Modeling of optimal green liquor pretreatment for enhanced biomass saccharification and delignification by distinct alteration of wall polymer features and biomass porosity in *Miscanthus*

Aftab Alam [a, b, c], Youmei Wang [a, b, d], Fei Liu [a, b, c], Heng Kang [a, b, c], Shang-wen Tang [c], Yanting Wang [a, b, c], Qiuming Cai [a, b], Hailang Wang [a, b], Hao Peng [a, b, c], Qian Li [a, b, c], Yajun Zeng [a, b, c], Yuanyuan Tu [a, b, c], Tao Xia [a, c, d], Liangcai Peng [a, b, c, *]

[a] Biomass & Bioenergy Research Centre, Huazhong Agricultural University, Wuhan, 430070, China
[b] College of Plant Science & Technology, Huazhong Agricultural University, Wuhan, 430070, China
[c] Laboratory of Biomass Engineering & Nanomaterial Application in Automobiles, College of Food Science & Chemical Engineering, Hubei University of Arts & Science, Xiangyang, China
[d] College of Life Science & Technology, Huazhong Agricultural University, Wuhan, 430070, China

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ABSTRACT

Miscanthus is a leading bioenergy crop with enormous biomass resource convertible into bioethanol and biochemicals. However, lignocellulose recalcitrance basically causes costly bioethanol production with potential secondary pollution to the environment. In this study, the green liquor (mixed sodium carbonate and sodium sulfide) pretreatments were optimized using response surface methodological modeling for enhancing biomass saccharification and delignification in *Miscanthus*. By comparison, the optimal saccharification approach led to relatively higher hexose yield of 87% (% cellulose) for bioethanol yield of 17.1% (% dry matter) with the sugar-ethanol conversion rate at 98%, whereas the optimal delignification approach could achieve the highest delignification rate at 93% potential for lignin-derived biofuel or value-added biochemicals. Notably, those two optimized pretreatments could distinctively extract hemicellulose-lignin complex and altered wall polymer features, leading to much increased cellulose accessibility for efficient biomass enzymatic hydrolysis. Exceptionally, the optimal delignification led to decreased biomass porosity accountable for relatively lower hexose yield, suggesting that its cellulose microfibrils may be aggregated from excessive non-cellulosic polymers extraction. Hence, this study has demonstrated two optional strategies for green-like and cost-effective biofuels and biochemical production in *Miscanthus* and other bioenergy crops.

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1. Introduction

Perennial grass crops with enormous growth potential in a variety of ecosystems, are promising lignocellulosic feedstock for biofuels and value-added chemicals, and in particular, *Miscanthus* has been regarded as a leading bioenergy crop, due to its extremely high lignocellulose yield and much less requirement of water and fertilizer supplies [1,2]. However, the intrinsic resistance of plant cell walls to biological deconstruction leads to an expensive conversion process along with production of hazardous by-products to the environment [3,4]. Among wall polymers, lignin has been regarded as the main barrier to achieving efficient biomass enzymatic saccharification on account of its 'glue-like' shield encapsulating the polysaccharides, and non-productive interactions with cellulase enzymes during enzymatic saccharification [5,6]. On the other hand, lignin has also received significant attention as a potential biofuel source and ideal bio-precursor for valuable products [7,8]. Therefore, efficient solubilization of polysaccharides and valorization of co-product lignin via cost-effective lignocellulose bioconversion platforms are desirable for a profitable and sustainable biorefinery.

The green liquor (the mixture of sodium carbonate and sodium sulfide) is the smelt solution initially generated in the recovery

boiler in a kraft pulp mill [9]. The green liquor pretreatment is a moderate alkaline delignification process selectively removing lignin from lignocellulosic biomass with maximum possible retention of polysaccharides (cellulose and hemicelluloses) in biomass residues [10]. An exciting feature of the green liquor pretreatment is that much less toxic or corrosive by-products such as furfural, acetic acid, and metal ions are produced that affect the fermentation process and damage the pretreatment equipment. Furthermore, in terms of the eco-economic perspective, the chemicals utilized in the green liquor pretreatment can be recycled entirely with proven technology [11]. Therefore, in terms of technical feasibility, capital costs, and investment risk, exploiting the green liquor pretreatment could prove an attractive lignocellulose bioconversion technology for bioethanol production.

While recent works have focused on the biomass composition, high sugar recovery and lignin removal during the green liquor pretreatments in hardwood [9], softwood [12,13] and agricultural residues [11,14–16], little has been reported about statistical optimization of the green liquor pretreatment for maximum carbohydrate output and lignin removal from agricultural feedstock, such as *Miscanthus*. Besides, the extracted lignin is regarded as residual waste, or it is burned for energy production, leading to underutilization of this valuable co-product in a competitive and sustainable biorefinery.

In this study, we used response surface methodology (RSM) approach to optimize green liquor pretreatments for enhancing either lignocellulose enzymatic saccharification or delignification efficiency in bioenergy *Miscanthus*. We also determined lignocellulose porosity and cellulose accessibility accountable for cellulolytic enzymes loading and digestion, with wall polymer features examined for understanding of how lignocellulose saccharification and delignification could be largely enhanced under optimal green liquor pretreatments. Furthermore, the extracted lignin was recovered as the value-added co-product from the green liquor pretreated slurry, suggesting a significantly intensified bioconversion technology for multiple biofuels production in bioenergy crops.

2. Methods and materials

2.1. Miscanthus sample collection

The mature *Miscanthus* straws were harvested from Huazhong Agricultural University experimental field. The stem tissues were then collected, inactivated at 105 °C for 10 min and dried at 50 °C until constant weight. The dried stem tissues were ground into the powders through a 40 mesh screen and stored in the container as biomass samples for all experiments performed in this study.

2.2. Wall polymers extraction and determination

Plant cell wall polysaccharides were extracted as previously described by Peng et al. [17] with minor modification by Li et al. [18]. After removal of soluble sugars, lipid and starch from successive extractions with phosphate buffer (pH 7.0), chloroform-methanol (1:1, v/v) and dimethyl sulphoxide (DMSO)/water (9:1, v/v), the crude cell wall pellets were incubated with 0.5% ammonium oxalate monohydrate (AO, w/v) for 1 h in a boiling water bath. After centrifugation, the supernatants were collected as pectin fraction. The remaining crude cell walls were suspended in 4 M KOH containing $NaBH_4$ (1.0 mg/mL), and the combined supernatants were used as KOH-extractable hemicelluloses fraction. The remaining non-KOH-extractable pellets were suspended in 67%

H_2SO_4 (v/v) for 1 h at 25 °C and supernatant was collected as cellulose fraction. Pectin content was calculated by measuring hexoses, pentoses and uronic acids in the AO-extractable fraction. Total hemicelluloses were calculated by measuring hexoses and pentoses of the KOH-extractable hemicellulose fraction and the pentoses of the remained non-KOH-extractable fraction. Hexose of the remained non-KOH-extractable fraction was determined as cellulose level. UV–VIS Spectrophotometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd., Shanghai, China) was employed for hexoses, pentoses and uronic acids assays. The anthrone/H_2SO_4, orcinol/HCl and *m*-hydroxybiphenyl/H_2SO_4 methods were respectively applied for hexose, pentose and uronic acid assays [1]. Regarding the high pentose content interfering the absorbance reading at 620 nm for hexose assay, the deduction from pentose reading at 660 nm was conducted for final hexose calculation, which was verified by GC/MS (SHIMADZU GCMS-QP2010 Plus) assay [1] with the analytical conditions: Restek Rxi-5ms, 30 m × 0.25 mm ID × 0.25 μm df column. Carrier gas: He. Injection Method: Split. Injection port: 250 °C, Interface: 250 °C. Injection Volume: 1.0 μL. The temperature program: from 170 °C (held for 12 min) to 220 °C (held for 8 min) at 3 °C/min. Ion source temperature: 200 °C, ACQ Mode: SIM. The mass spectrometer was operated in the EI mode with ionization energy of 70 eV. Mass spectra were acquired with full scans based on the temperature program from 50 to 500 *m/z* in 0.45 s.

Total lignin content of the acid-soluble and insoluble lignin was determined by the two-step acid hydrolysis method according to Laboratory Analytical Procedure of the National Renewable Energy Laboratory. The acid-insoluble lignin was calculated gravimetrically as acid-insoluble residue after correction for ash, and the acid-soluble lignin was measured by UV spectroscopy as previously described by Wu et al. [19]. Delignification of green liquor pretreatment with *Miscanthus* sample was calculated using Equation (1). All analyses were performed in independent triplicate.

$$\text{Delignification } (\%) = 1 - \frac{\text{lignin }_{\text{residues (g)}}}{\text{lignin }_{\text{raw (g)}}} \times 100 \quad (1)$$

lignin $_{\text{residues (g)}}$: lignin content in the *Miscanthus* residues after GL pretreatment (g);
lignin $_{\text{raw (g)}}$: lignin content in the raw material of *Miscanthus* biomass (g).

2.3. Green liquor lignin isolation

The supernatant (50 mL) obtained from the green liquor pretreatment was neutralized to pH 5–6 with 6 M HCl and then concentrated to about 30 mL on a rotary evaporator under reduced pressure. The concentrated supernatant was mixed with three volumes of 95% ethanol at room temperature. After settling for 24 h, the precipitated residue was centrifuged to obtain the green liquor-soluble hemicelluloses. The residue was washed twice with 20 mL 70% (v/v) ethanol, and all supernatant were collected. The supernatant was concentrated to about 30 mL to remove ethanol under reduced pressure, added with 6 M HCl to adjust pH up to 2 with agitation and precipitated for 24 h at 4 °C. The precipitated lignin was centrifuged and washed once with acidified water (HCl, pH 2) to remove salt, and freeze-dried (or vacuum-dried) for mass balance calculations and its FTIR characteristics were profiled using PerkinElmer Spectrophotometer (NEXUS 470, Thermo Fisher Scientific, Waltham, MA, USA) as previously described by Alam et al. [4].

The lignin recovery yield (%) relative to total initial lignin in raw

material was calculated using Equation (2) as follows:

$$\text{Lignin recovery } (\%) = \frac{W_{\text{recovered lignin}}}{W_{\text{biomass}} \times \text{Lignin}_{\text{raw}}} \times 100 \qquad (2)$$

$W_{\text{recovered lignin}}$: Dry weight of recovered lignin (g) from the supernatant after green liquor pretreatment;

W_{biomass}: Dry weight of *Miscanthus* biomass (g) used for pretreatment;

Lignin $_{\text{raw}}$: Lignin content (g/g biomass) in the raw *Miscanthus* material.

2.4. Detection of wall polymer features and polymer linkages

Cellulose crystallinity index (CrI) was determined using X-ray diffraction (XRD) method (Rigaku-D/MAX, Ultima III; Japan) as recently described [4]. Cellulose degree of polymerization (DP) was determined using the viscosity method [20]. Monosaccharides of hemicelluloses were determined by GC—MS (SHIMADZU GCMS-QP2010 Plus) as described above in section 2.2. Three monomers of lignin were analyzed by HPLC method (1525, Waters Corp., MA, USA) as described by Li et al. [21]. Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy was performed to observe the structural constituents and chemical linkages in the representative raw and pretreated *Miscanthus* samples using a PerkinElmer spectrophotometer (Nicolet Nexus 470, Thermo Fisher Scientific, Waltham, MA, USA) equipped with diamond—germanium ATR single reflection crystal. The sample spectra were recorded in absorption mode over 32 scans at a resolution of $4\,\text{cm}^{-1}$ in the range of 4000 to $400\,\text{cm}^{-1}$ region [4].

2.5. Biomass porosity measurement

Simons' stain (SS) was applied to measure overall biomass porosity using Direct Blue 15 (DB15, Phenamine Sky Blue A Conc) and Direct Yellow 11 (DY11) purchased from Pylam Products Co. Inc., Garden City, NY. The biomass samples (0.10 g) were added with 1 mL Alum saline solution (5 mM KAl(SO$_4$)$_2$ + 1.5 mM NaCl) in 15 mL polypropylene centrifuge tubes. A set of tubes with 1:1 solution of DB (blue dye) and DY (yellow dye) were prepared by adding the same amount of each dye solution (10 mg/mL) in a series of volumes (0.25, 0.50, 0.75, 1.0, 1.5 mL) to each sample. Distilled water was added to make the final volume up to 10 mL and incubated for 9 h at 70 °C and 200 rpm. After the solutions were cooled at room temperature and centrifuged at 8000×g, the absorbance of the supernatants was measured on UV-1100 spectrophotometer at 612.5 nm and 410.5 nm. The dyes adsorption capacity of biomasses was determined between the initial added dyes and the final free dyes in the supernatants, and the maximum DB and DY dyes adsorbed to the biomasses were calculated by monolayer Langmuir adsorption model [20].

Congo Red (CR) stain was applied to estimate the cellulosic surface area in lignocellulose samples. The samples (100 mg) were treated with dye solution in a series of increasing concentrations (0.25, 0.50, 0.75, 1.0, 1.50 mg/mL) in 0.3 M phosphate buffer (pH 6) with 1.4 mM NaCl and an incubation temperature of 60 °C for 24 h. After centrifugation at 8000×g, the absorbance of the supernatant was measured at 498 nm, and the maximum amount of adsorbed dye was calculated by subtraction of free dye in the supernatant from the initial added dye.

Cellulose accessibility was determined subjective to the maximum enzyme adsorption by the substrate under non-catalytic condition [4]. The samples were thoroughly mixed with 0.2 M Na-

acetate buffer (pH 4.8) containing different amounts of dissolved mixed-cellulase enzymes (Imperial Jade Biotechnology Co., Ltd. Ningxia 750002, China) ranging from 0.5 to 3 mg/mL with 1% substrate consistency. All samples were incubated at low temperature (4 °C) for 15 h to inhibit hydrolysis of the substrates with gentle shaking after every 3h to increase enzyme contact with the substrate. After centrifugation at 3000×g for 15 min, the supernatants were used for protein analysis by Bradford protein assay with bovine serum albumin (BSA) as a protein standard. The maximum enzyme adsorbed on biomass was measured from the monolayer adsorption isotherm.

2.6. Green liquor pretreatment

The green liquor solution was prepared by mixing Na$_2$S and Na$_2$CO$_3$ with a sulfidity (percent ratio of Na$_2$S to the sum of Na$_2$S and Na$_2$CO$_3$ on Na$_2$O basis) of 30%. Total titratable alkali (TTA, sum of Na$_2$S and Na$_2$CO$_3$, as Na$_2$O) charge on oven-dried biomass sample ranged from 7% to 35% (w/w). The pretreatment was carried out at solid:liquid ratio of 1:8 (w/v) in a lab-scale electrically heated oil bath containing 6 stainless steel bombs stirred at 15 rpm. The samples were first impregnated with green liquor solution at 60 °C for 30 min. Then temperature was raised at the rate of 3 °C/min to the target temperature (130—170 °C) and maintained for scheduled time (20—60 min). At the end of pretreatment, bombs were cooled to room temperature, and the pretreated residues were washed with distilled water for 3—5 times until pH 7.0 for following enzymatic hydrolysis.

2.7. Experimental design and statistical analysis for optimizing green liquor pretreatment

To optimize green liquor pretreatment, the central composite rotatable design (CCRD) of RSM was performed with TTA (X_1), residence time (X_2) and pretreatment temperature (X_3) as independent factors/variables, and delignification during pretreatment and hexose yield during enzymatic hydrolysis as response variables (Y). The actual and coded values of independent variables at five different levels of each variable was presented in Table S1. The experimental design was carried out with twenty trials, including six trials of the central point as shown in Table S2. All experiments were performed in triplicate to maintain accuracy and reproducibility of the model, and their means were taken as the response values. Linear regression analysis of experimental data was performed to fit the second-order polynomial equation for the response variables as given below:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1 X_2 + \beta_5 X_1 X_3 + \beta_6 X_2 X_3 + \beta_7 X_1^2 + \beta_8 X_2^2 + \beta_9 X_3^2 \qquad (3)$$

where Y is the dependent (response) variable; β_0 is a constant; β_1, β_2, and β_3 are linear coefficients; β_4, β_5, and β_6 are interaction coefficients; β_7, β_8, and β_9 are the quadratic coefficients of X_1, X_2, and X_3, respectively; and X_1, X_2, and X_3 are the coded values for TTA (%), time (min) and temperature (°C) respectively. The statistical significance of the model was determined by evaluating the *P*-value (<0.05) obtained from the analysis of variance (ANOVA). The quality of the model developed was evaluated by the coefficient of determination (R^2). The fitted polynomial equations obtained from the regression analysis were then expressed in the form of three-dimensional surface plots to illustrate the relationship between the responses and any two variables to be optimized, keeping the other variable at the center point (constant). Furthermore, the

Table 1
The model of response surface methodology for optimization of hexose yield and delignification in green liquor pretreated biomass residues of *Miscanthus*.

Exp. No.	TTA (%)[a]	Time (min)	Temperature (°C)	Hexose yield (% dry matter)		Delignification (% total lignin)	
				Observed	Predicted	Observed	Predicted
1	28	50	160	26.20	25.80	78.41	81.08
2	28	50	140	26.14	26.00	77.21	79.00
3	28	30	160	27.26	27.64	78.74	82.15
4	28	30	140	27.42	28.12	79.07	80.80
5	14	50	160	15.59	14.92	54.12	56.01
6	14	50	140	16.36	16.06	53.24	53.44
7	14	30	160	15.18	15.34	55.35	57.16
8	14	30	140	16.33	16.76	54.39	55.33
9	35	40	150	32.42	32.12	84.72	85.89
10	7	40	150	13.56	13.59	40.33	43.77
11	21	60	150	20.02	20.77	63.86	66.55
12	21	20	150	23.97	22.89	67.04	69.00
13	21	40	170	18.47	18.64	71.82	72.63
14	21	40	130	20.49	19.99	65.52	69.37
15	21	40	150	21.33	21.41	63.11	64.72
16	21	40	150	21.04	21.41	62.92	64.72
17	21	40	150	21.58	21.41	62.85	64.72
18	21	40	150	21.45	21.41	61.98	64.72
19	21	40	150	21.49	21.41	62.56	64.72
20	21	40	150	21.82	21.41	63.09	64.72

[a] TTA: Total titratable alkali (% dry biomass, w/w).

numerical optimization method was used to calculate the optimal conditions using MS Excel. Confirmatory experiments under optimized conditions for green liquor pretreatment were carried out in triplicate for both delignification and hexose yield after green liquor pretreatment in *Miscanthus* to confirm the authenticity of the model generated. The experimentally observed results of hexose yield and delignification were compared with those of the predicted values as shown in Table 1.

2.8. Enzymatic hydrolysis and yeast fermentation for bioethanol production

Enzymatic hydrolysis of the pretreated and raw material was performed using mixed-cellulase enzymes (Imperial Jade Biotechnology Co., Ltd. Ningxia 750002, China) in 0.2 M Na-acetate buffer (pH 4.8) as described by Li et al. [3]. The solid: liquid ratio during enzymatic hydrolysis was 1 : 20 (5% solid loading). The yeast fermentation was conducted using *Saccharomyces cerevisiae* strain (purchased from Angel Yeast Co., Ltd., Yichang, China) as previously described [22]. All experiments were performed with biological triplicate. The sugar-ethanol conversion rates were calculated by using the following equation.

$$S\text{-}E(\%) = E/(A \times H) \times 100$$

where *S-E(%)*: sugar-ethanol conversion rate; *E*: total ethanol weight (g) at the end of fermentation; *A*: the theoretical conversion rate at 51.11% (92/180) in the case when glucose is completely converted to ethanol according to the Embden-Meyerhof-Parnas pathway in *S. cerevisiae*; and *H*: total hexoses weight (g) at the beginning of fermentation. All experiments were performed in technological triplicate.

2.9. Statistical analysis

Statistical analysis (analysis of variance, regression coefficients, Spearman's rank correlation coefficient) was carried out using Superior Performance Software System (SPSS version 16.0, Inc., Chicago, IL). Pair-wise comparisons were conducted between two measurements by Student's t-test. The line graph, histogram, and regression analysis for the best fit curve were generated using

Origin 8.5 software (Microcal Software, Northampton, MA). The average values were calculated from the original triplicate measurements for these analyses.

3. Results and discussion

3.1. Modeling of optimal green liquor pretreatment for enhancing biomass saccharification and delignification

As the initial steps, various physical and chemical pretreatments have been applied for enhancing biomass enzymatic saccharification by partially extracting wall polymers [4,20]. However, it remains essential to find out a green-like and cost-effective pretreatment technology. Using RSM-based modeling approach, this study optimized the influential factors (TTA, time and temperature) of green liquor pretreatments for enhanced biomass saccharification and delignification in *Miscanthus*. As a result, analysis of variance (ANOVA) exhibited highly significant regression model with an extremely high coefficient of determination (R-square) values of 0.991 and 0.993, respectively, indicating 99.1% and 99.3% of variation in both hexose yield released during enzymatic saccharification and delignification ($P < 0.01$) (Table S3). The three-dimensional (3D) surface plots demonstrated the effect of individual factor and their interactive effect on the responses (hexose yield and delignification) keeping the other factor constant at their center points (Fig. 1). By comparison, TTA showed linear effect on both hexose yield and delignification (Fig. 1A; 1B; 1D and 1E). Furthermore, both the time and temperature demonstrated elliptical and parabolic effects on both hexose yield and delignification efficiency (Fig. 1C and 1F). However, the interactions between two factors keeping the other factor constant on both hexose yield and delignification were found to be non-significant during the optimization of green liquor pretreatment in *Miscanthus* (Table S3). Meanwhile, the final optimal values of all factors were calculated by numerical optimization in the MS Excel to achieve the maximum responses under green liquor pretreatment [23]. The optimized conditions for enhanced biomass saccharification were found to be TTA 32.77% (% oven dry biomass, w/w), time 23.18 min and temperature 150 °C with predicted hexose yield of 34.59% (% dry matter) (Table S4). Similarly, green liquor pretreatment of TTA 32.77% for 23.18 min at 166.82 °C was found optimum with
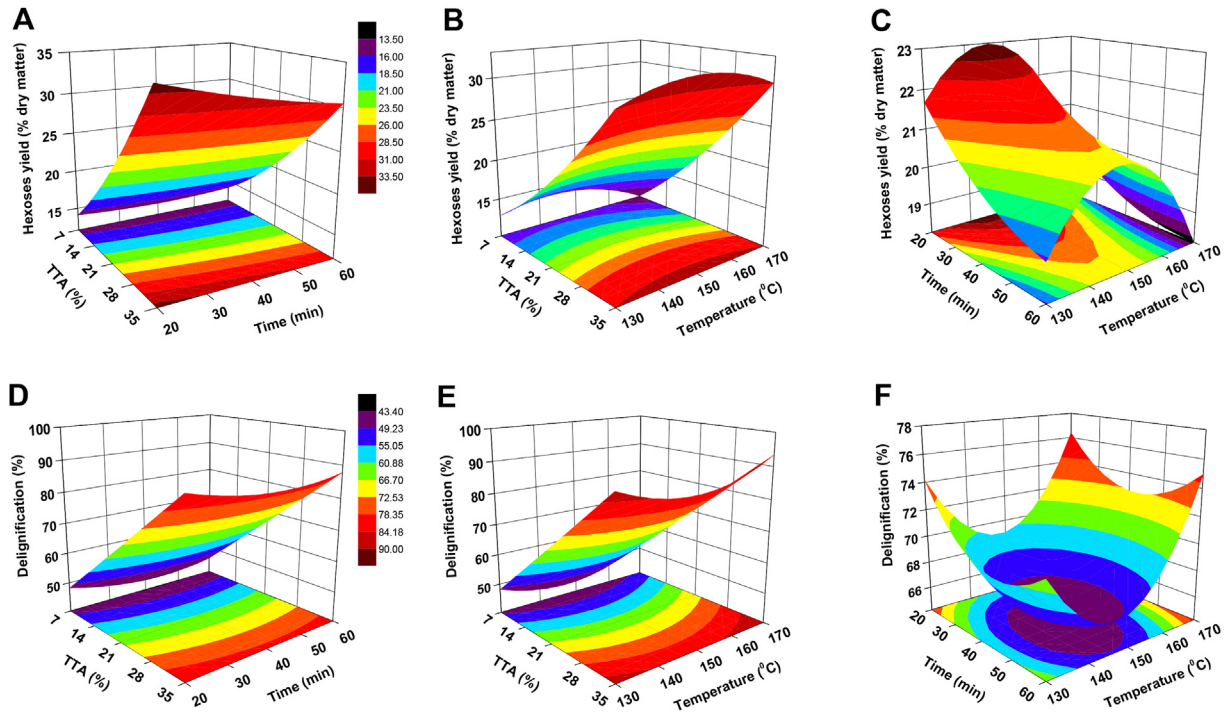
Fig. 1. Response surface plots showing effects on hexose yield (% dry matter) and delignification (% total lignin) after green liquor pretreatment. (A) Effects of TTA (%) and Time (min) on hexose yield; (B) Effects of TTA and Temperature (°C) on hexose yield; (C) Effects of Time and Temperature on hexose yield; (D) Effects of TTA and Time on delignification; (E) Effects of TTA and Temperature on delignification; and (F) Effects of Time and Temperature on delignification. The remaining factor is constant at zero level. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

predicted biomass delignification rate of 97.21% (% total lignin) in *Miscanthus*. Alternatively, hexose yield and delignification were also predicted to be 32.28% and 90.15% under two optimized green liquor conditions (Table S5).

Furthermore, confirmatory green liquor pretreatment was performed with optimized conditions to compare the results of hexose yield and delignification rate with the predicted values. Notably, the experimental results obtained for hexose yield were 34.22% and 31.18% (% dry matter), with delignification at 92.65% and 84.24% (% total lignin), which were very close to the predicted values with the small differences of 1.09%, 3.53%, 7.01% and 4.92%, indicating the authenticity of the model applied (Table S5). Meanwhile, Spearman's correlation analysis showed that the predicted and observed values in Table 1 were positively correlated from the green liquor

pretreatments at $P < 0.01$ level (n = 20) with extremely high coefficient values (r) at 0.98 (Fig. 2), revealing the reproducibility of experiments conducted, which was consistent with the previous findings [23–25]. Because lignin removal could facilitate cellulose accessibility to cellulolytic enzymes for saccharification, the delignification rate under green liquor pretreatment showed a positive correlation with hexose yields at $P < 0.01$ level with high coefficient value (n = 20).

3.2. Effective extraction and recovery of wall polymers for high biofuels production

While green liquor pretreatment was optimized to maximize biomass saccharification and delignification in *Miscanthus*, two
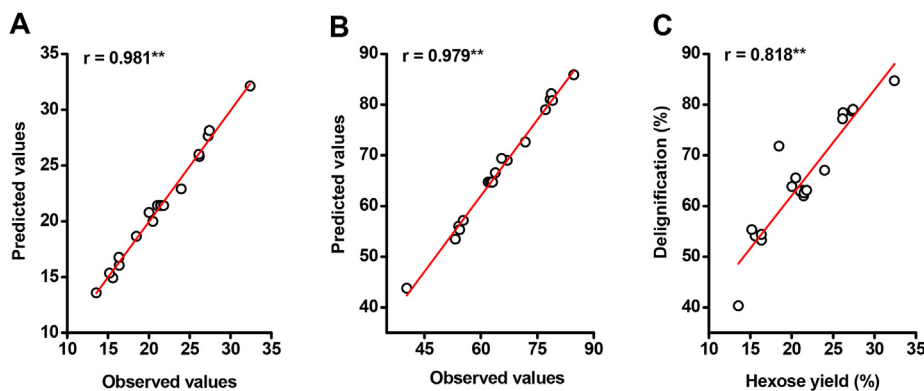

Fig. 2. Spearmen rank correlation analysis (n = 20). (A) Correlation analysis between the observed and predicted values of hexose yield (% dry matter); (B) Correlation analysis between the observed and predicted values of delignification (% total lignin); (C) Correlation analysis between hexose yield and delignification after green liquor pretreatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

optimized pretreatment conditions were identified termed as optimal saccharification for high hexose yield and optimal delignification for efficient removal of lignin from biomass feedstock. Meanwhile, this study analyzed the overall mass balance of two optimized pretreatment conditions, subsequent enzymatic saccharification, and yeast fermentation. The composition of resultant products was standardized to a common basis of 100g dried raw *Miscanthus* biomass as the starting material. Compared to the raw material, optimal green liquor pretreatment recovered the cellulose-rich solid residues at 65.99% and 56.47% of the initial biomass (Fig. 3), indicating that green liquor pretreatment is cellulose-benign bioconversion process preserving almost all cellulose content of the biomass. Furthermore, optimal saccharification distinctively removed lignin and pectin with considerably lower residual lignin level (22.0% vs. 3.6%, respectively) and pectin (1.03% vs. 0.15%, respectively), however, no significant change was observed in hemicelluloses (30.5% vs. 29.3%, as calculated on the relative weight basis of recovered residues, Table S6), which should be the partial cause of enhanced lignocellulose enzymatic saccharification under optimal green liquor pretreatment. By contrast, optimal delignification largely extracted lignin and pectin

with only 1.6% residual lignin and 0.10% pectin in the biomass residues, leading to the highest delignification efficiency (93%) in *Miscanthus*, compared to the previous reports [13,15,16]. Overall, the results suggest that optimal green liquor pretreatment at high temperature may specifically solubilize ferulate cross-linked hemicelluloses and lignin by dissociating lignin-carbohydrate complex linkages, consistent with the previous reports in *Miscanthus* and other grasses [4,10,14].

Meanwhile, the pretreated residues generated after green liquor pretreatments were subjected to subsequent enzymatic hydrolysis using commercial mixed-cellulase enzymes. Notably, the optimal saccharification pretreatment led to hexose yield of 86.6% (% cellulose), whereas optimal delignification could lead to relatively low hexose yield at 78.9% (Fig. 3; Table S7), probably due to the excessive extraction of non-cellulosic polymers (lignin and hemicelluloses) that caused cellulose microfibrils to aggregate and collapse, thereby limiting cellulose accessibility to cellulases for hydrolysis [26]. Also, sugar oxidation caused by high temperature may be another factor of low hexose yield, consistent with previous reports in *Miscanthus* and other grasses [22]. Using hexose yields from enzymatic hydrolysis of the pretreated biomass residues,
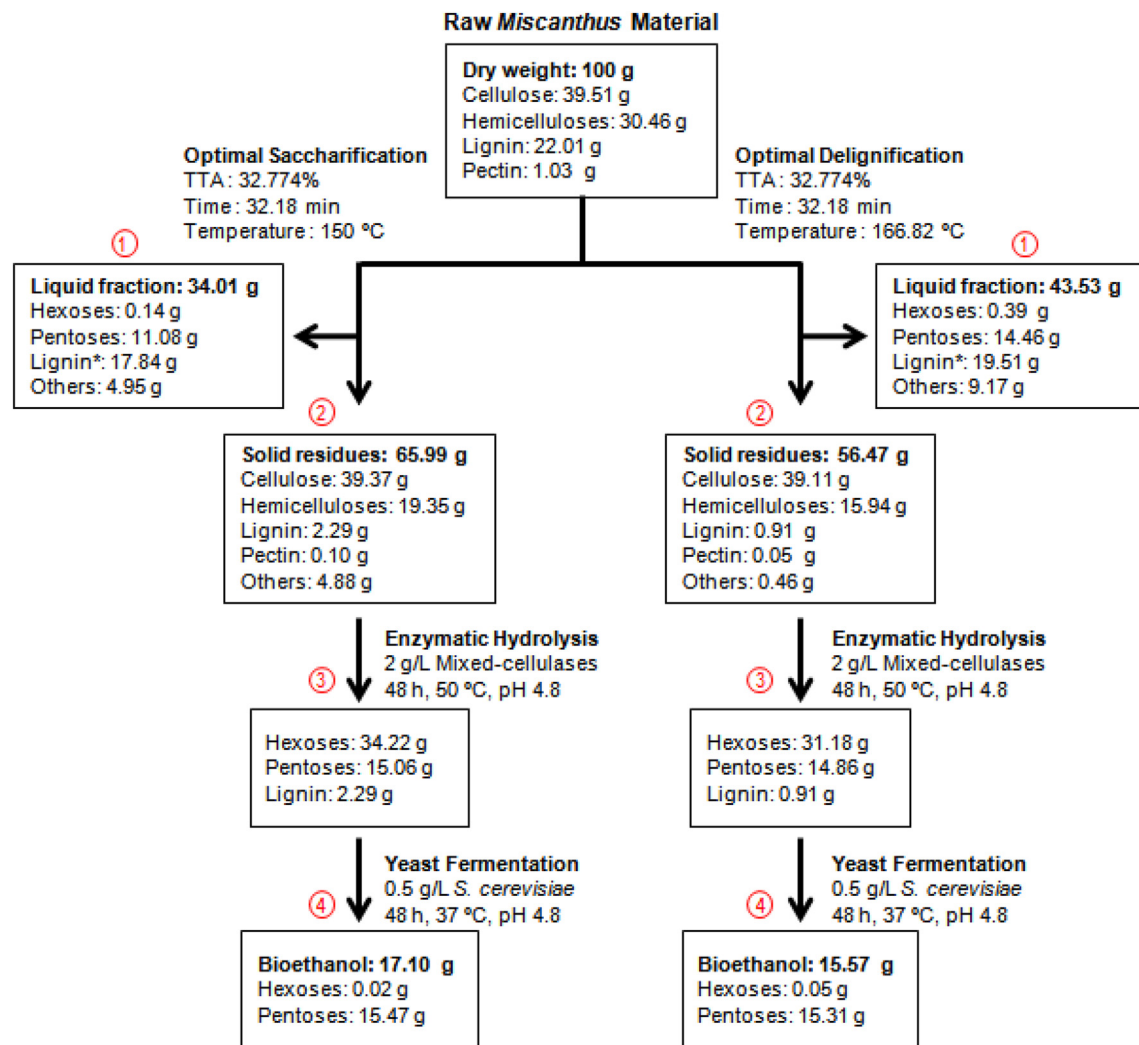

Fig. 3. Mass balance flow chart of *Miscanthus* biomass residues for bioethanol yields (on basis of 100 g) by optimal saccharification (left) and optimal delignification (right) under the green liquor pretreatment, sequential simultaneous enzymatic saccharification and final yeast fermentation processing. *Lignin recovered from the pretreatment liquor (supernatant) by acidic precipitation method (g/100 g biomass). Mass amount of soluble sugars (hexoses and pentoses) and ethanol yield were presented in streams 1, 3, and 4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

yeast fermentation was finally performed to obtain bioethanol production. As a result, the optimal green liquor pretreatments led to the bioethanol yields of 15.6—17.1% (% dry matter), with much high sugar-ethanol conversion rates at 98% (Fig. 3; Table S7), suggesting that the optimal green liquor pretreatments may release little inhibitors to yeast fermentation.

As lignin is a major wall polymer of lignocellulose, it has potential for upgrading to fuels and value-added chemical products. In this study, we isolated lignin-rich liquid hydrolysate streams during optimal green liquor pretreatments, which contained 84.2—92.7% of the total lignin present in *Miscanthus*. Notably, the results showed that about 81.1% of the initial lignin in *Miscanthus* was recovered in the liquid fraction after optimal saccharification approach, whereas optimal delignification approach recovered 88.7% of total lignin present in the raw material of *Miscanthus* (Fig. 3; Table S5), suggesting an efficient lignin valorization for producing fuels and valuable chemicals in a consolidated biorefinery. As the liquid hydrolysate streams may contain other products, i.e. polysaccharides, proteins, lipids, etc. extracted during optimal pretreatments, detail characterization of such products will be invested in the future experiments.

3.3. Remarkably reduced wall polymer features under optimal green liquor pretreatment

To understand why high hexose yield and effective lignin removal were achieved under the optimal green liquor pretreatments, this study examined major wall polymer features in biomass residues of *Miscanthus*. Compared to the raw material, the optimal green liquor pretreatments led to largely reduced three lignin monomers (H, G, S) in particular for H-monomer, indicating vigorous de-polymerization of lignin under the optimal pretreatments (Table 2). As β-aryl ether and carbon-carbon (C—C) bonds are the major inter-unit linkages between lignin monomers in plant cell walls [6], H-lignin with no methoxy functional groups has more C—C linkages and less β-O-4 linkages, resulting in the production of relatively low molecular weight lignin as compared to the mono-/di-methoxylated G and S lignin monomers [27,28]. These features of low molecular weight lignification could increase lignin susceptibility to deconstruction during the pretreatment stage. Although alkaline pretreatments have been regarded as efficient delignification strategies, this study observed the highest delignification rate with much reduced residual H-lignin, compared to the previous reports in *Miscanthus* and other energy crops [5,14,21], consistent with the previous findings that lignin removal could largely enhance biomass enzymatic saccharification in *Miscanthus*. Meanwhile, hemicellulosic monosaccharide composition showed that arabinose (Ara), xylose (Xyl) and galactose reduced while mannose and glucose levels increased in the pretreated biomass residues, compared to the raw material (Table 3). Since Ara substitution degree in hemicelluloses (reverse of Xyl/Ara ratio) has been characterized as a positive factor on biomass enzymatic saccharification [29], the branched Ara inter-linked with amorphous regions of cellulose microfibrils should be mostly extracted

at elevated temperature, suggesting that the reduced Ara level may be the factor accounting for lower hexose yield in optimal delignification stage. The cellulose degree of polymerization (DP), a well-characterized negative factor on biomass enzymatic saccharification [18], significantly reduced by 38% and 52% in the pretreated biomass residues, indicating that green liquor pretreatments should facilitate de-polymerization of cellulose microfibrils (Table 4).

Furthermore, this study determined cellulose crystalline index (CrI), which has been regarded as a major negative factor on lignocellulose enzymatic hydrolysis [18,30]. Despite that the optimal green liquor pretreatments opened the biomass ultrastructure by extracting amorphous regions of hemicelluloses and lignin, total biomass CrI apparently increased due to the increased cellulose content in the pretreated residues, compared to the raw material (Table 4). However, the increased biomass CrI should not be linked with relative cellulose level, because the hexose in hemicelluloses increases the apparent cellulose level resulting in an overestimation of CrI determined by the X-ray diffraction method (Fig. S1). Therefore, the ratio of CrI to the cellulose content (CrI/cellulose ratio) has been introduced to estimate the real impact of green liquor pretreatment on cellulose CrI [31]. Notably, CrI of cellulose itself decreased to 0.94 and 0.84 after pretreatment compared to 1.24 of raw material (Table 4), indicating that green liquor pretreatment may cause modification of native crystalline cellulose to different crystal structures [32]. It also suggests that the CrI/cellulose ratio may be a legitimate indicator on cellulose CrI under different pretreatments.

3.4. Altered wall polymers linkages under optimal green liquor pretreatment

With respect to the effective wall polymers extraction under the green liquor pretreatment, this study observed alteration of wall polymer linkages in the pretreated residues using ATR-FTIR spectroscopy including hydroxyl (O—H), ether (C—O—C), alkene (C=C), ketone (C=O), ester (—COO—), aromatics (Ar) etc (Fig. 4; Table S8). The characteristic peaks at 898, 1033, 1160, 1320, 1371, 1423, 2896 and 3343 cm^{-1} were associated with C—O—C, C—O, CH$_2$, C—H, O—H linkages between cellulose microfibrils and hemicelluloses [33,34], whereas the peaks at 834, 1508 and 1604 cm^{-1} were assigned to C—H and C=C in lignin [8,35]. Absorption spectra at 1733 cm^{-1} constituting ester-linked acetyl, feruloyl and *p*-coumaroyl groups between lignin and hemicelluloses largely decreased in the pretreated biomass residues, suggesting that optimal green liquor pretreatment could effectively cleave ferulate and *p*-coumarate cross-linkages, being essential moieties responsible for lignin-carbohydrate complex cross-linking in plant cell wall. Furthermore, removal of lignin led to reduction of absorption spectra in the aryl-alkyl ether linkages (C—O—C) at 1240 cm^{-1} between lignin and hemicelluloses in the optimal pretreated residues. Notably, the declined aromatic skeletal of lignin constituting conjugated C=C, Aryl substituted C=C, and alkenyl C=C groups resulted in reduced intensity at 1508 and 1604 cm^{-1}, indicating vigorous delignification

Table 2
Lignin monomer composition in the optimal green liquor pretreated biomass residues of *Miscanthus*.

Pretreatment	Lignin monomers (μmole/g biomass)				Lignin monomers distribution		
	H	G	S	Total	H/G	S/G	S/H
Raw material	210.61	216.82	207.46	634.90	0.97	0.96	1.02
Optimal saccharification	3.92 (-98%)[a]	37.88 (-83%)	29.78 (-86%)	71.58	0.10	0.79	0.13
Optimal delignification	2.94 (-99%)	33.69 (-84%)	23.85 (-89%)	60.48	0.09	0.71	0.12

[a] Percentage of decreased level between the raw material and pretreated residues by subtraction of two values divided by value of the raw material.

Table 3
Hemicellulose monosaccharides composition in the optimal green liquor pretreated residues of *Miscanthus*.

Pretreatment	Hemicellulose monosaccharides (μmole/g biomass)								Xyl/Ara
	Rha	Fuc	Ara	Xyl	Man	Glc	Gal	Total	
Raw material	0.00	0.00	80.39	2193.32	0.87	133.19	10.38	2418.13	27.28
Optimal saccharification	0.00	0.03	62.74 (−22%)[a]	1628.40 (−26%)	17.80 (1953%)	235.02 (76%)	4.53 (−56%)	1948.53	25.95
Optimal delignification	0.00	0.00	55.27 (−31%)	1560.47 (−29%)	17.15 (1878%)	315.01 (137%)	2.18 (−79%)	1950.08	28.23

[a] Percentage of increased or decreased level between the raw material and pretreated residues by subtraction of two values divided by value of raw material.

Table 4
Cellulose crystallinity and degree of polymerization in the optimal green liquor pretreated residues of *Miscanthus*.

Optimal condition	Cellulose DP	Cellulose CrI by XRD		Cellulose CrI by FTIR	
		CrI[a]	CrI/cellulose	Lateral order index[b]	Total CrI[c]
Raw material	1408 ± 8.84	0.49	1.24	1.01	0.92
Optimal saccharification	866 ± 4.12[d]	0.56	0.94	0.94	0.76
Optimal delignification	679 ± 4.62[d]	0.58	0.84	0.93	0.77

[a] Cellulose crystalline index (CrI) detected in raw material sample.
[b] Lateral order index was calculated from the spectral ratios A1423/A898.
[c] Total CrI was calculated from the spectral ratios A1371/A2896.
[d] Significant difference between the raw material and residues of *Miscanthus* samples after optimal pretreatment by *t*-test at $p < 0.01$ (n = 3).
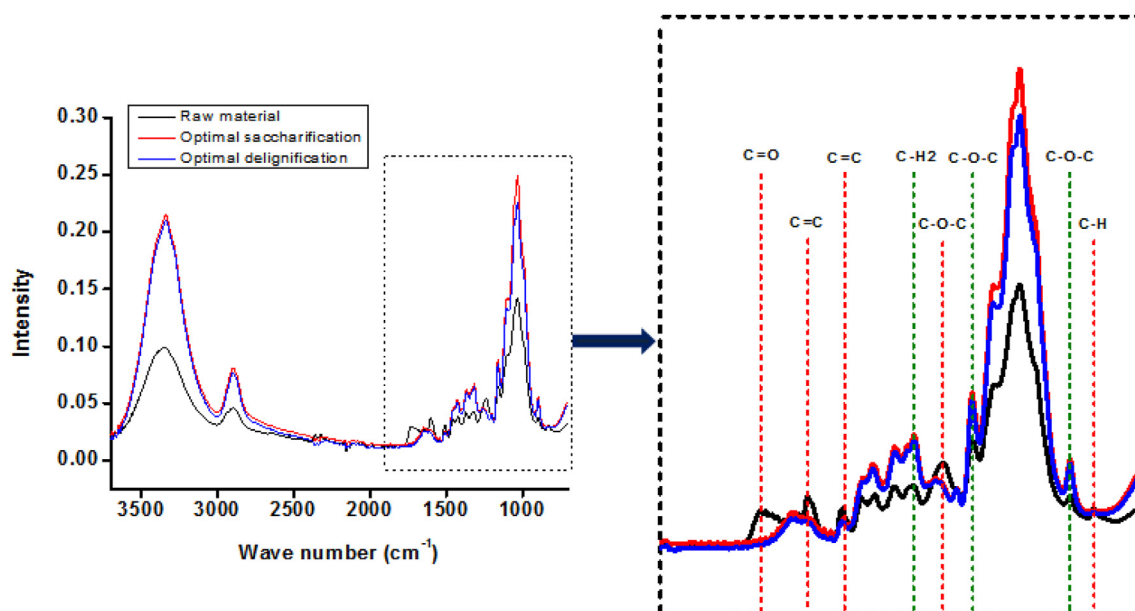

Fig. 4. Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopic profiling of the raw materials (black) and the optimal green liquor pretreated (Optimal saccharification-red, Optimal delignification-blue) biomass residues of *Miscanthus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

rate under optimal green liquor pretreatment. Similarly, the signals from the C—H group in H-monomer of lignin were observed at 834 cm^{-1} [8]. Predominantly, absorption spectra corresponding for typical β-glycosidic linkages and intermolecular hydrogen bonds in polysaccharides relatively increased after the optimal pretreatment, indicating that the green liquor pretreatment is cellulose-benign technology preserving cellulose in the pretreated biomass residues. Meanwhile, FTIR spectroscopy of the recovered lignin was conducted (Fig. S2), but further characterization and functionalities would be progressed in future work.

Since the optimal green liquor pretreatments distinctively altered the native lignocellulose ultrastructure, this study estimated cellulose crystallinity from the intensity measured during ATR-FTIR spectroscopy. The crystallinity index or lateral order index (LOI) and total crystallinity index (TCI) of cellulose were calculated based on the spectral ratios A1423/A898 and A1371/A2896 according to the previous reports [35]. Compared to the raw material, both LOI and TCI indices reduced at 8% and 17%, respectively, consistent with their CrI/cellulose ratio under XRD pattern during the optimal green liquor pretreatment (Table 4). The results indicated that the optimal pretreatment should lead to decreased crystalline and increased amorphous regions of the cellulosic network. It also confirmed that green liquor pretreatment was more efficient in cellulose de-crystallization by dissociating intra- and intermolecular hydrogen bonding in lignocellulose architecture.

3.5. Significantly increased lignocellulose accessibility from green liquor pretreatment

Because the optimal green liquor pretreatment led to effective

Table 5
Biomass porosity measured by Simons stain, Congo red stain, and enzyme adsorption in optimal green liquor pretreated biomass residues of *Miscanthus*.

Optimal conditions	Simons' stain (mg/g cellulose)				CR stain (m²/g cellulose)	Enzyme adsorption (mg/g biomass)	
	Yellow dye (DY)	Blue dye (DB)	Total dye (DY + DB)	DY/DB ratio	CR	E_{max}	K_{ads}[#]
Raw material	106.06 ± 4.14	109.22 ± 5.32	215.28 ± 9.46	0.97 ± 0.01	107.49 ± 2.02	83.97 ± 3.66	14.78 ± 1.02
Optimal Saccharification	149.91 ± 3.98	117.01 ± 1.62	266.92 ± 2.35	1.28 ± 0.05	130.00 ± 2.11	133.27 ± 2.60	18.23 ± 0.05
Optimal delignification	123.63 ± 2.49[*]	114.56 ± 1.70	238.18 ± 0.79	1.08 ± 0.04	121.41 ± 0.98[*]	114.02 ± 0.75[*]	21.20 ± 1.84

[*] As significant difference between the raw material and the biomass residues after optimal pretreatment by *t*-test at $p < 0.05$ (n = 3).

[#] K_{ads} as the binding affinity of enzymes to biomass (mL/mg); CR as Congo Red stain.

wall polymer extraction and polymer linkage alteration as described above, this study detected the biomass porosity and cellulose accessibility in the pretreated biomass residues of *Miscanthus* samples using multiple techniques such as Simons' stain, Congo red stain and cellulase enzyme adsorption (E_{max}) assay. In terms of Simons' stain, we detected significantly increased values of blue dye (DB), yellow dye (DY), total dye (DY + DB) and DY/DB (Y/B) ratio, particularly the DY with increased rates by 41% and 17% in the pretreated biomass residues, compared to the raw materials (Table 5), consistent with our previous findings that DY is an optimal parameter accounting for hexose yield [4,20]. Meanwhile, this study applied Congo red dye to quantify the specific surface area of cellulose under the optimal green liquor pretreatments. Because Congo red dye is tailored explicitly for high binding affinity with cellulose, the results revealed that the optimal green liquor pretreatments could distinctively extract non-cellulosic wall polymers, leading to relatively large cellulosic surface areas of biomass residues. In particular, the optimal saccharification approach led to higher CR values than those of the raw material and optimal delignification pretreatment (Table 5). To verify the enhancement of biomass porosity, this study further assessed the adsorption behavior of mixed-cellulase enzymes at non-catalytic condition (4 °C) in the pretreated biomass residues, which has been considered as a good inference of cellulose accessibility to cellulases [35]. Notably, the enzyme adsorption capacity was increased by 59% and 36% in the optimal green liquor pretreated biomass residues, compared to the raw material (Table 5). The Langmuir constant (K), another parameter determining the binding affinity between the enzyme and biomass, was also determined from adsorption kinetics analysis. As cellulase enzymes attach preferentially to cellulose than to lignin, high delignification led to a higher binding affinity (higher K value) between the enzyme and pretreated biomass residues. With respect to the optimal saccharification approach, the decrease of the K value is likely due to the difference in the chemical composition of pretreated biomass residues after the optimal pretreatments (Table S6). The results suggested that the optimal green liquor pretreatments may solubilize hydrophobic lignin to either produce more cellulose surfaces or enlarge pore sizes for enzyme loading. Despite large lignin and hemicellulose removal, the optimal delignification pretreatment showed relatively lower biomass porosity than that of the optimal saccharification as detected by Simons' stain, Congo red dye and enzyme adsorption assays, which may be due to the aggregation and collapse of neighboring cellulose microfibrils, thus restricting the interior accessible cellulose surfaces to cellulase enzymes for subsequent enzymatic hydrolysis [26]. It may also interpret why the optimal delignification approach had significantly lower hexose yield during enzymatic hydrolysis. Taken together, it confirmed that biomass porosity should be an integrative parameter accounting for biomass enzymatic saccharification in *Miscanthus* and other bioenergy crops.

3.6. Mechanism of enhanced biomass saccharification and delignification under optimal green liquor pretreatment

Based on the novel findings obtained in this study, a hypothetical model was thus proposed to elucidate integrated impacts on hexoses and bioethanol yields or lignin yield under the optimal green liquor pretreatment in *Miscanthus* (Fig. 5). (1) The optimal green liquor pretreatment initially reduced major wall polymers (lignin, pectin) levels and their major features in particular on cellulose CrI and DP. (2) The reduction of wall polymers levels and features consequently increased biomass porosity and cellulose accessibility, providing much more cellulase enzymes accession and loading for high biomass saccharification and bioethanol production. (3) The decreased lignin level, in particular on H-
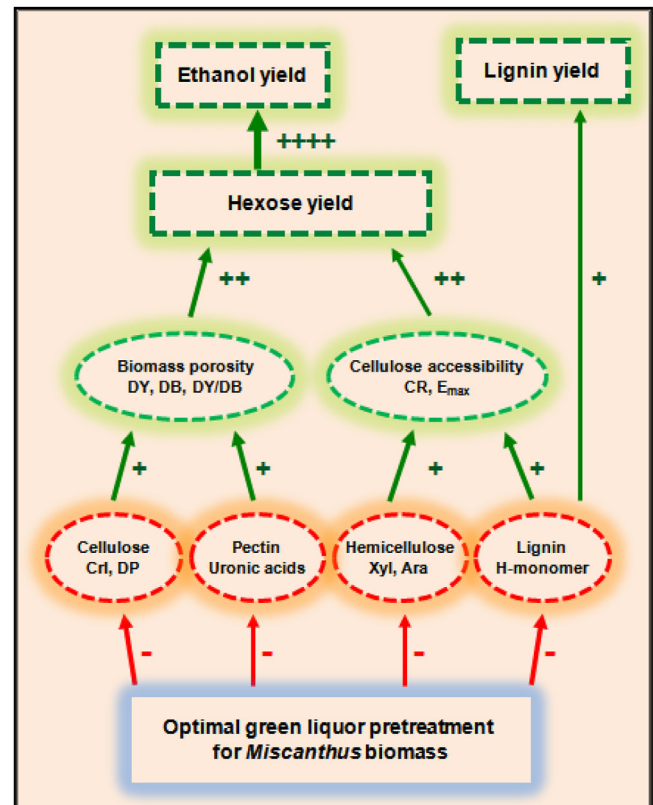

Fig. 5. A hypothetical model highlighting the integrated impacts on hexose and ethanol yields as well as the lignin recovery yield under green liquor pretreatment in bioenergy *Miscanthus*. Green/red arrows and "+"/"-" marks as positive/negative impacts, respectively. Xylose as Xyl and arabinose as Ara. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

monomer, led to an efficient lignin extraction with the optimal green liquor pretreatment. Therefore, this study could not only establish a green-like and cost-effective bioconversion technology for the maximum cellulosic ethanol production, but also provide a potential strategy for high lignin-derived biofuels and value-added biochemicals production with minimum waste release into the environment.

4. Conclusion

Using response surface methodological modeling, this study optimized green liquor pretreatments for enhancing biomass saccharification and delignification in *Miscanthus*. By comparison, the optimal saccharification approach led to relatively higher hexose yield with almost complete sugar-ethanol conversion rate, whereas the optimal delignification showed the highest delignification rate at 93% with lignin recovery of 89%. Two optimized pretreatments distinctively extracted lignin-carbohydrate complex, leading to significant alteration of wall polymer features and much increase of cellulose accessibility for effective enzymatic hydrolysis. Hence, this study has provided two optimal technologies respectively for bioethanol and lignin-derived biochemicals in bioenergy *Miscanthus* and beyond.

CRediT authorship contribution statement

Aftab Alam: Conceptualization, Investigation, Writing - original draft. **Youmei Wang:** Data curation, Resources. **Fei Liu:** Methodology. **Heng Kang:** Formal analysis, Software, Validation, Visualization. **Shang-wen Tang:** Formal analysis, Software, Validation, Visualization. **Yanting Wang:** Funding acquisition, Project administration, Supervision, Writing - review & editing. **Qiuming Cai:** Data curation, Resources. **Hailang Wang:** Data curation, Resources. **Hao Peng:** Methodology. **Qian Li:** Methodology. **Yajun Zeng:** Methodology. **Yuanyuan Tu:** Formal analysis, Software, Validation, Visualization. **Tao Xia:** Formal analysis, Software, Validation, Visualization. **Liangcai Peng:** Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

All authors agreed to state no conflict of the interest.

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Appendix A. Supplementary data

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