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Mild organosolv pretreatment of sugarcane bagasse with acetone/phenoxyethanol/water for enhanced sugar production†

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This study conducted mild organosolv pretreatment of sugarcane bagasse with acetone/phenoxyethanol/water (APW) solutions to improve sugar production in the subsequent enzymatic hydrolysis step. The Box–Behnken design was used to optimize pretreatment conditions based on lignin removal. Further examination of the data revealed that lignin removal was positively correlated (R^2 > 0.95) with the combined severity factor (CSF). Pretreatment under the optimal conditions (125 °C–120 min, 0.17 M H_2SO_4, liquid–solid ratio of 15) led to 98.1% lignin removal and 74.5% cellulose digestibility compared to a low digestibility of 9.3% with raw sugarcane bagasse (SCB). Moreover, the APW process showed effective fractionation of pine, corn stalk and bamboo, and lignin removal was over 90%. The recovered lignin was characterized by 2D-HSQC NMR and ^{31}P NMR, suggesting that the pretreatment resulted in breakage of β-O-4, total phenolic OH and H-units increased. However, the enzymatic hydrolysis efficiency (EHE) of samples with a lower lignin content (<4%) varied significantly. Correlations between various substrate-related factors of the pretreated SCB and EHE were analyzed to understand this observation. The results showed that the surface area of cellulose, lateral order index, CrI, and specific surface area were the predominant factors for enzymatic hydrolysis rather than lignin properties.

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Introduction

The concern about global warming urges the world to develop fuels and energies with zero- or low-carbon emissions. Renewable biofuels are one fuel option that could help deal with climate change issues.[1,2] Lignocellulosic biomass is the most abundant renewable terrestrial carbon source to produce biofuels, such as bioethanol. Lignocellulose is composed of cellulose, hemicellulose, and lignin, and the carbohydrate polymers in lignocellulose are utilized to produce biofuels.[3] A typical cellulosic ethanol production process comprises three key steps: biomass pretreatment, enzymatic hydrolysis of pretreated biomass to produce fermentable sugars, and sugar fermentation to produce ethanol. Pretreatment is a critical step that determines the sugar yield. *This is because* lignocellulosic biomass has a recalcitrant structure, which hinders the enzymatic hydrolysis of carbohydrate polymers from producing fermentable sugars.[4] Pretreatment improves the biomass porosity or specific surface area and/or removes lignin (and hemicellulose), and/or decreases the cellulose crystallinity. As a result, cellulose accessibility to cellulases is increased, leading to enhanced hydrolysis and sugar production.[5]

In the last two decades, various pretreatment methods have been developed, including dilute acid, alkali, ionic liquid, organosolv, deep eutectic solvents, steam explosion, liquid hot water, and combined treatment.[6–13] However, not all pretreatment methods can effectively fractionate biomass components, especially lignin, to maximize the value of lignocellulose. Of various pretreatments, organosolv pretreatment is attractive as it can fractionate lignocellulose into carbohydrate

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polymers and lignin with the selection of solvents and pretreatment conditions.[14,15]

Organic solvents such as alcohols, esters, phenol, and acetone have been employed in the treatment of biomass.[16–18] Islam *et al.* proposed an acidic pentanol–water biphasic system for the pretreatment of lignocellulose (*Acacia Confusa* wood) at 170 °C with H_2SO_4 as the aqueous phase, and over 70% of lignin was removed into the organic phase, leaving a solid residue rich in cellulose.[14] The mass transfer efficiency of monophasic systems is obviously higher than that of biphasic systems.[9] However, the biphasic system can effectively fractionate lignocellulose biomass, as hemicellulose was decomposed and then dissolved in the aqueous phase. Lignin is distributed in the organic phase with cellulose left as the residue.

In order to improve the selectivity for different major components of biomass, several ternary organic solvent systems have been developed. For example, a ternary system of acetone/butanol/ethanol was used to fractionate *Salix schwerinii* at 200 °C,[19] achieving 98.5% of hemicellulose removal and 58.2% delignification, while 53.8% xylose and 12.1% of glucose could be recovered from the liquor after pretreatment. NREL proposed a clean fractionation method using a ternary organosolv system composed of acetone/MIBK/H_2O to fractionate corn stover. It was found that 77.2% lignin and 74% hemicellulose from the corn stover could be removed using the acetone/MIBK/H_2O solvent system at 120 °C for 40 min while avoiding cross-contamination of biopolymers.[15,20] However, lower lignin removal limited its application. Recently, our research group developed a ternary solvent system composed of acetone, phenoxyethanol, and water (APW).[9] The APW system has the advantages of monophasic deconstruction and biphasic separation and can achieve effective biomass fractionation. With the APW system, *Amorpha* was effectively fractionated under the optimal conditions of 130 °C, 70 min, 0.15 M sulfuric acid, and 20 LSR (liquid–solid ratio), achieving 95.6% delignification and 98.39% hemicellulose removal, retaining 80.48% of cellulose in the solid fraction. Nonetheless, lignocellulosic biomass pretreated with APW is not widely studied, and its dynamic characteristics of delignification remain unclear.

This study applied APW pretreatment to fractionate sugarcane bagasse (SCB). The effects of temperature, cooking time, liquid–solid ratio, and sulfuric acid concentration were investigated using the Box–Behnken design (BBD). The correlation of lignin removal with a combined severity factor was studied. Then, the APW pretreatment performance of various lignocellulosic biomass (SCB, corn stalk, pine and bamboo) and the characteristics of isolated lignin were also investigated. Subsequently, the cellulose-rich residue was directly hydrolyzed by cellulase. Finally, the interactions between 9 substrate-related factors and enzymatic hydrolysis efficiency (EHE) were comprehensively characterized through correlation analysis to investigate the predominant factors for enzymatic hydrolysis when the lignin content in the residue is low.

Experimental

Materials

SCB was kindly provided by Guangxi Baiguitang Food Technology Co., Ltd, China. Pine, corn stalk and bamboo were collected from our test field in Guangzhou, China. The cellulose, hemicellulose and lignin contents of SCB, pine, corn stalk, and bamboo are shown in Table 1. Before testing, the SCB was air-dried for three days, ground, and screened through a 20–60 mesh, followed by drying at 60 °C in an oven until a constant weight was reached. All reagents in this study were analytical grade and used without any purification. Solid-state cellulase was purchased from Jade Bio-technology Co., Ltd, China, with a filter paper activity of 191.7 FPU per g. The milled-wood lignin (MWL) of SCB was obtained referring to the previous report.[21]

APW pretreatment

The pretreatment was performed in thick-walled pressure bottles. The oil bath was first heated to the target temperature. The substrate was then added to acidified acetone/phenoxyethanol/water mixture, with a solid–liquid ratio of 1 : 10, and heated at 110 °C for 80 min. After pretreatment, the mixture was filtered using a sand funnel (G3) and then the solid part was washed with ethanol and water until pH = 7. Lignin was precipitated from the organic phase with isopropyl ether and named APWL.

Optimize conditions

The Box–Behnken design (BBD) model was employed to optimize APW pretreatment.[22] The BBD was applied with four variables at three levels to study the optimal conditions of deconstruction and the effects of critical factors on lignin removal. Time (X_1, 30, 75, 120 min), temperature (X_2, 80, 105, 130 °C), H_2SO_4 concentration (X_3, 0.05, 0.175, 0.3 M), and liquid–solid ratio (X_4, 10, 15, 20) were selected as variables (Table S1†), and the removal of lignin was used as the response value. Twenty-nine preliminary groups were obtained. The pretreatment procedure was carried out as previously described by Chen *et al.*[9] To obtain mild reaction conditions and figure out an indicator for effective lignin removal in the APW system, the combined severity factor (CSF), which combined temperature, time, and pH into a single parameter R_0, was introduced to further optimize the pretreatment conditions.[23]

$$\mathrm{CSF} = \log R_0 - \mathrm{pH}$$

$$R_0 = t \times e^{\frac{T_r - T_b}{14.75}}$$

where t is the cooking time (min), T_r is the pretreatment temperature (°C), and T_b is the base temperature (100 °C).

Table 1 Chemical composition of raw biomass

Material	Cellulose/%	Hemicellulose/%	Lignin/%
Sugarcane bagasse	40.51	23.56	22.64
Corn stalk	33.50	20.04	15.72
Pine	43.42	18.32	28.55
Bamboo	41.83	19.47	21.17

Enzymatic hydrolysis

The pretreated SCB residues and raw SCB as enzymatic substrates were mixed with 4 ml of 0.05 M sodium acetate buffer (pH 4.8) to form solutions of 5% solid concentration. All enzymatic hydrolysis tests were performed at 50 °C for 72 h, with a cellulase loading of 20 FPU per g cellulose. The supernatants were sampled and centrifuged at 10 000 rpm for further sugar analysis. The enzymatic hydrolysis efficiency (EHE) was calculated as follows:

$$\mathrm{EHE}(\%) = \frac{\text{glucose mass} \times 0.9}{\text{cellulose mass}} \times 100$$

where cellulose mass is the weight of SCB cellulose, 0.9 is the dehydration coefficient of glucan being converted to glucose.

All the experiments were repeated three times.

Analytical methods

The chemical composition of pretreated and raw SCB was measured according to NREL/TP-510-42618. Cellobiose, glucose and xylose were detected at 50 °C by an HPLC system (Waters 2698) equipped with a sugar column (SH1001, Shodex). The flow rate of the mobile phase (5 mM sulfuric acid) was 0.5 ml min^{-1}.

Morphological features of the biomass before and after pretreatment were observed using a field emission SEM (S4800, Hitachi) (FESEM) at an accelerating voltage of 2.0 kV. The untreated and pretreated SCB were sprayed with gold before FESEM analysis.

The correlations between 9 substrate-related factors related to different pretreatment conditions and enzymatic hydrolysis were analyzed through statistical analysis by the correlation analysis method. The 9 substrate-related factors included cellulose content, surface area of cellulose (SAC), specific surface area (SA), crystallinity index (CrI), CrI/cellulose, O/C ratio, lateral order index (LOI), surface lignin content (SL) and lignin content.

The cellulose surface area (SAC) was estimated by staining with Congo Red. The measurement procedure was reported by Sipponen *et al.*[24] The Langmuir maximum absorption capacity (X_{mc}) was determined by fitting the Langmuir isotherm. SAC was calculated based on the following formula:

$$\mathrm{SAC} = \text{surface of cellulose}\left(\frac{m^2}{\text{g material}}\right) = 1.055 \times X_{\mathrm{mc}}$$

The specific surface area (SA) was determined by the Barrett–Emmett–Teller (BET) method.

X-ray diffraction (XRD) (PW3040/60, Philips, Holland) was used to detect cellulose crystallinity. The cellulose crystallinity index (CrI) was evaluated as follows:[25]

$$\mathrm{CrI} = \frac{I_{002} - I_{\mathrm{am}}}{I_{002}} \times 100$$

where I_{002} is the diffraction (002) plane intensity at around 22.5° and I_{am} represents the baseline intensity at around 18.4°.

CrI/cellulose was introduced to correlate CrI and cellulose content to analyze the actual changes in cellulose crystallinity.

The surface lignin content of SCB was determined by X-ray photoelectron spectroscopy (XPS, ESCALAB 250Xi) using the following equation:

$$\mathrm{SL} = [5 - 6 \times (\mathrm{O/C})]/[1.68 + 3.66 \times (\mathrm{O/C})],$$

where SL is the mol fraction of lignin on the substrate surface and O/C is the ratio of oxygen to carbon atoms determined by XPS.

Fourier transform infrared (FTIR) spectroscopic analyses of untreated and pretreated SCB were conducted to reveal changes in the functional groups using a Bruker Tensor 27 FTIR spectrometer. The lateral order index (LOI = A1430 cm^{-1}/A898 cm^{-1}) was applied to evaluate structural changes in cellulose.

The quantitative 2D HSQC NMR and ^{31}P NMR experiments were carried out according to a previous publication.[26,27]

Results and discussion

Optimization of APW pretreatment of SCB for lignin removal with BBD

The Box–Behnken design (BBD) is a multi-factor nonlinear experimental optimization method, which uses multiple quadratic regression equation to fit the functional relationship between various factors and response values, to evaluate the interaction among various factors, and then to determine the optimal experimental conditions. This design method has fewer test times and is a convenient application. After APW pretreatment, the main components in sugarcane bagasse were fractionated into cellulose-enriched solid residue, an aqueous stream, and an organic stream. Material balance was performed to assess the fractionation efficiency, and the results are shown in Table 2. The equation below describes the relationship between variables and lignin removal in coding units.

$$\begin{aligned}\text{Lignin removal} = {} & -546.5 + 1.00X_1 + 7.96X_2 + 575X_3 \\ & + 5.53X_4 - 0.00314X_1X_2 - 1.491X_1X_3 \\ & + 0.0086X_1X_4 - 2.05X_2X_3 - 0.0189X_2X_4 \\ & + 2.96X_3X_4 - 0.00185{X_1}^2 - 0.02778{X_2}^2 \\ & - 527{X_3}^2 - 0.161{X_4}^2\end{aligned}$$

where X_1, X_2, X_3 and X_4 are time (X_1), temperature (X_2), H_2SO_4 concentration (X_3) and liquid–solid ratio (X_4), respectively.

As shown in Table S2,† lignin removal was strongly affected by the temperature ($P < 0.0001$), sulfuric acid concentration ($P < 0.0001$), and reaction time ($P < 0.0001$), while the liquid–solid ratio ($P = 0.758 > 0.05$) had a lower impact. The value of R^2 in the model is 0.9575, indicating great agreement between the experimental value and the expected value. Statistical analysis confirmed that the proposed model is appropriate for the proof of data and offers a comprehensive overview of the relationship between variables and responses. The optimal process parameters for APW pretreatment are as follows: liquid–solid ratio of 15 : 1, 125 °C, 120 min, and 0.17 M H_2SO_4

Table 2 Box–Behnken designed APW treatment of SCB with the response values

No.	A: *T* (min)	B: Tem. (°C)	C: acid conc. (M)	D: LSR	Glucan retention (%)	Xylan removal (%)	Y: Lignin removal (%)
1	75	105	0.175	15	86.59	69.31	84.00
2	120	105	0.175	20	75.29	72.42	89.46
3	75	105	0.175	15	89.15	69.64	81.98
4	75	80	0.3	15	91.57	47.05	34.98
5	75	105	0.175	15	88.38	69.57	83.58
6	30	105	0.175	20	93.03	57.33	58.51
7	30	130	0.175	15	90.59	82.92	84.43
8	75	80	0.05	15	90.99	6.27	11.46
9	75	105	0.05	10	73.06	26.19	56.55
10	75	130	0.3	15	75.10	100.00	93.12
11	75	130	0.175	10	82.77	92.96	94.91
12	75	80	0.175	20	88.61	28.78	34.50
13	75	130	0.05	15	85.88	82.88	95.21
14	75	105	0.05	20	90.26	48.09	48.90
15	120	105	0.05	15	81.07	66.37	78.51
16	120	80	0.175	15	85.91	36.33	49.41
17	75	105	0.175	15	92.51	68.84	84.17
18	30	105	0.05	15	97.67	38.10	34.08
19	30	80	0.175	15	90.69	11.86	26.58
20	30	105	0.175	10	88.18	59.54	67.43
21	120	105	0.3	15	85.44	85.22	92.68
22	75	105	0.3	20	77.95	80.41	91.58
23	75	105	0.175	15	91.61	66.61	87.14
24	120	105	0.175	10	89.93	77.31	90.66
25	120	130	0.175	15	77.92	93.50	93.14
26	75	130	0.175	20	82.45	100.00	95.07
27	75	105	0.3	10	94.05	75.82	91.84
28	75	80	0.175	10	92.16	25.93	24.89
29	30	105	0.3	15	84.53	69.59	81.80

Table 3 Pretreatment efficiency based on BBD optimization (SCB-1, SCB-2) and CSF = ~1.86 (SCB-3, SCB-4, SCB-5)

Experiment	Tem. (°C)	Time (min)	Acid conc. (M)	LSR	CSF	Solid remaining(%)	Cellulose content (%)	Glucan retention(%)	Xylan removal (%)	Lignin content (%)	Lignin removal (%)
SCB-1	125	120	0.17	15	2.52	35.33	91.68	85.59	98.15	1.23	98.12
SCB-2	110	120	0.15	10	1.98	41.29	79.58	81.10	91.83	0.53	99.03
SCB-3	110	110	0.10	10	1.83	49.27	70.49	78.53	81.41	0.58	96.54
SCB-4	110	80	0.20	10	1.87	42.10	77.97	87.87	86.99	1.99	94.94
SCB-5	110	90	0.15	10	1.86	48.62	70.01	84.77	81.52	3.90	91.55

(reaching complete lignin removal) (SCB-1). The actual lignin removal of SCB pretreated under the optimal condition was 98.12% (Table 3), which was close to the quadratic model prediction, indicating that the model obtained by the surface response design was applicable to the prediction of lignin removal. According to the response surface methodology results, the liquid–solid ratio has little impact on lignin removal, so the solid–liquid ratio is selected as 1 : 10 to improve the pretreatment capacity. Based on the response surface regression equation, at a solid–liquid ratio of 1 : 10, the lowest pretreatment temperature was 110 °C (every 5 °C as an interval) with a target of over 90% lignin removal (shown in Fig. 1). The optimal conditions under this limit were 0.15 M acid for 120 min (SCB-2), which predicted a corresponding lignin removal of 91.51%. The actual lignin removal was 99.03%, slightly higher than the predicted value, indicating that the model obtained using the surface response design was appropriate.

Optimization of APW pretreatment of SCB for lignin removal using the combined severity factor

Taking lignin removal as the response value, the reaction time, reaction temperature, acid concentration and solid–liquid ratio were optimized according to the BBD experimental design. The conclusion was that the solid–liquid ratio was the only factor that had no significant influence on lignin removal. As cooking time, reaction temperature, and acid concentration considerably impact the fractionation efficiency, CSF combining cooking time, sulfuric acid concentration (pH), and temperature was introduced to assess the correlation between pretreatment severity and delignification. As shown in Fig. 2, lignin removal increases with CSF. Lignin removal can reach 90% when the CSF equals 1.86. According to the observation of the scatter plot of lignin removal ratio and CSF, there is a platform in the figure. Then in order to better describe the relationship between the two parameters and find the turning

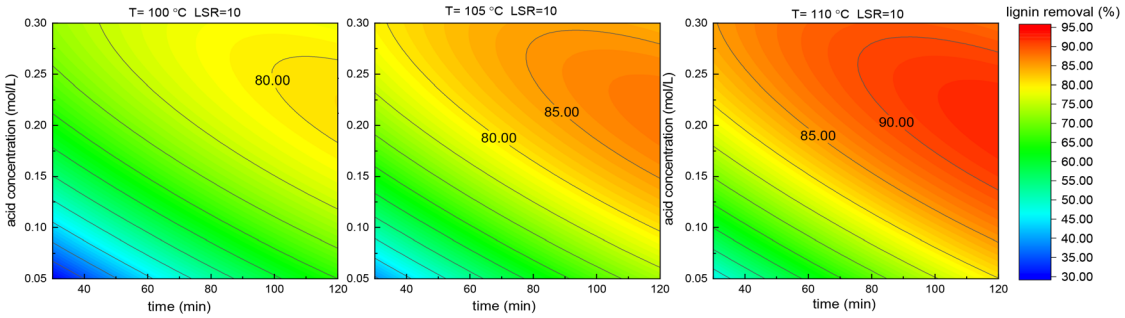

Fig. 1 Contour plots of time vs acid concentration, lignin removal rate under different temperatures (A:100 °C, B:105 °C, C:110 °C) with LSR is 10.

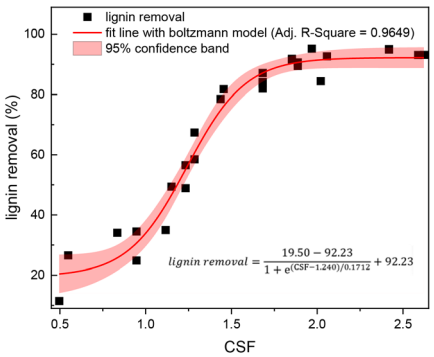

Fig. 2 Lignin removal as a function of CSF.

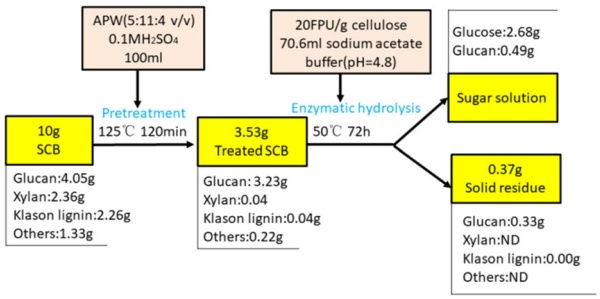

Fig. 3 Mass balance of SCB converted to sugars based on APW pretreatment.

point, we use the Boltzmann function for fitting, and the equation obtained as follows:

$$\text{lignin removal} = \frac{19.50 - 92.23}{1 + e^{(\text{CSF}-1.240)/0.1712}} + 92.23$$

To further evaluate the feasibility of pretreatment optimization using CSF, three process parameters with a CSF of around 1.86 for APW pretreatment were tested as shown in Table 3: (1) 110 °C, 110 min, 0.1 M H_2SO_4 (SCB-3, CSF = 1.83); (2) 110 °C, 80 min, 0.2 M H_2SO_4 (SCB-4, CSF = 1.87); and (3) 110 °C, 90 min, 0.15 M H_2SO_4 (SCB-5, CSF = 1.86). The actual lignin removal of SCB treated under these conditions (CSF with values of 1.83, 1.87, and 1.86) was 95.27%, 94.94%, and 91.55%, respectively. This result suggests that CSF = ~1.86 can be used as an indicator for effective lignin removal in the APW system and is useful in figuring out a relatively mild condition for the fractionation of lignocellulose biomass, allowing a more straightforward experimental design than using BBD.

Mass balance and performance comparison of APW

The mass balance in SCB during the process of fractionation was calculated as shown in Fig. 3. After pretreatment, 3.53 g of residue remained from 10 g of bagasse. The residue contained 3.23 g glucose, which means that the cellulose content reached 91.68%. It can also be concluded that 85.59% glucan was retained, 98.12% lignin and 98.15% xylan were removed after APW pretreatment. After enzymolysis, 74.52% of glucan was converted into glucose, and a sugar solution with a glucose content of 37.89 g L^{-1} was obtained, which contained 2.68 g glucose and 0.49 g polysaccharide. Therefore, APW pretreatment can enrich the cellulose substrate and improve the enzymatic hydrolysis rate of the residue.

In addition, the fractionation effect of various lignocellulosic biomass, including sugarcane bagasse, corn stalk, pine and bamboo, pretreated by APW was also studied to investigate the adaptability of the system, as shown in Table 4. The delignification and hemicellulose removal rates of these biomass were both over 90%, lignin retention in residue was less than 7% and cellulose content in the residue was over 85%. It is worth mentioning that this solvent system can reach over 90% lignin removal of pine. Haykir *et al.* studied the pine pretreatment with PIL–water (4 : 1) at 170 °C for 3 h, and only achieved 52% lignin removal.[28] Therefore, APW pretreatment showed excellent fractionation effect on different biomass.

For chemical pretreatment, organic solvents with acid were used, according to Table S3,† chemical pretreatment resulted in a higher delignification rate when compared to the initial concentration in the biomass. Compared to the pretreatment with water/phenoxyethanol,[23] adding acetone to water/phenoxyethanol significantly improved delignification from 63.16% to 94.94%. The mixture of 50% ethanol and butylated hydroxytoluene (BHT) was applied to enhance the treatment efficiency of SCB, achieving 45.28% lignin removal at 121 °C.[29] Kawamata

Table 4 The fractionation effect of various lignocellulosic biomass under the given conditions (125 °C–120 min–0.17 MH_2SO4-liquid–solid ratio 15)

Material	Solid remaining/%	Cell./%	Hemi./%	Lign./%	Cellulose retention/%	Hemicellulose removal/%	Delignification/%
Sugarcane bagasse	35.33	91.68	1.23	1.45	85.59	98.15	98.12
Corn stalk	29.74	87.58	ND	0.66	68.82	100.00	98.74
Pine	41.37	85.54	3.74	6.45	93.50	91.56	90.65
Bamboo	28.22	92.50	ND	2.38	68.97	100	96.83

ND: not detected.

et. al. employed water/1-buthanol (4.0 mol mol^{-1}) solvent for treatment of SCB at 200 °C and achieved 67% delignification rate.[30] Furthermore, 60% pentanol could separate 85% lignin from corn stalk at 160 °C, 60 min.[31] Making a comparison with these biphasic solvents or pure solvents, the APW system gave high delignification under mild conditions.

Characterization of recovered lignin

2D-HSQC NMR spectra of MWL and APWL were recorded to identify the structural changes of lignin after APW pretreatment, and the correlated peaks in the 2D-HSQC were assigned as described previously;[23] the assignments are listed in Table S4† and the 2D-HSQC spectra of MWL and APWL are displayed in Fig. 4.

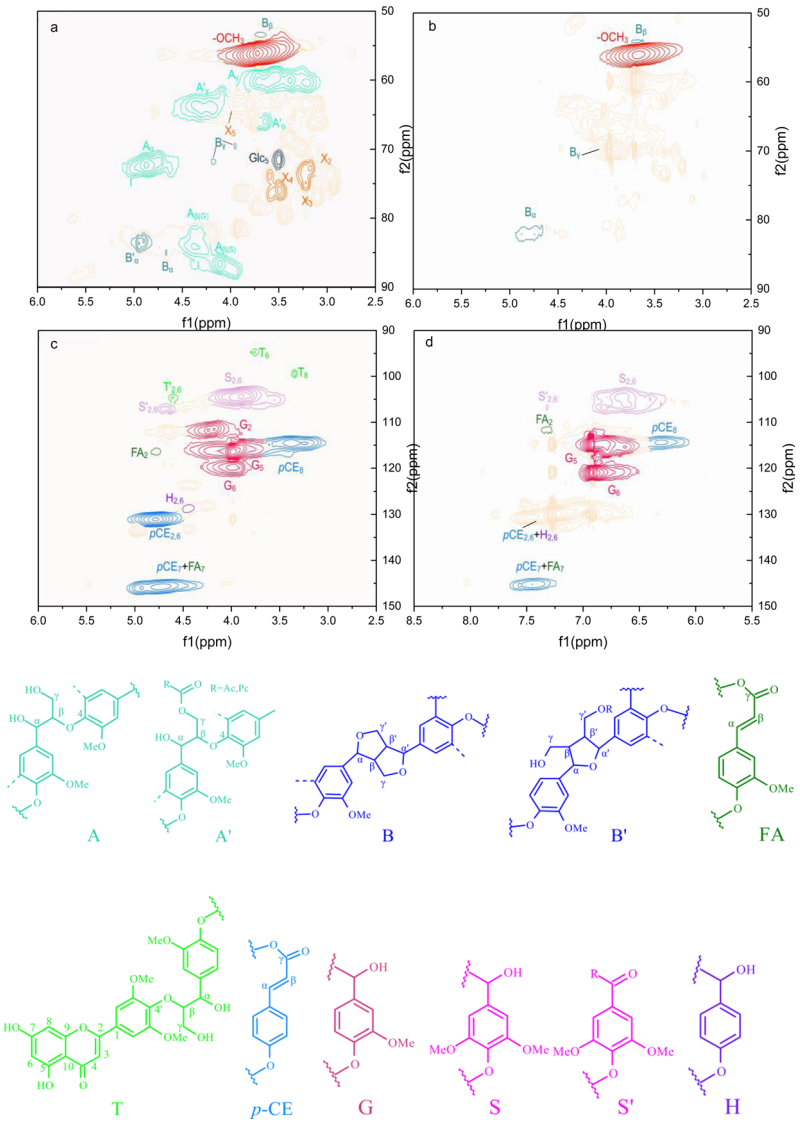

Fig. 4 Side chain region (δ_C/δ_H 54.00–88.00/2.80–5.50) and aromatic region (δ_C/δ_H 92.00–147.00/6.10–7.70) in 2D HSQC NMR spectra of MWL (a) and (c) and APWL (b) and (d).

In the side-chain regions (δ_C/δ_H 50–90/2.5–6.0 ppm) of the spectra (Fig. 4a), the xylan backbone at X_2 (δ_C/δ_H 72.71/3.21 ppm), X_3(δ_C/δ_H 74.94/3.25 ppm), X_4(δ_C/δ_H 76.14/3.53 ppm) and C_5–H_5 in cellulose (Glc_5, δ_C/δ_H 71.47/3.51 ppm) were indicative of the presence of carbohydrates. However, these signals for polysaccharide cross-peak signals were removed after APW pretreatment, suggesting that the obtained lignin has high purity. As shown in spectra (Fig. 4a and b), the signals of methoxy groups (–OCH_3) presented the most prominent signals, implying that these lignin fractions were rich in S- and G-type units. Additionally, the main interunit linkages of MWL were β-O-4 arylethers (A) and β–β resinol (B). Signals corresponding to both β-O-4 and β–β decreased after pretreatment, especially for the β-O-4 linkage which is predominant in MWL but became trace in APWL. β-O-4 linkage is susceptible to breakage under acidic conditions,[32] it is speculated that the APW pretreatment facilitated the cleavage of β-O-4. It was found that the C_α–H_α and C_β–H_β in β-β (tetrahydrofuran) (B′) with the cross signal at δ_C/δ_H 83.57/4.97 ppm and δ_C/δ_H 50.49/2.62 ppm, respectively, was detected for MWL and absent for APWL, suggesting that the cleavage of the β–β linkage could take place after APW pretreatment. These results suggest that the proposed APW pretreatment resulted in the cleavage of both C–O bond and C–C bonds.

In the region of aromatic substructures (δ_C/δ_H 90–140/6.0–8.0 ppm) of the 2D-NMR spectra (Fig. 4c and d), the correlated peaks of syringyl (S), guaiacyl (G) and *p*-hydroxypheny (H) can be obviously distinguished. As shown in Fig. 4c, the correlated signals at δ_C/δ_H 104.53/6.72 are indicative of the presence of sypringyl(S) unit ($S_{2,6}$), besides the oxidized S unit ($S'_{2,6}$) at δ_C/δ_H 107.13/7.34. After APW treatment, the signals of main substructure, such as $C_{2,6}$-$H_{2,6}$ in syringyl units (S) and the oxidized S unit, were weakened. Interestingly, the $H_{2,6}$ (δ_C/δ_H 129.65/7.16 ppm) correlation of H unit presented a stronger signal in APWL than in WML. Besides, it's of great importance to note that there is no signal of condensed structure occurred in the APWL even though condensed G_2 and $S_{2,6}$ easily appeared after pretreatment.[33] Moreover, given the breakage of β-O-4 linkage and no signal of condensed structure, we hypothesize that the condensation was inhibited by the reaction between solvent and the fractionated lignin during the reaction, but further studies are needed. In addition, the signal of tricin units can only be detected in the spectra of MWL, labeled as T_3 and T_8 correlation at δ_C/δ_H 105.45/7.05, 99.38/6.25, respectively, which was on account of that tricin was easily degraded in acidic organic solvents.[23] Additionally, the S/G ratio in lignin was an important implement to evaluate the structural changes during APW pretreatment. The S/G ratio of MWL was 0.59, and that of APWL was 0.13. Previous work has indicated that the decreased S/G ratio of the substrates after the pretreatment was primarily because lignin was depleted in S units and enriched in H units.[34] In short, it was found that lignin containing less carbohydrates and enriched in H-units can be obtained after APW pretreatment as compared to that of MWL.

To further determine the changes of different OH groups in lignin before and after APW pretreatment, the quantitative ^{31}P NMR technology was used. The spectra of the lignin fractions were recorded based on the previous methods,[27] and the results are listed in Fig. S4 and Table S5.† The content of aliphatic OH was reduced after APW pretreatment as compared with that (8.60 mmol g^{-1}) of MWL, indicating that the aliphatic OH groups in SCB carbohydrate fractions were removed after the pretreatment process, which is in agreement with the 2D-HSQC NMR of the MWL sample (Fig. 4). In addition, the content of S and G-type phenolic hydroxyl groups significantly increased after APW pretreatment, which was ascribed to the cleavage of β-O-4 linkages and free phenolic hydroxyl groups released. Phenolic OH is the main factor affecting the redox activity of lignin because of its strong ability to capture and neutralize free radicals.[35] The total phenolic OH content of MWL was 2.41 mmol g^{-1}. In comparison with MWL, APWL exhibited higher total phenolic OH contents, which was calculated to be 3.04 mmol g^{-1}, resulting from the dissociation of β-O-4 ether linkages. In short, the lignin induced by APW pretreatment contained higher total phenolic OH contents and less aliphatic OH compared with MWL.

The morphology and properties of treated SCB

Biomass pretreatment improves the accessibility of enzymes to cellulose which facilitates carbohydrate digestion to fermentable sugars.[36] The impact of APW pretreatment on SCB under selected conditions was investigated. Compared to raw SCB, APW pretreated SCB showed an increase in glucose yields ranging from 9.35% to 67.76%–78.06%. The morphology of SCB is an important factor in understanding the enzymatic digestibility of residual carbohydrates. According to SEM analysis of raw SCB and SCB pretreated under different conditions (Fig. S1†), the surface of the raw biomass was closely arranged with smooth morphologies. At the same time, scattered clusters of cells and vascular bundles were observed after pretreatment. In addition, the intensity of etching and rough morphologies were dependent upon increasing temperature from 110 °C to 120 °C. These observations agree with the pretreatment performance shown in Table 3, most of the lignin and hemicellulose were removed. Complete separation of cells was observed in samples treated at 125 °C for 120 min with 0.17 M acid (Table 3). Comparing the FTIR spectrum of untreated and pretreated SCB (Fig. S2†), it can be speculated that the intensity of the absorbance peak at 3330 cm^{-1} increased after pretreatment. It indicates that a significant part of cellulose was recovered after the APW pretreatment due to the 3330 cm^{-1} peak corresponding to hydrogen bonding or the hydroxyl group of cellulose. All these observations are consistent with removing a substantial amount of lignin and hemicellulose while most of the cellulose is retained in the solid residue.

Correlation analysis of substrate-related properties on enzymatic hydrolysis

The effective bioconversion of cellulose to sugars is essential for the valorization of lignocellulose. Therefore, it is of great importance to understand the relationship between substrate-related properties with enzymatic digestibility. Statistical ana-

lysis for this has been developed over many years.[37]One of the commonly used method is correlation analysis.[38]

In our study, after APW pretreatment, with the lignin content being 1.23%, 0.53%, 0.58%, 1.99%, and 3.90% in SCB-1, SCB-2, SCB-3, SCB-4, and SCB-5, the EHE values were 74.52%, 63.67%, 68.62%, 74.15%, and 67.76%, respectively. Therefore, it indicated that the enzymatic hydrolysis efficiency varied greatly even when most of the lignin was removed. Since many substrate-related factors could affect enzymatic hydrolysis efficiency,[37,38] we conducted a correlation analysis to understand the properties of substrates that affect enzymatic hydrolysis with 9 participatory hydrolysis-related factors including the cellulose content, surface area of cellulose (SAC), specific surface area (SA), crystallinity index (CrI), CrI/cellulose, O/C ratio, lateral order index (LOI), surface lignin content (SL) and lignin content, as shown in Table.5.

Cellulose-based analysis

The organosolv pretreatment induced delignification and hemicellulose removal with concomitant changes in cellulose crystallinity and exposed more surface area of cellulose. To understand the impact of APW pretreatment on cellulose, the relationship between EHE and cellulose conversion, cellulose content, CrI of pretreated SCB, CrI/cellulose value, lateral order index (LOI), surface area of cellulose (SAC), and specific surface area (SA) which related to the changes of cellulose after different APW pretreatment runs was investigated.

As illustrated in Table 5, the cellulose content of untreated and pretreated SCB varied from 70.01% to 91.68%. FTIR spectra (Fig. S2†) showed that the intensity of the characteristic peaks of cellulose increased significantly after pretreatment under different conditions, such as bands at 898 cm^{-1} and 1165 cm^{-1}, which were attributed to β-(1–4)-glycosidic bond (C–O–C), and the wide band at 3423 cm^{-1} which was associated with O–H stretching of the hydrogen bonds of cellulose. However, the cellulose content implicitly correlated with EHE (r = 0.49, p > 0.05). Since the absorbance at 1430 cm^{-1} and 897 cm^{-1} are assigned to the crystal structure of cellulose, the absorbance ratio A1430/A897 or lateral order index (LOI) has been used to reflect the cellulose I fraction in the cellulose structure. The higher value of LOI indicates that it contains a higher fraction of cellulose I. The correlation between LOI and EHE was positive (r = 0.94, p < 0.05), indicating that higher cellulose I content is favorable for enzymatic hydrolysis. At the same time, delignification and hemicellulose removal of the APW pretreatment increased the content of cellulose I.[39] For SAC, the increase in exposed SAC is supposed to improve EHE due to the effective contact between cellulase and the cellulose surface.[24] This study found a significant positive correlation between EHE and SAC (r = 0.98, p < 0.05). Many studies have reported that biomass porosity increased after pretreatment. In contrast, in the present study, the specific surface area (SA) of the treated substrates was lower than that of the raw material, as indicated by BET tests. This can be attributed to the fractionation of the cell wall and the separation of the major biopolymers, leading to the blockage of the fiber cell cavities and pits by the cell wall fragments, subsequently reducing the pore volume.[40] It is also worth noting that drying the substrate could lead to a partial collapse of biomass pores. For instance, the pore diameter of native hardwoods decreased considerably due to thermal drying at 200 °C for 4 h.[41] To avoid this collapse, organic solvent exchange drying was used in surface area measurements.[42] EHE was significantly positively correlated with SA (r = 0.97, p > 0.05). XRD quantified the CrI of pretreated SCB (Fig. S3†), and the results ranged from 54.62% to 61.82%. The correlation between cellulose CrI and EHE was positive (r = 0.92, p < 0.05). It was confirmed from the results that a lower value of CrI has high resistance to enzymatic hydrolysis.[39,43] These results show that there is no increase in the trend of enzymatic hydrolysis with a decrease in crystallinity. This effect is probably due to only the relative CrI cannot illustrate the correlation between crystallinity and enzymatic hydrolysis. Since it was observed that cellulose CrI always increases with cellulose content increasing, a parameter (CrI/cellulose value) was adopted to associate cellulose CrI and cellulose content to analyze the actual changes in cellulose crystallinity.[44] The CrI/cellulose value of the residue decreased significantly after pretreatment. This observation could be ascribed to the fact that a stronger pretreatment condition facilitates the degradation of amorphous cellulose, leaving cellulose with a more crystalline region. However, the correlation between CrI/cellulose and EHE was indefinite (r = 0.23, p > 0.05).

Residual lignin

Generally, lignin is a primary inhibitor of enzymatic hydrolysis due to its unproductive binding to the enzyme, which physically limits the interaction between the enzyme and polysaccharide.[45] According to a previous study, the effect of lignin's nonproduc-

Table 5 Characteristics of SCB and treated SCB including the cellulose content, surface area of cellulose (SAC), specific surface area (SA), crystallinity Index (CrI), CrI/cellulose, O/C ratio, lateral order index (LOI), surface lignin content (SL) and lignin content

	EHE (%)	Cellulose content (%)	LOI	SAC (m^2 g^{-1})	SA (m^2 g^{-1})	CrI	CrI/cellulose value	Lignin content (%)	O/C	SL
SCB	9.25	40.51	1.24	151.81	6.139	41.39	1.02	22.64	0.32	1.08
SCB-1	74.52	91.68	1.04	220.01	5.79	66.7	0.73	1.23	0.72	0.16
SCB-2	63.67	79.58	0.98	174.07	3.63	54.62	0.69	0.53	0.58	0.40
SCB-3	68.62	70.49	1.01	192.27	4.16	56.97	0.81	0.58	1.69	0.40
SCB-4	74.15	77.97	1.07	213.54	5.94	61.82	0.79	1.99	0.54	0.48
SCB-5	67.76	70.01	1.01	181.29	4.63	58.18	0.83	3.90	0.59	0.38

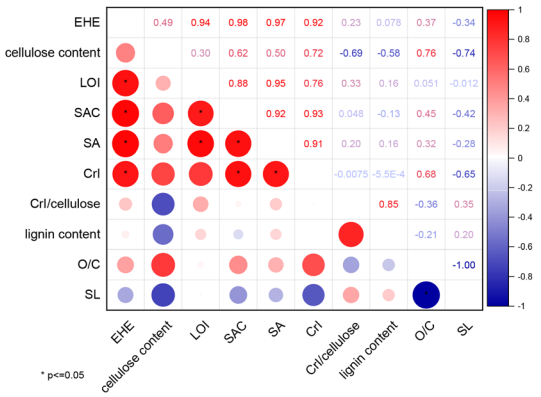

Fig. 5 Metrix of correlation coefficients among all factors. Larger redder (bluer) plots display strong positive (negative) correlations.

tive binding to cellulase is a less significant factor affecting enzymatic hydrolysis when compared to the steric hindrance of lignin.[46] Although the unproductive absorption and steric hindrance of lignin are important factors influencing enzymatic hydrolysis, the complete removal of lignin is not necessary to achieve high enzymatic hydrolysis yields.[47] To understand the extent of the impact of residual lignin on enzymatic hydrolysis after APW pretreatment, we further investigated the correlation between residual lignin and cellulose conversion (Fig. 5).

The results showed that the correlation between EHE and the residual lignin content was indefinite (r = 0.12, p > 0.05). After different runs of APW pretreatment, the lignin content ranged from 0.53% to 3.90% (Table 3). Previous studies found that the residual lignin on the surface of the substrate had more impact on cellulose conversion than the bulk lignin.[37] Mooney *et al.* reported that the decreased surface distribution of lignin on biomass materials positively influenced the enzymatic hydrolysis of woody biomass.[48] Therefore, it was imperative to investigate the surface compositions and lignin coverage of pretreated SCB and their relationship with cellulose conversion. The surface carbon (C_{1s}) and oxygen (O_{1s}) of pretreated SCB were determined by XPS, and then the surface-lignin content (SL) was identified by the O/C ratio. The theoretical O/C ratios of lignin and cellulose were 0.33 and 0.83, respectively.[38,49] Therefore, a higher O/C ratio indicated that lignin was less distributed than cellulose on the surface. However, the O/C ratio exhibited a poor correlation with EHE (r = −0.086, p > 0.05), while the correlation between EHE and SL was also indefinite (r = −0.34, p > 0.05). These results suggest that the surface coverage of lignin on SCB was not an essential factor affecting fermentable sugar yield when the lignin content was low in the substrate.

Conclusions

In conclusion, APW pretreatment was optimized by BBD combined with CSF. CSF as an indicator was a more straightforward and easier optimization method because it considers only one parameter. Under the optimized conditions, the removal of lignin and hemicellulose reached 98.12% and 98.15%, respectively, with 85.59% of cellulose retained in the solid residue, leading to an enzymatic digestibility of 74.52%. The results show that a CSF of ~1.86 facilitated lignin removal. Furthermore, this system showed good universality, and lignin removal over 90% can be obtained from SCB, pine, corn stalk, and bamboo. Moreover, compared to SCB MWL, the content of total phenolic OH and H units in precipitated lignin was increased, in which β-O-4 bond was broken and without condensation structure. Additionally, fermentable sugar yields are affected by the surface area of cellulose, lateral order index, CrI, and specific surface area when the lignin content in the pretreated biomass is low.

Author contributions

Wuhuan Li: investigation, formal analysis, and writing – original draft. Xuesong Tan: methodology, validation, and writing – review & editing. Changlin Miao: methodology. Zhanying Zhang: resources. Yunxuan Wang: writing – review & editing. Arthur J. Ragauskas: resources. Xinshu Zhuang: conceptualization, supervision, and writing – review & editing.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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