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Lignin extraction distinctively enhances biomass enzymatic saccharification in hemicelluloses-rich *Miscanthus* species under various alkali and acid pretreatments

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HIGHLIGHTS

- One-step pretreatment with 4% NaOH has the highest hexoses yields in *Miscanthus*.
- 2% NaOH followed by 1% H_2SO_4 is optimal for high biomass saccharification.
- Hemicelluloses-rich *Miscanthus* samples show largely enhanced biomass digestibility.
- Lignin extraction predominately determines hexoses yields upon various pretreatments.
- Suggest the potential applications in energy crop breeding and biomass process.

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ABSTRACT

In this study, one- and two-step pretreatments with alkali and acid were performed in the three *Miscanthus* species that exhibit distinct hemicelluloses levels. As a result, one-step with 4% NaOH or two-step with 2% NaOH and 1% H_2SO_4 was examined to be optimal for high biomass saccharification, indicating that alkali was the main effecter of pretreatments. Notably, both one- and two-step pretreatments largely enhanced biomass digestibility distinctive in hemicelluloses-rich samples by effectively co-extracting hemicelluloses and lignin. However, correlation analysis further indicated that the effective lignin extraction, other than the hemicelluloses removals, predominately determined biomass saccharification under various alkali and acid pretreatments, leading to a significant alteration of cellulose crystallinity. Hence, this study has suggested the potential approaches in bioenergy crop breeding and biomass process technology.

1. Introduction

Plant cell walls represent an attractive and enormous biomass resource for biofuels and chemicals (Ragauskas et al., 2006; Chen and Peng, 2013). Bioethanol derived from lignocellulose feedstock has been regarded as clean and renewable biofuels (Himmel et al., 2007; Xie and Peng, 2011). Principally, lignocellulose conversion into bioethanol involves three main steps: chemical and physical pretreatments for plant cell wall destruction, enzymatic hydrolysis toward fermentable sugar releasement, and yeast fermentation into ethanol production (Himmel et al., 2007; Rubin, 2008; Peng, 2011). However, plant cell wall composition and features basically determine biomass recalcitrance, leading to a costly biomass conversion (Himmel et al., 2007; Wang et al., 2014; Xie and Peng, 2014). As genetic modification of plant cell walls has been considered as a promising solution (Peng, 2011; Wang et al., 2014), it becomes important to characterize the plant cell wall polymers

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that distinctively affect biomass enzymatic digestibility under various physical and chemical pretreatments.

Plant cell walls are mainly composed of cellulose, hemicelluloses and lignin. Cellulose is the crystalline β-1,4-glucans, accounting for 28%–45% of dry matter in higher plants (Himmel et al., 2007; Peng et al., 2002), and cellulose crystallinity is the key factor negatively affecting biomass enzymatic digestibility in *Miscanthus* and other plants (Xu et al., 2012; Wu et al., 2013; Jia et al., 2014; Li et al., 2014a,b; Wang et al., 2014; Zhang et al., 2013; Huang et al., 2015). Hemicelluloses are branched non-cellulosic polysaccharides with various monosaccharides (Scheller and Ulvskov, 2010). Despite hemicelluloses positively affect biomass saccharification under alkali (NaOH) or acid (H_2SO_4) pretreatments in *Miscanthus* (Xu et al., 2012), much remains unknown about mechanism of hemicelluloses impact in biomass pretreatments and sequential enzymatic digestions. Lignin is a hydrophobic polymer consisting of three major monolignols: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (Sun et al., 2013). It has been reported that lignin plays dual roles in biomass enzymatic digestions, due to distinct monolignol proportions in different plant species (Ragauskas et al., 2006; Studer et al., 2011; Xu et al., 2012; Li et al., 2014d). In particular, lignin is examined as the negative factor on biomass saccharification in *Miscanthus* (Xu et al., 2012). Hence, it remains to investigate the predominate effect of hemicelluloses or lignin on biomass digestibility under various chemical pretreatments in *Miscanthus*.

As pretreatment is the initial step for biomass process, it becomes essential to find out the optimal pretreatments that enhance biomass saccharification. Generally, different pretreatments appear to have distinct action mechanisms by either increasing the porosity and accessibility of biomass particles or decreasing lignocellulose crystallinity or selectively removing hemicelluloses and lignin (Hendriks and Zeeman, 2009; Macdonald et al., 1983; Saha et al., 2005; Zheng et al., 2009). A variety of pretreatments have been applied to various lignocellulosic materials. Acid and alkali chemicals such as H_2SO_4 and NaOH are extensively used for biomass pretreatments (Mosier et al., 2005). Principally, alkali pretreatment can mostly lead to the lignin extraction by breaking hydrogen and other covalent bonds, whereas acid pretreatment mainly causes the hemicelluloses release by splitting strong chemical bonds under high temperature (Mosier et al., 2005; Zheng et al., 2009; Macdonald et al., 1983; Xu et al., 2012). In this study, one- and two-step pretreatments with NaOH and H_2SO_4 were applied for understanding the mechanism of plant cell wall polymer destructions by alkali and acid chemicals, rather than for developing biofuel technology.

Miscanthus is a leading candidate bioenergy crop with high biomass yield and well adaptation to various environmental conditions (Lygin et al., 2011; Xie and Peng, 2011). Since *Miscanthus* is originally derived from East Asia, large populations of *Miscanthus* natural accessions with rich and stable germplasm resource were collected in China (Huang et al., 2012; Xu et al., 2012; Li et al., 2013; Zhang et al., 2013; Wang et al., 2014). In the present study, three *Miscanthus* species were selected that exhibited distinct hemicelluloses contents, and their biomass enzymatic digestibility was also detected in order to find out hemicelluloses and lignin effects on biomass saccharification under one- and two-step pretreatments with NaOH and H_2SO_4. Hence, this study could identify that the hemicelluloses-rich *Miscanthus* species were effective at lignin extraction under various chemical pretreatments, leading to a significant alteration of cellulose crystallinity.

2. Methods

2.1. Plant materials

Miscanthus samples were collected from Hunan experimental field in 2011 season. The collected mature stem tissues were dried at 50 °C, ground through a 40 mesh screen and stored in a dry container until use.

2.2. Plant cell wall fractionation

The plant cell wall fractionation procedure was described by Peng et al. (2000) with minor modification (Li et al., 2014c). The soluble sugar, lipids, starch and pectin of the samples were successively removed with the potassium phosphate buffer (pH 7.0), chloroform–methanol (1:1, v/v), dimethyl sulphoxide (DMSO)–water (9:1, v/v) and 0.5% (w/v) ammonium oxalate. The remaining pellet was extracted with 4 M KOH containing 1.0 mg/mL sodium borohydride for 1 h at 25 °C and washed with distilled water until the soluble sugars were undetectable. The combined supernatant was neutralized, dialyzed and lyophilized as KOH-extractable hemicelluloses. The remaining residues were then extracted with 2 M trifluoroacetic acid (TFA) at 120 °C in an autoclave for 1 h, and washed the residues with distilled water. The combined supernatants were collected as the non-KOH-extractable hemicelluloses, and combined with the KOH-extractable as total hemicelluloses. The remaining residues were sequentially extracted with acetic acid-nitric acids-water (8:1:2, v/v/v) for 1 h in a boiling water bath and the remaining pellet was defined as cellulose. All samples were conducted in biological triplicate.

2.3. Total hexoses and pentoses assay

A UV/VIS Spectrometer (V-1100D, MAPADA Instruments Co., Ltd., Shanghai, China) was applied for total hexoses and pentoses assay as described by Wu et al. (2014) and Li et al. (2014a). The anthrone/H_2SO_4 method was used for determination of total hexoses (Fry, 1988), and the orcinol/HCl assay was for total pentoses (Dische, 1962). The standard curves for hexoses and pentoses were drawn using D-glucose and D-xylose as standard, respectively. Both anthrone/H_2SO_4 and orcinol/HCl methods were used to measure total hemicelluloses levels and also employed for total sugars released from pretreatment and enzymatic hydrolysis of biomass samples. Regarding the high pentoses level effect on the absorbance reading at 620 nm for hexoses account, the deduction from pentoses reading at 660 nm was conducted for final calculation of hexoses level, verified by GC–MS analysis. All of the samples resulted from biological triplicates.

2.4. Total lignin analysis

Total lignin contents of the raw samples and the residues obtained from pretreatment were determined by two-step acid hydrolysis method according to Laboratory Analytical Procedure of the National Renewable Energy Laboratory (NREL; Sluiter et al., 2008). The acid-insoluble lignin was accounted gravimetrically after correction for ash. The acid-soluble lignin was measured by UV spectroscopy. The details of the two-type of lignin assay were previously described by Xu et al. (2012). All samples were performed in biological triplicate.

2.5. Cellulose crystalline index (CrI) detection

Cellulose crystallinity was characterized by measuring crystalline index (CrI) of samples using X-ray diffraction (XRD) method as described by Xie et al. (2013) and Zhang et al. (2013). The biomass samples were analyzed by means of wide-angle X-ray diffraction on a Rigaku-D/MAX instrument (Uitima III, Japan) with 0.0197°/s from 10° to 45°. The crystallinity index was estimated by using the height of the 200 peak (I_{200}, $\theta = 22.5°$) and height at the minimum between the 200 and 110 peaks (I_{AM}, $\theta = 18.5°$), based on the equation: $CrI = 100 \times (I_{200} - I_{AM})/I_{200}$. I_{200} represents

both crystalline and amorphous materials while I_{AM} represents amorphous material. Standard error of the CrI method was detected at ±0.05–0.15 using five representative samples in triplicate.

2.6. Pretreatment and enzymatic hydrolysis

Alkali and acid pretreatments and biomass enzymatic hydrolysis were performed as previously described by Xu et al. (2012) with minor modifications on chemical concentrations as described below.

2.6.1. One-step pretreatment by H_2SO_4 and NaOH

The well-mixed biomass powders were pretreated by H_2SO_4 and NaOH. For one-step pretreatment with acid, the biomass sample was added with H_2SO_4 at various concentrations (%, w/w) in a plastic centrifuge tube, and treated at 121 °C for 20 min in an autoclave. The tube was then cooled down, and shaken at 150 rpm and 50 °C for 2 h. The sample was finally centrifuged at 3000*g* for 5 min, and the supernatant was collected for determination of free hexoses and pentoses while the residues were collected for plant cell wall composition analysis. For one-step pretreatment with alkali, the biomass sample was added with NaOH at various concentrations (%, w/v) in a plastic centrifuge tube, and shaken under 150 rpm at 50 °C for 2 h. The treated sample was centrifuged at 3000*g* for 5 min, and the supernatant was collected for assay of hexoses and pentoses, and the residues were collected for plant cell wall composition analysis. The biomass sample added with distilled water was shaken for 2 h at 50 °C as the control, and all samples were carried out in biological triplicate.

2.6.2. Two-step pretreatments by NaOH and H_2SO_4

The one-step NaOH or H_2SO_4 pretreated samples were rinsed with distilled water until pH at 7.0. The biomass residues were then added with H_2SO_4 or NaOH at various concentrations as the second step pretreatments under the conditions as described above. All samples were carried out in biological triplicate.

2.6.3. Enzymatic hydrolysis

The remaining residues from various pretreatment were washed with distilled water until pH at neutral, and once with the mixed-cellulases reaction buffer (0.2 M acetic acid-sodium acetate, pH 4.8). The washed samples were added with mixed-cellulases (containing β-glucanase $\geqslant 5.96 \times 10^4$ U and cellulase $\geqslant 596$ U and xylanase $\geqslant 9.6 \times 10^4$ U, purchased from Imperial Jade Bio-technology Co., Ltd., China) with the final enzyme concentration at 3.2 g/L (64 mg/g dry matter), and shaken under 150 rpm at 50 °C for 48 h. After the enzymatic hydrolysis, the biomass samples were centrifuged at 3000*g* for 10 min, and the supernatants were collected for determination of pentoses and hexoses. All samples were carried out in biological triplicate.

2.7. Statistical calculation of correlation coefficients

Correlation coefficients were generated by performing spearman rank correlation analysis for all the measured traits across the three species of *Miscanthus* from different pretreatments (Xu et al., 2012; Li et al., 2013). The analysis used average values calculated from all original determinations values.

3. Results and discussion

3.1. Three Miscanthus species distinctive in hemicelluloses levels

In the previous studies, total 200 *Miscanthus* accessions have been determined with a diverse plant cell wall composition and biomass enzymatic digestibility under one-step pretreatments with H_2SO_4 or NaOH (Huang et al., 2012; Xu et al., 2012; Li et al., 2013, 2014c; Zhang et al., 2013). In this study, three *Miscanthus* species with distinct hemicelluloses levels were selected in order to distinguish hemicelluloses and lignin effects on biomass digestibility under various chemical pretreatments (Table 1). In general, the selected *Miscanthus* species had similar cellulose contents ranged from 31.02% to 31.49% of dry matter, but showed significantly different hemicelluloses levels from 29.24% to 35.38% of dry matter at $p < 0.01$ levels ($n = 3$). In particular, the Msa32 sample from *Miscanthus sacchariflorus* exhibited hemicelluloses level higher than that of the Mlu03 sample from *Miscanthus lutarioriparius* by 12 %, but it had less hemicelluloses content than that of the Msi69 from *Miscanthus sinensis* by 8% at $p < 0.01$ (Table 1). Despite Msi69 and Msa32 had a similar lignin level, both samples showed a significantly lower lignin content than that of Mlu03 by less than 5% at $p < 0.01$. Hence, the three *Miscanthus* samples may be applicable for investigating hemicelluloses and lignin distinct roles in lignocellulose enzymatic digestions under various chemical pretreatments performed in this study.

3.2. Biomass saccharification under one-step pretreatment

Biomass saccharification (digestibility) has been defined (Huang et al., 2012) by measuring either hexoses yield (% of cellulose) released from hydrolysis by a crude cellulase mixture of lignocellulose after pretreatment, or the total sugar yield (hexoses and pentoses/dry weight) from both pretreatment and enzymatic hydrolysis. In the present work, three *Miscanthus* samples were initially pretreated with NaOH or H_2SO_4 at series concentrations from 0.25% to 8% (Fig. 1 and Table S1). As a result, three *Miscanthus* species exhibited a maximum increasing velocity of biomass saccharification from 0.25% to 2% NaOH pretreatments (Fig. 1A). However, Msi69 and Msa32 samples could reach to the highest hexoses yields up to 100% (cellulose) at 4% NaOH, whereas Mlu03 had the top hexoses yield at 8% NaOH (Table S1). As a comparison, three *Miscanthus* samples under H_2SO_4 pretreatments exhibited much lower hexoses (or total sugars) yields than that of NaOH (Fig. 1B), consistent with the previous observations in *Miscanthus* and other plants (Xu et al., 2012; Wu et al., 2013; Jia et al., 2014). Notably, three *Miscanthus* samples showed a distinct biomass saccharification under both NaOH and H_2SO_4 pretreatments. For instance, Msi69 had much higher hexoses (or total sugars) yields than that of Msa32 from 0.25% to 2% NaOH and H_2SO_4 pretreatments, while Mlu03 showed lower hexoses (or total sugars) yields than that of Msa32 under various concentrations of NaOH and H_2SO_4 pretreatments (Table S1). Hence, the results were in agreement with the previous findings that biomass saccharification is either positively affected by hemicelluloses or negatively influenced by lignin in *Miscanthus* (Xu et al., 2012). However, it remains unclear about which plant cell wall polymer (hemicelluloses or lignin) plays a predominant role in biomass enzymatic digestions.

3.3. Biomass digestibility upon two-step pretreatments

As the one-step pretreatments with alkali (NaOH) or acid (H_2SO_4) could distinctively enhance biomass saccharification in three *Miscanthus* species, the two-step pretreatments with dilute alkali and acid were performed in this study (Fig. 2, Tables S2 and S3). In terms of the biomass saccharification with maximum velocity rate as described above, the four concentrations (0.25%, 0.5%, 1% and 2%) of NaOH and H_2SO_4 were applied for the two-step pretreatments (Fig. 2A). Among the three *Miscanthus* species, Msi69 sample exhibited the highest hexoses (or total sugars) yields, whereas Mlu03 had the lowest yields under various two-step pretreatments. Pretreated with 2% NaOH followed by 1%

Table 1
Cell wall composition (% dry matter) in the three *Miscanthus* species.

Sample	Cellulose		Hemicelluloses		Lignin	
Msi69 (H)	31.02 ± 0.67	0.04%[@]	35.38 ± 0.52[**]	8.02%	25.33 ± 0.27	0.87%
Msa32 (M)	31.04 ± 2.20	1.47%[#]	32.75 ± 0.40[**]	12.01%	25.55 ± 0.13[**]	4.43%
Mlu03 (L)	31.49 ± 1.45		29.24 ± 0.66		26.69 ± 0.11	

(H), (M), (L): indicated the *Miscanthus* sample with relatively high (H), medium (M) and low (L) biomass digestibility. All data as means ± SD (n = 3).
[**] A significant difference between two *Miscanthus* species by t-test at $p < 0.01$ level (n = 3).
[@] Increased rate between Msi69 and Msa32: subtraction of two samples divided by low value.
[#] Increased rate between Msa32 and Mlu03: subtraction of two samples divided by low value.

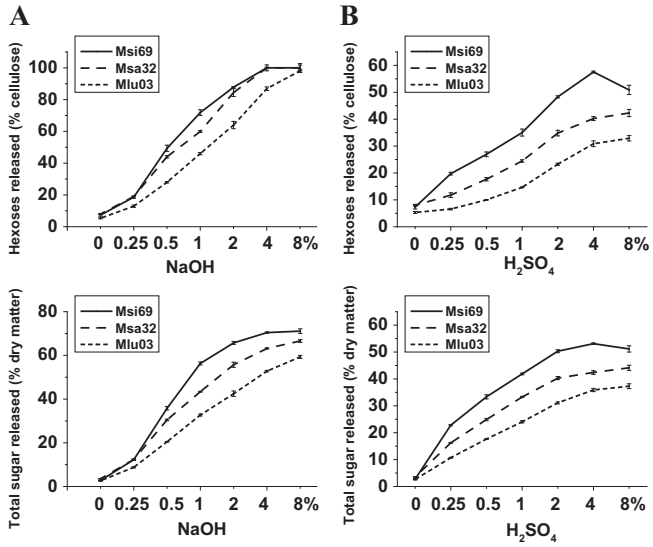

Fig. 1. Biomass digestibility of three *Miscanthus* species under one-step pretreatment with NaOH (A) or H_2SO_4 (B) at various concentrations: hexoses yields (% cellulose) released by mixed-cellulases after pretreatment; Total sugar (hexoses and pentoses) yields (% dry matter) released from both pretreatment and sequential enzymatic hydrolysis; The bar indicated means ± SD (n = 3).

H_2SO_4 (2% NaOH + 1% H_2SO_4), three *Miscanthus* samples showed the highest biomass saccharification among all two-step pretreatments performed in the study. By comparison, all samples had the lowest biomass digestibility under the 0.25% NaOH and 0.25% H_2SO_4 pretreatment. On the other hand, pretreated with H_2SO_4 followed by NaOH (H_2SO_4 + NaOH), three *Miscanthus* samples exhibited much lower hexoses (or total sugars) yields (Fig. 2B), compared with the two-step pretreatments of alkalis followed by acids (NaOH + H_2SO_4) (Fig. 2A). In particular, the three samples appeared to have a relatively higher biomass digestibility under the 0.25% H_2SO_4 + 2% NaOH pretreatment than that of the other two-step pretreatments with H_2SO_4 followed by NaOH (H_2SO_4 + - NaOH). Hence, the data suggested that the alkali (2% NaOH), other than the acid, should be the predominant effecter for enhancing high biomass saccharification among various two-step pretreatments performed in this work.

3.4. Comparison of hexoses yields between one- and two-step pretreatments

As described previously, both one- (4% NaOH) and two-step (2% NaOH + 1% H_2SO_4) pretreatments could largely enhance biomass enzymatic saccharification. As a comparison, however, three *Miscanthus* samples pretreated with 4% NaOH, exhibited significantly higher hexoses yields (of dry matter) than that of the two-step pretreatment (2% NaOH + 1% H_2SO_4) at $p < 0.05$ and 0.01 (Table 2). For instance, Msi69 under two-step pretreatment showed less hexoses yields than that of the one-step by 14%, whereas Msa32 and Mlu03 respectively had the reduced hexoses yields by 27% and 28%, suggesting that the one-step alkali pretreatment with 4% NaOH consistently remained slightly higher biomass saccharification compared with the two-step pretreatments (2% NaOH + 1% H_2SO_4).

3.5. Hemicelluloses and lignin extractions under various pretreatments

To compare hemicelluloses and lignin extractions under one- and two-step pretreatments, the extracted plant cell wall polymers were determined in three *Miscanthus* samples under four pretreatments (Table 3). In general, three *Miscanthus* samples exhibited the highest hemicelluloses extraction rates ranged from 78% to 87% under 4% H_2SO_4 pretreatment, whereas they had much low extracted rates from 33% to 44% under 4% NaOH pretreatment, or from 31% to 48% under two-step pretreatments with 0.25% NaOH and 0.25% H_2SO_4. By comparison, the two-step pretreatments with 2% NaOH and 1% H_2SO_4 also remained high hemicelluloses extraction rates from 55% to 70% in three *Miscanthus* samples. Notably, due to their distinct hemicelluloses levels in raw stalks, three *Miscanthus* samples showed a quite different hemicelluloses extraction rate among the four pretreatments (Table 3). The results suggest that both one- and two-step pretreatments could relatively extract more hemicelluloses in the hemicelluloses-rich *Miscanthus* samples.

With respect to lignin extraction, the one-step pretreatment with 4% H_2SO_4 could lead to the lowest lignin extraction rates ranged from 17% to 31% in three *Miscanthus* samples, contrast to the hemicelluloses extraction. Meanwhile, the two-step pretreatments with 0.25% NaOH and 0.25% H_2SO_4 remained much low extraction rates from 23% to 39% (Table 3). Notably, both one-step pretreatment with 4% NaOH and two-step pretreatments with 2% NaOH followed by 1% H_2SO_4 caused similar and high lignin extraction rates in the Msi69 at 72% and 73% or in the Msa32 at 65% and 63%. In the Mlu03 sample, both one-step pretreatment with 4% NaOH and two-step pretreatments with 2% NaOH followed by 1% H_2SO_4 also remained high lignin extraction rates at 51% and 45% (Table 3). Hence, the data indicated that one-step pretreatment with 4% H_2SO_4 and 4% NaOH respectively lead to predominant hemicelluloses and lignin extractions, confirming that alkali and acid pretreatments have distinct mechanism of plant cell wall polymer extractions (Hendriks and Zeeman, 2009). It was the first time to report that the two-step pretreatments with dilute alkali and acid could also largely extract both hemicelluloses and lignin in the hemicelluloses-rich *Miscanthus* samples. Hence, it suggests that hemicelluloses level should also positively affect both hemicelluloses and lignin extractions under the two-step pretreatments with dilute alkali and acid.

3.6. Mechanism of hemicelluloses and lignin distinct effects on biomass digestibility

Hemicelluloses and lignin have been respectively examined to be the positive and negative factors affecting biomass saccharification under one-step pretreatment with alkali or acid in *Miscanthus*

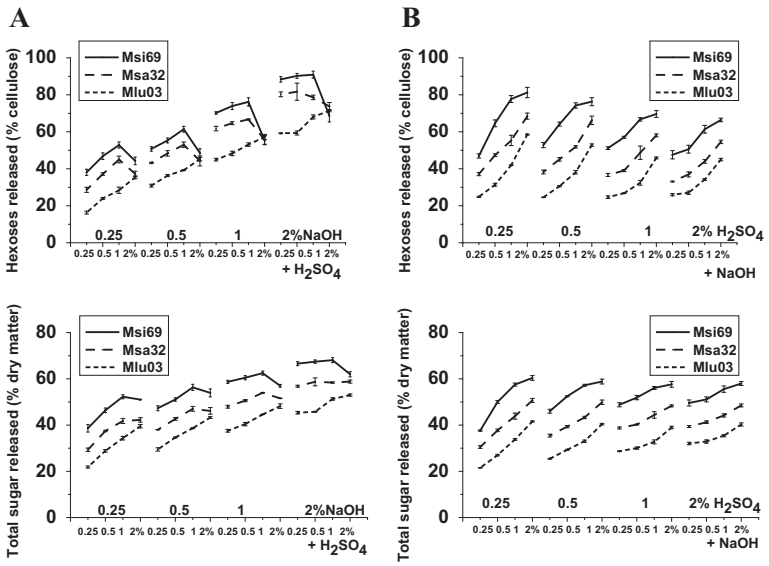

Fig. 2. Biomass digestibility of three *Miscanthus* species under two-step pretreatments (A) with NaOH followed by H_2SO_4 or (B) with H_2SO_4 followed by NaOH at various concentrations: hexoses yields (% cellulose) released by mixed-cellulases after pretreatment; total sugar (hexoses and pentoses) yields (% dry matter) released from both pretreatment and sequential enzymatic hydrolysis; The bar indicated means ± SD (n = 3).

Table 2
Hexoses yields (% dry matter) released under one- and two-step pretreatments in three *Miscanthus* species.

	4% NaOH	2% NaOH + 1% H_2SO_4	Increased rate (%)
Msi69 (H)	33.60 ± 0.79	29.39 ± 0.61	14.31*
Msa32 (M)	32.59 ± 0.24	25.55 ± 0.39	27.56**
Mlu03 (L)	28.66 ± 0.19	22.41 ± 0.30	27.88**

* and ** Indicated significant different hexoses yields released from pretreatment and sequential enzymatic hydrolysis by t-test at $p < 0.05$ and $p < 0.01$ levels between one-step pretreatment with 4% NaOH and the two-step pretreatment with 2% NaOH followed by 1% H_2SO_4; The increased rate was calculated by subtraction of two pretreatments values divided by low value. All data as means ± SD (n = 3).

(Xu et al., 2012), however, it remains unclear about mechanism of two plant cell wall polymer contrast effects on biomass enzymatic digestibility in plants. As alkali and acid agents could distinctively extract hemicelluloses and lignin, a correlation analysis was performed between the extracted plant cell wall polymers and the hexoses yields released from enzymatic hydrolysis in three *Miscanthus* species under the four pretreatments (Fig. 3). Despite the hemicelluloses levels in the raw stalks have a positive correlation with biomass saccharification in *Miscanthus* (Xu et al., 2012), such correlation was not observed either on the hemicelluloses levels of biomass residues after the four pretreatments, or the hemicelluloses extracted amounts/rates with the four pretreatments (Fig. 3A). Notably, the lignin levels of biomass residues after the four

Table 3
Hemicelluloses and lignin extraction rates from four pretreatments in three *Miscanthus* species.

Sample	Pretreatment	Hemicelluloses level* after pretreatment	Extracted rate# (% of control)	Lignin level* after pretreatment	Extracted rate# (% of control)
Msi69 (H)	Control (raw material)	35.38 ± 0.52		25.33 ± 0.27	
	4% H_2SO_4	4.55 ± 0.06	87.14	17.52 ± 0.20	30.85
	4% NaOH	19.68 ± 0.40	44.37	7.01 ± 0.96	72.33
	2% NaOH + 1% H_2SO_4	10.73 ± 0.12	69.67	6.81 ± 0.12	73.14
	0.25% NaOH + 0.25% H_2SO_4	18.35 ± 0.31	48.13	15.47 ± 0.44	38.94
Msa32 (M)	Control	32.75 ± 0.40		25.55 ± 0.13	
	4% H_2SO_4	5.54 ± 0.14	83.07	18.35 ± 0.33	28.20
	4% NaOH	18.85 ± 0.19	42.43	8.90 ± 0.62	65.18
	2% NaOH + 1% H_2SO_4	12.35 ± 0.30	62.28	9.44 ± 0.24	63.06
	0.25% NaOH + 0.25% H_2SO_4	18.70 ± 1.04	42.90	16.98 ± 0.37	33.54
Mlu03 (L)	Control	29.24 ± 0.66		26.69 ± 0.11	
	4% H_2SO_4	6.58 ± 0.17	77.50	22.06 ± 0.42	17.34
	4% NaOH	19.61 ± 0.35	32.92	13.01 ± 0.57	51.23
	2% NaOH + 1% H_2SO_4	13.10 ± 0.18	55.19	14.78 ± 1.72	44.63
	0.25% NaOH + 0.25% H_2SO_4	20.18 ± 0.32	30.99	20.65 ± 0.26	22.64

* Indicated the hemicelluloses and lignin levels (% dry matter) in the biomass residues after pretreatment. All data as means ± SD (n = 3).
# Indicated the hemiciellulloses and lignin extraction rates: subtraction between hemicelluloses/lignin level of biomass residue with the control value (raw material) divided by control value.

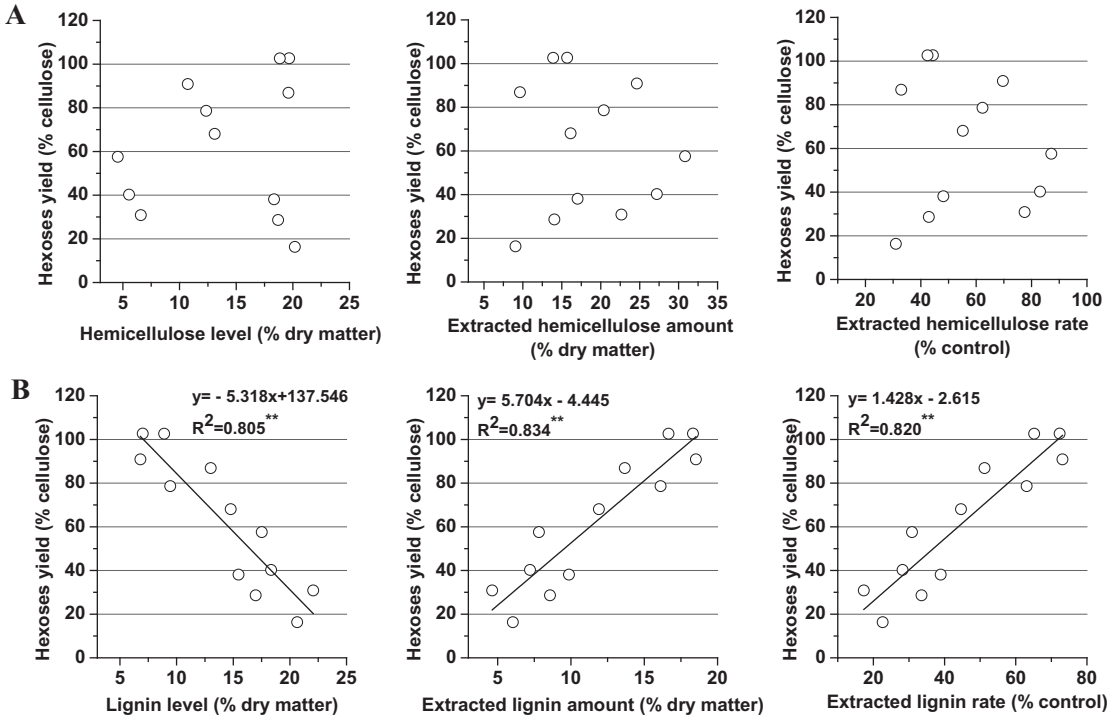

Fig. 3. Correlations between hexoses yields and plant cell wall polymers extractions under four pretreatments: (A) hemicelluloses; (B) lignin; hemicelluloses and lignin levels (% dry matter) were defined by measuring the biomass residues from four pretreatments (Table 3); extracted hemicelluloses and lignin amounts (% dry matter) were subjective to the subtractions of raw materials with the biomass residues from four pretreatments; extracted hemicelluloses and lignin rates (% control) were defined by calculating the extracted polymers amounts divided by control (raw materials). ** Indicated significant correlation level at $p < 0.01$ ($n = 12$).

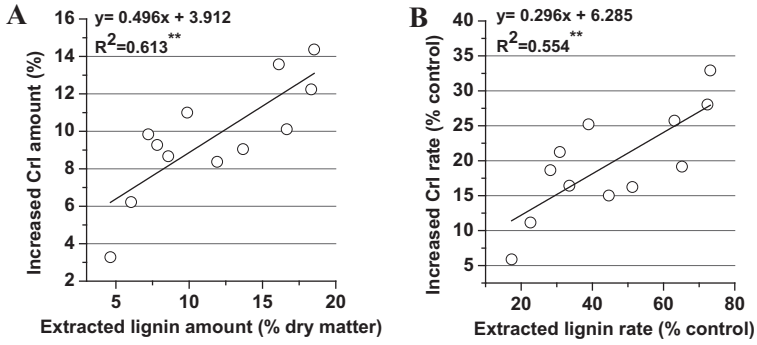

Fig. 4. Correlation between the increased cellulose CrI and the extracted lignin amount (A) and rate (B) under four pretreatments. ** Indicated significant correlation level at $p < 0.01$ ($n = 12$).

pretreatments exhibited a significantly negative correlation with the hexoses yields at $p < 0.01$ levels ($n = 12$; Fig. 3B), consistent with the previous findings that the lignin levels in the raw stalks negatively affect biomass saccharification under alkali and acid pretreatments in *Miscanthus* (Xu et al., 2012). As a contrast, the lignin extracted amounts and rates from the four pretreatments were positively correlated with the hexoses yields at $p < 0.01$ levels (Fig. 3B). Hence, the results indicated that the lignin extraction, other than the hemicelluloses removals, should predominately affect biomass enzymatic digestibility in *Miscanthus* under various one- and two-step pretreatments with alkali and acid. On the other hand, as the hemicelluloses-rich samples showed relatively high lignin extractions in this study, it also suggests that the hemicelluloses-positive effects on biomass saccharification should be due to an effective lignin extraction with hemicelluloses under the four pretreatments, consistent with the assumption that there is a tight association between hemicelluloses and lignin in *Miscanthus* (Xu et al., 2012; Li et al., 2014c).

Furthermore, a correlation was performed between the extracted lignin and the altered cellulose CrI (Table S4) in three *Miscanthus* samples under the four pretreatments. Significantly, the extracted amounts and rates of lignin exhibited a positive correlation with the increased amounts and rates of cellulose CrI at $p < 0.01$ levels (Fig. 4). As cellulose CrI is the main factor negatively affecting biomass enzymatic digestibility in the raw stalks of *Miscanthus* (Xu et al., 2012; Zhang et al., 2013), the data interpreted the lignin-negative effects should be due to its increasing cellulose crystallinity in the raw biomass. In other words, despite that cellulose CrI of the raw biomass materials is the negative factor on biomass saccharification in *Miscanthus*, this study indicated that cellulose CrI of the biomass residues obtained from various pretreatments could not be used to account for biomass enzymatic digestibility. However, the

altered amount and rate of cellulose CrI from pretreatments could significantly indicate biomass enzymatic saccharification in *Miscanthus*.

Taken all together, the effective lignin extraction under one- and two-step pretreatments with alkali and acid has a predominate enhancement on biomass enzymatic saccharification in *Miscanthus*. In addition, the hemicelluloses-rich biomass could positively affect biomass enzymatic digestibility by an effective co-extraction of hemicelluloses and lignin under various alkali and acid pretreatments.

4. Conclusions

Hemicelluloses-rich *Miscanthus* species have exhibited the enhanced biomass saccharification under either one-step pretreatment with 4% NaOH or two-step pretreatment with 2% NaOH followed by 1% H_2SO_4. Hence, the lignin removal predominately determines the biomass enzymatic digestibility distinctive in the hemicelluloses-rich samples under various alkali and acid pretreatments by effectively co-extracting hemicelluloses and lignin polymers, leading to a significant alteration of cellulose crystallinity. It has also suggested the potential approaches on plant cell modification in bioenergy crops and biotechnology in biomass process.

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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.2015.02.031.

References

Chen, P., Peng, L., 2013. The diversity of lignocellulosic biomass resources and their evaluations for use as biofuels and chemicals. In: Sun, J., Ding, S.-Y., Peterson, J.D. (Eds.), Biological Conversion of Biomass for Fuels and Chemicals. The Royal Society of Chemistry.

Dische, Z., 1962. Color reactions of carbohydrates. In: Whistler, R.L., Wolfrom, M.L. (Eds.), Methods in Carbohydrate Chemistry, vol. 1. Academic Press, New York, pp. 477–512.

Fry, S.C., 1988. The Growing Plant Cell Wall: Chemical and Metabolic Analysis. Longman, London.

Hendriks, A.T., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100, 10–18.

Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315, 804–807.

Huang, J., Xia, T., Li, A., Yu, B., Li, Q., Tu, Y., Zhang, W., Yi, Z., Peng, L., 2012. A rapid and consistent near infrared spectroscopic assay for biomass enzymatic digestibility upon various physical and chemical pretreatments in *Miscanthus*. Bioresour. Technol. 121, 274–281.

Huang, Y., Wei, X., Zhou, S., Liu, M., Tu, Y., Li, A., Chen, P., Wang, Y., Zhang, X., Tai, H., Peng, L., Xia, T., 2015. Steam explosion distinctively enhances biomass enzymatic saccharification of cotton stalks by largely reducing cellulose polymerization degree in *G. barbadense* and *G. hirsutum*. Bioresour. Technol. 181, 224–230.

Jia, J., Yu, B., Wu, L., Wang, H., Wu, Z., Li, M., Huang, P., Feng, S., Chen, P., Zheng, Y., Peng, L., 2014. Biomass enzymatic saccharification is determined by the non-KOH-extractable wall polymer features that predominately affect cellulose crystallinity in corn. PLoS One 9, e108449.

Li, F., Ren, S., Zhang, W., Xu, Z., Xie, G., Chen, Y., Tu, Y., Li, Q., Zhou, S., Li, Y., Tu, F., Liu, L., Wang, Y., Jiang, J., Qin, J., Li, S., Li, Q., Jing, H.C., Zhou, F., Gutterson, N., Peng, L., 2013. Arabinose substitution degree in xylan positively affects lignocellulose enzymatic digestibility after various NaOH/H_2SO_4 pretreatments in *Miscanthus*. Bioresour. Technol. 130, 629–637.

Li, F., Zhang, M., Guo, K., Hu, Z., Zhang, R., Feng, Y., Yi, X., Zou, W., Wang, L., Wu, C., Tian, J., Lu, T., Xie, G., Peng, L., 2014a. High-level hemicellulosic arabinose predominately affects lignocellulose crystallinity for genetically enhancing both plant lodging resistance and biomass enzymatic digestibility in rice mutants. Plant Biotechnol. J. http://dx.doi.org/10.1111/pbi.12276.

Li, M., Feng, S., Wu, L., Li, Y., Fan, C., Zhang, R., Zou, W., Tu, Y., Jing, H.C., Li, S., Peng, L., 2014b. Sugar-rich sweet sorghum is distinctively affected by wall polymer features for biomass digestibility and ethanol fermentation in bagasse. Bioresour. Technol. 167, 14–23.

Li, M., Si, S., Hao, B., Zha, Y., Wan, C., Hong, S., Kang, Y., Jia, J., Zhang, J., Li, M., Zhao, C., Tu, Y., Zhou, S., Peng, L., 2014c. Mild alkali-pretreatment effectively extracts guaiacyl-rich lignin for high lignocellulose digestibility coupled with largely diminishing yeast fermentation inhibitors in *Miscanthus*. Bioresour. Technol. 169, 447–454.

Li, Z., Zhao, C., Zha, Y., Wan, C., Si, S., Liu, F., Zhang, R., Li, F., Yu, B., Yi, Z., Xu, N., Peng, L., Li, Q., 2014d. The minor wall-networks between monolignols and interlinked-phenolics predominantly affect biomass enzymatic digestibility in *Miscanthus*. PLoS One 9, e105115.

Lygin, A.V., Upton, J., Dohleman, F.G., Juvik, J., Zabotina, O.A., Widholm, J.M., Lozovaya, V.V., 2011. Composition of cell wall phenolics and polysaccharides of the potential bioenergy crop – *Miscanthus*. GCB Bioenergy 3, 333–345.

Macdonald, D.G., Bakhshi, N.N., Mathews, J.F., Roychowdhury, A., 1983. Alkali treatment of corn stover to improve sugar production by enzymatic hydrolysis. Biotechnol. Bioeng. 25, 2067–2076.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96, 673–686.

Peng, L., 2011. Fundamental solution for biofuel production in China. J. Huazhong Agric. Univ. (Social Sciences Edition) 2, 1–6.

Peng, L., Hocart, C.H., Redmond, J.W., Williamson, R.E., 2000. Fractionation of carbohydrates in Arabidopsis root cell walls shows that three radial swelling loci are specifically involved in cellulose production. Planta 211, 406–414.

Peng, L., Kawagoe, Y., Hogan, P., Delmer, D., 2002. Sitosterol-beta-glucoside as primer for cellulose synthesis in plants. Science 295, 147–150.

Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick Jr., W.J., Hallett, J.P., Leak, D.J., Liotta, C.L., Mielenz, J.R., Murphy, R., Templer, R., Tschaplinski, T., 2006. The path forward for biofuels and biomaterials. Science 311, 484–489.

Rubin, E.M., 2008. Genomics of cellulosic biofuels. Nature 454, 841–845.

Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V., 2005. Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. Biotechnol. Prog. 21, 816–822.

Scheller, H.V., Ulvskov, P., 2010. Hemicelluloses. Annu. Rev. Plant Biol. 61, 263–289.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of Structural Carbohydrates and Lignin in Biomass. in: National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures (LAP) for Standard Biomass Analysis. Available at: <http://www.nrel.gov/biomass/pdfs/42618.pdf>.

Studer, M.H., DeMartini, J.D., Davis, M.F., Sykes, R.W., Davison, B., Keller, M., Tuskan, G.A., Wyman, C.E., 2011. Lignin content in natural Populus variants affects sugar release. Proc. Natl. Acad. Sci. U.S.A. 108, 6300–6305.

Sun, H., Li, Y., Feng, S., Zou, W., Guo, K., Fan, C., Si, S., Peng, L., 2013. Analysis of five rice 4-coumarate:coenzyme A ligase enzyme activity and stress response for potential roles in lignin and flavonoid biosynthesis in rice. Biochem. Biophys. Res. Commun. 430, 1151–1156.

Wang, Y.T., Xu, Z.D., Peng, L.C., 2014. Research progress in the groove structures of plant cell walls and biomass utilizations. Sci. Sin. Vitae 44, 766–774. http://dx.doi.org/10.1360/052014-99.

Wu, Z., Hao, H., Zahoor, Tu, Y., Hu, Z., Wei, F., Liu, Y., Zhou, Y., Wang, Y., Xie, G., Gao, C., Cai, X., Peng, L., Wang, L., 2014. Diverse cell wall composition and varied biomass digestibility in wheat straw for bioenergy feedstock. Biomass Bioenergy 70, 347–355.

Wu, Z., Zhang, M., Wang, L., Tu, Y., Zhang, J., Xie, G., Zou, W., Li, F., Guo, K., Li, Q., Gao, C., Peng, L., 2013. Biomass digestibility is predominantly affected by three factors of wall polymer features distinctive in wheat accessions and rice mutants. Biotechnol. Biofuels 6, 183.

Xie, G., Peng, L., 2011. Genetic engineering of energy crops: a strategy for biofuel production in China. J. Integr. Plant Biol. 53, 143–150.

Xie, G., Peng, L., 2014. Genetic engineering of bioenergy crops. In: Wang, L. (Ed.), Sustainable Bioenergy Production. CRC Press Taylor & Francis Group.

Xie, G., Yang, B., Xu, Z., Li, F., Guo, K., Zhang, M., Wang, L., Zou, W., Wang, Y., Peng, L., 2013. Global identification of multiple OsGH9 family members and their involvement in cellulose crystallinity modification in rice. PLoS One 8, e50171.

Xu, N., Zhang, W., Ren, S., Liu, F., Zhao, C., Liao, H., Xu, Z., Huang, J., Li, Q., Tu, Y., Yu, B., Wang, Y., Jiang, J., Qin, J., Peng, L., 2012. Hemicelluloses negatively affect lignocellulose crystallinity for high biomass digestibility under NaOH and H_2SO_4 pretreatments in *Miscanthus*. Biotechnol. Biofuels 5, 58.

Zhang, W., Yi, Z., Huang, J., Li, F., Hao, B., Li, M., Hong, S., Lv, Y., Sun, W., Ragauskas, A., Hu, F., Peng, J., Peng, L., 2013. Three lignocellulose features that distinctively affect biomass enzymatic digestibility under NaOH and H_2SO_4 pretreatments in *Miscanthus*. Bioresour. Technol. 130, 30–37.

Zheng, Y., Pan, Z., Zhang, R., Wang, D., 2009. Enzymatic saccharification of dilute acid pretreated saline crops for fermentable sugar production. Appl. Energy 86, 2459–2465.