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# Mild alkali-pretreatment effectively extracts guaiacyl-rich lignin for high lignocellulose digestibility coupled with largely diminishing yeast fermentation inhibitors in *Miscanthus*

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## HIGHLIGHTS

- G-rich *Miscanthus* sample has high biomass digestibility under alkali pretreatments.
- S- and H-rich samples show relatively low biomass saccharification.
- G-rich sample displays effective extraction of lignin–hemicelluloses complex.
- G-rich sample is in favor of diminishing lignin inhibitors to yeast fermentation.
- Minor monolignin modification for cost-effective biofuel production in *Miscanthus*.

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## ABSTRACT

In this study, various alkali-pretreated lignocellulose enzymatic hydrolyses were evaluated by using three standard pairs of *Miscanthus* accessions that showed three distinct monolignol (G, S, H) compositions. Mfl26 samples with elevated G-levels exhibited significantly increased hexose yields of up to 1.61-fold compared to paired samples derived from enzymatic hydrolysis, whereas Msa29 samples with high H-levels displayed increased hexose yields of only up to 1.32-fold. In contrast, Mfl30 samples with elevated S-levels showed reduced hexose yields compared to the paired sample of 0.89–0.98 folds at $p < 0.01$. Notably, only the G-rich biomass samples exhibited complete enzymatic hydrolysis under 4% NaOH pretreatment. Furthermore, the G-rich samples showed more effective extraction of lignin–hemicellulose complexes than the S- and H-rich samples upon NaOH pretreatment, resulting in large removal of lignin inhibitors to yeast fermentation. Therefore, this study proposes an optimal approach for minor genetic lignin modification towards cost-effective biomass process in *Miscanthus*.



## 1. Introduction

Plant cell walls represent an abundant and renewable biomass resource for biofuels and chemical products (Ragauskas et al., 2006; Chen and Peng, 2013). In particular, lignocellulose-derived ethanol has been considered to be a clean liquid fuel worldwide (Zaldivar et al., 2001). Principally, lignocellulosic ethanol conversion involves the following three major steps: physical and chemical pretreatments for cell wall disassociation, enzymatic digestion for soluble sugar release, and yeast fermentation for ethanol production (Himmel et al., 2007; Rubin, 2008).

However, plant cell wall recalcitrance represents a significant determinant of the costly biomass process, which releases numerous inhibitory compounds that inhibit the yeast fermentation process. To address this issue, the genetic modification of plant cell walls has been proposed for the reduction of biomass recalcitrance

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in bioenergy crops (Xie and Peng, 2011). Therefore, it is essential to identify the key determinants of plant cell walls that can be exploited to improve both biomass enzymatic digestion and ethanol conversion following physical and chemical pretreatments. As the initial step for biomass degradation, it is important to determine the mild pretreatments that not only enhance biomass enzymatic digestibility but also aid in the removal of yeast fermentation inhibitors.

Plant cell walls are mainly composed of cellulose, hemicelluloses and lignin. Cellulose crystallinity has been reported to be negative factor in terms of biomass enzymatic digestibility, whereas hemicelluloses can positively affect biomass saccharification by reducing cellulose crystallinity in *Miscanthus* (Xu et al., 2012; Zhang et al., 2013). Recently, the degree of arabinose substitution in xylans has been characterized as a positive factor on biomass digestibility (Li et al., 2013).

By comparison, lignin is a stable and complex polymer with the following three major phenylpropane units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Sun et al., 2013). These three monomers are linked by ether, ester and C-C bonds that are irregularly repeated (Ralph et al., 2004). Lignin has been proposed to be associated with other cell wall polymers via ester- and ether-interlinked phenolics to form cell wall networks that might act as barriers that hinder enzyme penetration into cellulose surfaces (Achyuthan et al., 2010). However, due to structural diversity and heterogeneity, lignin has been recently suggested to elicit dual effects on biomass enzymatic hydrolysis (Fu et al., 2011; Studer et al., 2011; Xu et al., 2012; Wu et al., 2013). For instance, the S/G ratio is a negative factor on biomass enzymatic digestibility in *Miscanthus*, whereas the H/G could positively affect lignocellulose saccharification in rice and wheat (Xu et al., 2012; Wu et al., 2013). Furthermore, lignin represents a source of aromatic compounds that can inhibit hydrolase enzymes and fermentative organisms used in the production of ethanol (Klinke et al., 2004; Palmqvist and Hahn-Hägerdal, 2000a,b).

In recent years, acid- and alkali-based chemical pretreatments have been broadly used for biomass processes, although both pretreatments appear to represent distinct processes for plant cell wall destruction. Generally, alkali pretreatments predominantly dissociate entire wall polymers by extracting ferulate-crosslinked hemicelluloses and lignin (Sun and Sun, 2002; Macdonald et al., 1983; Mosier et al., 2005), whereas acid pretreatments induce the partial release of monosaccharides and monolignins by splitting strong chemical bonds (Xu et al., 2012). Recently, alkali pretreatments have been reported to result in relatively higher biomass enzymatic digestibility than resulting from acid pretreatments in *Miscanthus* and other plants (Huang et al., 2012; Li et al., 2013; Wu et al., 2013). Accordingly, a wide range of yeast fermentation inhibitory compounds can be released from or form as a result of acid pretreatments compared to that of alkali pretreatments. The inhibitors include the following three major groups: weak acids, furan derivatives and phenolic compounds. However, much remains unknown in terms of the inhibitors during pretreatment that can affect yeast fermentation, particularly in terms of monolignins. Hence, this study focused to evaluate how mild alkali pretreatment can effectively extract lignin–hemicelluloses complexes for a cost-effect lignocellulose enzymatic digestibility and yeast fermentation in *Miscanthus*.

*Miscanthus* is a fast growing perennial crop that exhibits a significantly high biomass yield for biofuels. Due to its original center in East Asia, more than one thousand natural *Miscanthus* germplasm accessions were collected in China, and hundreds of representative samples have been determined with diverse cell wall compositions and variable biomass enzymatic digestibility upon chemical pretreatments (Huang et al., 2012; Li et al., 2013; Zhang et al., 2013). In this study, three standard pairs of *Miscanthus* accessions were characterized with distinct monolignol compositions, leading to identification of the G-rich lignin that could be effectively extracted by mild alkali pretreatments for a cost-effective biofuel production.

## 2. Methods

### 2.1. Biomass sample collection

The *Miscanthus* samples were selected from the natural *Miscanthus* germplasm accessions collected in China in 2007. Mature stem tissues of *Miscanthus* samples were harvested from Hunan experimental fields in 2009 season (Huang et al., 2012; Xu et al., 2012). The collected mature stem tissues were dried at 50 °C, ground through a 40 mesh screen, and stored in a dry container until further use. All of the *Miscanthus* samples were collected from 5–10 individual mature stems, and the ground powders were mixed thoroughly prior to cell wall composition analysis, biomass pretreatment and enzymatic hydrolysis as described below.

### 2.2. Plant cell wall fractionation

The plant cell wall fractionation method previously described by Peng et al. (2000) was used to extract cellulose and hemicelluloses with minor modification suggested by Wu et al. (2013). The soluble sugars, lipids, starches and pectins of the biomass samples were successively removed using potassium phosphate buffer (pH 7.0), chloroform–methanol (1:1, v/v), DMSO-water (9:1, v/v) and 0.5% (w/v) ammonium oxalate. The remaining pellets were extracted using 4 M KOH with 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 supernatants were then neutralized, dialyzed and lyophilized as KOH-extractable hemicelluloses. The remaining pellets were extracted using 2 M TFA for 1 h at 120 °C and washed with distilled water. The combined supernatants were dried under air as non-KOH-extractable hemicelluloses. Accordingly, the remaining pellets from the 4 M KOH extractions were treated with acetic-nitric acids (acetic acid/nitric acid/water: 8:1:2) for 1 h at 100 °C, and the remaining residues were defined as cellulose. All of the experiments were conducted in biological triplicate.

### 2.3. Total hexose and pentose assays

A UV–vis spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai, China) was used for assaying total hexose and pentose levels. Hexoses were detected using the anthrone/$H_2SO_4$ method (Fry, 1988), whereas pentoses were tested using the orcinol/HCl method (Dische, 1962). The standard curves for hexoses and pentoses were drawn using D-glucose and D-xylose as standards, respectively. As the high pentose levels can affect the absorbance reading at 620 nm for hexose content, 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 biological triplicate.

### 2.4. Total lignin measurement

Total lignin content of the raw material and the residue samples obtained from the NaOH pretreatments (0.5%, 1%, 4%; w/v) was determined using the two-step acid hydrolysis method according to the Analytical Procedure of the National Renewable Energy Laboratory (Sluiter et al., 2008). Acid-insoluble lignins were

measured gravimetrically after correction for ash. Acid-soluble lignins were measured by UV spectroscopy. The details of the two-type of lignin assay were previously described by Wu et al. (2013). All of the samples resulted from biological triplicates.

### 2.5. Analysis of monolignin compounds

The monolignin composition of the raw materials and the residue samples obtained from the NaOH pretreatments (0.5%, 1%, 4%, w/v) were determined by HPLC as described by Wu et al. (2013). Standard chemicals: *p*-hydroxybenzaldehyde (H), vanillin (G) and syringaldehyde (S) were purchased from Sinopharm Chemical Reagent Co., Ltd. All samples were extracted with benzene-ethanol (2:1, v/v) in a Soxhlet for 4 h, and the remaining pellets were collected as cell wall residue (CWR). Nitrobenzene oxidation of lignin was conducted as follows: 0.05 g CWR was added with 5 mL 2 M NaOH and 0.5 mL nitrobenzene, and a stir bar was put into a 25 mL Teflon gasket in a stainless steel bomb. The bomb was sealed tightly and heated at 170 °C (oil bath) for 3.5 h and stirred at 20 rpm. Then, the bomb was cooled with cold water. The chromatographic internal standard (ethyl vanillin) was added to the oxidation mixture. This alkaline oxidation mixture was washed 3 times with 30 mL $CH_2C1_2$/ethyl acetate mixture (1:1, v/v) to remove nitrobenzene and its reduction by-products. The alkaline solution was acidified to pH 3.0–4.0 with 6 M HCl, and then extracted with $CH_2Cl_2$/ethyl acetate ($3 \times 30$ mL) to obtain the lignin oxidation products which were in the organic phase. The organic extracts were evaporated to dryness under reduced pressure 40 °C. The oxidation products were dissolved in 10 mL chromatographic pure methanol.

HPLC analysis: The solution was filtered with membrane filter (0.22 μm). 20 μL solution was injected into HPLC (Waters 1525 HPLC) column Kromat Universil C18 (4.6 mm × 250 mm, 5 μm) operating at 28 °C with $CH_3OH$:$H_2O$:HAc (25:74:1, v/v/v) carrier liquid (flow rate: 1.1 mL/min). Calibration curves of all analytes routinely yielded correlation coefficients 0.999 or better, and the detection of the compounds was carried out with a UV-detector at 280 nm. In addition, as nitrobenzene oxidation at 170 °C for 3.5 h could not distinguish ferulic acid and G-monomer or *p*-coumaric acid and H-monomer (Billa et al., 1996), the G- and H-levels should respectively contain small amounts of ferulic acid and *p*-coumaric acid in this study.

### 2.6. Total hemicellulose assay and the determination of monosaccharide composition

Total hemicelluloses were subjected to hexose and pentose assays, as described above. The monosaccharide compositions of the hemicelluloses and the residue samples obtained from 4% NaOH were determined by GC–MS as described by Wu et al. (2013), with minor modification. The well-mixed biomass powder samples were pretreated with 4% NaOH, and the remaining residues were washed with distilled water until a pH of 7.0 was reached. Then, the residue samples were hydrolyzed by 2 M TFA for free monosaccharide release within a sealed tube at 121 °C in an autoclave for 1 h. *Myo*-inositol (200 μg) was added as the internal standard for GC–MS (SHIMADZU GCMS-QP2010 Plus) analysis.

To determine the total hemicellulose monosaccharide composition, the combined supernatants from the 4 M KOH fraction were dialyzed for 36 h after neutralization with acetic acid. The sample from the dialyzed KOH-extractable supernatant or the non-KOH-extractable residue was hydrolyzed by 2 M TFA for free monosaccharide release within a sealed tube at 121 °C in an autoclave for 1 h. Both the non-KOH-extractable and the KOH-extractable hemicelluloses were combined as total hemicelluloses. *Myo*-inositol (200 μg) was added as the internal standard for GC–MS (SHIMADZU GCMS-QP2010 Plus) analysis.

GC–MS analytical conditions: Restek Rxi-5 ms, 30 m × 0.25 mm ID × 0.25 μm df column. Carrier gas: Helium. Injection method: split. Injection port: 250 °C, Interface: 250 °C. Injection volume: 1.0 μL. Temperature program: from 155 °C (maintained for 23 min) to 200 °C (held for 5 min) at 3.8 °C/min and then from 200 °C to 300 °C (maintained for 2 min) at 20 °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. The mass spectra were acquired with full scans based on the temperature program from 50 to 500 m/z in 0.45 s. The calibration curves of all analytes routinely yielded correlation coefficients of at least 0.999.

### 2.7. Pretreatment and enzymatic hydrolysis

Alkali pretreatment and enzymatic hydrolysis have been previously described by Wu et al. (2013). The NaOH pretreatment was performed as follows: the well-mixed biomass powder samples (0.3 g) were added to 6 mL of NaOH at four concentrations (0.5%, 1%, 4%, 8%, w/v), shaken at 150 rpm for 2 h at 50 °C, and centrifuged at 3000*g* for 5 min for soluble sugar collection. The resulting pellets were washed 3–5 times with 10 mL of distilled water until all of the soluble sugars were fully extracted and were subsequently stored at −20 °C for enzymatic hydrolysis. All of the supernatants were combined for the determination of total sugars (pentoses and hexoses); control samples containing 6 mL of distilled water were shaken for 2 h at 50 °C. All samples were derived from biological triplicates.

The enzymatic hydrolysis was performed as follows: the remaining residues from NaOH pretreatments were washed twice with 10 mL of distilled water and once with 10 mL of a mixed-cellulase reaction buffer (0.2 M acetic acid-sodium acetate, pH 4.8). The washed residues were incubated with 6 mL (2.0 g/L) of a mixed-cellulase buffer containing β-glucanase ($\geqslant 3.60 \times 10^4$ U), cellulase ($\geqslant 3.60 \times 10^2$ U) and xylanase ($\geqslant 6.00 \times 10^4$ U; from Imperial Jade Bio-technology Co., Ltd) at 0.20% (w/w) concentration for NaOH-pretreated samples. During enzymatic hydrolysis, the samples were shaken at 150 rpm at 50 °C for 48 h. After centrifugation at 3000*g* for 10 min, the supernatants were collected for determination of the levels of pentose and hexose released by enzymatic hydrolysis. Control samples containing 6 mL of reaction buffer were shaken for 48 h at 50 °C. All of the samples were derived from biological triplicates.

### 2.8. Scanning electron microscopy (SEM)

Scanning electron microscopy was previously described by Wu et al. (2013), with minor modifications. The well-mixed biomass powder sample was pretreated with 1% or 4% NaOH at 50 °C for 2 h; the residue obtained from 1% NaOH treatment was sequentially hydrolyzed with mixed-cellulases at 50 °C for 48 h, as described above. These two parallel remaining residues were rinsed with distilled water, dried under air, and sputter-coated with gold in a JFC-1600 ion sputter (Mito City, Japan). The surface morphology of the treated samples was visualized by SEM (JSM-6390/LV, Hitachi, Tokyo, Japan), and representative images of each sample were acquired from 5–10 views.

### 2.9. Fermentation with three standard monolignins

*Saccharomyces cerevisiae* (Angel yeast Co., Ltd., Yichang, China) was used in all of the fermentation reactions, and the yeast powder was dissolved in 0.2 M pH 4.8 phosphate buffer for fermentation.

The fermentation medium was supplemented with 10.00 g/L peptone, 20.00 g/L yeast extract and 200.00 g/L glucose, and the substances were dissolved in 0.2 M phosphate buffer (pH 4.8), sterilized in autoclave at 0.15 Mpa, and 121 °C for 20 min. The three standard monolignins Vanillin (G), Syringaldehyde (S) and

*p*-Hydroxybenzaldehyde (H) were added separately to the fermentation medium up to concentrations of 30, 60, and 120 μM, respectively. The stock solutions of monolignin were dissolved in 0.2 M phosphate buffer (pH 4.8) and sterile filtered prior to use.

The fermentation reactions were performed in 50-mL wide mouth Erlenmeyer flasks with a working volume of 20.00 mL. The flasks were inoculated to an initial cell mass concentration of 2.00 g/L (cell dry weight) and incubated at 37 °C for 48 h. Samples (300 μL) were withdrawn prior to inoculation and after 4, 8, 16, 32 and 48 h for measurement of optical density (OD) at 600 nm and of residual hexose. The fermentation liquid was distilled after 48 h for the determination of the ethanol content. All of the experiments were performed in biological triplicate.

### 2.10. Fermentation of hydrolysates extracted from NaOH pretreatments

The well-mixed biomass powder samples (0.5 ± 0.0001 g) mixed with 10.00 mL of three concentrations of NaOH (0.5%, 1%, 4%; w/v) and shaken at 150 rpm for 2 h at 50 °C. The supernatants were collected by centrifugation at 3000*g* for 5 min. The residues were washed twice with 5.00 mL of distilled water, and the combined supernatants were neutralized to pH 4.8 using appropriate amounts of HCl, and 0.2 M phosphate buffer (pH 4.8), which were added up to 20.00 mL for yeast fermentation. The supernatants (20.00 mL) were transferred to 30-mL glass test tubes together with appropriate amounts of glucose to final hexose concentrations at 200.00 g/L in all fermentation vessels and then sterilized in autoclave at 0.15 Mpa, and 121 °C for 20 min.

The fermentation reactions were performed in 30-mL glass test tubes that were sealed with rubber plugs. The flasks were inoculated to an initial cell mass concentration of 2.00 g/L (cell dry weight) and incubated at 37 °C for 48 h. The fermentation liquid was distilled after 48 h for determination of ethanol content. All of the experiments were performed in biological triplicate.

### 2.11. Determination and calculation of the ethanol content

Determination of the ethanol content was performed using the dichromate oxidation method (Fletcher and van Staden, 2003) with modifications. The fermentation liquid was distilled at 100 °C for 15 min to produce ethanol liquor. The appropriate amount of ethanol sample in 2.00 mL of 5.00% potassium dichromate ($K_2Cr_2O_7$) (5.00 g $K_2Cr_2O_7$ dissolved in 90.00 mL distilled water and 10.00 mL 98% sulfuric acid) was heated for 10 min in a boiling water bath. After cooling, distilled water was added to a final volume of 10.00 mL, and the absorbance was measured at 600 nm. Absolute ethanol was used as the standard.

The sugar-ethanol conversion rate at the end of the fermentation reaction was calculated according to the following formula: S-E = E/A/H × 100% [S-E: sugar-ethanol conversion rate; E: total ethanol weigh (g) at the end of fermentation; A: the conversion rate at 51.11% (92/180) in the case that glucose is completely conversed to ethanol according to the Embden–Meyerhof–Parnas pathway (EMP pathway) in *S. cerevisiae*; and H: total hexoses weigh (g) at the beginning of fermentation]. All of the experiments were performed in biological triplicate.

## 3. Results and discussion

### 3.1. Monolignin effects on biomass digestibility upon alkali pretreatment

Lignins are primarily composed of three predominant monomers. However, given their structural diversity and chemical heterogeneity, it becomes difficult to determine effects of the three monolignins on biomass enzymatic digestibility (Xie and Peng, 2011; Fu et al., 2011). In this study, three standard pairs of biomass samples were selected with a distinct monolignin constitution (Table 1). Based on a *t*-test, each pair exhibited significant differences in single monolignins ($p < 0.01$, $n = 3$) but had similar compositions in terms of the three major cell wall polymers (cellulose, hemicelluloses, and lignin; details can be found in Electronic Annex Table A.1). By comparison, pair I exhibited a G-monomer difference at 9.25%, whereas pairs II and III showed S- and H-monomer variations at 28.85% and 44.07%, respectively. Although pairs I and II displayed one more monomer difference at $p < 0.05$, they had significantly lower variation rates of 3.26% and 6.53%, respectively (Table 1). Thus, the three pairs of biomass samples can be applied to test for single monolignin effects on biomass enzymatic digestibility of *Miscanthus* samples.

Biomass enzymatic digestibility or saccharification is defined by the hexoses yield (% cellulose) released from hydrolysis using a crude cellulase mixture of lignocellulose after pretreatment (Li et al., 2013; Wu et al., 2013). In this study, a series of alkali pretreatments were performed by using four concentrations (0.5%, 1%, 4%, and 8%) of NaOH at mild conditions (50 °C for 2 h). As a result, all *Miscanthus* samples exhibited significantly higher rates of hexoses yields after pretreatments with various concentrations of NaOH ranging from 0.5% to 4%, whereas almost all of the samples exhibited only slightly increased the hexoses rates from 4% to 8% NaOH pretreatments (details can be found in Electronic Annex Table A.2). At pair I, the G-level of the Mfl26 sample increased by 9.25% (Table 1), exhibiting hexoses yields that were significantly higher (by 1.24- to 1.61-fold) than those of Mfl22 at 0.5%, 1%, and 4% of NaOH pretreatments (details can be found in Electronic Annex Table A.2). Notably, all of the lignocellulosic residues of the Mfl26 sample were completely hydrolyzed by cellulases into soluble sugars after the 4% NaOH pretreatment (details can be found in Electronic Annex Table A.2). By comparison, the H-level of the Msa29 sample increased by 44% for pair III (Table 1), demonstrating that the hexoses yields were increased by 1.2- to 1.32-fold compared to its paired sample (Mfl04) (details can be found in Electronic Annex Table A.2). Furthermore, Msa29 exhibited complete hydrolysis of lignocellulose residues only after the 8% NaOH pretreatment. By contrast, the S level of the Mfl30 sample increased by 28.85% for pair II (Table 1), displaying relatively reduced (by 0.91- and 0.89-fold) hexoses yields compared to its paired sample (Mfl35) after 1% NaOH and 4% NaOH pretreatments, respectively (details can be found in Electronic Annex Table A.2). Under 8% NaOH pretreatment, Mfl30 and Mfl35 exhibited only 90% and 96% hydrolysis of lignocellulose residues, respectively.

Therefore, the minor increases in G-level could result in significantly raised biomass enzymatic digestibility after alkali pretreatment, and the G-rich biomass samples, rather than the H- or S-rich samples, can be completely hydrolyzed by cellulase enzymes into soluble sugars after the 4% NaOH pretreatment at 50 °C for 2 h.

### 3.2. Lignin extraction with alkali pretreatment

To understand the distinct effects of the three monolignins on biomass enzymatic digestibility, the lignin levels were detected from NaOH pretreatments in the three pairs of *Miscanthus* samples. Generally, the Mfl26, Mfl30, and Msa29 samples exhibited significantly increased lignin levels extracted from three concentrations of NaOH ($p < 0.01$ or 0.05) compared with the paired samples (details can be found in Electronic Annex Table A.3). In particular, Mfl26 exhibited increased lignin rates from 14.96% to 28.49% for pair I, Mfl30 exhibited increased rates from 6.94% to 8.48% for pair II, and Msa29 was augmented from 10.79% to 18.07% for pair III (details can be found in Electronic Annex Table A.3). The lignin may have been more significantly extracted from the high G-levels

**Table 1**
Monolignin composition in the *Miscanthus* samples.

| Pair | Sample | Three monolignins (μmol/g) | | | | | |
|---|---|---|---|---|---|---|---|
| | | G | | S | | H | |
| I | Mfl22 | **690.88 ± 7.09**** | **9.25%**[a] | 388.70 ± 3.99* | 3.26% | 536.50 ± 5.50 | 0.86% |
| | Mfl26 | **754.82 ± 7.75** | | 376.43 ± 3.87 | | 531.91 ± 5.46 | |
| II | Mfl35 | 592.17 ± 9.25 | 4.28% | **263.37 ± 4.11**** | **28.85%** | 566.12 ± 8.84* | 6.53% |
| | Mfl30 | 567.85 ± 10.65 | | **339.36 ± 6.37** | | 603.07 ± 11.31 | |
| III | Mfl04 | 754.19 ± 13.35 | 1.42% | 398.73 ± 7.06 | 1.77% | **422.13 ± 7.47**** | **44.07%** |
| | Msa29 | 743.60 ± 18.02 | | 405.80 ± 9.84 | | **608.18 ± 14.74** | |

* and **, A significant difference at pair by *t*-test at $p < 0.05$ and 0.01 ($n = 3$).
[a] Percentage of the increased or decreased level at pair: subtraction of two samples divided by low value at pair.

of the *Miscanthus* samples than the samples with high S- and H-levels from alkali pretreatments. Thus, the increased biomass enzymatic digestibility in the G-rich samples was attributed to efficient lignin extraction from the alkali pretreatments. These results can explain the previous finding that the S/G ratio is a negative factor in terms of biomass enzymatic saccharification in *Miscanthus* (Xu et al., 2012).

Furthermore, the extracted three monolignin levels were examined from alkali pretreatments (Fig. 1, details can be found in Electronic Annex Table A.4). The Mfl26 sample exhibited higher levels of all three monolignins for pair I (G, S, H) compared to that of Mfl22 at three concentrations of NaOH pretreatments, whereas for pair II, the Mfl30 sample exhibited two monolignin (S and H) levels higher than that of its paired sample at significant levels ($p < 0.01$ or 0.05). Notably, pair III had a significant variation in H-level but insignificant changes in G- and S-levels (details can be found in Electronic Annex Table A.4). The data were consistent with the finding that G-rich biomass could be effectively extracted for lignins with alkali pretreatments for high lignocellulosic enzymatic digestibility. Moreover, the three monolignins exhibit distinct linkage styles. In particular, G-monomers might elicit a central function in cell wall structural networks.

As the three monolignins are held together by different linkage styles (Hatfield and Vermerris, 2001), two predominant interlinked phenolics (*p*-CA and FA) were detected from the alkali pretreatments in the three pairs of samples (details can be found in Electronic Annex Fig. A.1). As a result, for pair I, two interlinked phenolic levels were more detectable in the Mfl26 sample than in the Mfl22 sample, whereas for pair II, the Mfl30 sample only exhibited more *p*-CA compound than its paired sample at the three concentrations of NaOH pretreatments. Notably, pair III samples displayed variation in the two interlinked phenolics (details can be found in Electronic Annex Fig. A.1). The results support the above findings that the three monolignins may have distinct linkage styles in the cell wall structural networks.

Taken together, the results indicate that a G-rich sample is favorable for the effective alkali extraction of lignin structural complexes, enhancing accessibility for enzymes to cellulose surfaces for increased lignocellulose digestibility in *Miscanthus*.

### 3.3. Hemicelluloses extraction with alkali pretreatment

Lignin is directly associated with hemicelluloses that form cell wall networks (Gübitz et al., 1998; Choi et al., 2007). Given that alkali pretreatments can facilitate hemicellulose extraction by dissociating hydrogen bonds with cellulose microfibers (Chang and Holtzapple, 2000; Mosier et al., 2005; Xu et al., 2012), the extracted hemicellulose levels were also detected from three concentrations of NaOH pretreatments in three pairs of *Miscanthus* samples. Notably, for pair I, Mfl26 exhibited an increased amount of extracted hemicelluloses by 44% to 100% compared to its paired samples (Mfl22) at three concentrations of NaOH pretreatments (details can be found in Electronic Annex Table A.5). By comparison, the Mfl30 sample at pair II displayed only slightly more hemicelluloses by 15% to 24% than its paired sample (Mfl35). Thus, pairs I and II of the *Miscanthus* samples showed consistent extraction of lignin–hemicelluloses complexes. For pair III, Msa29 also exhibited significantly higher hemicellulose levels by 54% to 416% than its paired sample (Mfl04), suggesting that H-rich lignins should have more interactions with hemicelluloses in *Miscanthus*.

Xylans are major hemicelluloses in *Miscanthus* (Xu et al., 2012). The Xyl/Ara ratio of xylans (as a reverse indicator of the degree of arabinose substitution) is a main factor that can negatively affect biomass enzymatic digestibility (Li et al., 2013). In this study, the Xyl/Ara values were compared between the raw material and the biomass residues obtained from 4% NaOH pretreatments (details can be found in Electronic Annex Table A.6). Although the Mfl26 and Msa29 samples in pairs I and III exhibited higher Xyl/Ara values than those of their paired samples by 17% and 26%, respectively, in the raw materials, significantly reduced Xyl/Ara values

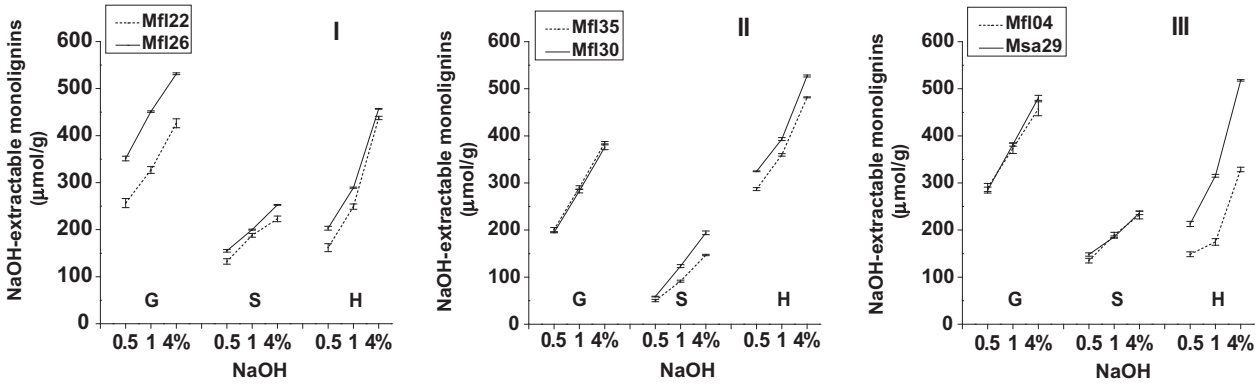


**Fig. 1.** The three monolignins extracted from various NaOH pretreatments in three pairs of *Miscanthus* samples; bar indicates SD ($n = 3$).

were found in the biomass residues of Mfl26 and Msa29 by 55% and 48%, respectively, after the 4% NaOH pretreatment (details can be found in Electronic Annex Table A.6). The cell wall features of G- and H-rich biomass samples were significantly altered from alkali pretreatment because of the effective extraction of lignin–hemicellulose complexes. By contrast, for pair II, the Mfl30 sample displayed higher Xyl/Ara values than its paired sample (Mfl35) in both raw materials and 4% NaOH residues, suggesting that the S-rich biomass samples might only be slightly affected by alkali pretreatment because of significantly less efficient lignin and hemicellulose extractions. In addition, the results support that Xyl/Ara is a negative factor in terms of biomass enzymatic digestibility after chemical pretreatments in *Miscanthus* and other plants (Li et al., 2013; Wu et al., 2013).

### 3.4. Lignocellulosic residue images from alkali pretreatment

In terms of altered cell wall features resulting from the alkali pretreatments, the lignocellulosic residues were observed in the three pairs of *Miscanthus* samples after a 4% NaOH pretreatment using a scanning electron microscope. However, each pair did not exhibit significant differences in biomass residue surfaces after a 4% NaOH pretreatment (data not shown). Further, the biomass residues were observed from the enzymatic hydrolysis after NaOH pretreatments. Given that the Mfl26 sample was completely hydrolyzed by cellulases after a 4% NaOH pretreatment (details can be found in Electronic Annex Table A.2), lignocellulosic residues were observed in the three pairs of *Miscanthus* samples after a 1% NaOH pretreatment and sequential enzymatic hydrolysis using a scanning electron microscope. The Mfl26 and Msa29 samples with high biomass digestibility for pairs I and III exhibited coarse biomass residue surfaces, whereas their paired samples (Mfl22 and Mfl04) displayed relatively smooth surfaces, similar to previous observations in *Miscanthus* and other plants (Xu et al., 2012; Zhang et al., 2013; Li et al., 2013; Wu et al., 2013). In particular, for pair I, the Mfl26 sample exhibited relatively rougher surfaces than that of Msa29 for pair III (data not shown) due to their distinct hexose yields of 82% and 64%, respectively (details can be found in Electronic Annex Table A.2). By comparison, pair II samples did not exhibit significant differences due to their similar biomass enzymatic saccharification rates of 66% and 60% (details can be found in Electronic Annex Table A.2). Thus, the relatively rougher surfaces of G-rich biomass residues should be attributed to effective enzymatic hydrolysis after alkali pretreatments.

### 3.5. Monolignin inhibition of ethanol fermentation

Phenolic compounds are major inhibitors of yeast fermentation. However, little is known regarding the distinct effects of the three monolignins. In this study, an ethanol fermentation course was performed using *S. cerevisiae* and the monolignin-mediated inhibition was examined by adding three concentrations of standard G-, S-, and H-compounds to the yeast culture media. The degree of inhibition was negatively defined by accounting for the sugar-ethanol conversion rate (Table 2). After supplementation with 30 μM of the three monolignins, yeast fermentation was only slightly affected for the ethanol products but was significantly inhibited at 60 μM of the S- and H- compounds compared with the control ($p < 0.05$). Furthermore, at 120 μM, all of the three monolignins affected yeast fermentation at similar rates, whereas the H-monomer did not display differences at 60 μM (Table 2). To verify this experiment, the related time course of *S. cerevisiae* growth in the culture media supplied with 120 μM of the monolignin (Fig. 2A) and also the sugar consumption rates (Fig. 2B) were assessed. Thus, to varying extents, the three monolignins can inhibit yeast fermentation by affecting ethanol production.

**Table 2**
Effects of three standard monolignins on the sugar-ethanol conversion rate.

| Pairs | Concentration (μM) | Sugar-ethanol conversion rate (%) | |
|---|---|---|---|
| Control | 0 | 78.05 ± 0.48 | |
| G | 30 | 77.28 ± 0.82 | −0.99%[a] |
| | 60 | 76.81 ± 0.19 | −1.59% |
| | 120 | 76.20 ± 0.11* | −2.37% |
| S | 30 | 77.20 ± 0.29 | −1.09% |
| | 60 | 76.43 ± 0.61* | −2.08% |
| | 120 | 75.96 ± 0.66* | −2.68% |
| H | 30 | 76.89 ± 0.79 | −1.49% |
| | 60 | 76.01 ± 0.51* | −2.61% |
| | 120 | 76.08 ± 0.48* | −2.52% |

* A significant difference between the sample and control by *t*-test at $p < 0.05$ ($n = 3$).

[a] Percentage of the increased or decreased level between the sample and control: subtraction of the sample and control divided by control.

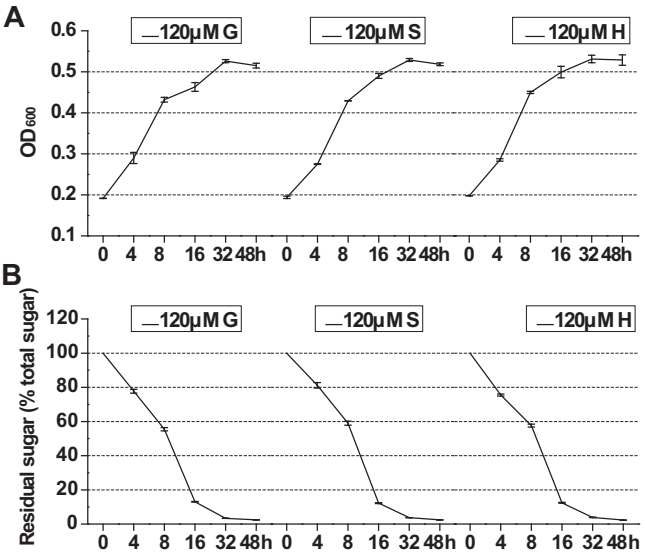


**Fig. 2.** Effects of three standard monolignins on time-course yeast fermentation: (A) Observation by reading $OD_{600}$ of *Saccharomyces cerevisiae* growth; (B) Detection of the unused residual sugars in the culture media supplied with 120 μM monolignins (G: vanillin, S: syringaldehyde, H: *p*-hydroxybenzaldehyde); bar indicates SD ($n = 3$).

### 3.6. Lignin inhibition of yeast fermentation from alkali pretreatment

As the standard monolignins were determined to inhibit yeast fermentation, the effects of the alkali extracts on yeast fermentation were investigated in three pairs of *Miscanthus* samples (Fig. 3). As a result, for pair I, the Mfl26 sample exhibited significantly increased inhibition of yeast fermentation than that of its paired sample (Mfl22), with increased inhibition from 28.51% to 34.95%, respectively (Fig. 3A, details can be found in Electronic Annex Table A.7), consistent with more efficient extraction of lignins at the three concentrations of NaOH pretreatments (details can be found in Electronic Annex Table A.3). By comparison, although for pair II the Mfl30 samples exhibited slightly higher lignin levels than those of its paired samples (Mfl35) at the three concentrations of NaOH pretreatments (details can be found in Electronic Annex Table A.3), it also displayed significantly increased inhibition levels from 21.67% to 29.36%, respectively (Fig. 3A, details can be found in Electronic Annex Table A.7). By contrast, for pair III, the Msa29 sample only exhibited increased inhibition levels compared with its paired sample (Mfl04) from 8.95% to 15.73%, respectively, which was inconsistent with their

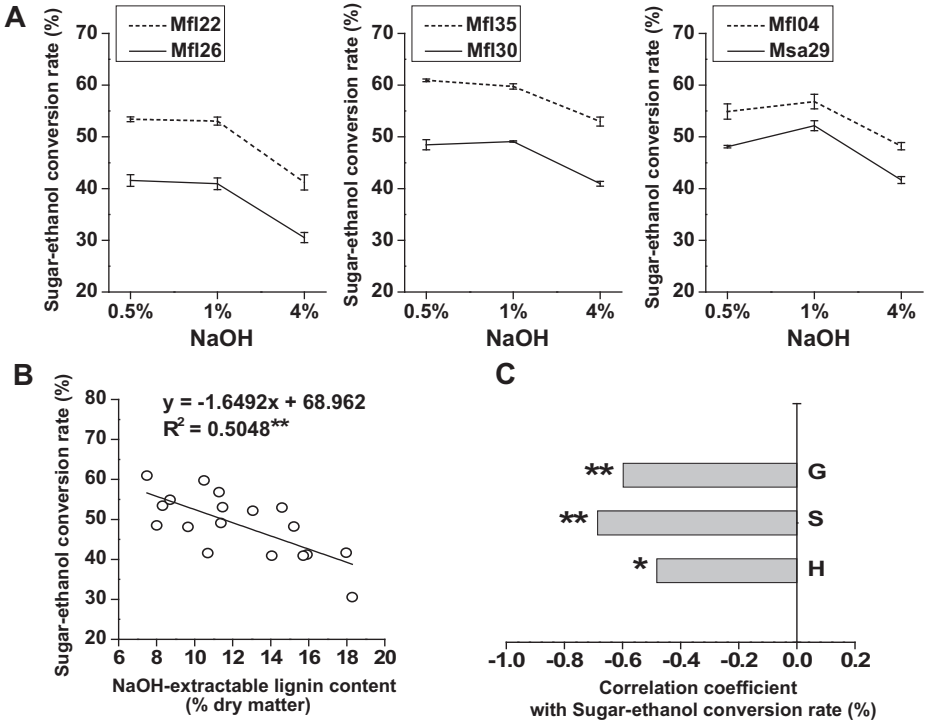


**Fig. 3.** Effects of the lignins extracted from NaOH pretreatments on yeast fermentation in the *Miscanthus* samples: (A) Detection of sugar-ethanol conversion rate by yeast fermentation in the culture media supplied with the extracts from three concentrations of NaOH pretreatments; (B) Analysis of correlative coefficients between the sugar-ethanol conversion rates and total lignin levels extracted from the NaOH pretreatments; (C) Determination of correlative coefficients between the sugar-ethanol conversion rates and three monolignins levels extracted from the various NaOH pretreatments; * and **, highlighted as significant differences by *t*-test at $p < 0.05$ and 0.01, respectively ($n = 18$); bar indicates SD ($n = 3$).

lignin levels at the three concentrations of NaOH pretreatments (details can be found in Electronic Annex Table A.3). This result might be attributed to the increased number of H-monomer associations with hemicelluloses that reduce lignin inhibition. With regard to the distinct monolignin composition of the three pairs of biomass samples, the results suggest that both lignin levels and monolignol composition are important inhibitory factors of yeast fermentation from alkali pretreatments.

To confirm the lignin inhibitory effects on yeast fermentation, a correlation analysis was performed between sugar-ethanol conversion rates and total lignin levels at three concentrations of NaOH pretreatments in three pairs of *Miscanthus* samples (Fig. 3B). Notably, a significantly negative correlation was found ($p < 0.01$) with a coefficient $R^2$ value of 0.505, indicating that the lignin level was an important inhibitory factor in ethanol production. Furthermore, a negative correlation was observed between the three monolignins levels and the sugar-ethanol conversion rates (Fig. 3C). However, the H-monomer exhibited a significantly lower correlative coefficient value ($p < 0.05$) than that of the other two monomers ($p < 0.01$), whereas the S-monomers displayed a more significantly higher coefficient value than the G-monomers. In terms of the similar degree of inhibition by the three standard monolignins *in vitro*, the results suggest that the three monolignins may occur at the different linkage styles during alkali pretreatments in the three pairs of *Miscanthus* samples, resulting in their distinct inhibition of yeast fermentation. Since alkali pretreatment predominantly causes the dissociation of entire wall polymers, including most lignins and some hemicelluloses (Macdonald et al., 1983; Zheng et al., 2009; Xu et al., 2012), the data also support the previous assumption that the three monolignins have distinct linkages in the cell wall structural network *in vivo* in *Miscanthus* (Xu et al., 2012). Thus, the G-rich biomass samples are favorable in terms of enhanced biomass enzymatic digestibility and reduced lignin-mediated inhibition, due to the efficient alkali extraction of lignin–hemicellulose complexes in *Miscanthus*.

### 3.7. Minor lignin modifications and mild alkali pretreatment for cost-effective biomass process

Genetic modification of plant cell walls represents a promising solution for the issue of lignocellulosic recalcitrance in biofuel production. Reducing lignin levels and altering monolignin composition for enhancing biomass digestibility in transgenic plants have been previously reported (Baucher et al., 1999; Chen and Dixon, 2007). However, as a major cell wall polymer, lignin elicits an important biological function in plant mechanical strength and stress responses. Thus, large-scale lignin modifications might result in defective plant growth and reduce biomass production. Both minor lignin modifications and mild pretreatments are crucial for cost-effective biomass digestibility and yeast fermentation. In this study, the natural *Miscanthus* accession (Mfl26) with moderately increased G-levels (less than 10%) exhibited complete enzymatic hydrolysis of lignocelluloses after a mild 4% NaOH pretreatment at 50 °C for 2 h (Table 1, details can be found in Electronic Annex Table A.2). This finding is attributed to the G-monomer, which has a central linkage function in cell wall structural networks that influences the effective extraction of entire cell wall polymers during the alkali pretreatment. As mild alkali pretreatments can result in extraction of more than 70% of lignin in the G-rich Mfl26 accession (Details can be found in Electronic Annex Table A.8), it could also significantly diminish lignin inhibitors of yeast fermentation. Meanwhile, given that more than 50% of the hemicelluloses were not extracted by the alkali pretreatments in the Mfl26 accession (Details can be found in Electronic Annex Table A.8), they nonetheless could be used for biofuel and other chemical production. Hence, the results illustrate an optimal

approach for potential minor genetic modifications of lignin in transgenic plants and provide insights into cost-effective pretreatments for the removal of lignin–hemicellulose complexes from *Miscanthus* and other plants.

## 4. Conclusion

Three major monolignins elicit distinct effects on biomass enzymatic digestibility upon NaOH pretreatments of *Miscanthus* samples. The G-rich biomass sample exhibits increased biomass saccharification compared to the S- and H-rich samples from mild alkali pretreatment due to effective alkali extraction of lignin–hemicelluloses complexes. Furthermore, the G-rich sample appears favorable for the large-scale removal of lignin inhibitors of yeast fermentation, suggesting that the G-monomer may elicit a central function in wall structural networks. Hence, the slight genetic modification of three monolignins proportions could significantly enhance biomass enzymatic digestibility for increased ethanol production 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.2014.07.017.

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