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Arabinose substitution degree in xylan positively affects lignocellulose enzymatic digestibility after various NaOH/H_2SO_4 pretreatments in *Miscanthus*

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HIGHLIGHTS

- Xylan effect on biomass digestion remains unknown in *Miscanthus* and other grasses.
- Hemicelluloses positively affect biomass digestibility upon various pretreatments.
- Arabinose substitution degree in xylan is the key factor on biomass saccharification.
- Arabinose association with cellulose negatively affects cellulose crystallinity.
- Provide biomass digestion mechanism and a goal for xylan genetic modification.

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ABSTRACT

Xylans are the major hemicelluloses in grasses, but their effects on biomass saccharification remain unclear. In this study, we examined the 79 representative *Miscanthus* accessions that displayed a diverse cell wall composition and varied biomass digestibility. Correlation analysis showed that hemicelluloses level has a strong positive effect on lignocellulose enzymatic digestion after NaOH or H_2SO_4 pretreatment. Characterization of the monosaccharide compositions in the KOH-extractable and non-KOH-extractable hemicelluloses indicated that arabinose substitution degree of xylan is the key factor that positively affects biomass saccharification. The xylose/arabinose ratio after individual enzyme digestion revealed that the arabinose in xylan is partially associated with cellulose in the amorphous regions, which negatively affects cellulose crystallinity for high biomass digestibility. The results provide insights into the mechanism of lignocellulose enzymatic digestion upon pretreatment, and also suggest a goal for the genetic modification of hemicelluloses towards the bioenergy crop breeding of *Miscanthus* and grasses.

1. Introduction

Lignocellulose, the most abundant biomass on earth, represents a major source of carbon for biofuels and other chemical compounds (Ragauskas et al., 2006). Non-food perennial grasses (such

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as *Miscanthus*) make up the bulk of lignocellulosic resources and their utilization does not compete with food supplies. However, plant biomass has evolved a complex structure for resisting breakdown by mechanical and microbial forces (Himmel et al., 2007). Genetic modification of plant cell walls was proposed as a promising solution for efficient biomass processing, which reduces biomass recalcitrance (McCann and Carpita, 2008; Vega-Sanchez and Ronald, 2010; Xie and Peng, 2011). Hence, the effort would be greatly aided by understanding of the fundamental relationship between cell-wall composition and sugar release through pretreatment and enzymatic hydrolysis.

Hemicelluloses are a heterogeneous class of polysaccharides with various sugar units (Scheller and Ulvskov, 2010). In the secondary cell walls of grasses, xylans are the major hemicelluloses, which have the common feature of a β-(1 → 4)-linked xylose chain backbone. Xylans are most commonly substituted by α-L-arabinofuranosyl units on the C2- and/or C3-position in arabinoxylan, and α-D-glucopyranosyl uronic units or its 4-O-methyl derivative side chains on the C2-position in glucuronoarabinoxylan (GAX) (Girio et al., 2010). The most important biological role of hemicelluloses is their contribution to the cross-linked interaction with cellulose and lignin. This interaction strengthens the cell wall and embeds the crystalline cellulose elementary fibrils. In grasses, some of α-(1–3)-linked arabinofuranosyl residues can be esterified with *p*-coumaric or ferulic acid, with the latter forming cross-links with other GAX chains or with lignin. Cross-linking through ferulate esters renders the cell wall recalcitrant to digestion (Scheller and Ulvskov, 2010). However, the functional significance of the detail structure of arabinoxylans in grasses (e.g., degree of branching and spatial arrangement of arabinosyl substituents) is widely unknown.

Effective bioconversion of bioresources is currently based on pretreatment technologies, which reduces biomass recalcitrance. Numerous pretreatment processes have been studied over the past years (Saha et al., 2005; Garlock et al., 2011; Heiss-Blanquet et al., 2011; Huang et al., 2012). In particular, pretreatments with diluted acid and alkaline using various processes have been well studied. The main reaction that occurs during dilute acid pretreatment is the hydrolysis of hemicelluloses. However, strong acids can break the glycosidic linkages of polysaccharides, freeing individual monosaccharide components (Saha et al., 2005; Galbe and Zacchi, 2007). On the other hand, alkali pretreatment breaks down the intermolecular ester bonds that cross-link lignin with hemicelluloses, thereby solubilizing lignin and making cellulose more accessible to enzymes during hydrolysis (Macdonald et al., 1983; Hendriks and Zeeman, 2009).

It has been reported that the removal of hemicelluloses increases the ability of cellulolytic enzymes to hydrolyze cellulose (Palonen et al., 2004; Himmel et al., 2007), Systematic removal of hemicelluloses via acidic or enzymatic processes reportedly results in a marked reduction in the cellulase load required to convert cellulose (Torget et al., 1992; Yang and Wyman, 2004). Furthermore, the removal of acetyl groups in xylan increases biomass digestibility (Chang and Holtzapple, 2000; Zhang et al., 2011). The side chains of hemicelluloses are presumed to be cross-linked with lignin in lignin–carbohydrate complexes, and reducing the number of side chains have been suggested to increase cellulose accessibility and hydrolysis (Abramson et al., 2010). By contrast, the addition of hemicelluloses side chains may increase water solubility and decreases hydrogen bonding with cellulose microfibrils (Pauly and Keegstra, 2008). More recently, hemicelluloses are reported as the dominant factor positively determining biomass enzymatic digestibility after pretreatments with NaOH and H_2SO_4 (Xu et al., 2012). However, much remains unknown about the effects of hemicelluloses monosaccharides composition on biomass saccharification in plants.

Miscanthus is a typical C4 perennial grass with an averaged biomass yield at 420 million Mg/year in China, and is currently regarded as a leading candidate for biofuel feedstocks (Lygin et al., 2011; Xie and Peng, 2011). Given the high adaptability of *Miscanthus* to various environmental conditions, 1400 natural *Miscanthus* accessions that presented various ecological types and germplasms in China were obtained (Xie and Peng, 2011). Then, up to 200 typical samples that displayed diverse cell wall compositions were identified (Huang et al., 2012). In the present work, an integrative analysis was performed between hemicelluloses composition and biomass digestion using 79 representative *Miscanthus* accessions. The degree of arabinose substitution in xylan was found to be the key factor that enhances the enzymatic digestibility of lignocellulose after various pretreatments. Individual enzyme hydrolysis analysis was also conducted to determine the mechanism of biomass enzymatic digestibility in *Miscanthus* and other grasses.

2. Methods

2.1. Plant materials

The *Miscanthus* samples were typically selected from *Miscanthus* germplasm accessions collected in China. The samples were harvested from an experimental field in Hunan. The rice, wheat, maize and sweet sorghum were harvested from Wuhan. The mature stem tissues were collected and dried at 50 °C after inactivation at 105 °C for 30 min. The dried tissues were ground through a 40 mesh screen and stored in a dry container until use.

2.2. Plant cell wall fractionation and polymer determination

2.2.1. Plant cell wall fractionation

The plant cell wall fractionation method was used to extract cellulose and hemicelluloses, as described by Peng et al. (2000). The crude cell wall material was suspended in 0.5% (w/v) ammonium oxalate and heated for 1 h in a boiling water bath, and the supernatants were combined as total pectin. The remaining pellet was suspended in 4 M KOH containing 1.0 mg/mL sodium borohydride for 1 h at 25 °C, and the combined supernatant was neutralized, dialyzed and lyophilized as hemicelluloses. The non-KOH-extractable residue defined as crude cellulose, was further extracted with acetic–nitric acids–water (8:1:2) for 1 h at 100 °C and the remaining pellet was regarded as crystalline cellulose. All experiments were carried out in triplicate.

2.2.2. Hemicelluloses monosaccharide determination by GC–MS

Trifluoroacetic acid (TFA) and myo-inositol were purchased from Aladdin Reagent Inc. Acetic anhydride and acetic acid were obtained from Sinopharm Chemical Reagent Co., Ltd. 1-Methylimidazole was purchased from Sigma–Aldrich Co. LLC. Monosaccharide standards including L-rhamnose, L-arabinose, L-fucose, D-xylose, D-galactose, D-glucose and D-mannose, were obtained from Sinopham Chemical Reagent Co., Ltd.

The sample preparation and GC–MS running were previously described by Xu et al. (2012) with minor modification.

2.2.2.1. Acid hydrolysis. The combined supernatants from 4 M KOH fraction were dialyzed for 36 h after neutralization with acetic acid. Prior to GC–MS running, the sample from the neutralized KOH-extractable supernatant or the non-KOH-extractable residues was hydrolyzed by 2 M TFA to release free monosaccharides in the sealed tube at 121 °C in autoclave (15 psi) for 1 h. Myo-inositol (200 μg) was added as the internal standard. The supernatant was dried under vacuum at 38 °C to remove TFA.

2.2.2.2. Derivatisation of monosaccharides to alditoal acetates. Distilled water (800 μL) and a freshly prepared solution of $NaBH_4$ (400 μL, 100 mg/mL in 6.5 M aqueous NH_3) were added to each sample. Sample was incubated at 40 °C for 30 min, and the excess $NaBH_4$ was decomposed by adding acetic acid (800 μL). Four hundred microliters of sample was moved 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, mixed gently, and centrifuged (2000*g*, 10 s) for phase separation. The collected lower phase was dehydrated by adding with anhydrous sodium sulfate and stored at −20 °C until analyzed by GC–MS (SHIMADZU GCMS-QP2010 Plus).

2.2.2.3. GC–MS analytical conditions. Restek Rxi-5 ms, 30 m × 0.25 mm ID × 0.25um 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 for12 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. Calibration curves of all analytes routinely yielded correlation coefficients 0.999 or better.

2.3. Determination of biomass digestibility

2.3.1. Biomass pretreatment and enzymatic hydrolysis

Chemical pretreatments and the residues enzymatic hydrolysis from pretreatments were performed as previously described by Huang et al. (2012).

2.3.2. Colorimetric assay of total hexoses and pentoses

UV–VIS Spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd., Shanghai, China) was used for the absorbance reading. Hexoses were detected using the anthrone/H_2SO_4 method (Fry, 1988), and pentoses were measured using the orcinol/HCl method (Dische, 1962). Anthrone was purchased from Sigma–Aldrich Co., LLC., and ferric chloride and orcinol were obtained from Sinopharm Chemical Reagent Co., Ltd. The standard curves for hexoses and pentoses were drawn using D-glucose and D-xylose as standards (purchased from Sinopharm Chemical Reagent Co., Ltd.), respectively. For cellulose content assay, sample was dissolved in 67% H_2SO_4 and total hexoses were determined by the anthrone/H_2SO_4 method. Total sugar yield from pretreatment and enzymatic hydrolysis was subject to the sum total of hexoses and pentoses. Considering the high pentoses level can affect the absorbance reading at 620 nm for hexoses content by anthrone/H_2SO_4 method, the deduction from pentoses reading at 660 nm was carried out for final hexoses calculation. A series of xylose concentrations were analyzed for plotting the standard curve referred for the deduction, which was verified by GC–MS analysis. All experiments were carried out in triplicate.

2.4. Scanning electron microscopic (SEM) observation

The biomass samples were pretreated with 1% NaOH or 1% H_2SO_4, and hydrolyzed with the mixed-cellulases. The remaining residues were washed with distilled water until pH 7.0. The surface morphology of the sample was sputter-coated with gold and observed by scanning electron microscope (SEM JSM-6390/LV, Hitachi, Tokyo, Japan).

2.5. Enzymatic-digestion analysis of crude cellulose

Cellobiohydrolase I (CBH I) from *Trichoderma longibrachiatum*, endo-1,4-β-glucanase II (EG II) from *Asperillus niger*, β-glucosidase from *Agrobacterium* sp. and endo-1,4-β-xylanase (xylanase) from *Thermotoga maritime* were purchased from Megazyme International Ireland Ltd. The mixed-cellulases enzyme containing β-glucanase ($\geqslant 6 \times 10^4$ U), cellulase ($\geqslant$600 U) and xylanase ($\geqslant 1.0 \times 10^5$ U) was purchased from Imperial Jade Bio-technology Co., Ltd. (Ningxia 750002, China). Bovine serum albumin (BSA) was obtained from Aladdin Reagent Inc. The methods for enzymatic analysis were shown in Fig. S1, and all experiments were carried out in triplicate.

2.6. Detection of cellulose crystallinity

X-ray diffraction (XRD) method was previously described by Xie et al. (2013) for cellulose crystallinity index (CrI) detection using Rigaku-D/MAX instrument (Uitima III, Japan). The powders of cellulose samples were laid on the glass sample holder (35 × 50 × 5 mm) and were analyzed under plateau conditions. Ni-filtered Cu Kα radiation (k = 0.154056 nm) generated at voltage of 40 kV and current of 18 mA, and scanned at speed of 0.0197°/s from 10° to 45°. The crystallinity index (CrI) was estimated using the intensity of the 200 peak (I200, h = 22.5°) and the intensity at the minimum between the 200 and 110 peaks (I_{am}, h = 18.5°) as the follow: $CrI = 100 \times (I200 - I_{am})/I200$. I200 represents both crystalline and amorphous materials while I_{am} represents amorphous material. The standard error of the CrI method was detected at ±0.05–0.15 (n = 5).

2.7. Statistical calculation of correlation coefficients

Correlation coefficients were generated by performing Spearman rank correlation analysis for all pairs of measured traits across the whole population. This analysis used average values calculated from all original determinations for a given traits pair.

3. Results

3.1. Diverse cell wall composition and varied biomass digestibility in Miscanthus

Among the C4 perennial grasses, *Miscanthus* displayed superior biomass yields and high adaptability to various environments. Considering natural *Miscanthus* accessions include various ecological types and genetic germplasms (Xie and Peng, 2011), 79 representative *Miscanthus* samples that showed a large variation of plant cell wall composition were selected, which include cellulose content ranging from 23.22% to 42.74% (% dry matter), hemicelluloses from 15.85% to 28.46%, lignin from 19.28% to 31.10%, and pectin from 0.46% to 2.48% (Fig. 1A).

Due to the diverse compositions of plant cell walls, biomass enzymatic digestion was analyzed after various chemical pretreatments. The biomass digestibility was measured by accounting for either the hexoses yield (hexoses/cellulose) released from hydrolysis by a crude cellulase mixture of lignocellulose after chemical pretreatment, or the total sugar yield (hexoses and pentoses/dry weight) from both pretreatment and enzymatic hydrolysis. After treatment with NaOH (0.5%, 1%, and 4%) or with H_2SO_4 (0.25%, 1%, and 4%), the 79 *Miscanthus* samples exhibited largely varied biomass digestibility either as hexoses yield released from enzymatic hydrolysis (Fig. 1B) or as total sugar yield obtained from both pretreatment and sequential enzymatic hydrolysis (Fig. 1C). This finding is consistent with their diverse cell wall compositions as

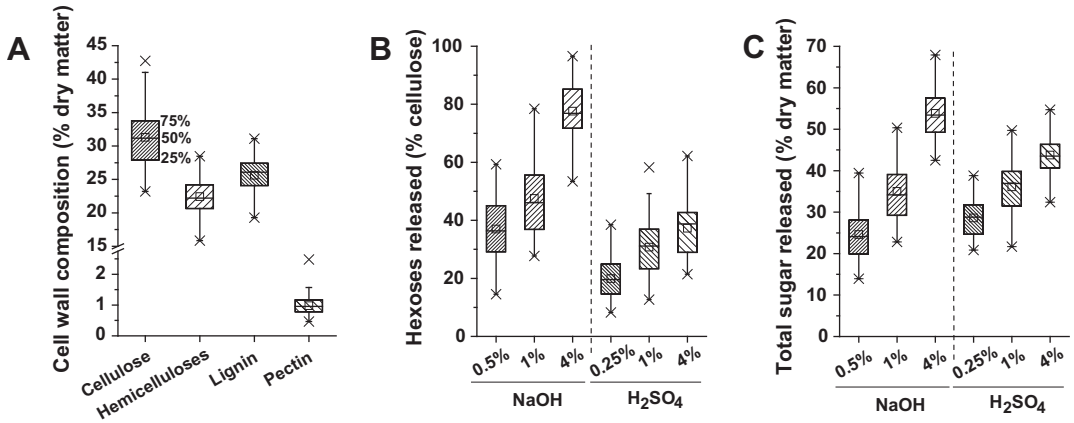

Fig. 1. Variation of cell wall composition and biomass digestibility of *Miscanthus* samples. The line and square within the box presented the median and mean values of all data; the bottom and top edges of the box indicated 25 and 75 percentiles of all data, respectively; and the bottom and top bars presented maximum and minimum values of all data, respectively. (A) Cell wall components ($n = 79$); (B) hexoses yield (%cellulose) released from enzymatic hydrolysis after pretreatment ($n = 79$); (C) total sugar yield (%dry matter) released from both pretreatment and enzymatic hydrolysis ($n = 79$).

described above. In particular, several *Miscanthus* samples had their hexoses yield of 96% or total sugar yield of 68% upon pretreatment with 4% NaOH, whereas the samples showed a maximum hexoses yield of less than 63% or a total sugar yield of 55% after pretreatment with 4% H_2SO_4.

3.2. Correlation between hemicelluloses and biomass saccharification

Although the effects of cellulose and lignin on biomass digestibility have been reported (Puri, 1984; Baucher et al., 1999; Chen and Dixon, 2007; Yoshida et al., 2008; Studer et al., 2011), little is known about the hemicelluloses composition in plants. In the current work, the monosaccharide compositions of two types hemicelluloses (KOH-extractable and non-KOH-extractable) were determined in the selected *Miscanthus* samples (Table 1). The two types of hemicelluloses showed a typical xylan composition with two major monosaccharides (xylose and arabinose). Considering the xylose/arabinose (Xyl/Ara) ratio can negatively account for the degree of arabinose substitution of xylan, we observed that the KOH-extractable hemicelluloses had a much higher Xyl/Ara (10.93) than the non-KOH-extractable hemicelluloses (3.43). Hence, the two types of hemicelluloses in *Miscanthus* have distinct xylan structures.

An in-depth statistical analysis was performed to characterize the correlations between hemicelluloses and biomass digestibility. Initially, a positive correlation was observed between the hemicelluloses level and the hexoses yield from enzymatic hydrolysis after pretreatment with NaOH or H_2SO_4 (Fig. 2). Despite the low R^2 values of the correlation equations (0.1–0.185), all of the correlation coefficients reached extremely significant levels at $p < 0.01$ ($n = 79$), as well as at $p < 0.05$ except for the 4% NaOH pretreatment (Fig. 2). Hence, the increase in total hemicelluloses level resulted in enhanced biomass digestibility in *Miscanthus*. The correlation coefficients were also calculated between the two major monosaccharides (xylose and arabinose) in the two types of hemicelluloses and the biomass digestibility. The *Miscanthus* samples displayed a significantly positive correlation between arabinose level and hexoses yield in the two types of hemicelluloses after pretreatment with three concentrations of NaOH (Fig. 3). Although the xylose level positively correlated with the KOH-extractable hemicelluloses, a significantly negative correlation ($p < 0.01$) was observed between the Xyl/Ara ratio and biomass digestibility in the two types of hemicelluloses. An extremely similar correlation was found in the H_2SO_4-pretreated *Miscanthus* samples (Fig. 4), which suggests that the degree of arabinose substitution in xylan is the key factor that positively affects biomass digestibility of *Miscanthus* after various pretreatments.

Table 1
Variation of two-types of hemicelluloses in *Miscanthus* samples ($n = 79$).

	Rha	Fuc	Ara	Xyl	Man	Glu	Gal	Total	Xyl/Ara
KOH-extractable[a]	0.59 (0.05%)[c]	0.01 (0.001%)	100.40 (8.44%)	1058.53 (88.95%)	0.69 (0.06%)	23.95 (2.01%)	5.83 (0.49%)	1189.99 (80.18%)	10.93
	(ND[d]∼2.69)	(ND∼0.27)	(44.86–146.59)	(741.51–1421.69)	(0.00–4.04)	(6.16–75.62)	(1.76–13.45)	(827.95–1591.76)	
Non-KOH-extractable[b]	2.85 (0.97%)	0.04 (0.01%)	63.82 (21.72%)	210.80 (71.67%)	1.95 (0.66%)	ND	14.59 (4.96%)	294.12 (19.82%)	3.43
	(1.21–5.24)[e]	(ND∼0.30)	(31.13–105.94)	(125.96–336.21)	(0.77–7.16)		(6.09–31.06)	(177.62–465.72)	
Total	3.44 (0.23%)	0.06 (0.01%)	164.29 (11.07%)	1269.33 (85.53%)	2.64 (0.18%)	23.95 (1.61%)	20.41 (1.38%)	1484.12 (100%)	7.97
	(1.24–6.55)	(ND∼0.40)	(75.99–231.84)	(886.0–1629.70)	(0.77–9.83)	(6.16–75.62)	(8.98–39.83)	(1048.58–1883.43)	

[a] Supernatant of 4 M KOH extraction.
[b] Residue of 4 M KOH extraction.
[c] Mean value and percentage.
[d] Not detectable.
[e] Minimum and maximum values.

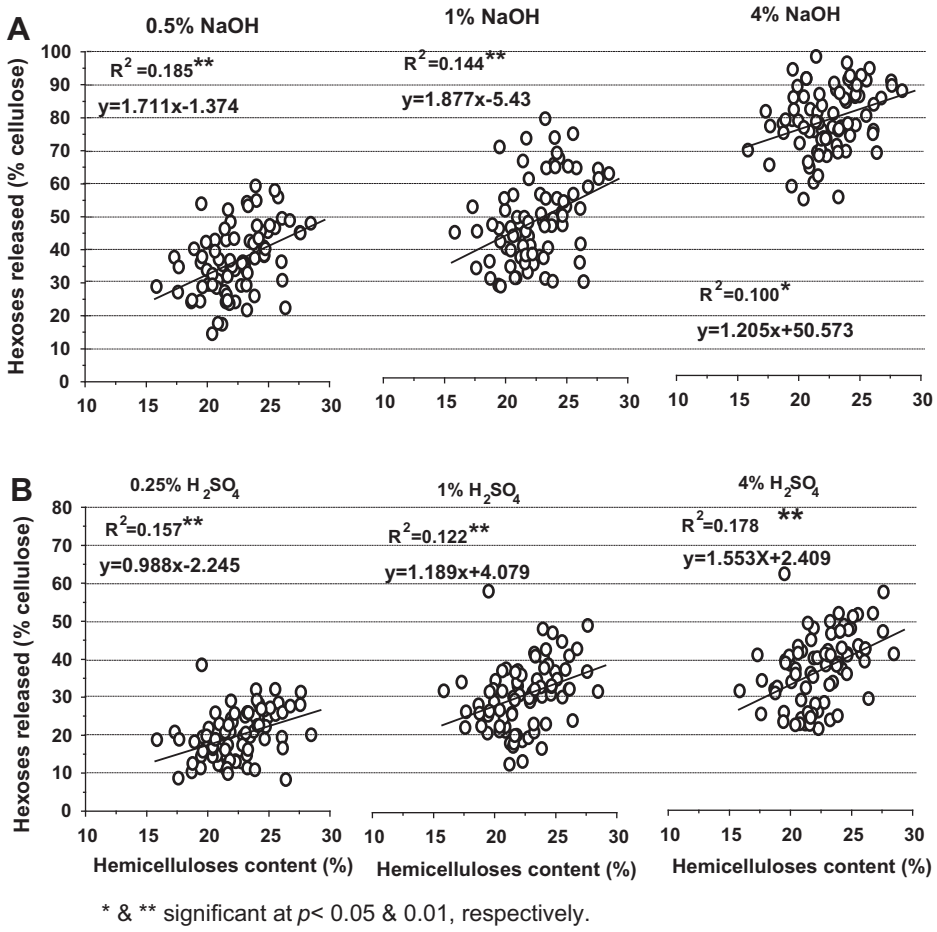

Fig. 2. Correlation analysis between total hemicelluloses content and lignocellulose enzymatic digestibility after pretreatments with three concentrations of (A) NaOH and (B) H_2SO_4 (n = 79).

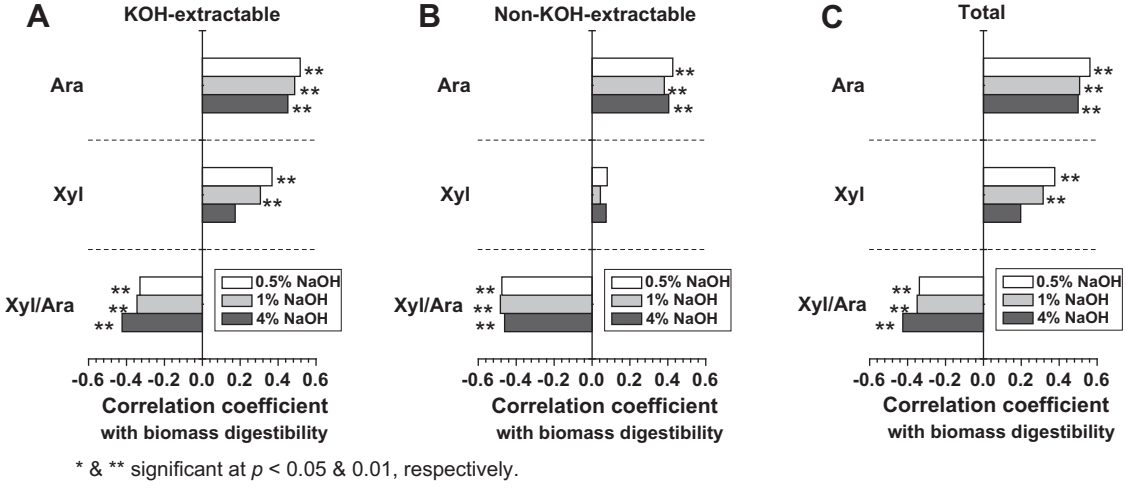

Fig. 3. Correlation analysis between xylan and lignocellulose enzymatic digestibility after pretreatments with three concentrations of NaOH (n = 79). (A) 4 M KOH-extractable xylan; (B) non-4 M KOH-extractable xylan; (C) total xylan.

3.3. Mechanism of lignocellulose enzymatic hydrolysis

To understand the effects of Xyl/Ara on lignocellulose digestibility, individual enzyme digestion analysis of the crude cellulose substrates obtained from 4 M KOH extraction of the biomass (Fig. S1) was performed using typical *Miscanthus* samples (Msi56 and Msa01) and other grass plants (rice, wheat, maize and sweet sorghum) that presented different cell wall compositions in the mature tissues (Table S1). By comparison, the Msi56 sample exhibited higher biomass enzymatic digestibility than Msa01 after pretreatment with NaOH, H_2SO_4, or 4 M KOH (Fig. 5A). Under scanning electron microscopy, the Msi56 sample exhibited a rougher

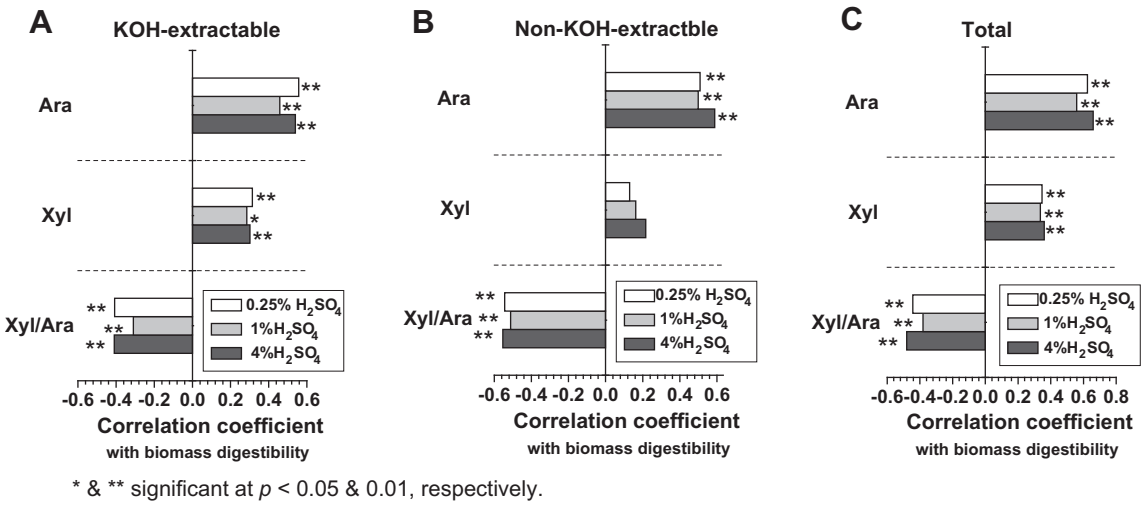

Fig. 4. Correlation analysis between xylan and lignocellulose enzymatic digestibility after pretreatments with three concentrations of H_2SO_4 ($n = 79$). (A) 4 M KOH-extractable xylan; (B) non-4 M KOH-extractable xylan; (C) total xylan.

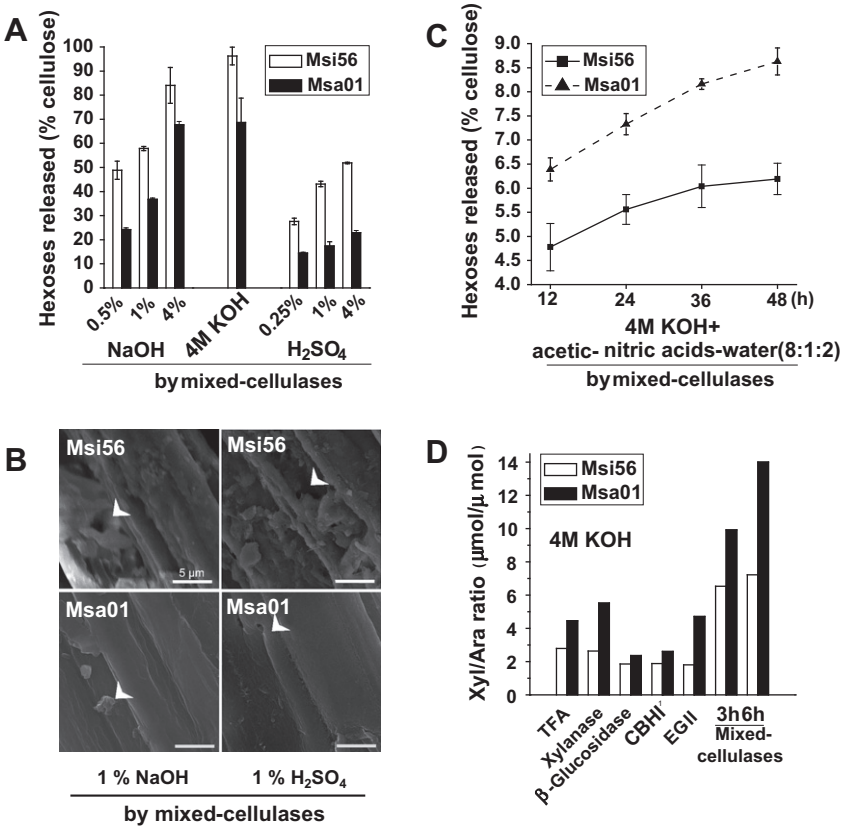

Fig. 5. Individual-enzyme digestion analysis of the crude cellulose substrates obtained after 4 M KOH extraction of biomass. (A) Hexoses yield released by the mixed-cellulases hydrolysis after pretreatments of the raw biomass in the selected *Miscanthus* samples. (B) SEM imagine of the residue obtained from biomass pretreatments and sequential mixed-cellulases hydrolysis. The arrow indicated the rough surface. (C) Hexoses yield released by the mixed-cellulases hydrolysis of the crystalline cellulose obtained from 4 M KOH extraction followed with acetic–nitric acids–water (8:1:2). (D) Xyl/Ara ratio of hemicelluloses released by individual enzymatic-digestion of the crude cellulose substrate in the selected *Miscanthus* samples.

surface than the Msa01 after pretreatment with 1% NaOH or 1% H_2SO_4 and sequential enzymatic hydrolysis (Fig. 5B). However, both samples showed extremely low lignocellulose enzymatic digestibility after pretreatment with 4 M KOH followed with acetic–nitric acid–water (8:1:2) (Fig. 5C). Considering the strongly positive correlation on biomass enzymatic digestibility among various chemical pretreatments (including 4 M KOH) of the 79 *Miscanthus* samples (Table S2), the biomass residue extracted with 4 M KOH, other than the crystalline cellulose obtained from 4 M KOH and acetic–nitric acid extraction, was the desired crude cellulose substrate for individual enzyme digestion analysis (Fig. S1).

The three typical cellulases, β-glucosidase, cellobiohydrolase I (CBH I), endo-1,4-β-glucanase II (EG II) each was incubated with the crude cellulose substrate to release soluble extract in the supernatant, and the extract was then determined by GC–MS for monosaccharide composition analysis after 2 M TFA hydrolysis. The

extract by each type enzyme contained glucose (Glu), Xyl, Ara, and other minor monosaccharides (Figs. 5D and S2, Tables S3–S5). Because the three cellulase enzymes were detected to show non-activity with xylan substrate (data not shown), the Xyl, Ara and other minor monosaccharides released in the extract should be derived from the xylan that was dis-associated with cellulose by the each cellulase digestion. This finding was supported by the comparison that the Xyl/Ara ratios from three cellulase enzymes digestion were lower than that of xylanase in *Miscanthus* and other grass samples (Figs. 5D and S2, Tables S3–S5). The lower Xyl/Ara ratios (or relative high Ara level) further suggested that partial Ara of xylan in the crude cellulose substrate should be associated with cellulose because it was released by β-glucosidase/CBH I/EG II digestion with cellulose, other than by xylanase with xylan. Furthermore, all three cellulases (β-glucosidase, CBH I, and EG II) digestions, compared with the xylanase or TFA hydrolysis, resulted in similar low Xyl/Ara ratios in all samples (Figs. 5D and S2, Tables S3–S5), which suggests that each cellulase member digestion should initially and preferentially take place from the same and specific region of cellulose. This finding is consistent with the recent reports about the limited interactions of cellulase with cellulose (Igarashi et al., 2011). Given that CBH I is specific for releasing the reduced form of cellobiose (Taylor et al., 2008), digestion with all three member cellulases should start from the amorphous regions of cellulose, which are associated mainly with Ara-rich xylan (lower Xyl/Ara ratio). Digestion with the mixed cellulases (containing β-glucanase $\geqslant 6 \times 10^4$ U, cellulase $\geqslant 600$ U and xylanase $\geqslant 10 \times 10^4$ U) produces a much higher Xyl/Ara ratio than with a single cellulase (β-glucosidase or CBH I or EG II). This finding indicates that the high amounts of cellulase and xylanase in the mixed cellulases extends the digestion from the amorphous cellulose towards the adjacent regions (crystalline cellulose). Notably, the *Miscanthus* Msi56 sample (with high biomass digestibility) showed a consistently lower Xyl/Ara ratio than the Msa01 sample (with low biomass digestibility) in all individual enzyme digestions and mixed cellulase hydrolysis assays. The results confirm that the Xyl/Ara ratio negative affects the biomass enzymatic digestibility of *Miscanthus*.

3.4. Hemicelluloses composition effect on cellulose crystallinity

Cellulose crystallinity (CrI) is reportedly a negative factor affecting biomass hydrolysis (Yoshida et al., 2008; Xu et al., 2012). Provided the partial Ara in xylan is associated with the amorphous region of cellulose as just described, the Ara level or the Xyl/Ara ratio should affect cellulose crystallinity. Correlation analysis between the hemicelluloses level/composition and cellulose CrI was performed (Fig. 6). Only the non-KOH-extractable hemicelluloses showed a significantly negative correlation with cellulose CrI, which suggests that this type hemicelluloses are strongly bound to cellulose by hydrogen bonds and others. However, the Ara level and the Xyl/Ara ratio of the two types of hemicelluloses were significantly correlated with the cellulose CrI. Hence, the degree of Ara substitution of xylan is the key factor that positively affects biomass enzymatic digestibility because Ara negative affects cellulose crystallinity.

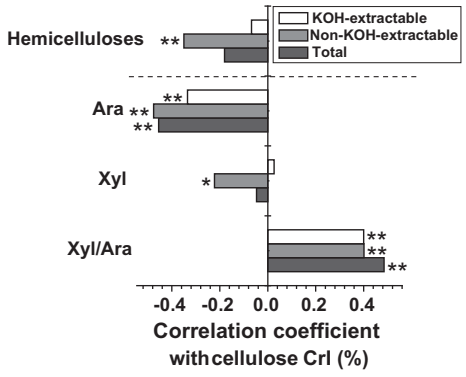

Fig. 6. Correlation analysis between hemicelluloses level/composition and cellulose crystallinity (CrI) in *Miscanthus* samples ($n = 79$).

4. Discussion

Miscanthus is a C4 perennial plant that has tremendous potential in biofuel production, thereby mitigating CO_2 production (Qin et al., 2011; Xie and Peng, 2011). Plant biomass consists of different cell types with extremely diverse cell wall compositions. Therefore, identifying specific types of plant cell walls that are suitable as substrates for biofuel production using small-scale samples is difficult (Pauly and Keegstra, 2010). One practical approach is to analyze of large populations for biomass samples and to correlate these findings with recalcitrance. A total of 79 geographically distributed *Miscanthus* accessions, which have considerable natural variations in cell wall composition, were collected to determine the fermentable sugar released from enzymatic hydrolysis after pretreatment with NaOH or H_2SO_4. Hence, the diversity of cell wall composition and the related variations in biomass digestibility provides high availability for the correlation analysis between cell wall components and biomass enzymatic digestibility. In the present study, a strongly positive correlation was observed on the hexoses yield released from enzymatic hydrolysis among various pretreatments of NaOH (0.5%, 1%, 4%), H_2SO_4 (0.25%, 1%, 4%) and 4 M KOH (Table S2). The correlation results suggested that biomass enzymatic digestibility is fundamentally determined by plant cell wall structures, although the desirability of a few traits may depend upon the type of pretreatment employed.

Over the past years, the effects of cellulose features (DP and CrI) and lignin composition on biomass digestibility have been reported (Puri., 1984; Chen and Dixon, 2007; Yoshida et al., 2008; Studer et al., 2011; Xie et al., 2013). However, little is known about hemicelluloses. In commelinid monocots, xylans are the major hemicelluloses in the secondary cell walls. Xylans usually contain different arabinose oligosaccharide residues attached to the backbone (Scheller and Ulvskov, 2010). In this work, KOH-extractable and non-KOH-extractable hemicelluloses were subjected to an in-depth correlation analysis between xylan level/composition and biomass enzymatic digestibility. Although non-KOH-extractable hemicelluloses comprise less than 20% of the total hemicelluloses (Table 1), they are tightly associated with cellulose, which negatively affects cellulose crystallinity (Fig. 6). On the other hand, the non-KOH-extractable hemicelluloses have a much lower Xyl/Ara ratio than KOH-extractable hemicelluloses, indicating that the crude cellulose obtained via KOH extraction is the ideal substrate for individual enzyme digestion analysis.

Acid and alkali pretreatments have distinct mechanisms for biomass depolymerization (Mosier et al., 2005; Xu et al., 2012). Acid pretreatment involves the hydrolysis of hemicelluloses by breaking the glycosidic linkages of polysaccharides (Saha et al., 2005; Galbe and Zacchi, 2007). Alkali pretreatment breaks down the intermolecular ester bonds that cross-link lignin with hemicelluloses, thereby solubilizing lignin (Macdonald et al., 1983). The removal of hemicelluloses increases the mean pore size of the substrate, which facilitates the hydrolysis of cellulose (Palonen et al., 2004; Adani et al., 2011). Although acid (H_2SO_4) pretreatment results in a lower biomass saccharification rate than the base (NaOH) pretreatment (Fig. 1B and C), the Ara level and the Xyl/Ara ratio of the xylan showed a strongly significant correlation with the enzymatic digestibility of lignocellulose after pretreatment with acid or with alkali. This correlation indicates that the degree of arabinose substitution of xylan fundamentally determines the enzymatic digestibility of

Miscanthus biomass no matter which pretreatment (acid or base) is employed. The mechanism of biomass enzymatic digestibility could be explored by analyzing the Xyl/Ara ratio of the crude cellulose substrate after hydrolysis with individual enzymes, and by calculating the correlation between Xyl/Ara and cellulose crystallinity (CrI). The findings are also consistent with the current hypothesis on the cross-linking of hemicelluloses with cellulose via hydrogen bonds, which negatively affects cellulose crystallinity (Scheller and Ulvskov, 2010). In addition, the Xyl/Ara ratio of the substrate after hydrolysis with individual enzymes is similar to that in four other grasses, which suggests that the Xyl/Ara ratio is an indicator for the biomass enzymatic digestibility of grasses. To our knowledge, this is the first study to correlate the degree of arabinose substitution of xylans with biomass digestibility in plants.

Genetic modification of plant cell walls is considered as a promising solution to biomass recalcitrance for high biofuel productions (Xie and Peng, 2011). The positive correlation among various pretreatments (Table S2) confirmed that plant cell wall composition and structure fundamentally determines biomass enzymatic digestibility. Recently, reducing lignin levels or altering lignin composition in transgenic plants was found to enhance biomass saccharification (Baucher et al., 1999; Chen and Dixon, 2007). However, little is known about the genetic modification of hemicelluloses in plants. Hence, this study provides a goal for the genetic engineering of hemicelluloses level and composition towards energy crop breeding. Furthermore, the study also indicates the appropriate approaches for cost-effective biomass degradation and biofuel conversion in the future.

5. Conclusions

A large population of *Miscanthus* germplasm accessions have been attributed a diverse cell wall composition that leads to a wide lignocellulose digestibility. Correlation analysis indicated that the degree of arabinose substitution of xylan positively affects lignocellulose digestibility after pretreatments with NaOH or H_2SO_4. Analysis via digestion with individual enzymes demonstrated that the arabinose in xylan negatively affects cellulose crystallinity for the high biomass saccharification of *Miscanthus* and grasses. The findings could be used to develop a goal for the genetic manipulation of bioenergy crops and cost-effective biomass processing.

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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.2012.12.107.

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