

Effect of dilute acetic acid hydrolysis on xylo-oligosaccharides production and inhibitory effect of cellulolytic enzyme lignin from poplar

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Abstract

Acetic acid (AC) hydrolysis has been reported to prepare xylo-oligosaccharides (XOS) from poplar. However, the influence of AC hydrolysis on the lignin structure changes is not clear, which is important for the following enzymatic hydrolysis of poplar. Herein, AC was used to produce XOS and the cellulases adsorption on cellulolytic enzyme lignin (CEL) from AC-pretreated poplar and its inhibitory effect on two commercial cellulase preparations were investigated. The AC hydrolysis gave a 39.8% XOS yield from poplar. After AC hydrolysis at 170 °C, the hydrophobicity and zeta potential of CEL were decreased to 2.3 L/g and 14.8 mV, respectively. The adsorption strengths of CTec2 on CELs were not increased by AC hydrolysis. And the inhibitory effect of CEL on Celluclast 1.5L and β -glucosidase were observed, but not on CTec2. CELs improved the LPMO activity of the enzymatic hydrolysis by CTec2. After CELs were added in enzymatic hydrolysis, the free filter paper activity of Celluclast 1.5L and β -G retained in enzymatic hydrolysate was decreased from 60.5% to 29.3%–42.9%. The addition of CELs in enzymatic hydrolysis could not decrease the free filter paper activity of CTec2 retained in enzymatic hydrolysate. In the enzymatic hydrolysis with CELs, higher glucose yields were obtained by CTec2 than that by Celluclast 1.5L and β -glucosidase. This work will help the understanding of the structural and inhibitory of AC-CELs and guide for the development of AC hydrolysis for production of XOS and monosaccharides from poplar.

Keywords: Poplar; Acetic acid hydrolysis; Cellulolytic enzyme lignin; Inhibition; Adsorption

Introduction

Poplar is widely used to produce chemicals such as lactic acid, ethanol, and xylo-oligosaccharides (XOS) by bio-refinery to maximize the utilization of poplar resources.¹⁻³ In recent reports, acetic acid (AC) hydrolysis has been employed to degrade poplar xylan into XOS.²⁻⁴ More than 50% XOS yields can be obtained by AC hydrolysis with the conditions of 170 °C, 5% AC, and 30 min, which proved AC hydrolysis can be considered as an efficient method for preparation of XOS.²⁻⁴ However, after the preparation of XOS by AC hydrolysis, the removal of xylan resulted in a high lignin content (more than 30%) in AC-pretreated poplar, which greatly limited the hydrolysis yield (less than 55%) of poplar by cellulase.^{3,4} Some reports have been used sodium chlorite, peroxide-acetic acid, and sodium hydroxide to remove the lignin of AC-pretreated poplar, which greatly improved the glucose yield of poplar by enzymatic hydrolysis.²⁻⁴ These reports confirmed that lignin limits the enzymatic hydrolysis of AC-pretreated poplar. Hence, it is necessary to explore the effect of lignin on the enzymatic hydrolysis of AC-pretreated poplar.

Normally, non-productive binding and physical barrier have been confirmed as the two main reasons for lignin inhibition, which reduce the accessibility of cellulase to cellulose.⁵⁻⁷ Non-productive adsorption of cellulase on lignin is mainly caused by hydrophobic and electrostatic.⁸⁻⁹ Hydrophobic interaction is the leading attractive force between cellulase and lignin.¹⁰ A higher hydrophobicity of lignin can exhibit the stronger cellulases adsorption capacity.¹¹ Electrostatic interactions play an important role in non-productive binding between cellulase and lignin. Lignin has high sample surface charges is more hydrophilic or less hydrophobic, thus reflects a poorer cellulase adsorption property.¹² Both the lignin hydrophobicity and electrostatic interactions with cellulase essentially are governed by the structure of lignin.¹³

Physical barrier of lignin prevents the bind of cellulase on cellulose due to the complex structure of biomass feedstock.^{6, 8, 14} Surface lignin is considered as the major factor in the evaluation of physical barrier of lignin, which can block the access of cellulase to cellulose.^{6, 15-17} Within a certain range, the lignin removal by pretreatment was positively correlated with the monosaccharide yield,^{2, 3, 18} which confirms lignin acts as a physical barrier in the enzymatic hydrolysis of biomass.^{6, 18} Previous work has confirmed that AC hydrolysis with the condition of 170 °C, 5% AC, and 30 min increases the surface lignin and total lignin of poplar and lignin removal benefits to the hydrolysis yield of poplar.² However, the knowledge about the effect of AC hydrolysis on inhibitory effect of poplar lignin is quite limited.

Inhibitory effects of isolated lignin on enzymatic hydrolysis have been proved in many reports.^{7, 15, 17} The previous report showed lignin in AC-pretreated poplar can be extracted by dioxane.¹⁹ However, the lignin isolated by solvent from lignocellulose may only represent the part of lignin dissolved in the solvent²⁰. Cellulolytic enzyme lignin (CEL) can maintain the native lignin structure and considered to represent native lignin in pretreated lignocelluloses.^{6, 15, 21-23} Therefore, CEL is more suitable for exploring the structure and inhibitory of lignin in AC-pretreated poplar. However, the effects of AC hydrolysis on the structure and inhibitory effect of CEL from poplar have not been reported.

In this work, AC hydrolysis was used to prepare XOS from poplar. To understand the effects of AC hydrolysis on lignin structure and its inhibitory effects, CEL was prepared from AC-pretreated poplar by cellulase hydrolysis. The lignin from ball-milled poplar was used as control to compare with the lignin from AC-pretreated poplar. X-ray photoelectron spectra (XPS), X-ray diffraction (XRD), Fourier transform infrared (FTIR), scanning electron microscopy (SEM), thermo gravimetric

(TG), 2D heteronuclear single quantum-nuclear magnetic resonance (HSQC-NMR), and pyrolysis-gas chromatography/mass spectrometry (PY-GC/MS) were employed to characterize the structure of CEL. The hydrophobicity, zeta potentials, and adsorption capacity of CEL were evaluated. The inhibitions of CELs to two commercial cellulases (CTec2 and Celluclast 1.5L/ β -glucosidase) were compared. The aim of this research is to explore the inhibitory effects of CELs in AC-pretreated poplar comprehensively, and to provide a reference for realizing efficient hydrolysis of AC-pretreated poplar.

Materials and methods

Materials

Poplar was obtained from Suqian, Jiangsu Province, China. The air-dried poplar was ground to pass an 80 mesh (≤ 0.178 mm) sieve.

Cellic CTec2 (Novozymes A/S, Bagsværd, Denmark) had an activity of 147 filter paper units (FPU)/mL (382 mg protein/mL).²⁴ Celluclast 1.5 L had an activity of 74.7 FPU/mL (169.6 mg protein/mL). The β -glucosidase (β -G) (2256.57 nkat/mL) was obtained from *Aspergillus niger* (Sigma–Aldrich, St. Louis, MO, USA). Protein was quantified by the Lowry method.²⁵ Endoglucanase, cellobiohydrolase, β -glucosidase activities were quantified by those methods in previous report.²⁶ The activity of lytic polysaccharide monooxygenases (LPMO) was determined by according to a method previously reported.²⁷ The glucose oxidation was calculated by the gluconic acid concentration in enzymatic hydrolysis.²⁸

Ball milling

The ball milling (BM) of poplar (25 g) was conducted with 500 mL zirconia bowl with zirconia milling balls of three diameters (2 large balls, 20 mm diameter; 200 medium balls, 10 mm diameter; 1000 small balls, 5 mm diameter). The vessel was

fixed into planetary ball mill system, operated at 500 rpm frequency at room temperature for 6 h working time. After 15 minutes of work, the ball mill system will stop for 15 minutes for pause to dissipate heat.

Acetic acid hydrolysis

The AC hydrolysis conditions were referred to our previous research.² The poplar sample was pretreated by 0%, 5%, and 10% (v/v) AC, respectively. The system was heated from 25 °C to 160–180 °C, and temperature was kept at the preset temperatures for 10, 30, and 50 min, respectively. The collections of hydrolysis liquid and the pretreated solid were consistent with previous report.²

Preparation of cellulolytic enzyme lignin

CELs were isolated and purified from the ball-milled and AC-pretreated poplar after enzymatic hydrolysis.²⁹ The ball-milled and AC-pretreated poplar were hydrolyzed using CTec2 (100 FPU/g dry material (DM)) in for 48 h with 0.02% NaN₃ for inhibition of bacterial growth. The enzymatic hydrolysis was repeated two times under the same conditions with fresh CTec2 to thoroughly remove hemicellulose and cellulose compounds. And then CELs were purified by protease. The obtained enzymatic hydrolysis residuals were washed until no glucose was released. Further purification was carried out using saturated NaCl (1:1) and acidic water (pH 2.5) with HCl. Then, CELs were washed and freeze-dried. CELs samples were prepared from the ball-milled and AC-pretreated poplar (5% AC, 160–180 °C, and 30 min) by cellulase hydrolysis, which were labeled as BM-CEL and AC-CELs, respectively. The AC-CELs from the poplar pretreated by AC at 160 °C, 170 °C and 180 °C were labeled as 160-CEL, 170-CEL and 180-CEL, respectively.

Enzyme adsorption isotherm

The enzyme adsorption onto poplar, CELs, and Avicel samples were performed at 4 °C for 1 h at 2% substrate loading. The CELs loading of adsorption experiment was 100 mg/g Avicel. The various protein concentrations of CTec2 were added in a range of 10–400 mg protein of enzyme/g DM. After 1 h, the supernatant was separated by centrifugation and collected for the analysis of non-adsorbed protein. The adsorbed protein of enzymatic was determined by the lost protein in solution. The adsorption parameters are calculated by Langmuir adsorption isotherms.¹¹

Enzymatic hydrolysis

The method of enzymatic hydrolysis referred to the method in previous report.² The 2% DM content of substrate was hydrolyzed at 50 °C and 200 rpm. The cellulases (CTec2 or Celluclast 1.5L) loading in hydrolysis was 20 FPU/g DM. The β -G loading was 500 nakt/g DM. Furthermore, 2 g/L CELs was loaded in the hydrolysis of Avicel. The statistical analyses were performed by Microsoft Excel 2010. The data of enzymatic hydrolysis was evaluated for significance

Structure characterization

The surface hydrophobicity of ball-milled poplar, AC-pretreated poplar, and CELs samples were according to the method described by Müller et al.³⁰ Two replications were measured for each sample.

The Zeta potentials of CELs samples were determined by a Zetasizer (Malvern Instruments Ltd, UK). Three replications were measured for each sample.

The XPS analyses were carried out using a Physical Electronics PHI Quantum 2000 ESCA instrument (Physical Electronics, USA) equipped with a monochromatic AlK α

X-ray source. The surface coverage by lignin (S_{lig}) were calculated by the oxygen to carbon ratios, according to the Eqs reported by Mou et al.³¹

XRD was used to measure the cellulose crystallinity index (CI) by Rigaku D/max-3C generator (Rigaku Corporation, Japan). The CI was calculated by the formula via reported previously.³²

FTIR analysis was performed on a FTIR spectrophotometer (Thermo Fisher, Nicolet iS10, USA).

SEM (Hitachi, S-4800 SEM, Japan) was used to analyze the surface morphology of the poplar and CELs samples. The representative images of lignin samples were acquired with a 10 kV accelerating voltage.

Py-GCMS analysis were carried out in a micro-furnace single shot pyrolyzer (Frontier Laboratories, EGA/PY-3030D, Japan) connected to a GC/MS (Shimadzu, QP-2010 Ultra, Japan).

The 2D HSQC NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer (Bruker Instruments, Switzerland) with a 5 mm Broadband Observe probe. The CELs (50 mg) were dissolved in deuterated dimethyl sulfoxide (0.5 mL). Spectra were acquired with a sweep width of 190 ppm in F1 (¹³C) with 256 data points and 11 ppm in F2 (¹H) with 2048 data points. The experiments were run for 128 scans with a relaxation delay of 1 s.

Analysis methods

The chemical compositions of samples were determined by the National Renewable Energy Laboratory analytical procedure.³³

The methods of monosaccharides analysis were consistent with those reported in previous literatures.²

Calculations

The glucose, xylose and XOS (DP 2 to 6) yields were calculated by these following formulas:

$$\text{XOS yield (\%)} = \frac{\text{XOS in acid hydrolysate}}{\text{Initial xylan content in the substrate}} \times 100 \quad (1)$$

$$\text{Glucose yield (\%)} = \frac{\text{Glucose in hydrolysate} \times 0.9}{\text{Initial glucan content in substrates}} \times 100 \quad (2)$$

$$\text{Xylose yield (\%)} = \frac{\text{Xylose in hydrolysate} \times 0.88}{\text{Initial xylan content in the substrate}} \times 100 \quad (3)$$

Results and discussion

Production of XOS by AC hydrolysis

Table 1 Chemical compositions of raw, ball-milled and AC-pretreated poplar. AC hydrolysis was performed with ball-milled poplar by 5% AC (w/v) at 160–180 °C for 30 min.

AC hydrolysis temperature (°C)	Glucan (%)	Xylan (%)	Acid-insoluble lignin (%)	Acid-soluble lignin (%)	Solid recovery (%)	Removal (%)		
						Glucan	Xylan	Lignin
Raw	41.7±1.4	16.8±0.1	24.7±1.9	4.8±0.1	-	-	-	-
BM	43.5±0.3	15.6±0.1	26.2±0.3	4.2±0.0	-	-	-	-
160	52.7±0.5	5.3±0.1	32.8±0.3	2.8±0.0	66.2	16.3	79.0	20.0
170	58.0±1.9	3.0±0.2	34.8±0.1	2.2±0.0	59.7	16.8	89.5	25.2
180	59.0±0.3	1.6±0.1	36.4±0.1	1.9±0.0	57.0	19.2	94.7	26.0

The compositions of the non-pretreated and ball-milled poplar were very similar (Table 1). After hydrolysis with 5% AC at 160 °C, 170 °C, and 180 °C for 30 min, the xylan contents of poplar decreased from 15.6% to 5.3%, 3.0%, and 1.6%, respectively, which was due to the formation of XOS, xylose, etc, from xylan in poplar.^{2,34} The high removal of xylan fraction (79.0%–94.7%) resulted in the increase of the relative contents of glucan and lignin. Finally, high total lignin contents (35.7%–38.3%) were

noticed in AC-pretreated poplar samples.

The 5% AC hydrolysis at 170 °C gave a XOS yield of 18.1% from ball-milled poplar, which was lower than the yield (39.8%) from raw poplar (Table 2).²

Meanwhile, under such conditions, the XOS yields of ball-milled poplar were lower than that from raw poplar, which might be due to part of xylan degrades into other by-products, such as furfural.³⁴ The high efficiency of conversion to XOS resulted in the increase of lignin content, which could affect the subsequent hydrolysis by cellulase.²⁻⁴

Table 2 Xylose and XOS (DP 2–6) yields from raw and ball-milled poplar by AC hydrolysis. The XOS yield was based on the xylan content in raw poplar.

Substrates	AC hydrolysis condition			Xylose (%)	Xylobiose (%)	Xylotriose (%)	Xylotetraose (%)	Xylopentaose (%)	Xylohexaose (%)	XOS yield (% DP 2–6)
	AC concentration (%)	Time (min)	Temperature (°C)							
Ball-milled poplar	5	30	160	4.0	2.7	2.4	3.4	2.1	2.3	12.9
Ball-milled poplar	5	30	170	17.4	5.7	3.6	5.4	1.8	1.5	18.1
Ball-milled poplar	5	30	180	21.8	4.9	2.1	4.5	0.6	0.5	12.7
Non-pretreated poplar	5	30	170	23.4	10.4	8.2	11.3	5.4	4.7	39.8

The data of XOS (DP 2–6) yields from non-pretreated poplar was the data reported by Wen et al.²

Characterization of acetic acid-pretreated poplar

After BM, the CI of the poplar was greatly decreased from 48.4% to 6.1% (Table 3) and such clear CI decrease in cellulose by BM has been reported in other reseach.³⁵ The reduction of CI indicated that crystalline form of cellulose in poplar was destroyed as the chemical compositions of poplar had no big change before and after BM of poplar (Table 1).³⁵ The destruction of cellulose would be helpful for the subsequent preparation of CEL.³⁶ With AC hydrolysis temperature increased from 160 °C to 180 °C, the CI of poplar improved from 42.5% to 51.5% (Table 3), which was attributed to the removal of hemicellulose in poplar.^{12, 37}

The O/C ratios of poplar samples were provided (Table 3). Low O/C ratio means more lignin is covered on the surface of samples.¹² The O/C ratio of ball-milled poplar (0.67) was closed to that of non-pretreated poplar, indicating that BM did not change the surface lignin content of poplar.¹² After AC hydrolysis, the O/C ratio of AC-pretreated poplar decreased to 0.59–0.57. Meanwhile, the surface lignin of poplar improved from 31.2% to 47.6%–52.1% after AC hydrolysis. The increase of C1 peak intensity data also confirmed that AC hydrolysis increased the surface lignin of poplar.² These data could be attributed to the xylan removal and the redeposition of lignin on the surface of poplar after AC hydrolysis,¹² which was consistent with our previous conclusion.²

Compared with raw poplar, ball-milled poplar showed higher hydrophobicity (1.3 g/L). The increase of hydrophobicity might be related to the decrease of particle size of ball-milled poplar.^{35, 38} The AC-pretreated poplar samples showed higher hydrophobicities (0.6–0.9 L/g) than raw poplar. It was confirmed that lignin had more hydrophobicity than glucan.³⁷ Hence, this result could be due to the AC-pretreated poplar had higher lignin content.^{2, 37}

Table 3 XPS, CI, and hydrophobicity analysis of ball-milled and AC-pretreated (5% AC, 160–180 °C, 30 min) poplar.

AC hydrolysis temperature (°C)	O/C	C1 (%)	C2 (%)	C3 (%)	S _{lig} (%)	S _{carb} (%)	CI (%)	Hydrophobicity (L/g)
Raw	0.68±0.1	53.9±0.5	34.8±0.2	11.4±0.2	30.0±0.1	70.0±0.1	48.4±0.9	0.4±0.0
BM	0.67±0.0	20.9±0.2	64.7±0.5	14.4±0.9	31.2±0.0	68.8±0.1	6.1±0.2	1.3±0.0
160	0.59±0.0	29.7±0.4	57.1±0.7	13.3±0.2	47.6±0.1	52.4±0.0	42.5±0.7	0.9±0.0
170	0.59±0.1	31.0±0.1	56.7±0.1	12.3±0.1	48.6±0.1	51.4±0.0	43.7±0.6	0.7±0.0
180	0.57±0.1	35.9±0.8	53.8±0.1	10.3±0.4	52.1±0.1	47.9±0.1	51.5±0.8	0.6±0.0

The S_{lig} and S_{carb} are the surface lignin and carbohydrate contents, respectively.

The surfaces images of poplar were characterized by SEM. Some spherical droplets were appeared on the surfaces of the poplar samples after AC hydrolysis at 170 °C and 180 °C (Supporting Information: Fig. S1). This phenomenon was consistent with the lignin droplets in AC hydrolysis of raw poplar (without BM) in prior report.² This result confirmed that AC hydrolysis improved the surface lignin of poplar (Table 3). Lignin droplets could limit the hydrolysis of AC-pretreated poplar by its steric hindrance.³⁹

Enzymatic hydrolysis of acetic acid pretreated poplar

Celluclast 1.5L is produced by Novozymes (Bagsværd, Denmark), which obtained from *Trichoderma reesei*. β -glucosidase (β -G) always used with Celluclast 1.5 L in enzymatic hydrolysis to relieve the inhibition caused by cellobiose. When Celluclast 1.5 L and β -G was used to hydrolyze the poplar samples, the BM significantly ($p < 0.0001$) improved glucose yield from 2.6% to 70.3% (Fig. 1). It was due to that BM destroyed the physical barrier of biomass, especially the crystalline region of cellulose,³⁸ which greatly improved the access of enzymes to cellulose and enhanced hydrolysis efficiency.³⁵ It was found that hydrolysis yields of ball-milled and AC-pretreated poplar by Celluclast 1.5L and β -G were lower than those of

ball-milled poplar (Figs. 1a), which could be due to the AC hydrolysis increased the lignin content in poplar by the removal of xylan.

Cellic CTec2 also is a commercial *Trichoderma reesei* cellulase formulation, which contains plenty of β -G.⁴⁰ After poplar samples were hydrolyzed by CTec2, the glucose yields of ball-milled and AC-pretreated poplar were 83.7%–86.8% (Figs. 1b). Importantly, the hydrolysis yields of AC-pretreated poplar by CTec2 were higher than that by Celluclast 1.5L and β -G.

Compared with CTec2, the activity of the cellulase mixture (Celluclast 1.5L/ β -G) is more easily inhibited by the inhibitors in the hydrolysate.⁴¹ After enzymatic hydrolysis for 72 h, the free cellulase activities (filter paper activity) retained in the hydrolysates of poplar by CTec2 were 35.2%–56.4%, which were higher than those by Celluclast 1.5L and β -G (Supporting Information: Fig. S2). Therefore, the higher free cellulase activities in the hydrolysate could be a reason that higher glucose yields can be obtained from poplar by CTec2. Furthermore, it has been confirmed that the LPMO enhances the hydrolysis of cellulose.²⁸ In the enzymatic hydrolysis of poplar by CTec2, the initial LPMO activity was $0.28 \mu\text{mol L}^{-1} \text{min}^{-1}$. However, the LPMO activity in the hydrolysis of poplar by Celluclast 1.5L/ β -G was below the limit of detection. This could be a reason that the glucose yields of AC-pretreated poplar by CTec2 were higher than that by Celluclast 1.5L and β -G.

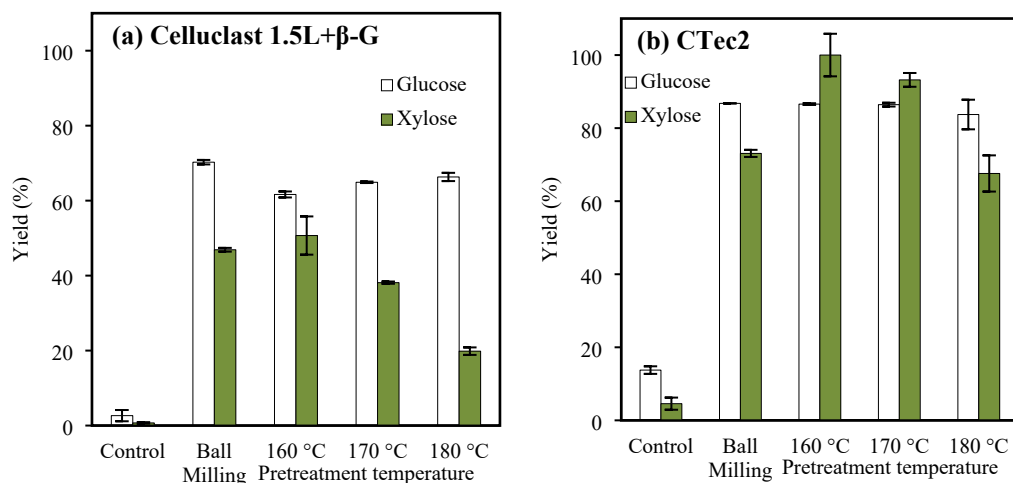


Fig. 1 Enzymatic hydrolysis of 2% ball-milled and AC-pretreated (5% AC, 160–180 °C, 30 min) poplar by different cellulases at 50 °C and pH 5.0 for 72 h. **(a)** Hydrolysis of poplar by Celluclast 1.5L (20 FPU/g DM) and β-G (500 nakt/g DM), and **(b)** Hydrolysis of poplar by CTec2 (20 FPU/g DM). Data indicated the mean ± SD of triplicates. All the BM and AC-pretred poplar data in Fig. 1 showed $p < 0.0001$ vs Control.

Cellulase adsorption on acetic acid-pretreated poplar

The hydrolysis yields of AC-pretreated poplar by CTec2 were higher than those by Celluclast 1.5L. Hence, it is necessary to further explore the adsorption of AC-pretreated poplar by CTec2 for the efficient hydrolysis. Adsorption isotherms of cellulase on BM and AC-pretreated samples were provided (Table 4 and Fig. 2). The adsorption of cellulase on ball-milled poplar showed the highest maximum adsorption capacity (301.8 mg/g), which was higher than the AC-pretreated poplar. This data might be owed to higher hydrophobicity of ball-milled poplar could exhibit a stronger cellulases adsorption capacity.^{2, 42} When the AC hydrolysis temperature increased from 160 °C to 180 °C, the maximum adsorption capacity of AC-pretreated poplar decreased from 211.1 mg/g to 74.3 mg/g. This data could be due to AC hydrolysis increased the surface lignin of poplar and led the adsorption of cellulase on poplar decreased because cellulase is more easily adsorb on cellulose.¹⁶ This phenomenon was consistent with the previously reported that lower the lignin content in

lignocellulosic substrates had higher the adsorption ability.⁴³ The cellulase adsorption on AC-pretreated poplar confirmed that decrease the hydrophobicity of poplar could reduce cellulase adsorption on substrate.^{12, 16}

Table 4 Adsorption parameters of cellulase (CTec2) on ball-milled and AC-pretreated (5% AC, 160–180 °C, 30 min) poplar.

AC hydrolysis temperature (°C)	$P_{ads,m}$ (mg/g)	K_p (mL/mg)	A(mL/g)	R^2
BM	301.8±1.8	0.2±0.0	65.7±1.0	0.94
160	211.1±1.2	0.4±0.0	81.0±1.3	0.99
170	86.3±0.7	0.8±0.0	72.3±0.7	0.99
180	74.3±0.2	1.1±0.1	80.2±1.1	0.99

$P_{ads, m}$ is the maximum amount of adsorbed CTec2 (mg CTec2/g solid).

K_p is the adsorption equilibrium constant (ml/mg CTec2).

The adsorption strength of enzyme (A) is calculated from $P_{ads, m}$ and K_p ($A = P_{ads, m} \times K_p$).

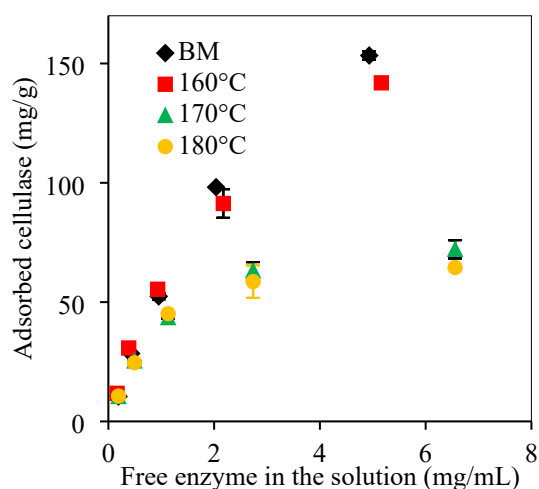


Fig. 2 The Langmuir adsorption isotherms of cellulases on ball-milled and AC-pretreated poplar.

The experiment was carried out in 2% poplar with CTec2 at 4 °C for 1 h.

Chemical compositions of cellulolytic enzyme lignin

Only 4.7%–7.4% glucan and 0.9%–5.3% xylan were found in CELs samples

(Table 5). The total lignin contents of AC-CELs were higher than 76.0%. The data showed that most of carbohydrates in ball-milled poplar were removed by enzymatic

hydrolysis regardless of poplar was pretreated by AC. Some glucan and xylan contents still remained in CELs, which was consistent with other reports.^{6, 21} After hydrolysis by cellulase, the total lignin contents increased from 29.5%–38.3% to 69.7%–85.7% (CELs) (Tables 1 and 5). The data suggested that the carbohydrates in CELs could not be completely removed by cellulase due to the physical blockage of the access of cellulase to the cellulose in poplar.⁶ Furthermore, lignin-carbohydrate complex cannot be hydrolyzed by cellulase, which also could be a reason that carbohydrates retained in CELs.³⁸

Table 5 Chemical compositions of CELs.

CEL	Glucan (%)	Xylan (%)	Acid-insoluble lignin (%)	Acid-soluble lignin (%)
BM-CEL	6.5±0.2	5.3±0.4	64.5±0.1	5.2±0.2
160-CEL	7.4±0.5	2.9±0.3	72.8±0.0	4.1±0.1
170-CEL	4.7±0.0	0.9±0.0	79.3±0.2	2.9±0.1
180-CEL	5.1±0.5	1.7±0.4	83.2±0.5	2.5±0.1

The sample of BM-CEL was prepared by enzymatic hydrolysis from the ball-milled poplar. The samples of 160-CEL, 170-CEL and 180-CEL were prepared by enzymatic hydrolysis from the AC-pretreated poplar.

Characterization of cellulolytic enzyme lignin

The O/C ratios of CELs samples were 0.39–0.41 (Table 6), which were close to the theoretical O/C ratio 0.33 of pure lignin.¹² S_{lig} of AC-CELs samples were 84.0%–88.0%, which were close to the 86.0% of BM-CEL. This data demonstrated that the S_{lig} contents of CELs were nearly intact by AC hydrolysis. The C1, C2, and C3 peak intensities of the BM-CEL, 160-CEL and 170-CEL were similar. At 180 °C, the C1 and C2 peak intensities showed that the C–O content of CELs surfaces decreased to 44.2% and the C–C content increased to 45.3%.³⁷

Table 6 XPS, hydrophobicity, and zeta potential of CELs.

CEL	O/C	C1 (%)	C2 (%)	C3 (%)	S _{lig} (%)	S _{carb} (%)	Hydrophobicity (L/g)	Zeta potential (mV)
BM-CEL	0.40±0.0	40.3±1.1	48.4±0.5	11.3±0.1	86.0±0.0	14.0±0.0	4.6±0.6	-15.1±0.7
160-CEL	0.41±0.0	40.9±0.7	50.1±0.7	9.0±0.5	84.0±0.0	16.0±0.0	2.5±0.3	-12.9±0.3
170-CEL	0.40±0.0	39.3±0.5	51.4±1.3	9.3±0.2	86.0±0.0	14.0±0.0	2.3±0.2	-14.8±0.6
180-CEL	0.39±0.0	45.3±0.3	44.2±0.4	10.6±0.7	88.0±0.0	12.0±0.0	1.8±0.1	-16.1±0.2

Lignin structures of CELs samples were analyzed by FT-IR spectra and the corresponding assignments of bonds were assigned. After AC hydrolysis, the peak represented acetyl group in hemicelluloses was diminished at 1738 cm⁻¹ (Supporting Information: Fig. S3). The decrease of acetyl group could improve the adsorption of cellulase.^{2,3} The decrease of peak at 1660 cm⁻¹ could be due to the removal of *p*-hydroxybenzoate.⁴⁴ The 1124 cm⁻¹ was ascribed to aromatic C–H deformation of syringyl unit (S) (Supporting Information: Table S1). The signal at 1327 cm⁻¹ was ascribed to C–O stretching vibration of syringyl (S), which slightly weakened with the severity of hydrolysis at 160 °C and 180 °C. The peaks for C–O stretching vibration of guaiacyl (G) rings were assigned at 1272⁻¹ and 1228 cm⁻¹. Besides, the formation of hydrogen bonding affects the signal of –OH groups.^{45,46} The peak related to –OH of BM-CEL was at assigned 3407 cm⁻¹. After AC hydrolysis, the peaks for –OH of all AC-CELs shifted to 3420 cm⁻¹ (Supporting Information: Table S2), which suggested that AC hydrolysis could destroy the hydrogen bond interactions of CELs. The fracture of hydrogen bond had a negative effect on adsorption between cellulases and CELs.⁴⁶

The thermal stability of CELs samples were analyzed by TG and their first derivation thermo gravimetric (DTG) that were presented (Supporting Information: Fig. S4, Table S3). The degradation between 150–400 °C was mainly attributed to the fragmentation of aryl ether bond linkages.⁴⁷ At 400 °C, the high temperature result in

the saturation of aromatic rings, fracture of C-C bond and release of H₂O, CO₂, and CO.⁴⁸ Methoxy cracking at about 400–600 °C and releases methane.⁴⁹ Above 500 °C, the weight loss changed slowly and decomposition rate became stability, which could be due to the decomposition of aromatic rings.⁴⁸ At 600 °C, the weights of char residues of AC-CELs were higher than that of BM-CEL. This result might be caused by the formation of condensed structures.²²

Table 6 showed that zeta potentials of all CELs were negative, which was attributed to those electro negative groups (–OH and –COOH).¹² The value of potential of BM-CEL was 15.1 mV, which was higher than 12.9 mV of 160-CEL. It meant that AC hydrolysis at 160 °C decreased the electrostatic repulsion between lignin and enzymes.⁴² With the hydrolysis temperature increased to 180 °C, the value of zeta potential increased to 16.1 mV, indicating the higher hydrolysis temperature resulted in stronger electrostatic repulsion and negative effect on non-productive adsorption between cellulases and CELs.

Hydrophobicity is believed to be another important parameter affecting the adsorption of cellulases on CEL.⁴² The hydrophobicities of CELs were 1.8–4.6 L/g (Table 6), which were much higher than the hydrophobicities of ball-milled and AC-pretreated poplar samples. This result could be due to the enzymatic hydrolysis increased the lignin content of CELs.¹² Normally, acid pretreatment could decrease the hydrophilic group and increase the hydrophobicity of lignin.^{50, 51} Interestingly, in this work, the hydrophobicities of AC-CELs (1.8–2.5 L/g) were lower than that of BM-CEL (4.6 L/g), which could be due to AC hydrolysis can increase the hydrophilic group (such as –OH and –COOH) of lignin in substrate.⁵²

Compared with the BM-CEL, the SEM images of AC-CELs showed more serious surface damage. With the AC hydrolysis temperature increased, the size of AC-CELs

particles decreased. This phenomenon indicated that AC hydrolysis affected the size of AC-CELs.

The pyrolysis performance of CELs samples were analyzed by Py-GC/MS. Three major volatile chemical compounds (phenols, phenol derivatives and aromatic hydrocarbons) were pyrolyzed and identified (Supporting Information: Table S4). The chemical compounds included higher content of syringyl(S) and guaiacyl(G) while lower content of *p*-hydroxyphenyl (H) unit, which were similar to previous research.⁵³ The abundance of the H-, G-, S-lignin units and S/G ratios was shown in Table 7. Compared with BM-CEL, the AC-CELs had higher contents of S-lignin while lower content of G-lignin, which resulted in that AC-CELs samples had higher S/G ratio (2.09–2.56) than BM-CEL (1.2).⁵⁴ With AC hydrolysis temperature improved from 160 °C to 170 °C, the S/G ratio increased from 2.09 to 2.56, which might be due to G unit was degraded during AC hydrolysis.^{6, 44, 51} Because the lignin of G unit was more easily to degrade by AC hydrolysis to form soluble chemicals.^{51, 55} Decrease in G unit led an increased contents of S units, as reflected from the 2D-HSQC NMR spectra (Supporting Information: Fig. S5), in which the corresponding signal at δ_C/δ_H 104.4/6.70 ($S_{2,6}$) became intense. At 180 °C, the S/G ratio was slightly decreased to 2.50, which probably due to the presence of demethoxylation in the S-lignin.¹⁵

To further evaluate the detailed structural information of CELs, the main structural signal of CELs samples existed in side-chain region (δ_C/δ_H 50-90/2.5-6.0) and aromatic region (δ_C/δ_H 100-140/6.0-8.0) in 2D-HSQC NMR spectra were determined (Supporting Information: Fig. S5). In the side-chain region, three major interunit linkages of β -aryl ether (β -O-4, A), resinols (β - β , B) and phenylcoumaran (β -5, C) were identified by cross peaks at δ_C/δ_H 72.4/4.88 (A_α), 84.5/4.30 ($A_{\beta(G)}$), 86.5/4.15 ($A_{\beta(S)}$), 85.6/4.66(B_α), 54.2/3.07 (B_β), 71.5/3.83 (B_γ), 87.0/5.43 (C_α) and 62.0/4.11

(C_γ), respectively. As shown in Table 7, β-O-4 linkage was the major linkage of lignin samples ranged from 26.8/100 Ar to 67.3/100 Ar. When the hydrolysis temperature increased from 160 °C to 180 °C, the content of β-O-4 linkage decreased from 46.9/100Ar to 26.8/100Ar. This implied that the breakage of β-O-4 linkage was susceptible to treatment temperature.⁵⁵ After AC hydrolysis, the C-C linkage of β-β was increased to 1.5/100Ar–3.7/100Ar. It might be due to the depolymerization and polymerization of lignin by AC hydrolysis.⁵⁶ In the aromatic region, subunits (S and G) and p-hydroxybenzoate (PB) of CELs samples were observed by their cross peaks at δ_C/δ_H 104.4/6.70 (S_{2,6}), 111.6/7.01 (G₂), 115.7/6.80 (G₅), 119.4/6.83 (G₆) and 131.8/7.69 (PB), respectively. The PB content gradually decreased as the temperature increased from 160 °C to 180 °C, which was consistent with previous report.⁵¹ It showed that the conversion or degradation of PB unit occurred at higher temperatures. The data of S/G ratio determined by 2D-HSQC NMR spectra were the same as those from Py-GC/MS. The S/G ratio of AC-CELs (2.23-5.97) was higher than that of BM-CEL (1.60) and the 170-CEL had the highest S/G ratio (5.96), confirming that the AC hydrolysis improved the S/G ratio of CELs. This result also was corroborated in the lignin isolated from AC- pretreated sugarcane bagasse.⁵¹

Table 7 2D-HSQC NMR and py-GC/MS analysis of CELs samples.

CEL	β-O-4 ^a	β-β ^a	β-5 ^a	S/G ^b	S/G ^c
BM-CEL	67.3	1.1	3.8	1.60	1.20
160-CEL	46.9	1.5	4.5	2.23	2.09
170-CEL	35.0	2.5	4.7	5.96	2.56
180-CEL	26.8	3.7	5.1	3.77	2.50

^aResults expressed per 100 Ar on the basis of quantitative 2D-HSQC NMR spectra.

^bData was determined by 2D-HSQC NMR.

^c Data was determined by py-GC/MS.

Adsorption of cellulases on cellulolytic enzyme lignin

Non-productive adsorption between lignin and cellulase is a main factor for lignin inhibition.^{5, 7-9} Fig. 3 described the Langmuir adsorption isotherms of cellulases on different lignin samples. The maximum adsorption capacity of the BM-CEL was 80.9 mg/g (Table 8). After AC hydrolysis, the maximum adsorption capacity of AC-CELs increased to 112.4 mg/g (160-CEL), 102.0 mg/g (170-CEL), and 152.5 mg/g (180-CEL), respectively. This trend was consistent with the result in previous reports that acid pretreatment can increase the maximum adsorption capacity of lignin.⁵⁰ The 180-CEL exhibited the highest maximum adsorption capacity, which indicated lignin in 180 °C and 5% AC-pretreated poplar might have the strongest non-productive adsorption.²¹

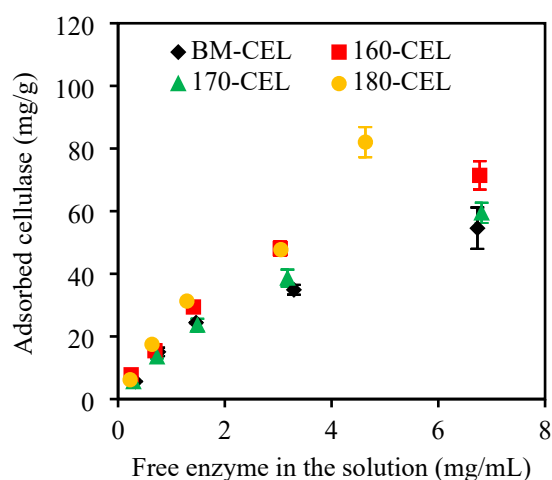


Fig.3 The Langmuir adsorption isotherms of cellulases on CELs. The experiments were performed in 2% CELs with CTec2 at 4 °C for 1 h.

Table 8 Adsorption parameters of cellulases (CTec2) on CELs.

CEL	$P_{ads,m}$ (mg/g)	K_p (mL/mg)	A (mL/g)	R^2
BM-CEL	80.9±0.8	0.3±0.0	22.4±0.8	0.97
160-CEL	112.4±1.7	0.3±0.0	28.9±0.7	0.98
170-CEL	102.0±0.5	0.2±0.0	20.4±0.7	0.99
180-CEL	152.5±1.5	0.2±0.0	26.9±0.9	0.94

Effect of cellulolytic enzyme lignin on cellulose hydrolysis

After the addition of CELs in hydrolysis system, the glucose yield of Avicel by Celluclast 1.5L and β -G significantly ($p < 0.001$) decreased from 53.3% to 43.0%–44.8% (Fig. 4a). The results indicated that both BM-CEL and AC-CELs could inhibit the hydrolysis of Avicel by Celluclast 1.5L and β -G. Meanwhile, after the addition of CELs in enzymatic hydrolysis, the retained endoglucanase, cellobiohydrolase, β -glucosidase, and filter paper activities in the enzymatic hydrolysate were decreased (Fig. 5a). Those data showed that the CELs inhibited the cellulase activities of Celluclast 1.5L and β -G. Previously, inhibitory effect of lignin from acid-pretreated biomass on Celluclast 1.5L and β -G has been reported,^{21, 23} which was consistent with the result here.

The glucose yield of Avicel by CTec2 was 46.3%, which was lower than 48.2%–54.6% of hydrolysis after addition of CELs (Fig. 4b). This positive effect of lignin has been observed in the hydrolysis of corn stover.^{57, 58} In a previous report, lignin isolated by γ -valerolactone from corn stover enhances the hydrolysis of Avicel.⁵⁷ Furthermore, another report showed the carboxylated and quaternized lignin can increase the β -glucosidase activity, which improves the hydrolysis yield of corn stover.⁵⁸

Lignin improves the activity of lytic polysaccharide monooxygenases, which is helpful to the enzymatic hydrolysis.²⁸ In hydrolysis process, glucose can be oxidized to gluconic acid by the LPMOs contained in CTec2.²⁸ Therefore, the glucose oxidation is used to reflect the LPMO activity.²⁸ After CELs were added in enzymatic hydrolysis of Avicel, the glucose oxidation was increased from 0.1% to 0.4%–0.5% (Fig. 6). Those data indicated that the LPMOs activity in CTec2 could be improved by CELs.²⁸ Meanwhile, after enzymatic hydrolysis for 0.5 h, the LPMO activity in

hydrolysis of Avicel with CELs were increased to 104.5%–114.4%, which were higher than (97.5%) without CELs (Fig. 7). This result also confirmed that adding CELs in enzymatic hydrolysis could improve the LPMO activity in CTec2. This might be the reason that CELs improved the hydrolysis of Avicel by CTec2.

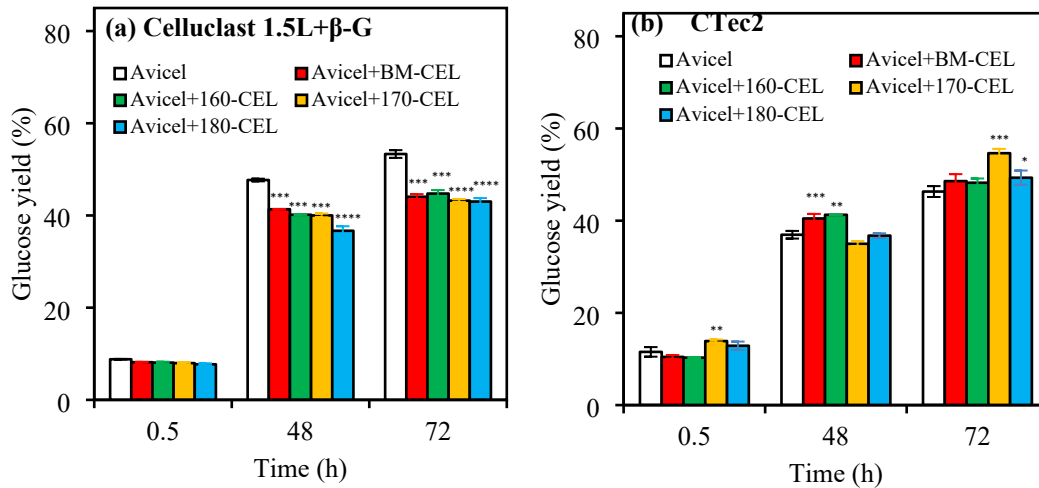


Fig. 4 Effects of CELs (100 mg/g DM) on hydrolysis of 2% Avicel by different cellulases at 50 °C and pH 5.0 for 72 h. **(a)** Hydrolysis of Avicel by Celluclast 1.5L (20 FPU/g DM) and β-G (500 nakt/g DM). **(b)** Hydrolysis of Avicel by CTec2 (20 FPU/g DM). Data indicated the mean ± SD of triplicates. * p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs Avicel.

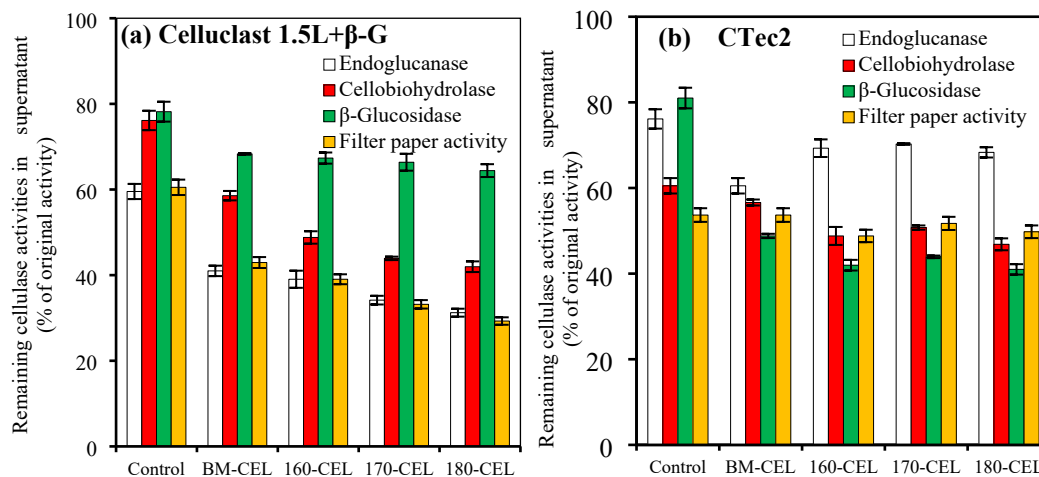


Fig. 5 Effects of CELs (100 mg/g DM) on the remaining cellulase activities (endoglucanase, cellobiohydrolase, β-glucosidase, and filter paper activity) in the enzymatic hydrolysis of 2% Avicel by different cellulases at 50 °C and pH 5.0 for 72 h. **(a)** Hydrolysis of Avicel by Celluclast

1.5L (20 FPU/g DM) and β -G (500 nakt/g DM). **(b)** Hydrolysis of Avicel by CTec2 (20 FPU/g DM).

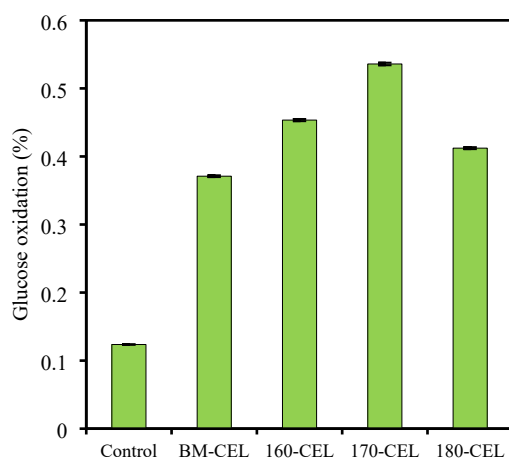


Fig. 6 Glucose oxidation in the hydrolysis of 2% Avicel by CTec2 with CELs (100 mg/g DM) at 50 °C and pH 5.0 for 72 h.

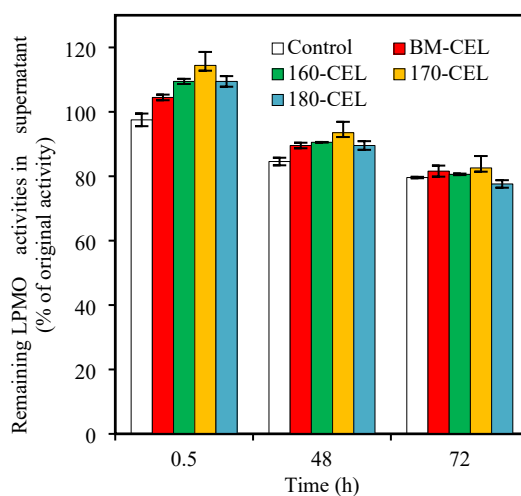


Fig. 7 Effects of CELs (100 mg/g DM) on the remaining LPMO activity in the hydrolysis of 2% Avicel by CTec2 (20 FPU/g DM) at 50 °C and pH 5.0 for 72 h. The initial LPMO activity in enzymatic hydrolysis was $0.28 \mu\text{mol L}^{-1}\text{min}^{-1}$.

Compared with Celluclast 1.5L, the CTec2 showed higher retention of filter paper activity after enzymatic hydrolysis with CELs (Fig. 5). This data could be a reason that the glucose yields by CTec2 with CELs were higher than those by Celluclast 1.5L. After the addition of CELs in enzymatic hydrolysis, the free β -glucosidase

activity retained in the enzymatic hydrolysis by CTec2 was greatly decreased from 81.0% to 42.0%-48.8%, which showed lower β -glucosidase activity than that of Celluclast 1.5L and β -G. This result could be due to the β -glucosidase in CTec2 was more easily attached to lignin.⁴⁰

Many reports had shown that lignin demonstrates an adverse factor in the hydrolysis of poplar, which was different from the plant of gramineae and required lignin in poplar need to be removed in pretreatment.^{2, 3, 18} However, this work showed that the poplar after BM and AC hydrolysis had high enzymatic hydrolysis yields (more than 86%) by CTec2 with high lignin content (more than 30%). The results might show that the lignin could not inhibit the enzymatic hydrolysis of poplar by CTec2. BM can destroy the physical barrier of biomass, especially the crystalline region of cellulose,³⁸ which can greatly increase enzymatic hydrolysis of carbohydrate.³⁵ Hence, we speculate that the complex physical barrier might be the main causes affecting the hydrolysis of poplar.

Lignin in AC-pretreated poplar showed different inhibitory effects by two cellulase preparations (Celluclast 1.5L and CTec2). Hence, the inhibitory effects of CELs from AC-pretreated poplar on other cellulase preparations (such as CTec3) can be further evaluated. To achieve a high yield monosaccharide production, the efficient hydrolysis of AC-pretreated poplar needs to be further explored. Furthermore, development of utilization of the lignin in AC-pretreated poplar can amplify the economic benefits of AC hydrolysis.

Conclusions

AC hydrolysis gave a 39.8% XOS yield from raw poplar. Surface lignin of poplar was improved by AC hydrolysis. After AC hydrolysis at 170 °C, the hydrophobicity and zeta potential of CEL were decreased. AC hydrolysis cannot increase the

adsorption strengths of CTec2 on CELs. AC-CELs improved the LPMO activity in CTec2. After the addition of CELs in enzymatic hydrolysis, the free filter paper activity of Celluclast 1.5L and β -G retained in enzymatic hydrolysate was decreased from 60.5% to 29.3%–42.9%. The addition of CELs in enzymatic hydrolysis could not decrease the free filter paper activity of CTec2 retained in enzymatic hydrolysate. All CELs could enhance the hydrolysis of cellulose by CTec2 but exhibited inhibitory effects toward Celluclast 1.5L and β -G. Hence, this report demonstrated the inhibitory effects of CELs from AC-pretreated poplar and guides the development of AC hydrolysis for the production of XOS and monosaccharides from poplar.

Note

The authors declare that they have no known competing financial interests. All authors approved the manuscript.

Acknowledgments

This work was supported by the National Key R&D Program of China (No.2017YFD0601001) and the National Natural Science Foundation of China (No.31670598).

Supplementary material

Supplementary data for this work can be found online.

Supplementary data including FT-IR analysis, TG and DTG analysis, Py-GCMS analysis, SEM images, remaining filter paper activity in the enzymatic hydrolysis, and 2D-HSQC NMR spectra.

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