Enhancement of Enzyme Hydrolysis by Increasing the Zeta Potential to Reduce Non-productive Cellulase Adsorption on Sugarcane Bagasse Treated with Liquid Hot Water

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The enhancement of enzymatic hydrolysis is important for the biorefinery industry of lignocellulose. Changing the pH of hydrolysis is a simple and direct way to improve hydrolysis efficiencies. In this study, the enzymatic hydrolysis efficiencies of sugarcane bagasse (SCB) treated with liquid hot water (LHW) were 56.7% and 65.5% at pHs of 4.8 and 5.5, respectively. The result of cellulase adsorption on the LHW treated SCB showed that the non-productive adsorption was smaller at pH 5.5, which might tend to enhance hydrolysis. The surface hydrophobicity of lignin was larger at pH 5.5. This suggested that the hydrophobic interaction was not dominant because a strong hydrophobicity force can cause more non-productive adsorption of cellulase with lignin. At pH 5.5, the surface negative charges of lignin and cellulase increased. Therefore, the electrostatic repulsive force between lignin and cellulase increased, leading to less of the non-productive adsorption of cellulase on lignin. In addition, the cellulase desorption from the LHW treated SCB also increased at pH 5.5. This was beneficial in increasing the possibility of cellulase re-adsorption in new binding sites on cellulose and promoting enzyme hydrolysis efficiency.

Keywords: Cellulase adsorption; Enzymatic hydrolysis; Hydrophobicity; Zeta potential; Sugarcane bagasse

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INTRODUCTION

The utilization of renewable biomass energy can alleviate the fossil energy crisis and environmental problems. Therefore, it has received more attention in recent years (Alya and Steven 2012). Lignocellulosic biomass has become an important raw material for renewable energy production due to the abundant reserves and re-application possibilities. However, the enzymatic hydrolysis efficiency of lignocellulose still needs further improvement. Characteristics of cellulase adsorption on lignocellulosic substrates and interactions between substrates and cellulase are the key influencing factors of enzymatic hydrolysis efficiency (Zheng *et al*. 2016; Zhai *et al*. 2018).

Cellulase activity is generally considered to be optimal at pH 4.5 to 5.0. However, some studies showed that pretreatment methods can affect the optimal pH of enzymatic hydrolysis of lignocellulose (Lan *et al.* 2013; Lai *et al*. 2018). Therefore, elevating the pH of enzymatic hydrolysis is efficient to improve hydrolysis efficiency (Lai *et al*. 2018). However, the effect mechanism of pH on hydrolysis is still not revealed clearly.

The objective of this study was to explore why the enzymatic hydrolysis efficiency of the LHW treated SCB at pH 5.5 was higher than at pH 4.8. This was accomplished by investigating cellulase adsorption profiles on the LHW treated SCB, the hydrophobicity and zeta potentials of lignin and cellulase, and the pore properties and chemical groups on the substrates' surface of hydrolysis residues. This study examined the effect of pH on the enzymatic hydrolysis of cellulose, adsorption profiles of lignocellulose and cellulase, and the surface characteristics of lignocellulosic substrates.

EXPERIMENTAL

Materials

Sugarcane bagasse was provided by the Guitang Sugar Refinery (Guigang, China). The size of sugarcane bagasse pieces was about from 0.1 × 0.1 × 0.1 to 0.1 × 1.0 × 2.0 cm^3. The cellulase CTec2 (147 FPU per mL, 84.43 mg protein per mL) was purchased from Novozymes (Tianjin, China). All chemicals were analytical grade.

Pretreatment

The SCB pieces were dried to the constant weight at 30 °C in an oven but not ground. The ratio of dried SCB to water was 1 to 20. The LHW pretreatment was conducted at 170 °C for 20 min in the rotating steam-jacketed pressure vessel (ZQS1-15, Machinery Works in Shanxi University of Science and Technology, Shanxi, China).

Enzymatic Hydrolysis

The enzyme hydrolysis of the LHW treated SCB was performed at 50 °C and 180 rpm for 72 h. This was performed in a 50 mM citric acid/citrate buffer (pH 4.8 or 5.5) with a substrate loading of 2% and an enzyme loading of 7.5 FPU per g of dried LHW treated SCB. The reducing sugar content in hydrolysate was determined by the dinitrosalicylic acid (DNS) method (Ghose 1987). The enzyme hydrolysis efficiency was calculated according to Eq. 1,

$$\textit{Hydrolysis efficiency}\ (\%) = (RS \times 0.9 \times 100\%) \div W_C \quad (1)$$

where RS (mg) is the reducing sugar weight in enzymatic hydrolysate, 0.9 is the conversion coefficient between glucose and glucan, and W_C is the carbohydrate weight in the dried LHW treated SCB used for enzymatic hydrolysis.

Enzyme Adsorption and Desorption

Productive and non-productive adsorption

The extraction of cellulose was performed according to the reported method (Jin *et al.* 2019). The lignin was isolated as described in the literature (Lou *et al*. 2013). The contents of the extracted cellulose and lignin were 88.55±0.17% and 75.32±0.59%, respectively. The composition was analyzed according to the protocol recommended by the National Renewable Energy Laboratory (Sluiter *et al.* 2012). The experiments of productive and non-productive adsorption of cellulase on the LHW treated SCB were conducted in 50 mM citric acid/citrate buffer (pH 4.8 or 5.5) at 4 °C using the extracted cellulose or isolated lignin as the substrate. The substrate loading was 1% (weight per volume). The substrate was kept in the buffer for 2 h, and then cellulase was added. The enzyme loading was 160 mg of protein per g of dry substrate. The mixture was incubated

at 4 °C and 180 rpm for 2 h and then centrifuged at 8000 rpm for 15 min. The protein content in the supernatant was determined by the Bradford method (Liu *et al*. 2017). The adsorbed protein was calculated by subtracting the free protein in the supernatant from the total used cellulase protein.

Desorption

The solid obtained after centrifugation in the adsorption experiment was put in 10 mL of 50 mM citric acid/citrate buffer solution, and then incubated at 4 °C and 180 rpm for 2 h. Next, it was centrifuged at 8000 rpm for 15 min. The free protein in the supernatant was the desorbed cellulase (Lou *et al*. 2013).

Adsorption kinetics

The experiment was carried out as described in the Productive and Non-Productive Adsorption section. The only difference was that before centrifugation, the incubation time was 5, 10, 20, 30, 60, 90, and 120 min, respectively. The fitting of the adsorption kinetics was based on the pseudo-first-order adsorption kinetic model (Guo and Wang 2019). This model was calculated according to Eq. 2,

$$(dq_t) \div dt = k_1 (q_e - q_t) \quad (2)$$

where k_1 (min^{-1}) is the first-order rate constant, q_e (mg protein per g of dry SCB) and q_t (mg protein per g of dry SCB) are the cellulase adsorption amounts at the adsorption equilibrium, and t (min) is the adsorption time, respectively.

Hydrophobicity

The experiment was performed according to the published literature (Huang *et al*. 2017). The isolated lignin of 1, 2, 3, 4, and 5 g per L was individually put in the 50 mM citric acid/citrate buffer solution (pH 4.8 or 5.5) containing rose bengal with 40 mg per L. The mixture was incubated at 50 °C and 180 rpm for 2 h. It was then separated by centrifugation at 8000 rpm for 15 min. The supernatant was determined at 543 nm using a UV-Visible spectrophotometer (UV-1800PC, Mapda Instrument Limited Company, Shanghai, China). The ratio of adsorbed rose bengal to free rose bengal in the supernatant was the ordinate, and the lignin content was the abscissa. The slope represented the hydrophobicity of lignin.

Zeta Potential

Cellulase and lignin of 0.1% (weight per volume) were prepared with citric acid/citrate buffer solutions at pH 4.8 or 5.5. Each experiment was done in triplicate using a zeta potentiometer (Zetasizer Nano ZS90, Malvern, Malvern, England) (Yang *et al*. 2017).

Brunauer-Emmett-Teller (BET) and Fourier Transform Infrared (FTIR)

The specific surface area, pore volume, and average pore diameter of hydrolysis residues were tested by a BET analyzer (ASAP 2460, Micromeritics, Georgia, USA). The chemical groups were determined by a FTIR spectrometer (TENSOR27, Bruker, Karlsruhe, Baden-Wurttemberg, Germany).

Statistical Analysis

Statistical analysis was conducted by a student's t test. Origin 8.5 (Origin8.5, OriginLab, Northampton, Massachusetts, USA) was used for the data analysis. A p-value less than 0.05 indicated a significant difference.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis

The optimal pH value of enzymatic hydrolysis of pure cellulose was pH 4.8, which has been widely accepted and used for the saccharification of lignocellulose. However, Lou *et al.* (2013) suggested that different conditions should be considered, since lignocellulose is different from pure cellulose without lignin. It was found that the enzymatic hydrolysis of lignocellulose at a pH value of higher than 5.5 was higher than that at pH 4.8. In Fig. 1, the enzymatic hydrolysis efficiencies of the LHW treated SCB at pH 4.8 and 5.5 are listed. The results showed that the enzymatic hydrolysis efficiency of the LHW treated SCB at pH 5.5 (65.46%) was higher ($P < 0.05$) than at pH 4.8 (56.70%). It has been reported that the enzymatic hydrolysis efficiency of pretreated lignocellulose was closely related to the lignocellulosic surface characteristics. This includes the adsorption and desorption profiles of cellulase with lignocellulose and the zeta potential and hydrophobicity of the lignocellulosic substrate (He *et al.* 2017; Lu *et al.* 2017a; Lai *et al.* 2018). Therefore, to discover the reason why the hydrolysis of the LHW treated SCB at pH 5.5 was higher than at pH 4.8, the surface characteristics of the LHW treated SCB were determined.

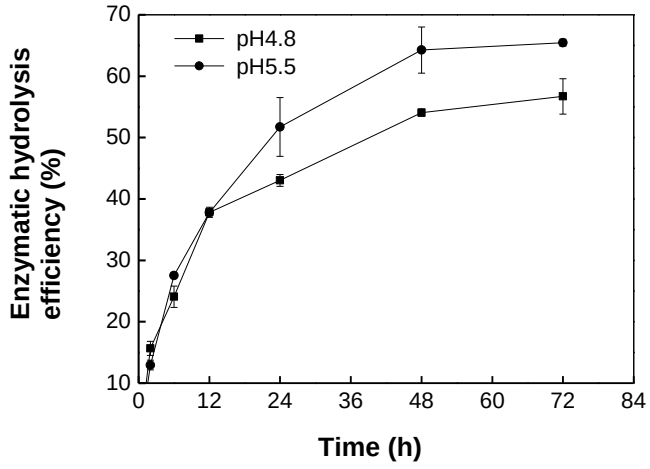

Fig. 1. Enzymatic hydrolysis efficiencies of the LHW treated SCB at pHs of 4.8 and 5.5

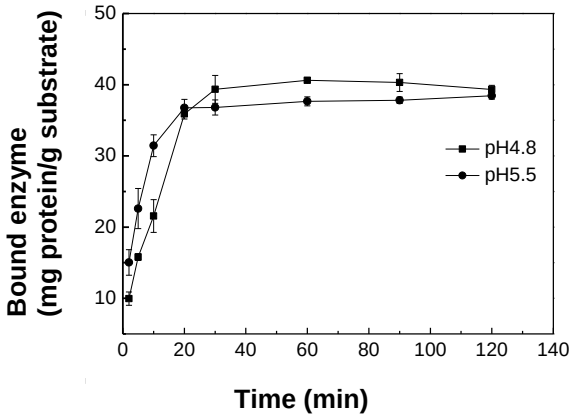

Fig. 2. Adsorption kinetics of cellulase with the LHW treated SCB at pHs of 4.8 and 5.5

Cellulase Adsorption and Desorption

In Fig. 2, the adsorption kinetics of the LHW treated SCB at pHs of 4.8 and 5.5 are demonstrated. In Table 1, the fitting parameters of pseudo-first-order cellulase adsorption kinetics of the LHW treated SCB are given. From Table 1, it was found that the cellulase adsorption profiles on the LHW treated SCB at two pHs were well fitted with the pseudo-first-order adsorption kinetics (R^2 approximated to 1). The values of k_1 at pH 4.8 and pH 5.5 indicated that the cellulase adsorption at a 5.5 pH was quicker than at a 4.8 pH, which might promote the enzymatic hydrolysis of the LHW treated SCB.

Additionally, the results from Fig. 2 showed that the maximum adsorption amounts of the LHW treated SCB at pHs of 4.8 and 5.5 were 40.6 and 38.4 mg protein per g of substrate at 30 and 20 min, respectively. This was extremely similar to the fitting values of 40.4 and 37.9 mg protein per g of substrate in Table 1. The hydrolysis efficiency at pH 5.5 was higher, but the adsorption amount of cellulase was lower. Therefore, it was assumed that the non-productive adsorption of cellulase at pH 5.5 would be lower.

Table 1. Fitting Parameters of Pseudo-First-Order Adsorption Kinetics of LHW Treated SCB

pH	q_e (mg protein / g dry LHW SCB)	k_1 (min^{-1})	R^2
4.8	40.40	0.10	0.99
5.5	37.89	0.20	0.97

The productive and non-productive adsorptions were subsequently determined in this study, representing the adsorptions of cellulase on cellulose and lignin extracted from the LHW treated SCB, respectively. In Fig. 3, the productive and non-productive adsorptions are presented. The results showed that the productive adsorption at these two pHs had no significant difference ($p > 0.05$), and the non-productive adsorption at pH 5.5 was smaller. In Fig. 3, the desorption amounts at the two pH levels are also shown. The desorption amount at the 5.5 pH level was larger. A higher productive adsorption amount and a lower non-productive adsorption amount of cellulase were helpful for the enzymatic hydrolysis efficiency of cellulose (Zheng *et al.* 2020). In addition, a larger desorption amount of cellulase was also beneficial to the hydrolysis of cellulose. This is because it was more possible for the desorbing cellulase to adsorb again on the new binding sites of cellulose with cellulase (Hao *et al.* 2019).

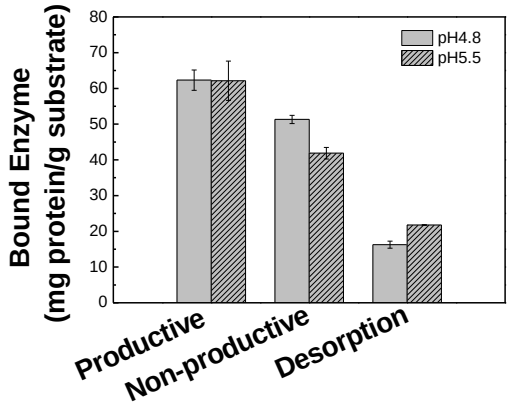

Fig. 3. Productive adsorption, non-productive adsorption, and desorption of cellulase

Non-productive adsorption of cellulase on lignin was mainly due to hydrophobic and electrostatic interactions (Huang *et al.* 2017). Thus, hydrophobicity and zeta potentials of the LHW treated SCB at the two pH levels of 4.8 and 5.5 should be further studied.

Hydrophobicity

The data on the hydrophobicity of lignin and cellulase are listed in Table 2. The hydrophobicity of lignin at the 5.5 pH level was 0.38 L per g, which was higher than 0.09 L per g at the 4.8 pH level. Additionally, the hydrophobicity of cellulase (3.64 L per g) at the 5.5 pH level was also higher than 2.02 L per g at the 4.8 pH level. A higher hydrophobicity of lignocellulose can produce a stronger hydrophobic interaction force between the substrate and cellulase (Lai *et al.* 2018). Therefore, the hydrophobic interaction between lignin and cellulase at the 5.5 pH level was higher than at the 4.8 pH level. However, the non-productive adsorption amount of cellulase on lignin at the 5.5 pH level was lower than that at the 4.8 pH level in this study. Therefore, the hydrophobic interaction did not play a dominant role in the non-productive adsorption of cellulase on lignin. The similar phenomenon was observed in other reported literatures (Lu *et al.* 2017b; Zhang *et al.* 2016). Therefore, the electrostatic interaction force between lignin and cellulase might be dominant. To verify the effect of electrostatic interaction on non-productive adsorption, zeta potentials of cellulase and lignin were measured, respectively.

Table 2. Hydrophobicity and Zeta Potential of Cellulase and Lignin

Samples	Hydrophobicity (L / g)		Zeta Potential (mV)	
	pH 4.8	pH 5.5	pH 4.8	pH 5.5
Cellulase	2.02 ± 0.16^{a}	3.64 ± 0.42^{a}	-0.75 ± 0.18^{a}	-4.84 ± 0.50^{b}
Lignin	0.09 ± 0.00^{a}	0.38 ± 0.03^{b}	-7.68 ± 0.60^{a}	-10.83 ± 0.61^{b}
* Data with the different superscripts denote a statistically significant difference ($p < 0.05$). The values following ± are standard deviations.				

Zeta Potential

The zeta potential values of cellulase and lignin are shown in Table 2. The zeta potentials of lignin and cellulase at two pHs were all negative. Therefore, there were electrostatic repulsion forces between lignin and cellulase. At the 5.5 pH level, the absolute values of the zeta potential of lignin and cellulase both increased, indicating that the electrostatic repulsion force between cellulase and lignin increased. The increase in the

repulsion force caused the decrease in the non-productive adsorption amount of cellulase on lignin. Therefore, in this study, the electrostatic interaction between lignin and cellulase might mainly be responsible for the decrease in the non-productive adsorption amount of cellulase on lignin at the 5.5 pH level. As a result, the enzymatic hydrolysis efficiency at the 5.5 pH level was improved.

BET Analysis

In order to explore the hydrolysis mechanism of lignocellulose, except for the adsorption profiles of cellulase, the hydrolysis residues of the LHW treated SCB should also be studied due to their importance (Pihlajaniemi *et al.* 2016). In Table 3, the specific surface area, pore volume, and average pore diameter of hydrolysis residues of the LHW treated SCB are shown. The specific surface area, pore volume, and average pore diameter of the LHW treated SCB at the 5.5 pH level were all higher than those at the 4.8 pH level, suggesting that the enzyme hydrolysis was more effective at a 5.5 pH.

Table 3. Specific Surface Area, Total Pore Volume, and Average Pore Diameter of the Enzymatic Hydrolysis Residues of the LHW-treated SCB

pH	Specific Surface Area (m^2 / g)	Pore Volume (cm^3 / g)	Average Pore Diameter (nm)
4.8	1.648	0.008	20.094
5.5	1.922	0.010	21.158

FTIR Analysis

In Fig. 4, the FTIR spectra of enzymatic hydrolysis residues of the LHW treated SCB are shown. The peaks at 2930, 1730, 1604, and 1083 cm^{-1} respectively represented the methoxy groups, the ester groups, the aromatic ring structure and the aromatic methyl ether bridges of lignin (Kang *et al*. 2012; Guo *et al*. 2014; Zehra *et al*. 2019). These groups are all hydrophobic (Li *et al.* 2018; Lavagna *et al.* 2019; Yu *et al.* 2019; Chai *et al.* 2020). The transmittance intensities at these peaks of the hydrolysis residue at the 5.5 pH level were smaller, suggesting that the contents of the hydrophobic groups of hydrolysis residue at pH 5.5 were higher than that at pH 4.8. The hydrophobic interaction was stronger between lignin and cellulase at the 5.5 pH level. This was consistent with the hydrophobicity results.

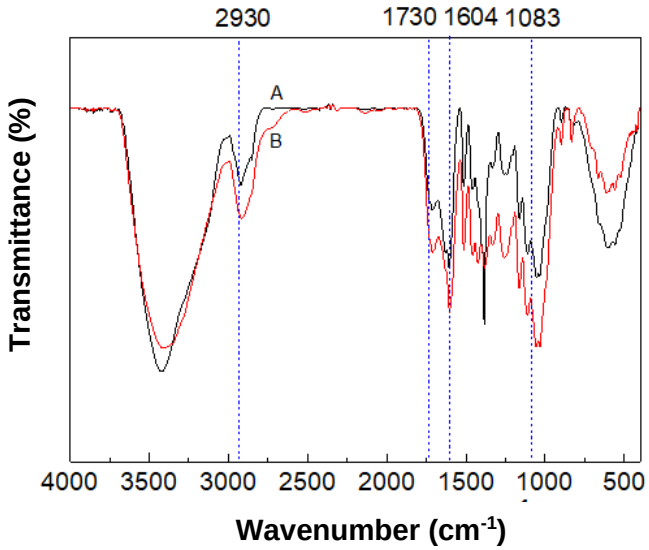

Fig. 4. FTIR spectra of enzymatic hydrolysis residues of the LHW treated SCB at pH (A) 4.8 and (B) 5.5

CONCLUSIONS

1. The enzymatic hydrolysis efficiencies of the LHW treated SCB were 56.7% and 65.5% at pH 4.8 and 5.5 respectively, with a significant difference ($P < 0.05$). This was due to the decrease in the non-productive adsorption of cellulose on the LHW treated SCB at the 5.5 pH level.
2. The increase in the hydrophobicity of lignin and cellulase at the 5.5 pH level was not the dominant reason for the improvement in the enzymatic hydrolysis. The increasing zeta potentials of lignin and cellulase was mainly responsible for the decrease in the non-productive adsorption of cellulase at the 5.5 pH level.

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