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Ammonium oxalate-extractable uronic acids positively affect biomass enzymatic digestibility by reducing lignocellulose crystallinity in *Miscanthus*

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HIGHLIGHTS

- Uronic acids-rich wall polymers positively affect biomass saccharification.
- Uronic acids of AO-extracted components play a major role in biomass digestion.
- Uronic acids negatively affect lignocellulose CrI for high biomass digestibility.
- Potential modification of uronic acids-rich polymers in bioenergy *Miscanthus*.

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ABSTRACT

Based on systems biology analyses of total 179 representative *Miscanthus* accessions, ammonium oxalate (AO)-extractable uronic acids could either positively affect biomass digestibility or negatively alter lignocellulose crystallinity at $p < 0.01$ or 0.05. Comparative analysis of four typical pairs of *Miscanthus* samples indicated that the AO-extractable uronic acids, other than hexoses and pentoses, play a predominant role in biomass enzymatic saccharification upon various chemical pretreatments, consistent with observations of strong cell tissue destruction *in situ* and rough biomass residue surface *in vitro* in the unique Msa24 sample rich in uronic acids. Notably, AO-extraction of uronic acids could significantly increase lignocellulose CrI at $p < 0.05$, indicating that uronic acids-rich polymers may have the interactions with β-1,4-glucan chains that reduce cellulose crystallinity. It has also suggested that increasing of uronic acids should be a useful approach for enhancing biomass enzymatic digestibility in *Miscanthus* and beyond.

1. Introduction

Plant cell walls represent abundant renewable biomass resource for biofuels and chemicals (Ragauskas et al., 2006; Pauly and Keegstra, 2008). However, lignocellulosic recalcitrance greatly leads to a costly biomass process (Himmel et al., 2007; Lynd et al., 2008). As a promising solution, genetic modification of plant cell walls has been proposed for reducing recalcitrance in bioenergy crops (Torney et al., 2007; Xie and Peng, 2010; Vega-Sánchez and Ronald, 2010). Hence, it becomes essential to identify the main factors of plant cell walls that affect biomass enzymatic digestibility (Zhu et al., 2008; DeMartini et al., 2013; De Souza et al., 2015). Recently, three major wall polymers (cellulose, hemicelluloses and lignin) have been characterized with distinct effects on biomass enzymatic saccharification in plants. In particular, cellulose crystallinity is the key factor negatively affecting biomass digestibility (Chang and Holtzapple, 2000; Laureano-Perez et al., 2005; Zhang et al., 2013). However, little is reported about the uronic acids-existed wall polymers effects on biomass enzymatic digestions.

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In grass, uronic acids exist in glucuronoarabinoxylans (GAX) and galactosyluronic acid-rich pectin (Kim and Carpita, 1992), which could be effectively extracted with ammonium oxalate (AO), a classic chelating agent (Peng et al., 2000). In principle, glucuronic acids (GlcA) occur in the non-reducing branches attached to xylan chains, while galacturonic acid (GalA) is found primarily in pectic substances (Roberts et al., 1968). It has been reported that hemicelluloses through its arabinose substitution degree of glucuronoarabinoxylans (GAX) could negatively affect lignocellulose crystallinity for high biomass digestibility in *Miscanthus* (Xu et al., 2012; Li et al., 2013). However, due to their low levels in mature biomass residues (Vogel, 2008; Mohnen, 2008), much remains unknown about the uronic acids-rich wall polymers effects on biomass enzymatic saccharification in plants (Xiao and Anderson, 2013).

Miscanthus is a typical C4 perennial grass with extremely high biomass yield, and thus has been considered as one of leading energy crops for biofuel feedstock (Lewandowski et al., 2003; Angelini et al., 2009; Xie and Peng, 2010). As *Miscanthus* has well adaptations to various environmental conditions, we have collected more than thousand of natural *Miscanthus* accessions nationwide and determined hundreds of the representative samples with diverse cell wall compositions (Huang et al., 2012; Zhang et al., 2013). In this study, we initially performed systems biology analysis of total 179 representative *Miscanthus* accessions and then examined four typical pairs of *Miscanthus* samples in order to find out the AO-extractable uronic acids role in biomass enzymatic digestions under various chemical pretreatments.

2. Methods

2.1. Plant sample collection

Total 179 *Miscanthus* samples from four major *Miscanthus* species (*Miscanthus sinensis*-Msi, *Miscanthus floridulus*-Mfl, *Miscanthus sacchariflorus*-Msa, and *Miscanthus lutarioriparius*-Mlu) were harvested from Hunan experimental field, and the mature stem were collected and dried at 50 °C after inactivated at 105 °C for 10 min. The dried tissues were ground through 40 mesh and stored in the dry-container until use.

2.2. Wall polymer fractionation

The procedure of plant cell wall fractionation was described by Peng et al. (2000) with minor modification by Huang et al. (2012). The well-mixed powder of biomass sample was initially ground under the potassium phosphate buffer (pH 7.0). The remaining pellets were consequentially extracted with chloroform–methanol (1:1, v/v) and DMSO–water (9:1, v/v) to removal lipid and starch. The remaining pellets, defined as total crude cell walls, were treated with 0.5% (w/v) ammonium oxalate (AO) for 1 h at 100 °C, washed with distilled water for 4–5 times, and combined as AO-extractable wall polymers. The remaining residues were further treated with 4 mol/L KOH and 1.0 mg/mL sodium borohydride for 1 h at 25 °C, and the combined supernatants were neutralized and lyophilized as KOH-extractable hemicelluloses. The remaining residues were hydrolyzed with 2 mol/L trifluoroacetic acid (TFA) at 121 °C in autoclave (15 psi) for 1 h, washed with distilled water for 2–3 times and all supernatants were combined as the non-KOH-extractable hemicelluloses. The remaining pellet was used as crystalline cellulose. All experiments were carried out in biological triplicate.

2.3. Colorimetric determination of hexoses, pentoses and uronic acids

UV–VIS Spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd., Shanghai, China) was used for hexoses, pentoses and uronic acids assay as described by Huang et al. (2015). Hexoses were detected by anthrone/H_2SO_4 method (Fry, 1988), pentoses were measured by orcinol/HCl (Dische, 1962), and total uronic acids were assayed by *m*-hydroxybiphenyl/NaOH (Fry, 1988). As the high pentose levels affect the absorbance reading at 620 nm for hexoses assay, the deduction from pentoses was carried out for a final hexoses calculation by using a series of xylose concentrations for plotting the standard curve referred for the deduction, which was verified by GC–MS analysis. All experiments were conducted in biological triplicate.

2.4. Detection of three wall polymers

Cellulose was measured by hexoses assay, and hemicelluloses were detected by calculating total hexoses and pentoses of hemicelluloses fractions. Lignin was determined using a two-step acid hydrolysis method according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory (Sluiter et al., 2008).

2.5. Determination of monosaccharides and uronic acids by GC–MS

2.5.1. Monosaccharides

The AO fraction was dialyzed for 36 h under 4 °C, and 2.5 mL desalted solution was hydrolyzed with 2 mol/L TFA at 121 °C in autoclave (15 psi) for 1 h. *Myo*-inositol (100 μg) was added as the internal standard. After TFA hydrolysis, the supernatant was dried under vacuum at 38 °C. Distilled water (800 μL) and a freshly prepared solution of $NaBH_4$ (400 μL, 100 mg/mL in 6.5 mol/L aqueous NH_3) were added to the dried sample. The sample was incubated at 40 °C for 30 min, and the excess $NaBH_4$ was decomposed by adding 800 μL acetic acid. 400 μL sample was transferred into a 25 mL glass tube, and the acetic anhydride (4 mL) was added and mixed well. 1-Methylimidazole (600 μL) was added, and excess acetic anhydride was decomposed by adding distilled water (10 mL). Dichloromethane (3 mL) was added and mixed gently, and the sample remained static for phase separation. The collected lower phase was dehydrated by adding anhydrous sodium sulfate and analyzed by GC–MS (SHIMADZU GCMS-QP2010 Plus).

2.5.2. Uronic acids

The silanization reaction was conducted for two types of uronic acids assay as described by Wojtasik et al. (2011) with minor modifications. The AO fraction was dialyzed for 36 h, and 2.5 mL desalted solution was hydrolyzed with 2 mol/L TFA (final concentration) at 121 °C in autoclave (15 psi) for 1 h. *Myo*-inositol (10 μg) was added as the internal standard. The supernatant (2 mL) was dried under vacuum at 38 °C to remove TFA, and pyridine (50 μL) was added under nitrogen and incubated at 37 °C for 90 min. The *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (50 μL) was added under nitrogen and incubated at 30 °C for 120 min. The solution (50 μL) was diluted in 500 μL pyridine for GC–MS analysis.

2.5.3. GC–MS 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. GC temperature program for monosaccharide determination: from 155 °C (held for 23 min) to 200 °C (held for 5 min) at 3.8 °C/min, then rise to 300 °C for 2 min at the rate of 20 °C/min. GC temperature program for uronic acids determination: 50 °C held for 2 min, rise to 155 °C

(at the rate of 50 °C/min) and held for 25 min, then rise to 200 °C (at the rate of 3.5 °C/min) for 5 min, then to 300 °C (at the rate of 20 °C/min) for 2 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. Calibration curves of all analytes routinely yielded correlation coefficients 0.999 or better.

2.6. Measurement of cellulose crystalline index

X-ray diffraction (XRD) method was applied for measurement of cellulose crystallinity index (CrI) using Rigaku-D/MAX instrument (Ultima III, Japan) as described by Zhang et al. (2013). Standard errors of the CrI method were estimated at ±0.05–0.15 using five representative samples in triplicate.

2.7. Scanning electron microscopic (SEM) observation

2.7.1. Observation of biomass residue in vitro

The well-mixed biomass powder was pretreated with 1% (w/v) NaOH at 50 °C for 2 h or 1% (v/v) H_2SO_4 at 121 °C for 20 min, and sequentially hydrolyzed with the 6 mL (1.6 g/L) mixed-cellulases at 50 °C for 48 h as described below (Section 2.8). The remaining residues were washed with distilled water until pH 7.0. The sample was sputter-coated with gold and observed by SEM (JSM-6390/LV, Hitachi, Tokyo, Japan) as described by Xu et al. (2012) and Li et al. (2013). Each sample was observed for 5–10 times and the representative imagines were photographed in the study.

2.7.2. Observation of plant tissues in situ

The young stem tissues were cross-sectioned, and added with 1% (w/v) NaOH or 1% (v/v) H_2SO_4 under shaken at 150 rpm for 2 h at 50 °C. The stem tissues were then washed with distilled water until pH 7.0 and hydrolyzed with the 0.5 g/L mixed-cellulases at 25 °C for 3 h for the NaOH-pretreated or for 6 h for the H_2SO_4-pretreated. Samples were washed again with distill water, dried under air, and sputter-coated with gold in a JFC-1600 ion sputter (Mito City, Japan). Surface morphology of the treated-samples were sputter-coated with gold and observed by SEM. Each sample was observed for 5–10 times and the representative imagines were photographed in the study.

2.8. Biomass pretreatment and enzymatic hydrolysis

Chemical pretreatments were previously described by Zhang et al. (2013), Huang et al. (2015) with minor modifications.

2.8.1. H_2SO_4 pretreatment

The well-mixed powder of biomass sample was added with 6 mL H_2SO_4 at three concentrations (0.25%, 1%, 4%, v/v). The sealed sample tube was heated at 121 °C for 20 min in autoclave (15 psi) after mixing well. The tube was shaken at 150 rpm for 2 h at 50 °C, and centrifuged at 3000*g* for 5 min. The supernatant was collected for the determination of total sugars released from the acid pretreatment. The pellet was washed 5 times with 10 mL distilled water until all soluble sugars were extracted (verified by colorimetric determination of hexoses and pentoses), and the sample was stored at −20 °C for enzymatic hydrolysis. The samples with 6 mL distilled water were shaken for 2 h at 50 °C as the control. All samples were carried out in biological triplicate.

2.8.2. NaOH pretreatment

The well-mixed powder of biomass sample was added with 6 mL NaOH at three concentrations (0.5%, 1%, 4%, w/v). The sample tube was shaken at 150 rpm for 2 h at 50 °C, and centrifuged at 3000*g* for 5 min. The supernatant was collected for the determination of total sugars released from the alkali pretreatment. The pellet was washed 5 times with 10 mL distilled water until all soluble sugars were extracted, and stored at −20 °C for enzymatic hydrolysis. The samples with 6 mL distilled water were shaken for 2 h at 50 °C as the control. All samples were carried out in biological triplicate.

2.8.3. Enzymatic hydrolysis

The remaining residues from pretreatments of H_2SO_4 and NaOH were washed with 10 mL mixed-cellulases reaction buffer (0.2 M acetic acid–sodium acetate, pH 4.8). The washed residues were incubated with 6 mL (3.2, 2.0 g/L for the enzymatic digestibility analysis of 1% H_2SO_4/1% NaOH pretreatment of the 179 *Miscanthus* samples) mixed-cellulases containing β-glucanase ($\geqslant 6 \times 10^4$ U), cellulase ($\geqslant$600 U) and xylanase ($\geqslant 10 \times 10^4$ U) from Imperial Jade Bio-technology Co., Ltd. The samples were under shaken at 150 rpm at 50 °C for 48 h. After centrifugation at 3000*g* for 10 min, the supernatants were collected for sugar determination. The samples with 6 mL reaction buffer were shaken for 48 h at 50 °C as the control. All samples were carried out in biological triplicate.

2.9. Statistical analysis of data

Superior Performance Software Systems software package (SPSS 17.0, Inc., Chicago, IL) was applied for the related statistical analysis. Correlation analysis was performed using Spearman's rank correlation analysis at the two-sided 0.05 level of significance ($^{*}p < 0.05$, $^{**}p < 0.01$). The variation and regression analysis are developed using Origin 8.0 software (Microcal Software, Northampton, MA) for the best fit curve from the experimental data.

3. Results and discussions

3.1. Correlation between AO-extractable components and biomass digestibility

In our previous studies, the representative *Miscanthus* accessions have been determined with diverse cell wall compositions including three major wall polymers: cellulose, hemicelluloses and lignin (Xu et al., 2012; Zhang et al., 2013; Li et al., 2013). In the present work, varied AO-extractable wall components (hexoses, pentoses, uronic acids) were examined in the selected 179 *Miscanthus* samples (Fig. 1). Compared with three major wall polymers (Li et al., 2013), the AO-extractable wall components showed extremely low levels, but remained much variations from 0.4% to 2.24% with variable coefficient (CV) value at 28.48% (S1 and Fig. 1A). Among the AO-extractable components, hexoses displayed high CV value at 54.42%, whereas pentoses and uronic acids had similar variations with CVs at 24.23% and 28.93%, respectively.

Biomass digestibility (or biomass saccharification) has been measured by calculating hexoses yield (% cellulose) released from hydrolysis by a crude cellulase mixture of lignocellulose after various physical and chemical pretreatments (Xu et al., 2012; Huang et al., 2015). In this work, total 179 *Miscanthus* samples exhibited a diverse biomass enzymatic digestibility after 1% (v/v) H_2SO_4 or 1% (w/v) NaOH pretreatment (Fig. 1B). As a comparison, the pretreatment with 1% (v/v) H_2SO_4 could result in the hexoses yields ranged from 9.95% to 59.26%, whereas the 1% (w/v) NaOH pretreatment had the varied hexoses yields from 17.54% to 69.39%. Hence, the 179 *Miscanthus* samples exhibited a varied biomass enzymatic digestibility, which suggests that those samples could be

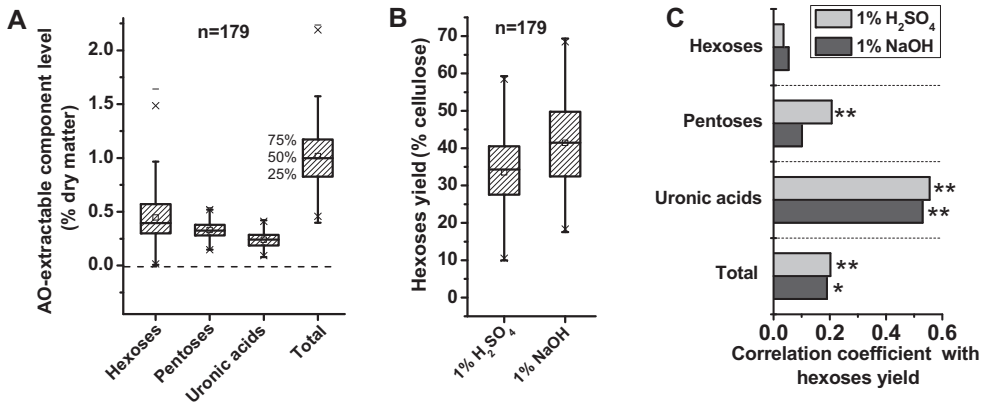

Fig. 1. Correlation between AO-extractable components and biomass digestibility in 179 *Miscanthus* accessions. (A) Variations of AO-extractable components; (B) variations of hexoses yields (% cellulose) released from enzymatic hydrolysis after 1% (v/v) H_2SO_4 and 1% (w/v) NaOH pretreatments; (C) correlation between AO-extractable components and hexoses yields ($n = 179$). * and ** indicated the significant correlations at $p < 0.05$ and 0.01, respectively. (A and B) The line and square within the box presented the median and mean values of all data; the bottom and top edges of the box indicated 25 and 75 percentiles of all data, respectively; and the bottom and top bars presented maximum and minimum values of all data, respectively ($n = 179$).

applicable for correlative analysis among biomass enzymatic digestibility and AO-extractable components.

Correlative analysis has been well applied into accounting for three major wall polymers effects on biomass saccharification in *Miscanthus* and other plants (Xu et al., 2012; Zhang et al., 2013; Li et al., 2013, 2014; Wu et al., 2013; Jia et al., 2014). Using 179 *Miscanthus* samples, correlative analysis was performed among AO-extractable components and biomass digestibility upon 1% (v/v) H_2SO_4 or 1% (w/v) NaOH pretreatment (Fig. 1C). In general, total AO-extractable components showed a positive correlation with biomass saccharification at significant levels ($p < 0.05$ or 0.01). Among three AO-extractable components, however, uronic acids exhibited much higher correlation coefficient values at 0.56 and 0.53 at $p < 0.01$ from 1% (v/v) H_2SO_4 and 1% (w/v) NaOH pretreatments, respectively (Fig. 1C). By comparison, pentoses were only positively correlated from 1% (v/v) H_2SO_4 pretreatment, and hexoses did not show any significant correlations. Therefore, the results suggested that the AO-extractable uronic acids should be the predominant factor positively affecting biomass enzymatic digestibility in *Miscanthus*.

3.2. Analysis of four typical pairs of Miscanthus samples

As the AO-extractable components cover much low proportion in cell wall composition, four typical pairs of *Miscanthus* samples were selected in this work. Each pair showed a similar composition of three major wall polymers (cellulose, hemicelluloses and lignin), but had significantly different AO-extractable constitutions including hexoses, pentoses and uronic acids (Table 1 and S2). Furthermore, each pair exhibited distinct monosaccharide and uronic acid compositions in the AO-extractable wall polymers (S3). As GalA and GlcA are respectively rich in the pectin and hemicellulosic glucuronoarabinoxylans, almost all samples (except Mlu01) had high proportions of GalA ranged from 62% to 95%, indicating that the AO-extracted wall polymers mainly contained pectin with minor GlcA. As each pairs exhibited relatively high and low biomass saccharification, they were applicable for testing AO-extractable components effect on biomass enzymatic digestions (Fig. 2 and S4). In details, Msi45 sample at Pair I had three AO-extractable components (hexoses, pentoses and uronic acids) significantly higher than that of its paired Mlu24 sample at $p < 0.01$ or 0.05 levels (Table 1 and S4). In terms of biomass saccharification, Msi45 showed much higher hexoses yield than that of Mlu24 at $p < 0.01$ or 0.05. Despite Pair II had pentoses and uronic acids alterations, other than hexoses, it also exhibited a significantly different biomass digestibility between two samples at $p < 0.01$ or 0.05 levels except 4% (v/v) H_2SO_4 pretreatment (Fig. 2, Table 1 and S4).

To demonstrate the uronic acids predominately roles in the AO-extractable components, Pairs III and IV samples were further examined, showing significant alterations (at $p < 0.05$) only on uronic acids levels, other than hexoses and pentoses (Table 1, S3 and S4). Despite Pairs III and IV had distinct GlcA and GalA proportions, the Msa03 and Msa24 samples with relatively high uronic acids levels, exhibited much higher hexoses yields than those of their paired samples (Mlu05 an Msi02) at significant levels ($p < 0.01$ or 0.05), except Pair IV from 4% (v/v) H_2SO_4 pretreatment. Notably, Pair III samples respectively showed the remarkably high hexoses yields increase rates by 104% and 203% from 0.25% (v/v) H_2SO_4 and 0.5% (w/v) NaOH pretreatments, compared with Pair I (52%, 43%) and Pair II (43%, 52%; S4). In addition, although Pair IV had much less uronic acids alteration by 38% than that of Pair III by 78%, the Pair IV samples exhibited higher hexoses yield increase rates (64%, 87%, 66%) that that of Pair I (46%, 33%, 8%) and Pair II (52%, 63%, 24%) from three concentrations of NaOH pretreatments. Therefore, the results supported that uronic acids were the predominant factor positively affecting biomass enzymatic digestibility under chemical pretreatments.

3.3. Observation of cell tissues in situ and biomass residues in vitro

To confirm uronic acids predominant role in biomass enzymatic digestion, stem cell tissues *in situ* and biomass residues *in vitro* were observed in Pair IV, which exhibited uronic acids alteration only (S5). Under scanning electron microscopy, the young stems of two samples at Pair IV did not show any visible differences, but Msa24 with relatively high biomass digestibility exhibited much more destructed and lost parenchyma cells than that of its paired Msi02 sample after pretreatment with 1% (v/v) H_2SO_4 and 1% (w/v) NaOH and sequential enzymatic hydrolyses. Hence, the effective destruction of parenchyma cells should be due to relatively high uronic acids levels in primary cell walls. Furthermore, Msa24 sample exhibited much rougher surface of biomass residues than that of Msi02 after 1% (v/v) H_2SO_4 and 1% (w/v) NaOH pretreatment and sequential enzymatic hydrolysis of the ground mature stem tissues, consistent with previous reports in *Miscanthus* (Xu et al., 2012; Zhang et al., 2013; Li et al., 2013). Therefore, both observations *in situ* and *in vitro* were strongly in

Table 1
AO-extractable components (% dry matter) in four typical pairs of *Miscanthus* samples.

Pairs	Samples	Hexoses		Pentoses		Uronic acids	
I	Msi45 (H)[#]	0.39 ± 0.02*	62.50%[@]	0.49 ± 0.02**	63.33%	0.23 ± 0.03*	21.05%
	Mlu24 (L)	0.24 ± 0.01		0.30 ± 0.02		0.19 ± 0.02	
II	Msi75 (H)	0.20 ± 0.02	−4.76%	0.83 ± 0.07**	295.24%	0.25 ± 0.04*	47.06%
	Mlu01 (L)	0.21 ± 0.04		0.21 ± 0.05		0.17 ± 0.03	
III	Msa03 (H)	0.46 ± 0.08	39.39%	0.50 ± 0.08	31.58%	0.16 ± 0.02*	77.78%
	Mlu05 (L)	0.33 ± 0.05		0.38 ± 0.05		0.09 ± 0.02	
IV	Msa24 (H)	0.29 ± 0.01	0%	0.39 ± 0.04	−9.30%	0.29 ± 0.02*	38.10%
	Msi02 (L)	0.29 ± 0.06		0.43 ± 0.04		0.21 ± 0.01	

* and ** indicated significant differences at pair by *t*-test at $p < 0.05$ and 0.01 ($n = 3$).
[#] Indicated the sample in the pair with relatively high (H) or low (L) biomass digestibility.
[@] Percentage of the increased or decreased level at pair.

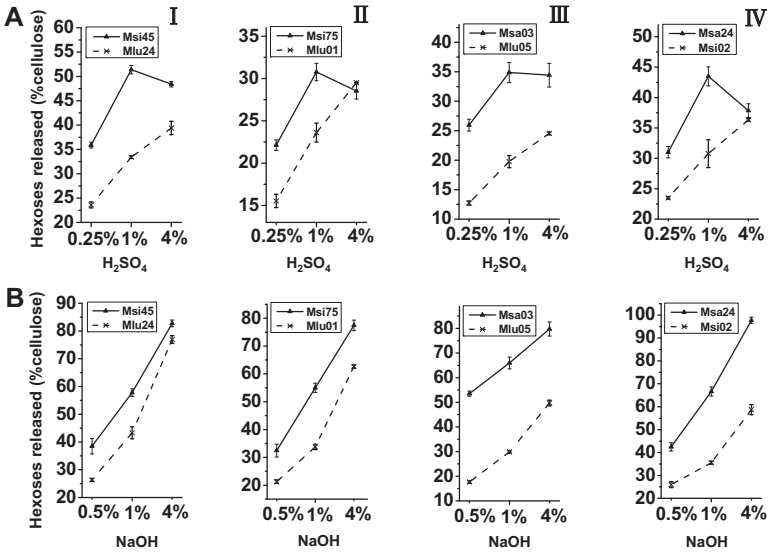

Fig. 2. Hexoses yields (% cellulose) released from enzymatic hydrolysis after pretreatments of NaOH (A) and H_2SO_4 (B) at three concentrations in the four typical pairs of *Miscanthus* samples. Three major wall polymers and AO-extractable components in four pairs of *Miscanthus* samples described in Table 1 and S2, and the values of hexoses yields were listed in S4. The bars indicated the means ± SD ($n = 3$).

supporting of findings about uronic acids predominant effect on biomass enzymatic saccharification upon various chemical pretreatments in *Miscanthus*.

3.4. Correlation between AO-extractable components and lignocellulose crystallinity

Lignocellulose crystallinity has been characterized as the key factor negatively affecting biomass enzymatic digestibility in *Miscanthus* and other plants (Chang and Holtzapple, 2000; Laureano-Perez et al., 2005; Xu et al., 2012; Zhang et al., 2013; Wu et al., 2013; Huang et al., 2015). To understand AO-extractable components effects on biomass saccharification, a correlation analysis was performed with lignocellulose crystalline index (CrI) by using 80 representative *Miscanthus* accessions (Fig. 3). Since the 80 *Miscanthus* accessions showed a normal distribution of lignocellulose CrI (Fig. 3A), it becomes applicable to calculate correlation coefficients. As a result, only AO-extractable uronic acids showed significantly negative correlation with cellulose CrI at $p < 0.01$ with high coefficient value at 0.59 (Fig. 3B) and R^2 value at 0.34 (Fig. 3C). By comparison, despite that hexoses were significantly correlated with CrI at $p < 0.05$, but the coefficient value remained much low at 0.23. The results indicated that AO-extractable uronic acids positively affect biomass saccharification probably by reducing lignocellulose crystallinity.

3.5. Mechanism on uronic acids predominate impacts on biomass saccharification

To find out direct evidence about uronic acids effects on lignocellulose crystallinity, cellulose CrI alterations were detected in four typical pairs of *Miscanthus* samples (Table 2). According to the cell wall fractionation procedure described in Materials and Methods, the crude cell walls were obtained after starch was extracted with DMSO fraction. As a result, the AO extraction from crude cell walls (DMSO residues) could significantly alter lignocellulose CrI with the increased rates (B − A/A) ranged from 1% to 6%. Notably, a significantly positive correlation was found between increased CrI rates and AO-extractable uronic acids levels at $p < 0.05$ in four pairs of *Miscanthus* samples with high coefficient value at 0.76 (Fig. 4A) and R^2 value at 0.58 (Fig. 4B). By comparison, both AO-extractable hexoses and pentoses did not show any significant correlation with increased CrI rates. The data thus indicated that AO-extraction of uronic acids could significantly increase lignocellulose crystallinity in *Miscanthus*.

Recently, it has been reported that hemicelluloses are the positive factor on biomass saccharification by negatively affecting lignocellulose crystallinity in *Miscanthus* (Xu et al., 2012). In particular, arabinose substitution degree of xylan plays a predominant role for hemicellulosic positive impacts, suggesting that the branched arabinose may have the interactions with β-1,4-glucan

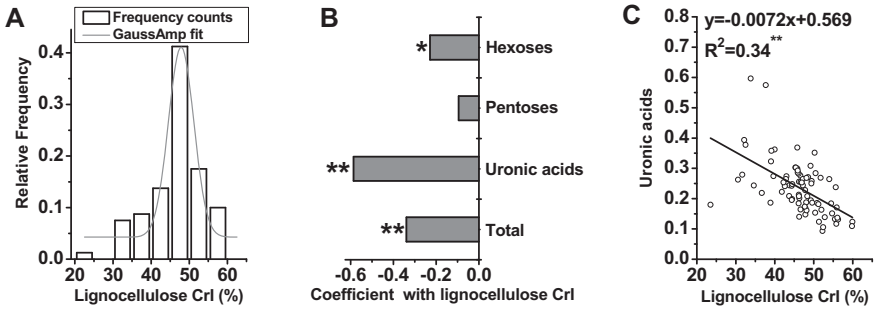

Fig. 3. Correlations between AO-extractable components and lignocellulose crystalline index (CrI). (A) Distribution of lignocellulose CrI values of 80 representative *Miscanthus* accessions; (B) correlation between AO-extractable components and lignocellulose CrI values of raw biomass materials in *Miscanthus* accessions ($n = 80$); (C) correlation distributions with coefficient R^2 values between AO-extractable uronic acids and lignocellulose CrI ($n = 80$); * and ** indicated the significant correlations at $p < 0.05$ and 0.01, respectively.

Table 2
Lignocellulose CrI alternation in four typical pairs of *Miscanthus* samples.

Pairs	Samples	CrI (%)*		Increase rate (%)
		DMSO residue (A)	AO residue (B)	(B − A)/A
I	Msi45 (H)#	50.08@	52.7	5.23
	Mlu24 (L)	59.23	60.87	2.77
II	Msi75 (H)	49.95	52.03	4.16
	Mlu01 (L)	60.26	62.04	2.96
III	Msa03 (H)	56.45	59.27	4.99
	Mlu05 (L)	58.68	59.44	1.29
IV	Msa24 (H)	50.82	53.97	6.18
	Msi02 (L)	49.69	51.98	4.60

* DMSO residue as total crude cell walls;tor 505(i75573.71l)-29rm(urum 28)Tj4082364-1.3426TDoxal6(rate)8326.7AO(B)Tf9(AO extraction2.386 1(of2-33599(crud,)-

chains that reduce lignocellulose crystallinity for high biomass digestibility (Li et al., 2013, 2014; Wu et al., 2013). Similarly, this work has also demonstrated that the AO-extractable uronic acids could positively affect biomass enzymatic digestibility by reducing lignocellulose crystallinity. There are rational evidences: (1) uronic acids have been examined with the highest correlative coefficient values with biomass enzymatic digestibility under various chemical pretreatments, compared with AO-extractable hexoses and pentoses; (2) the typical Pair IV sample showing high uronic acids only, has exhibited much stronger destruction of cell tissues *in situ* and rougher surface of biomass residues *in vitro* than those of its paired sample from chemical pretreatments and sequential cellulase digestions; (3) AO-extraction of uronic acids, rather than hexoses and pentoses, can significantly lead to an increased lignocellulose crystallinity at $p < 0.05$. Hence, the branched GlcA of xylans or GlaA of pectin extracted with AO should have an interaction with β-1,4-glucan chains that reduces lignocellulose crystallinity.

In addition, although cellulose microfibrils have been characterized with extensive interactions with pectin in *Arabidopsis* (Dick-Pérez et al., 2011), there are still not any evidences about how pectin interaction with cellulose. Hence, this study could conclude that the AO-extractable uronic acids that existed as pectic GalA and hemicellulosic GlcA may have the interactions with cellulose in *Miscanthus*, due to their significantly reducing of lignocellulose crystallinity. On the other hand, the non-AO-extractable uronic acids-existed wall polymers may mainly interact with major noncellulosic polymers (hemicelluloses and lignin), which remains under test about their effects on biomass enzymatic digestibility in the future.

3.6. Potential cell wall modification for enhancing biomass saccharification

Genetic modification of plant cell walls has been proposed as a promising solution for reducing biomass recalcitrance (Torney et al., 2007; Xie and Peng, 2010; Vega-Sánchez and Ronald, 2010). As plant biomass constitutes numerous different cell types with diverse wall components, identifying desirable cell walls is a priority work for enhancing biomass digestibility (Pauly and Keegstra, 2008; Knox, 2008). However, modification of three major wall polymers (cellulose, hemicelluloses, and lignin) is not a simple work, because it could largely affect plant growth and biomass yield (Pauly and Keegstra, 2008; Xie and Peng, 2010). In this study, we have found that the increased AO-extractable uronic acids in the natural *Miscanthus* accessions (Pairs III and IV), could enhance biomass enzymatic saccharification upon various chemical pretreatments. Since AO-extractable wall components cover extremely low proportions in plant cell walls, genetic modification of uronic acids-rich wall polymers may become a relatively simply approach for enhancing biomass digestibility in bioenergy crops.

Due to complicated structures of plant cell walls, however, minor modification of AO-extractable components may also cause cell wall network dynamic alteration. Hence, a correlation analysis was also performed between AO-extractable components and two

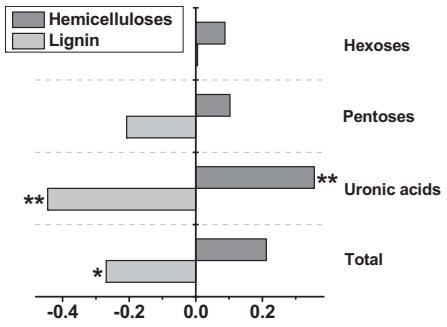

Fig. 5. Correlation between AO-extractable components and wall polymers (hemicelluloses and lignin) in 179 *Miscanthus* accessions * and ** indicated the significant correlations at $p < 0.05$ and 0.01, respectively.

major wall polymers (hemicelluloses and lignin) using 80 representative *Miscanthus* samples (Fig. 5). Notably, AO-extractable uronic acids showed significantly correlations either positively with hemicelluloses or negatively with lignin level at $p < 0.01$ ($n = 80$), whereas total AO-extractable components only displayed a negative correlation with lignin at $p < 0.05$. By comparison, AO-extractable hexoses and pentoses did not show any significant correlation. The data suggested that raising AO-extractable uronic acids in plant cell walls may coordinately result in hemicelluloses increase and lignin reduction in transgenic plants. As hemicelluloses and lignin have respectively been reported as positive and negative factors on biomass saccharification in *Miscanthus* (Li et al., 2013; Xu et al., 2012), genetic modification of plant cell walls by increasing uronic acids proportion should be a powerful approach for enhancing biomass enzymatic digestibility in bioenergy crops.

4. Conclusions

Using large population of *Miscanthus* accessions, AO-extractable uronic acids have been determined with positive effects on biomass enzymatic digestibility under various chemical pretreatments. Analysis of four typical pairs of *Miscanthus* samples has indicated that AO-extractable uronic acids, other than hexoses and pentoses, could significantly reduce lignocellulose crystallinity for high biomass saccharification. It suggests that uronic acids-existed wall polymer modification is a promising approach for enhancing biomass enzymatic digestibility in *Miscanthus*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.07.099.

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