS and the untreated CS. However, the difference in the K_i between the CS pretreated by H_2SO_4, H_2O_2, and NaOH and the untreated CS was larger than that between the CS pretreated with SE and enzyme and the untreated CS. These results were supported by previous studies (Xiao *et al.* 2014; Singh *et al.* 2015). The results further proved that SE could destroy the crystalline structure, decrease the DP of the cellulose, and increase the accessibility of cellulase to cellulose in the substrate. However, chemical pretreatment could decrease the loss and inactivation of the cellulase. The maximum $K_{obs,0}$ and minimum K_i were obtained from the saccharification reaction of the NaOH-pretreated CS. To further increase the Y_{trs}, the combination of the SE and NaOH pretreatments was used to pretreat the CS. The maximum Y_{trs} (803.91 mg/g) and $K_{obs,0}$ (0.3953 h^{-1}) and the minimum K_i (0.0545 h^{-1}) of the saccharification reaction were obtained from the CS pretreated under the conditions of 1.5 MPa and 400 s.

Table 5. Regressive Analysis of the Y_{trs} Obtained from the CS Pretreated with Various Methods *versus* Time after Saccharification

Method	Y_{trs}(%)	$K_{obs,0}$	K_i	R^2	Adj-R^2	F-value	p value
Untreated	38.817	0.2704	0.0590	0.996	0.995	1017.71	0.000
NaOH	74.098	0.3449	0.0552	0.979	0.973	184.654	0.000
H_2SO_4	50.546	0.2652	0.0575	0.979	0.974	191.036	0.000
H_2O_2	43.108	0.2653	0.0585	0.993	0.991	536.689	0.000
Fungal	39.199	0.2895	0.0592	0.997	0.996	1394.72	0.000
SE	46.957	0.3304	0.0588	0.995	0.994	873.246	0.000
SE + NaOH[1]	80.391	0.3953	0.0545	0.972	0.965	138.332	0.000
SE + NaOH[2]	77.959	0.3835	0.0550	0.936	0.920	58.751	0.002

To further confirm their relationship, a linear regression analysis was conducted using Y_{trs} as the dependent variable, and $K_{obs,0}$ and K_i as the independent variables. The following equation was developed,

$$Y_{trs} = \alpha K_{obs,0} + \beta K_i + \gamma \quad (9)$$

where α and β are the coefficients of the $K_{obs,0}$ and K_i, respectively, and γ is a constant.

Table 6. Regression Analysis between the Y_{trs}, $K_{obs,0}$, and K_i

Item	Coefficient	*t*-value	Significance
Constant	4084.863	28.625	0.000
$K_{obs,0}$	959.460	14.005	0.000
K_i	-66839.513	-29654	0.000

F-value = 1242.858, p = 0.000, R^2 = 0.994, and adj-R^2 = 0.993

Table 6 shows that the model fitting F-value and *p* value were 1242.858 and 0.000, respectively, which indicated that the correlation of the Y_{trs} with the $K_{obs,0}$ and K_i was significant, and the reliability of the analysis was high. The R^2 value was 0.994, which was higher than the R^2 value of 0.80 (Biswas *et al.* 2015). The adjusted R^2 value (0.993) was close to the R^2 value. These results suggested that the predictability and adaptability of this model were relatively more reliable. The coefficients for all of the parameters in Table 6 were significant (p = 0.000). Among them, the coefficient of the $K_{obs,0}$ was positive, and the coefficient of the K_i was negative; this indicated that the Y_{trs} significantly and positively correlated with the accessibility of the enzyme to the substrate ($K_{obs,0}$), while it significantly and negatively correlated with the gradual loss of the enzyme activity (K_i). Therefore, it was necessary to increase the accessibility for cellulase to the substrate and decrease the loss and inactivation of the enzyme to obtain the maximum saccharification efficiency. The same results obtained using a fractal model fitting empirical data of enzymatic saccharification of acid pretreated corn stover Wojtusik *et al*. (2016) and steam-exploded sugarcane bagasse (Aguiar *et al*. 2013).

SEM Analysis

Figure 1a shows that the untreated CS had smooth and well-ordered fibers, and the pores were irregularly distributed on the surface. These pores were believed to be the binding sites of cellulase. Lignin wraps cellulose and acts as a physical barrier, which hinders the accessibility of cellulase to cellulose.

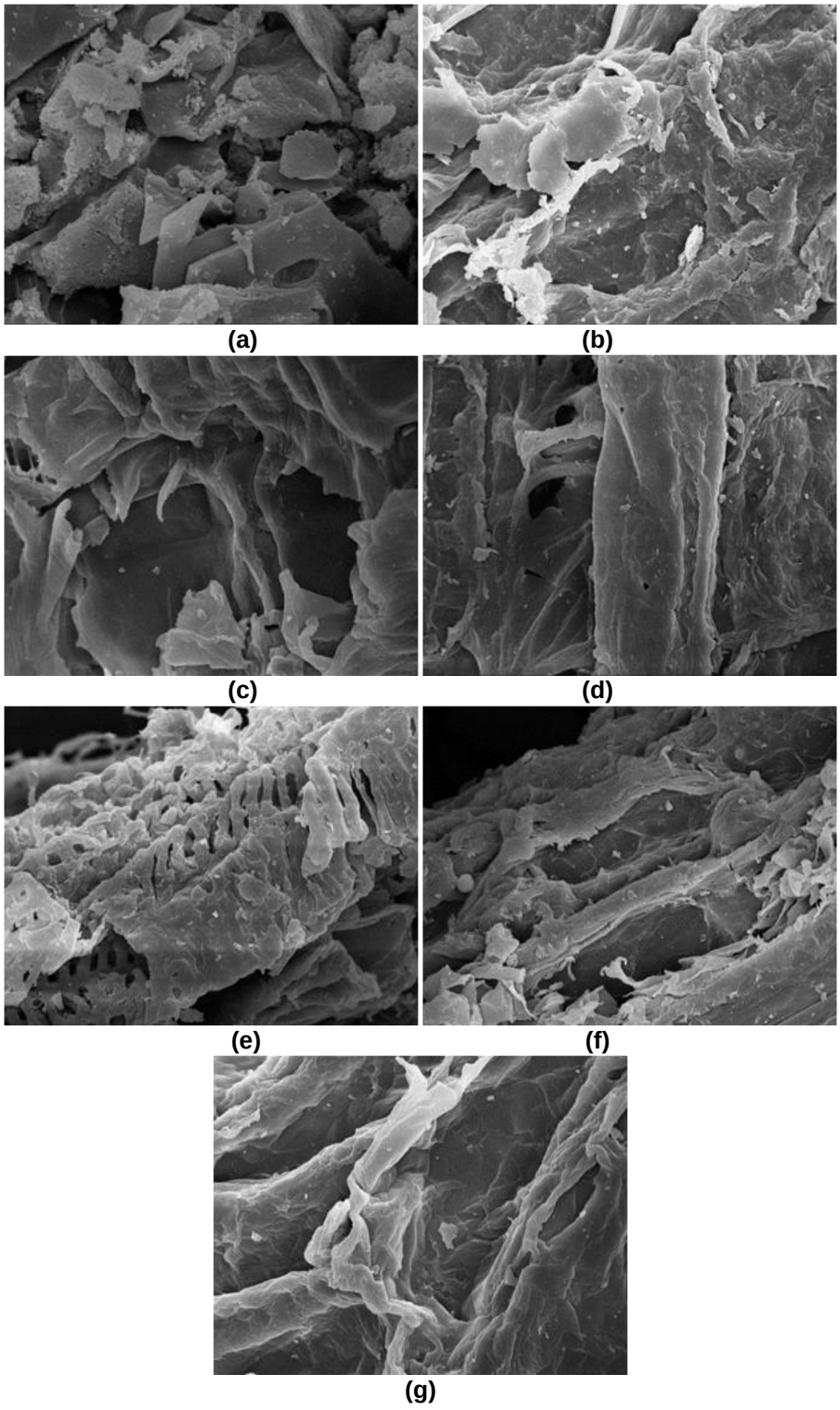

Fig. 1. Microscopic pictures of the various CS pretreatments: (a) untreated(5000×); (b) enzyme(2000×); (c) H_2O_2(2000×); (d) H_2SO_4(2000×); (e) NaOH(2000×); (f) SE(2000×); and (g) SE-NaOH(2000×)

Most studies have found that lignin negatively impacts biofuel production in the saccharification process (Zeng *et al.* 2014). Lignin causes irreversible adsorption on

cellulase, which greatly reduces its effectiveness (Fang *et al.* 2015). Figure 1 reveals that fractures only occurred on the surface of the untreated CS, that it was not disrupted on a deep level, and that the cellulose-hemicellulose-lignin structure remained stubborn.

Figure 1b shows that the substrate of the lignin that had been exposed to peroxidase pretreatment had undulating rough fibers and more lignin remained on the surface. However, the chemical pretreatment had a greatly destructive effect on the CS structure (Figs. 1c to 1e). In the H_2O_2-pretreated CS, the vascular bundles of the CS swelled at high temperatures and were partially degraded (Fig. 1c). The lignin residue was almost invisible on the surface of the CS pretreated with H_2SO_4 (Fig. 1d) and NaOH (Fig. 1e). The vascular bundle was exposed, especially during the NaOH pretreatment (Fig. 1e). After treatment, many pores and hollow network structures were formed. Consequently, the modified biomass structure resulted in favorable conditions for increased accessibility of cellulase to cellulose.

Figure 1f shows that residual lignin and many destroyed vascular bundles were on the surface of the SE-pretreated CS. This could have been the reason why the saturated steam penetrated the CS pores, tore the vascular bundle during rapid decompression, caused huge voids, and exposed the cellulose cavity. The SE-NaOH-pretreated CS showed the best effect. The lignin was almost completely removed from the CS surface and vascular bundles could be observed in a strip-like arrangement. The above-mentioned structural characteristics were explained by the fact that the SE-NaOH-pretreated CS had a high accessibility and low enzyme adsorption rate after enzymatic hydrolysis. This could have been because of the role that chemical high-temperature cooking plays in the degradation of hemicellulose and lignin, as well as the softening and exposing of cellulose (Yang *et al.* 2017).

XRD Analysis

Figure 2 shows the XRD images of the raw materials and pretreated samples. The peak intensities at 2θ values of 16° (002) and 22.8° (101) in all of the pretreated CS samples increased compared with that of the raw material, and the CrI of the pretreated samples also increased compared with that of the raw material.

The increased peak intensity at a 2θ value of 22.8° indicated that the chemical pretreatment changed the crystalline structure of the crystallization zone into irregular and non-crystalline structures (Figs. 2d, 2e, and 2f). Moreover, only the chemically pretreated CS showed a diffraction peak of the (040) crystal surface at a 2θ value of 35°, which suggested that the chemical pretreatment greatly affected the crystalline structure of the cellulose.

The cellulose in lignocellulosic biomass can be divided into crystalline structures and amorphous structures (Perez-Pimienta *et al.* 2015). The content of amorphous cellulose is positively related with the CrI value. Table 7 shows that the CrI value of the cellulose in the untreated CS (18.1%) was lower than that in the other pretreated CS. The increase in the CrI implied that pretreatment led to the conversion from crystalline cellulose to amorphous cellulose. The crystalline structure of cellulose and the lignin content have been reported to be two key factors that impact the Y_{trs} of pretreated lignocellulosic biomass after saccharification.

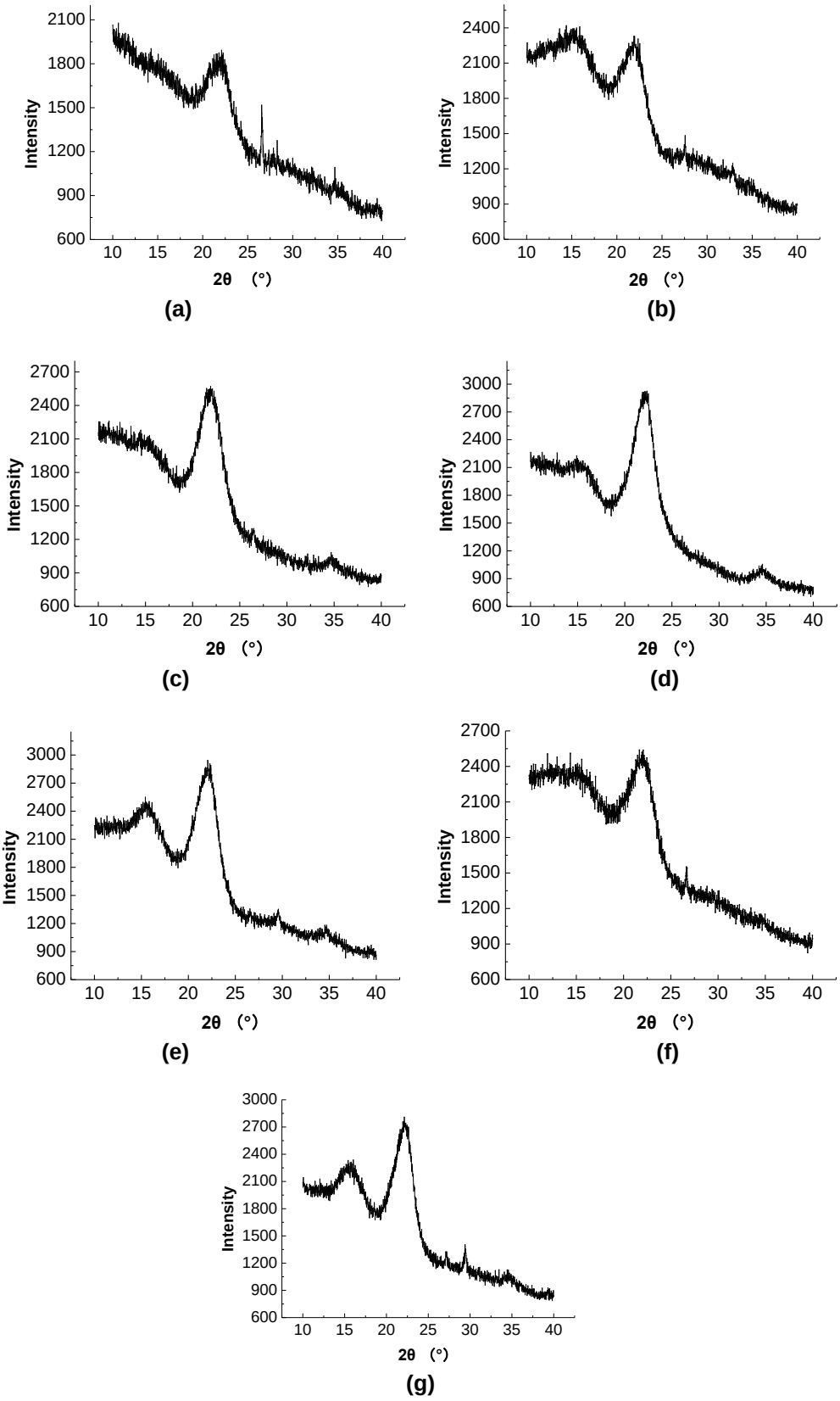

Fig. 2. XRD images of the (a) untreated; (b) enzyme; (c) H_2O_2; (d) H_2SO_4; (e) NaOH; (f) SE; and (g) SE-NaOH

To analyze the effects of the two factors on the Y_{trs}, non-linear regression analysis was performed using the Y_{trs} at 32 h in Table 4 as the dependent variable and the CrI and C_{lignin} in Table 7 as the independent variables with Eq. 10,

$$Y_{\mathrm{trs}} = \alpha e^{27\times \mathrm{CrI}} + \beta C_{\mathrm{lignin}} + \gamma \quad (10)$$

where α and β are the coefficients of the $e^{27\times CrI}$ and C_{lignin}, respectively, C_{lignin} is the content of the substrate (%), and γ is a constant.

Table 7. CrI and Lignin Content of the CS with Pretreated Various Methods

	Untreated	H_2O_2	Enzyme	H_2SO_4	SE	NaOH	SE-NaOH
I_{002}	1894	2544	2307	2926	2489	2944	2810
I_{am}	1551	685	1761	1577	1921	1821	1704
CrI (%)	18.11	33.77	23.67	46.70	22.82	38.15	39.36
C* (%)	19.05	16.40	17.82	29.62	18.81	7.94	7.92

*: C is the lignin content of the pretreated CS

The F-value and *p* value were 42.709 and 0.002, respectively, which indicated that the correlation of the Y_{trs} with the CrI and C_{lignin} was significant, and the reliability of the analysis was high (Table 8). The adjusted R^2 value (0.913) was close to the R^2 value (0.942), which showed that the model had a good adaptability.

The coefficients for all of the parameters in Table 8 were significant ($p < 0.01$). The coefficient of $e^{27\times CrI}$ was positive, which showed that the Y_{trs} had a positive correlation with the CrI. The Y_{trs} exponentially increased with an increase in the CrI. The phenomenon may be related to recrystallization when cellulose dried into water and crystallized cellulose is left after amorphous cellulose dissolved into water in the pretreatment process. The coefficient of the C_{lignin} was negative, which suggested that the Y_{trs} had a negative correlation with the C_{lignin}. Therefore, less residual lignin resulted in a better enzymatic hydrolysis effect and a higher Y_{trs}. Consequently, it was speculated that the effect of the CrI on cellulose saccharification was greater than that of the residual lignin.

Table 8. Regression Analysis of the Correlation between the Y_{trs}, CrI, and C_{lignin}

Item	Coefficient	*t*-value	Significance
Constant	947.832	16.785	0.000
$e^{27\times CrI}$	0.001	5.756	0.005
C_{lignin}	-29.319	-8.057	0.001

F-value = 32.636, p = 0.003, R^2 = 0.942, and adj-R^2 = 0.913

FTIR Analysis

The FTIR analysis was performed to evaluate the changes in the functional groups of the cellulose before and after the different pretreatments. The peak at 3404 cm^{-1} refers to the stretching vibration of the -OH group. Figure 3 shows that after the pretreatment, the intensity of this peak decreased, which indicated that pretreatment could destroy some hydrogen bonds in the cellulose. This was consistent with the increased Y_{trs} after pretreatment. The peaks at 1165 cm^{-1} and 1059 cm^{-1} were assigned to the β-1,4-glycosidic bonds present in the cellulose, which is a strong signal hinting at a high cellulose composition (Muñoz *et al.* 2018). The intensities of the two peaks at 1165 cm^{-1} and 1059 cm^{-1} after the various pretreatments did not change much (Fig. 3), which indicated that the pretreatments did not destroy the primary structure of the cellulose.

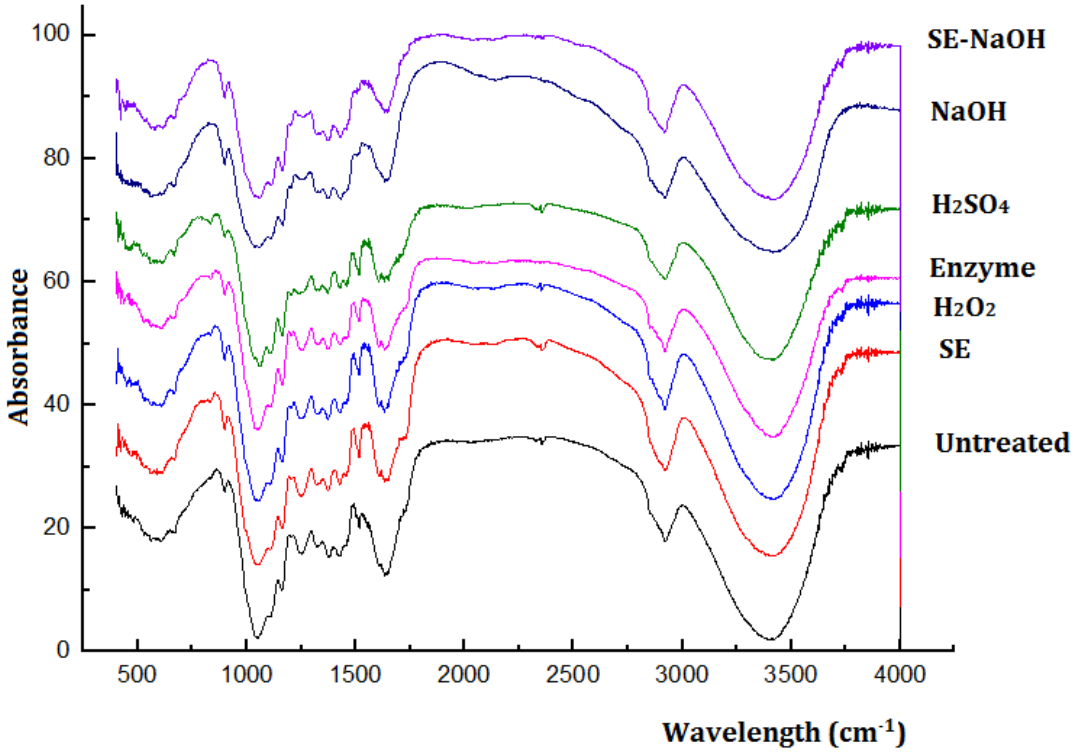

Fig. 3. FTIR spectra of the various pretreatments

The peak at 1731 cm^{-1} referred to the stretching vibration of the C=O group in the hemicellulose. After the NaOH and SE-NaOH pretreatments, the peak at 1731 cm^{-1} disappeared. The absence of a band at 1731 cm^{-1} could have been because of the removal of the C=O group (Kapoor *et al.* 2015), which indicated the removal of the acetyl and uronic ester groups from the hemicellulose. The peak intensities were changed after the other pretreatments. The peaks at 1515 cm^{-1} and 1650 cm^{-1} to 1515 cm^{-1} referred to the peaks of lignin aromatic ring vibration and aromatic ring C=C stretching in lignin. The disappearance of the peaks at 1515 cm^{-1} or 1650 cm^{-1} to 1515 cm^{-1} (Fig. 3) revealed that lignin was removed after chemical pretreatment, especially for the SE-NaOH pretreatment. According to the literature, phenolic lignin has more essential inhibitory effects compared with non-specific adsorption on cellulase (Xu *et al.* 2015). This was consistent with the increased availability of enzymes *versus* substrates, and the increased cellulase saccharification resulted in an increase in the $K_{obs,0}$ and Y_{trs}.

CONCLUSIONS

1. Corn stover was subjected to sequential steam explosion (SE) and NaOH pretreatment. The SE-NaOH pretreatment was found to be an effective method to improve the yield of total reducing sugars (Y_{trs}) by 106.6% after saccharification of the CS. Under the experimental conditions, the Y_{trs} and SE pressure had an exponential correlation and the pressure had a strong effect on the Y_{trs}. A higher pressure resulted in a higher Y_{trs}.

2. The chemical and SE pretreatments mainly increased the Y_{trs} by removing lignin and destroying the crystalline structure of the cellulose, respectively, according to the impeded Michaelis model (IMM), scanning electron microscopy (SEM), and X-ray diffraction (XRD) analyses. The Y_{trs} exponentially increased with an increase in the crystallinity index (CrI) and was negatively correlated with the C_{lignin}.
3. The SE-NaOH pretreatment removed most of the lignin in the samples and destroyed the crystalline structure of the cellulose.

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