Bioresource Technology 263 (2018) 67–74

Contents lists available at ScienceDirect

Bioresource Technology

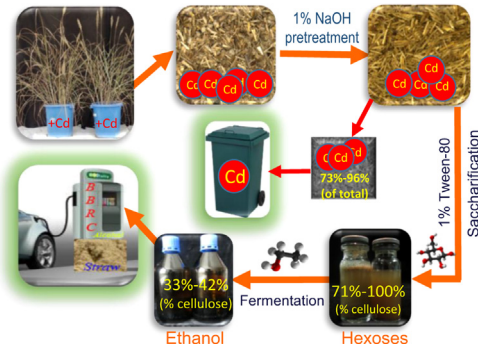
journal homepage: www.elsevier.com/locate/biortech

# *Miscanthus* accessions distinctively accumulate cadmium for largely enhanced biomass enzymatic saccharification by increasing hemicellulose and pectin and reducing cellulose CrI and DP

Shaozhe Cheng[a,b,1], Hua Yu[a,b,1], Meng Hu[a,b], Yue Wu[a,b], Liangliang Cheng[a,b], Qiuming Cai[a,b], Yuanyuan Tu[a,b], Tao Xia[a,c], Liangcai Peng[a,b,d,*]

[a] Biomass and Bioenergy Research Centre, Huazhong Agricultural University, Wuhan, China
[b] College of Plant Sciences and Technology, Huazhong Agricultural University, Wuhan, China
[c] College of Life Sciences and Technology, Huazhong Agricultural University, Wuhan, China
[d] School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

## GRAPHICAL ABSTRACT




## ARTICLE INFO

*Keywords:*
*Miscanthus*
Biomass saccharification
Alkali pretreatment
Wall polymer features
Cadmium accumulation

## ABSTRACT

In this study, total eight distinct *Miscanthus* accessions were collected from the cadmium (Cd)-supplied soil pots, and mild alkali pretreatments (0.5%, 1% NaOH) were then performed to enhance biomass enzymatic saccharification. Due to large Cd accumulation, all *Miscanthus* accessions showed significantly reduced cellulose levels and features (CrI, DP) with much increased hemicellulose and pectin contents in the mature stems. Under mild alkali pretreatments, all *Miscanthus* samples exhibited largely increased hexoses yields released from enzymatic hydrolysis, and one desirable accession had an almost complete biomass saccharification with the hexoses yield at 100% (% cellulose). Notably, the biomass residues remained from enzymatic hydrolysis upon 1% NaOH pretreatment could absorb 73–96% Cd (% of total), suggesting an applicable approach for Cd phyto-remediation. Hence, a hypothetic model was proposed to elucidate that the enhanced biomass saccharification should be mainly due to much reduced cellulose CrI and DP in the Cd-accumulated *Miscanthus* accessions.

* Corresponding author at: College of Plant Sciences and Technology, Huazhong Agricultural University, Wuhan, China.
*E-mail addresses:* lpeng@mail.hzau.edu.cn, pengliangcai2007@sina.com (L. Peng).
*URL:* http://bbrc.hzau.edu.cn (L. Peng).
[1] Equal contribution.

https://doi.org/10.1016/j.biortech.2018.04.031
Received 2 February 2018; Received in revised form 2 April 2018; Accepted 7 April 2018
Available online 19 April 2018
0960-8524/ 

## 1. Introduction

*Miscanthus* is a typical C4 perennial grass with extremely high lignocellulose yield for biofuels and chemical production. *Miscanthus* is a genus of about dozen species and distributes widely under various climate conditions (Lee and Kuan, 2015; Li et al., 2016). Due to a self-incompatible character, *Miscanthus* accessions have wind-dispersed pollens with diverse genetic germplasm, leading to large variations of cell wall compositions including cellulose, hemicellulose, lignin and pectin (Huang et al., 2012; Wang et al., 2015; Zhang et al., 2013).

As the second generation of biofuels, lignocellulose process into bioethanol mainly involves in three steps: initial physical and chemical pretreatments for wall polymer disassociation and destruction, sequential enzymatic hydrolysis for soluble sugar release, and final yeast fermentation for bioethanol production (Scott et al., 2013). Over the past years, physical and chemical pretreatments have been performed in biomass residues of different *Miscanthus* species for enhancing lignocellulose enzymatic saccharification and bioethanol production (Lee and Kuan, 2015; Li et al., 2016, 2014b). Meanwhile, it has been characterized that major wall polymer features distinctively affect hexoses yields released from enzymatic hydrolysis after various pretreatments in *Miscanthus* accession (BoakyeBoaten et al., 2016). As the major cellulose features, cellulose crystalline index (CrI) and degree of polymerization (DP) have been examined to negatively affect biomass enzymatic saccharification in *Miscanthus* and other grass plants (Li et al., 2017; Zahoor et al., 2017b). In comparison, hemicellulose levels could positively affect biomass enzymatic hydrolysis probably by interlinking with cellulose microfibrils via the hydrogen bonds that reduce cellulose CrI. In particular, the arabinose (Ara) substitution degree (reverse xylose/arabinose-Xyl/Ara) of xylans is the positive factor on biomass saccharificaition in *Miscanthus* accessions (Li et al., 2013; Xu et al., 2012). Despite that pectin is a minor wall polymer, it has been reported to positively affect biomass digestion in *Miscanthus* accessions (Wang et al., 2015). More recently, it has indicated that lignin may play dual roles in lignocellulose enzymatic hydrolysis, due to three monomer proportions distinctive in different plant species (Li et al., 2014a; Wu et al., 2013).

Cadmium (Cd) is one of toxic heavy metals occurred in soil, and it could be uptake by special plant species as a desired phyto-remediation technology for environmental protection (Guo et al., 2016; Santos et al., 2015). In particular, plant cell walls could largely accumulate heavy metals in the mature tissues of plants (Inoue et al., 2013; Sun et al., 2013). Despite *Miscanthus* can grow in the poor-quality soil with minimal inputs (CliftonBrown et al., 2001; Lewandowski et al., 2000; Sang and Zhu, 2011), little is reported about Cd accumulation in *Miscanthus* accessions. More importantly, it remains interesting to explore whether the accumulated Cd could be collected during biomass conversion into bioethanol production in *Miscanthus* accessions. Hence, this study collected the biomass samples of four major *Miscanthus* accessions grown in the soil pots co-supplied with Cd in green house, and then performed alkali pretreatment for enhancing biomass enzymatic saccharification and bioethanol production. Finally, this work sorted out how much Cd could be respectively restored in the supernatants and residues of *Miscanthus* accessions from the enzymatic hydrolysis and yeast fermentation.

## 2. Materials and methods

### 2.1. Plant samples

Total eight accessions derived from four *Miscanthus* species (*M. floridulus-Mfl*, *M. sacchariflorus-Msa*, *M. lutarioriparius-Mlu, M. sinensis-Msi*) were grown in the soil pots co-supplied with $CdCl_2$ (100 mg Cd/kg dry soil). Each pot was added with 35 kg soil and supplied with water at 2–3 cm above the soil surface for a month before *Miscanthus* seedlings were transferred. The mature stem and leaf were collected, dried at 50 °C and ground through a 40 mesh screen. The well-mixed powders were stored in a sealed dry container until use.

### 2.2. Biomass preparation and cadmium determination

The dry powder of biomass sample (0.1–0.2 g) was added into the crucible in the muffle furnace. The temperature of furnace was graduated raised to 200 °C for 0.5–1 h, and the temperature was then set to 600 °C for 6–8 h. The ashes was dissolved with 1% $HNO_3$(v/v), washed with 1% $HNO_3$ for 3 times, and all solutions were collected into a 25 mL volumetric flask. Atomic Absorption Spectrometer (Agilent 240Z GFAA) was used for detection of Cd content, and all samples were performed in biological triplicate.

### 2.3. Wall polymer extraction of biomass samples

The procedure of plant cell wall fractionation was used to extract pectin, hemicelluloses and cellulose as previously described by Peng et al. (2000) and Jin et al. (2016). The soluble sugars, lipid and starch were sequentially extracted using potassium phosphate buffer (pH 7.0), chloroform–methanol (1:1, v/v) and DMSO–water (9:1, v/v). The remaining crude cell walls were respectively extracted for pectin and hemicelluloses using ammonium oxalate 0.5% (w/v) and the 4 M KOH with 1.0 mg/mL sodium borohydride. The remaining non-KOH-extractable residues was dissolved with $H_2SO_4$ (67%, v/v), and the supernatants were collected for determination of free hexoses and pentoses as total cellulose and non-KOH-extractable hemicelluloses. All experimental analyses were conducted in biological triplicates.

### 2.4. Colorimetric assay of hexoses and pentoses and uronic acids

UV–VIS Spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd., Shanghai, China) was used for hexoses, pentoses and uronic acids assay as previously described by Huang et al. (2015). Hexoses and pentoses were respectively detected using the anthrone/$H_2SO_4$ (Fry, 1988) and orcinol/HCl (Dische, 1962) methods. Because the pentose could affect hexose readings at 620 nm, the deduction of pentose was conducted at 660 nm and a calibration curve was established to correct for hexose values. Total uronic acids were assayed by *m*-hydroxybiphenyl/NaOH method (Fry, 1988). For cellulose assay, sample was dissolved in 67% $H_2SO_4$ and total hexoses were calculated by the anthrone/$H_2SO_4$ method. The hemicelluloses were calculated by determining total hexoses and pentoses of the hemicelluloses fraction. The hexoses, pentoses and uronic acids of the pectin fraction were calculated as total pectin. All experimental analyses were performed in biological triplicates.

### 2.5. Total lignin assay

Two-step acid hydrolysis method was applied for total lignin assay according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory (Sluiter et al., 2008).

### 2.6. Hemicellulosic monosaccharide determination

GC-MS (SHIMADZU GCMS-QP2010 Plus) was applied for detection of monosaccharide composition of hemicellulose as previously described by Fan et al. (2017). Trifluoroacetic acid (TFA) and *myo*-inositol were purchased from Aladdin Reagent Inc. *1*-Methylimidazole was purchased from Sigma-Aldrich Co. LLC. Acetic anhydride and acetic acid were obtained from Sinopharm Chemical Reagent Co., Ltd.

### 2.7. Detection of degree of polymerization (DP) of cellulose

The dry biomass powders (0.2–1 g) were extracted with 4 M KOH (containing sodium borohydride at 1.0 mg/mL) at 25 °C for 1 h. After

centrifugation at 3000*g* for 5 min, the residues were washed once with 4 M KOH and five times with distilled water until pH at 7.0. The pellet was further extracted with 10 mL 8% $NaClO_2$ at 25 °C for 72 h (change $NaClO_2$ every 12 h). After centrifugation, the residues were washed five times with distilled water until pH at 7.0, and dried with vacuum suction filtration. The DP of crude cellulose sample was detected using the viscosity method (Puri, 1984) with minor modification (Li et al., 2017). All experiments were conducted in biological triplicate.

### 2.8. Detection of cellulose crystalline index (CrI)

The X-ray diffraction method was used for detection of cellulose CrI as previously described by Li et al. (2017) using the Rigaku-D/MAX instrument (Uitima III, Japan). Technical standard errors of the CrI values were measured at ± 0.05 ~ 0.15 using five representative samples in triplicate.

### 2.9. Alkali pretreatment and enzymatic hydrolysis

The well-mixed biomass powders were incubated with 20 mL NaOH at two concentrations (0.5%, 1%, w/v) for 2 h at 50 °C. After centrifugation at 3,000*g* for 5 min, the biomass residues were washed with 30 mL distilled water for 5–6 times until pH 7.0. The pretreated biomass samples were washed 5 ~ 6 times with 30 mL distilled water until pH 7.0, and once more with 30 mL cbuffer (0.2 M acetic acid-sodium acetate, pH 4.8). The washed residues were incubated with 20 mL (2.0 g/L) of mixed-cellulases containing β-glucanase (≥3.73 × 104 U), cellulase (≥373 U) and xylanase (≥6 × 104 U) purchased from Imperial Jade Bio-technology Co., Ltd and also co-supplied with 1% Tween-80. The samples were than shaken under 150 rpm for 48 h at 50 °C (solid–liquid ratio, 1:20). After centrifugation at 3,000*g* for 5 min, the supernatants were collected for total hexoses assay accounting for biomass enzymatic saccharification. The samples only added with 20 mL reaction buffer were shaken for 48 h at 50 °C and as the control. All experiments were carried out in biological triplicate.

### 2.10. Yeast fermentation and ethanol measurement

The yeast fermentation was conducted using *Saccharomyces cerevisiae* strain (purchased from Angel yeast Co., Ltd., Yichang, China) and ethanol was measured as previously described by Jin et al. (2016) and Zahoor et al. (2017b). All soluble sugars were used for yeast fermentation released from both pretreatment and enzymatic hydrolysis. The experiments were performed with biological triplicate.

### 2.11. Statistical calculation of correlation coefficients

Correlation coefficients were generated by using Spearman's rank correlation analysis for all measured traits across the two-sided level of significance (*p < 0.05, **p < 0.01). The line graph, histogram, other variation and regression analysis were performed using Origin 8.5 software (Microcal Software, Northampton, MA) from the experimental data for the best fit curve. The measured aspects were calculated from the average values of original triplications.

## 3. Results and discussion

### 3.1. Distinct Cd uptake in stem and leaf tissues of Miscanthus accessions

In this study, we collected the mature stem and leaf tissues of eight *Miscanthus* accessions derived from four major species (*Mfl*, *Msa*, *Mlu*, *Msi*) grown in the soil pots co-supplied with high concentration of Cd (100 mg $CdCl_2$ per kg dry soil). Based on chemical analysis, total eight *Miscanthus* accessions contained different Cd levels in both mature stem and leaf tissues (Fig. 1). In comparison, the stem tissues of eight accessions showed the Cd levels ranged from 1.99 to 5.12 ug/g dry weight, whereas the leaf tissues had the varied Cd contents from 0.63 to 1.86, consistent with the previous reports about relatively higher Cd levels in stems than those of leaves in other plant species examined (Kevrešan et al., 2003; Sun et al., 2015). Furthermore, despite the *Miscanthus* accessions contained relatively lower Cd levels than those of other plant species (Ok et al., 2011; Pinto et al., 2004), they were slightly affected by Cd supply with relatively high biomass yields, suggesting that the entire *Miscanthus* plant could totally accumulate high amounts of Cd in the biomass residues in this study. In addition, without Cd supply (CK), the *Miscanthus* accessions showed extremely low Cd levels from 0.00 to 0.15 ug/g dry weight. Hence, this study indicated that eight *Miscanthus* accessions were of different Cd accumulative capacity, partially due to their distinct cell wall compositions and wall polymer features (Astier et al., 2014; Douchiche et al., 2010).

### 3.2. Enhanced biomass saccharification in the Cd-accumulated Miscanthus accessions

Biomass enzymatic saccharification (digestibility) has been defined by measuring the hexoses yield (% cellulose) released from cellulases enzymatic hydrolysis of the pretreated biomass residues (Si et al., 2015; Wang et al., 2016). Because the *Miscanthus* leaf tissues contained much low Cd levels with small amounts of biomass residues, this study focused to determine biomass enzymatic saccharification in the mature stem tissues of *Miscanthus* accessions under alkali pretreatments (Fig. 2). Without any pretreatment, only three *Miscanthus* accessions showed significantly increased hexoses yields (% cellulose) released from enzymatic hydrolysis of the mature stem tissues harvested from the Cd-supplied soil pots at $p < 0.05$ and $p < 0.01$ levels, compared to their control (CK) samples without Cd supply (Fig. 2A). Exceptionally, one *Miscanthus* accession (Msa108) had reduced hexoses yield from the Cd-supplied pot. However, under 0.5% NaOH pretreatment, all eight *Miscanthus* accessions from the Cd-supplied pots exhibited significantly higher hexoses yields by 9–34% than those of their control samples at $p < 0.05$ and $p < 0.01$ levels (Fig. 2B). Furthermore, under 1% NaOH pretreatment, seven *Miscanthus* accessions remained further enhanced hexoses yields by 16–32%, compared with their control samples (Fig. 2C). Notably, one desirable accession (Msa108) had an almost complete biomass enzymatic saccharification with the hexoses yield at 100% (% cellulose), and other accessions showed the hexoses yields at 71–83% in the Cd-supplied pots. By comparison, all *Miscanthus* accessions had the hexoses yields at 61–86% in their control samples. Hence, the results indicated that the Cd accumulation in the *Miscanthus* accessions could largely enhance biomass enzymatic digestibility under alkali pretreatments. In addition, because one desirable accession has showed an almost complete biomass enzymatic hydrolysis under 1% NaOH, this study did not further perform NaOH pretreatment at higher concentration or other chemical pretreatments.

### 3.3. Varied bioethanol yields among the Cd-accumulated Miscanthus accessions

Using total soluble sugars released from both alkali pretreatment and sequential enzymatic hydrolysis of biomass samples, this study performed a classic yeast fermentation for bioethanol production in all *Miscanthus* accessions (Fig. 3). Without any pretreatment, six *Miscanthus* accessions from the Cd-supplied pots showed significantly higher bioethanol yields (% cellulose) than those of their control samples at $p < 0.05$ and $p < 0.01$ levels, but all *Miscanthus* accessions remained relatively low ethanol yields (Fig. 3A), due to their low hexoses yields (Fig. 2A). Furthermore, only two *Miscanthus* accessions from the Cd-supplied pots showed significantly enhanced bioethanol yields from 0.5% alkali pretreatment, whereas other five accessions had reduced bioethanol yields, compared to their control samples (Fig. 3B). Furthermore, under 1% NaOH pretreatment, six *Miscanthus* accessions did not show any significantly enhanced bioethanol yields from the Cd-

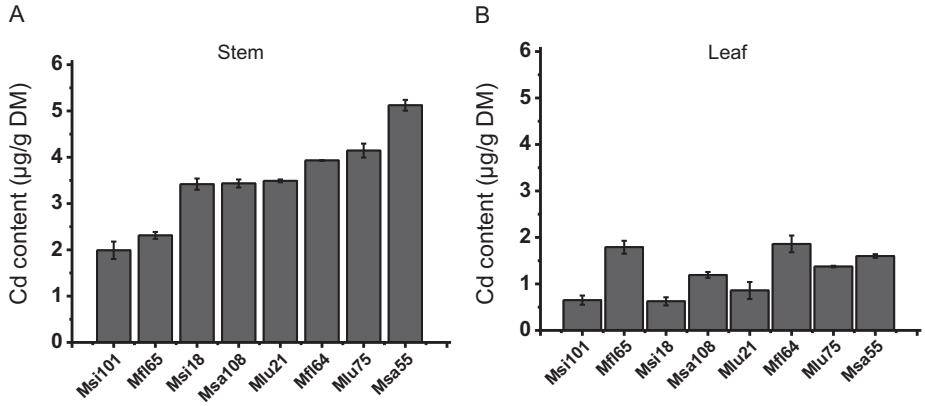



**Fig. 1.** Cd content (ug/g dry matter) in eight *Miscanthus* accessions collected from the soil pots supplied with 100 mg Cd/Kg dry soil. (A) Cd content in mature stem tissues; (B) Cd content in leaf tissues; Bar as means ± SD (n = 3).

supplied pots, but other two accessions from the Cd-supplied pots respectively had higher and lower bioethanol yields than those of their control samples at $p < 0.01$ levels (Fig. 3C). Hence, despite all *Miscanthus* accessions form the Cd-supplied pots had significantly enhanced hexoses yields upon alkali pretreatments (Fig. 2), they showed largely varied bioethanol yields compared to their control samples, suggesting that the Cd and other compounds released from the alkali pretreatment and enzymatic hydrolysis may inhibit yeast fermentation (de Souza Oliveira et al., 2012; Walker, 2004; White and Munns, 2013; Wysocki and Tamas, 2010). However, the desirable *Miscanthus* accessions from the Cd-supplied pots remained both increased hexoses yields and bioethanol production, indicating that they are applicable for Cd remediation and bioethanol production. In addition, it remains to find out extra step or other biomass process technology for removing the Cd and inhibition compounds prior to yeast fermentation in the future.

### 3.4. Altered cell wall composition in the Cd-accumulated Miscanthus accessions

To understand why the Cd accumulation could significantly enhance biomass enzymatic saccharification in all *Miscanthus* accessions examined in this study, we determined four wall polymer contents including cellulose, hemicelluloses, lignin and pectin in both Cd-supplied and control samples (Table 1). As a result, total eight *Miscanthus* accessions from the Cd-supplied pots had significantly lower cellulose contents by 6–13% than those of their control samples at $p < 0.05$ and $p < 0.01$ levels (n = 3), indicating that the Cd accumulation could largely inhibit cellulose biosynthesis in *Miscanthus* accessions. By contrast, all *Miscanthus* accessions from the Cd-supplied pots had much increased hemicellulose and pectin levels. In comparison, the hemicellulose levels were increased by 6–18%, whereas the pectin contents were raised by 13–45% (Table 1), suggesting that the Cd accumulation could relatedly enhance hemicellulose and pectin production, consistent with the previous reports in other plant species (Kan et al., 2016; Xiong et al., 2009; Zhu et al., 2013). Because hemicellulose and pectin levels have been characterized to positively affect biomass enzymatic saccharification under alkali and acid pretreatments in *Miscanthus* (Li et al., 2015; Wang et al., 2015; Xu et al., 2012), the increase of hemicellulose and pectin in the Cd-accumulated *Miscanthus* accessions should be the positive factors on biomass enzymatic hydrolysis. In addition, despite that lignin level negatively affects biomass enzymatic digestibility in *Miscanthus* (Xu et al., 2012), this study did not find out any significantly altered lignin levels in *Miscanthus* accessions from the Cd-supplied pots (Table 1), suggesting that the lignin level may not be the factor on biomass enzymatic hydrolysis in this study.

### 3.5. Reduced cellulose and hemicellulose features in the Cd-accumulated Miscanthus accessions

As cellulose features could largely affect biomass enzymatic saccharification under various pretreatments in different biomass residues (Huang et al., 2015; Jia et al., 2014; Zhang et al., 2013), this study detected cellulose CrI and DP values in *Miscanthus* accessions. In terms of reduced cellulose levels in the Cd-accumulated samples as described above (Table 1), all eight *Miscanthus* accessions from the Cd-supplied pots exhibited significantly decreased cellulose CrI and DP values at

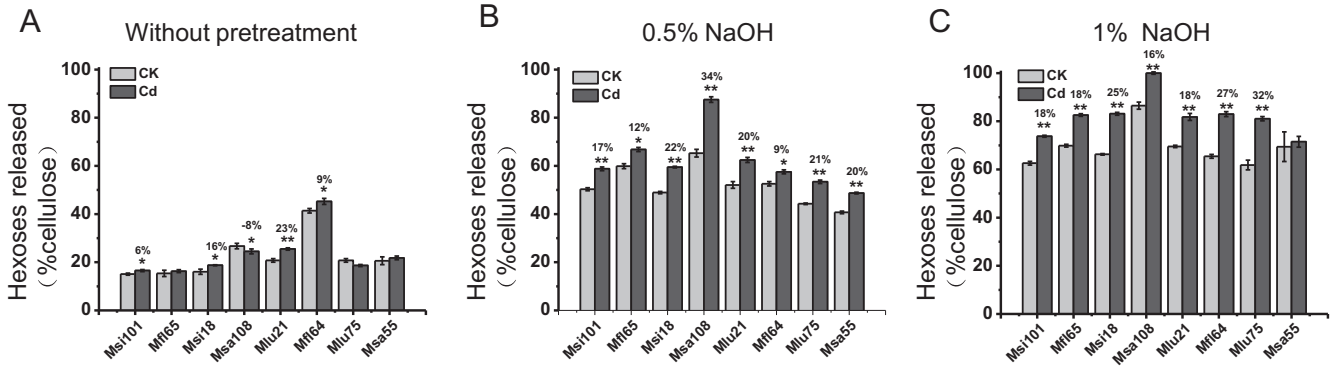



**Fig. 2.** Hexoses yields (% cellulose) released from enzymatic hydrolysis after alkali pretreatments in *Miscanthus* accessions. (A) Without pretreatment; (B) 0.5% NaOH pretreatment; (C) 1% NaOH pretreatment. Bars as means ± SD (n = 3); * and ** indicated significant difference between Cd-accumulated *Miscanthus* samples (Cd) and their control (CK) samples by *t*-test at $p < 0.05$ and $p < 0.01$ levels; Percentage was calculated by subtraction between Cd and CK values divided by CK value.

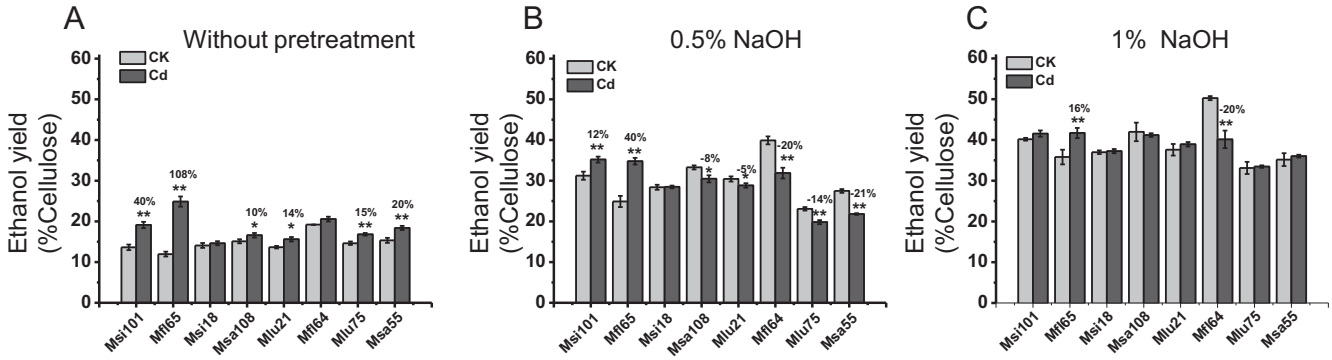



**Fig. 3.** Bioethanol yields (% cellulose) obtained by yeast fermentation using total sugars released from enzymatic hydrolysis and alkali pretreatment in *Miscanthus* accessions. (A) Without pretreatment; (B) 0.5% NaOH pretreatment; (C) 1% NaOH pretreatment. Bars as means ± SD (n = 3); * and ** indicated significant difference between Cd-accumulated *Miscanthus* samples (Cd) and their control (CK) samples by *t*-test at $p < 0.05$ and $p < 0.01$ levels; Percentage was calculated by subtraction between Cd and CK values divided by CK value.

$p < 0.01$ levels, compared to their control samples (Fig. 4A and B), suggesting that the inhibited cellulose biosynthesis may lead to a reduced cellulose DP in the Cd-accumulated *Miscanthus* accessions. Meanwhile, based on the previous reports (Huang et al., 2015; Jin et al., 2016; Li et al., 2014a; Zhang et al., 2013), the reduced cellulose DP and increased hemicelluloses should be combined to cause a decreased cellulose CrI in the Cd-accumulated *Miscanthus* accessions.

Because cellulose CrI and DP are the two key cellulose features negatively affecting biomass enzymatic hydrolysis in various biomass residues examined (Jiang et al., 2017; Li et al., 2014a), this study also performed a correlation analysis between cellulose CrI/DP values and hexoses yields released from enzymatic hydrolysis after alkali pretreatment. Significantly, both cellulose CrI and DP values were negatively correlated with the hexoses yields at $p < 0.05$ and $p < 0.01$ levels (n = 16), respectively (Fig. 4C and D), consistent with the previous reports in *Miscanthus* and other plant species (Huang et al., 2015; Wu et al., 2013; Zhang et al., 2013). Hence, the experimental data demonstrated that the significantly reduced cellulose CrI and DP should be the major causes for largely enhanced biomass saccharification in the Cd-accumulated *Miscanthus* accessions.

To confirm hemicellulose feature impact on biomass enzymatic saccharification in the Cd-accumulated *Miscanthus* accessions, this study determined monosaccharide composition of hemicellulose in all samples. As two major monosaccharides of hemicellulose, arabinose (Ara) levels were increased in all Cd-accumulated accessions, whereas xylose (Xyl) contents were relatively reduced, leading to much reduced Xyl/Ara ratio in the most Cd-accumulated *Miscanthus* accessions, compared to their control samples (Fig. 4E). However, the Xyl/Ara ratio did not show a significant correlation with the hexoses yields released from enzymatic hydrolysis in the *Miscanthus* accession (Fig. 4F), probably due to the insufficient sampling for statistical analysis. As the Xyl/Ara ratio has been well characterized to negatively affect biomass enzymatic hydrolysis in *Miscanthus* (Li et al., 2013), the reduced Xyl/Ara ratio should at least be a minor cause for enhanced biomass saccharification in the Cd-accumulated accessions.

### 3.6. Large Cd restored in the biomass residues from enzymatic hydrolysis

As described above, the *Miscanthus* accessions could largely accumulate Cd in the Cd-supplied soil pots. In this present work, we examined how much Cd could be recovery in the lignocellulose residues obtained from enzymatic hydrolysis upon alkali pretreatment in four representative *Miscanthus* accessions (Table 2). As a result, the four *Miscanthus* accessions maintained the biomass residues at 28–49% (of total dry matter) from enzymatic hydrolysis after 1% NaOH pretreatments, whereas they remained much high biomass residues at 73–89% from direct enzymatic hydrolysis without any pretreatment. Despite of small proportions of biomass residues obtained from 1% NaOH pretreatments, the four *Miscanthus* accessions contained the Cd levels at 3.47–9.68 ug per g of biomass, which were higher by 6–11 folds than those of the biomass residues from direct enzymatic hydrolysis without any pretreatments (Table 2). Notably, based on the enzymatic hydrolysis upon 1% NaOH pretreatment, the remained biomass residues of four *Miscanthus* accessions could restore the Cd at 73%-96% of total dry matter, leading to small proportions (4–27%) of Cd left in the supernatants, which suggested that the remained biomass residues may largely absorb the Cd released from enzymatic hydrolysis after alkali pretreatment (Kumar and Bandyopadhyay, 2006; Wan Ngah and

**Table 1**
Cell wall composition (% dry matter) of mature stem tissues in the Cd-accumulated *Miscanthus* accessions and control (CK) samples.

| Cell wall composition | Biomass sample | Msi101 | Mfl65 | Msi18 | Msa108 | Mlu21 | Mfl64 | Mlu75 | Msa55 |
|---|---|---|---|---|---|---|---|---|---|
| Cellulose | CK | 27.84 ± 0.24 | 27.39 ± 0.36 | 30.40 ± 0.14 | 25.67 ± 0.59 | 28.30 ± 0.26 | 25.71 ± 0.26 | 29.74 ± 0.40 | 25.92 ± 0.49 |
| | +Cd | 24.91 ± 0.29 | 24.96 ± 0.11 | 27.29 ± 0.21 | 22.71 ± 0.27 | 25.80 ± 0.18 | 22.86 ± 0.42 | 27.81 ± 0.05 | 22.63 ± 0.30 |
| | | **−10%**@** | **−8.00%*** | **−10.00%**** | **−11.5%**** | **−9.00%**** | **−11.00%**** | **−6.40%*** | **−12.70%**** |
| Hemicellulose | CK | 27.82 ± 0.10 | 29.48 ± 0.44 | 28.08 ± 0.26 | 26.40 ± 0.07 | 24.63 ± 0.24 | 27.41 ± 0.24 | 26.97 ± 0.12 | 28.55 ± 0.36 |
| | +Cd | 30.80 ± 0.02 | 31.07 ± 0.35 | 33.36 ± 0.22 | 28.09 ± 0.62 | 27.96 ± 0.67 | 28.96 ± 0.28 | 29.45 ± 0.34 | 30.41 ± 0.29 |
| | | **10.00%*** | **6.00%*** | **18.00%**** | **6.40%*** | **13.50%**** | **5.60%*** | **9.00%**** | **6.50%*** |
| Lignin | CK | 23.01 ± 0.53 | 18.04 ± 0.29 | 21.78 ± 0.56 | 21.29 ± 0.68 | 21.19 ± 0.54 | 17.07 ± 0.32 | 20.06 ± 0.39 | 22.76 ± 0.63 |
| | +Cd | 22.37 ± 0.15 | 18.51 ± 0.48 | 20.69 ± 0.34 | 22.98 ± 1.63 | 21.01 ± 0.77 | 17.96 ± 0.96 | 20.93 ± 0.25 | 23.33 ± 0.20 |
| Pectin | CK | 2.70 ± 0.03 | 2.01 ± 0.01 | 2.42 ± 0.05 | 3.23 ± 0.01 | 2.77 ± 0.05 | 2.94 ± 0.01 | 3.10 ± 0.02 | 3.49 ± 0.03 |
| | +Cd | 3.49 ± 0.01 | 2.92 ± 0.01 | 3.19 ± 0.03 | 3.84 ± 0.04 | 3.45 ± 0.02 | 3.33 ± 0.01 | 3.86 ± 0.03 | 4.16 ± 0.07 |
| | | **29.00%*** | **45.00%**** | **32.00%**** | **19.00%*** | **24.50%**** | **13.00%*** | **24.50%**** | **19.00%*** |

Data as means ± SD (n = 3); * and ** indicated significant difference between Cd-accumulated *Miscanthus* samples (+Cd) and their control (CK) samples by *t*-test at $p < 0.05$ and $p < 0.01$ levels; @ Percentage was calculated by subtraction between +Cd and CK values divided by CK value.

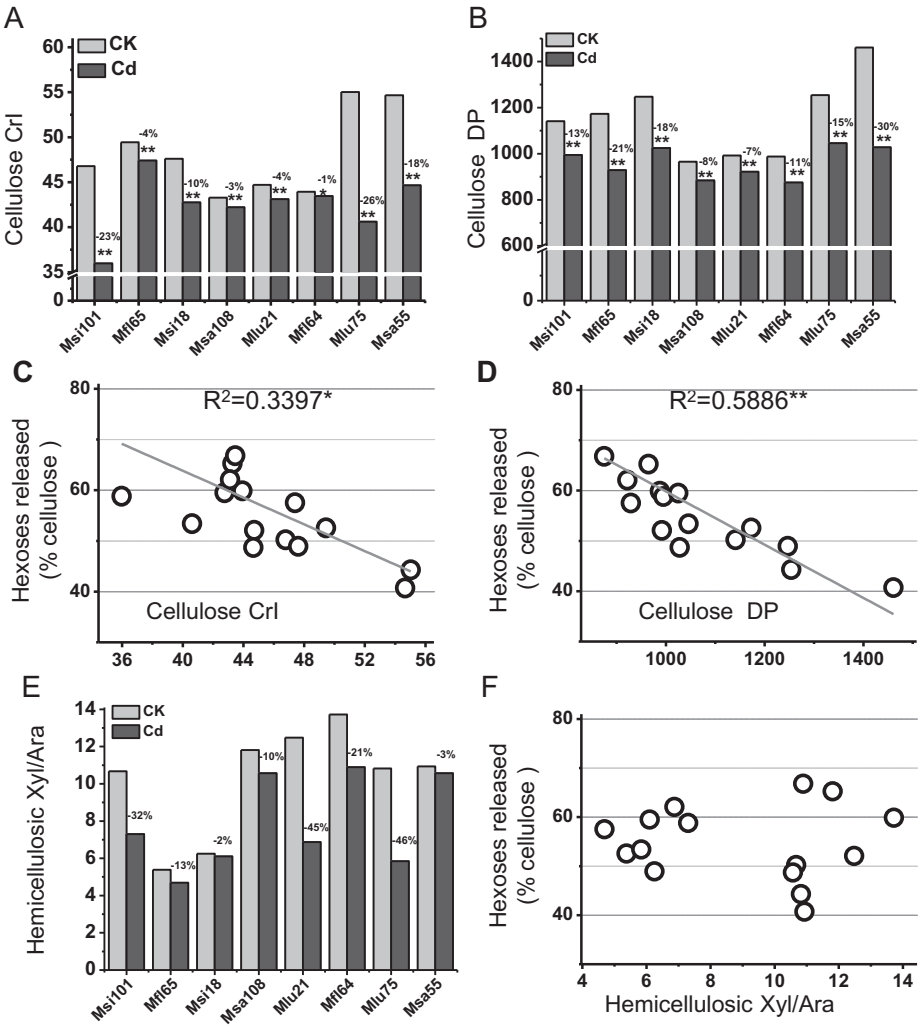



**Fig. 4.** Wall polymer feature impacts on biomass saccharificaiton in *Miscanthus* accessions. (A) Cellulose CrI; (B) Cellulose DP; (F) Hemicellulosic Xyl/Ara ratio; * and ** indicated significant difference between Cd-accumulated *Miscanthus* samples (Cd) and their control (CK) samples by *t*-test at $p < 0.05$ and $p < 0.01$ levels; Percentage was calculated by subtraction between Cd and CK values divided by CK value; (C, D, F) Correlation analysis between hexoses yields and wall polymer features (Cellulose CrI, DP and hemicellulosic Xyl/Ara); * and ** indicated significant correlation between Cd-accumulated *Miscanthus* samples (Cd) and their control (CK) samples at $p < 0.05$ and $p < 0.01$ levels.

**Table 2**
Cd level and proportion remained in the biomass residues after enzymatic hydrolysis with/without NaOH pretreatment in the *Miscanthus* accessions harvested from Cd-supply pots.

| Species | NaOH pretreatment | Biomass residues (% of total dry matter) | Cd level (ug/g biomass) | Cd proportion (% of total) |
|---|---|---|---|---|
| Msi101 | 0.0% | 86% | 0.75 | 32% |
| | 0.5% | 60% | 2.61 | 78% |
| | 1.0% | 38% | 4.25 | 81% |
| Mfl65 | 0.0% | 86% | 0.33 | 12% |
| | 0.5% | 70% | 2.25 | 68% |
| | 1.0% | 49% | 3.47 | 73% |
| Msa108 | 0.0% | 73% | 1.11 | 24% |
| | 0.5% | 38% | 6.32 | 70% |
| | 1.0% | 28% | 9.67 | 78% |
| Mlu75 | 0.0% | 89% | 1.17 | 25% |
| | 0.5% | 60% | 6.00 | 87% |
| | 1.0% | 41% | 9.68 | 96% |

Hanafiah, 2008). Therefore, the Cd accumulation in the *Miscanthus* accessions could not only significantly enhance biomass enzymatic saccharification under alkali pretreatments, but also it could be largely collected from the enzymatic hydrolysis.

### 3.7. Mechanism of Cd enhancements on biomass enzymatic saccharification

To sort out why CrI accumulation could largely enhance biomass enzymatic digestibility in the *Miscanthus* accession, we proposed a hypothetic model based on the previous reports and the experimental data obtained in this study (Fig. 5). (1) The Cd accumulation inhibited cellulose biosynthesis in all *Miscanthus* accessions, leading to significantly reduced cellulose CrI for largely enhanced biomass enzymatic hydrolysis, because cellulose CrI has been well examined to be the key negative factor on biomass saccharification under physical and chemical pretreatments in various biomass residues examined (Li et al., 2015; Zahoor et al., 2017a). (2) The Cd accumulation relatively increased hemicellulose and pectin contents, which have been characterized to positively affect biomass enzymatic hydrolysis by their Ara and uronic acids interlinking with cellulose microfibrils via the hydrogen bonds that reduce cellulose CrI in *Miscanthus* and other plant species (Li et al., 2013; Wang et al., 2016; Wang et al., 2015). (3) The reduced cellulose DP in the Cd-accumulated *Miscanthus* accession should increase the reducing ends of cellulose microfibrils for efficient cellulases enzyme accession and digestion, and it should also reduce cellulose CrI for high biomass saccharificaiton in this study.

## 4. Conclusions

Total eight distinct *Miscanthus* accessions were examined to accumulate Cd in the mature stem tissues, leading to significantly reduced cellulose content and features (CrI, DP) and much increased

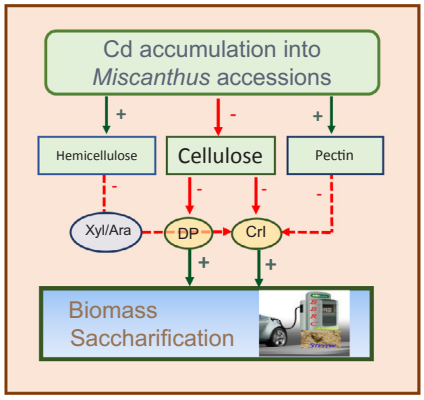



**Fig. 5.** A hypothetic model about how the Cd accumulation distinctively altered wall polymer levels and features for largely enhanced biomass enzymatic saccharification in *Miscanthus* accessions. The green/red arrows and "+"/"-" marks indicated significantly increased/decreased wall polymer levels/features and positively/negatively affected biomass enzymatic saccharification, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hemicellulose and pectin levels. Under mild NaOH pretreatments, the Cd-accumulated *Miscanthus* samples showed largely enhanced hexoses yields from enzymatic hydrolysis, and one desirable *Miscanthus* accession could even have a complete biomass saccharification. Based on correlation analysis and previous reports, a hypothetic model is hence proposed to interpret that the reduced cellulose features and increased wall polymer levels should mainly contribute for enhanced biomass saccharification in the Cd-accumulated *Miscanthus* accessions.

## Acknowledgements

This work was supported in part by grants from the National Key Research and Development Program (2016YFD0800804) and the National 111 Project of Ministry of Education of China (B08032).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.04.031.

## References

Astier, C., Gloaguen, V., Faugeron, C., 2014. Phytoremediation of cadmium-contaminated soils by young Douglas fir trees: effects of cadmium exposure on cell wall composition. Int. J. Phytoremediat. 16, 790–803.
BoakyeBoaten, N.A., Xiu, S., Shahbazi, A., Wang, L., Li, R., Mims, M., Schimmel, K., 2016. Effects of fertilizer application and dry/wet processing of miscanthus x giganteus on bioethanol production. Bioresour. Technol. 204, 98–105.
CliftonBrown, J.C., Lewandowski, I., Andersson, B., Basch, G., Christian, D.G., Kjeldsen, J.B., Jørgensen, U., Mortensen, J.V., Riche, A.B., Schwarz, K.U., 2001. Performance of 15 *Miscanthus* genotypes at five sites in Europe. Agron. J. 93 1013-1019.c.
de Souza Oliveira, R.P., Basso, L.C., Junior, A.P., Penna, T.C.V., Del Borghi, M., Converti, A., 2012. Response of Saccharomyces cerevisiae to cadmium and nickel stress: the use of the sugar cane vinasse as a potential mitigator. Biol. Trace Element Res. 145, 71–80.
Dische, Z., 1962. Color reactions of carbohydrates. Methods Carbohydr. Chem. 1, 477–512.
Douchiche, O., Driouich, A., Morvan, C., 2010. Spatial regulation of cell-wall structure in response to heavy metal stress: cadmium-induced alteration of the methyl-esterification pattern of homogalacturonans. Anna. Bot. 105, 481–491.
Fan, C., Li, Y., Hu, Z., Hu, H., Wang, G., Li, A., Wang, Y., Tu, Y., Xia, T., Peng, L., Feng, S., 2017. Ectopic expression of a novel *OsExtensin-like* gene consistently enhances plant lodging resistance by regulating cell elongation and cell wall thickening in rice. Plant Biotechnol. J.
Fry, S.C., 1988. The Growing Plant Cell Wall: Chemical and Metabolic Analysis. Longman, London, pp. 95–97.
Guo, H., Hong, C., Chen, X., Xu, Y., Liu, Y., Jiang, D., Zheng, B., 2016. Different growth and physiological responses to Cadmium of the three *miscanthus* species. PLoS ONE 11, e0153475.
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.
Inoue, H., Fukuoka, D., Tatai, Y., Kamachi, H., Hayatsu, M., Ono, M., Suzuki, S., 2013. Properties of lead deposits in cell walls of radish (Raphanus sativus) roots. J. Plant Res. 126, 51–61.
Jia, J., Yu, B., Wu, L., Wang, H., Wu, Z., Li, M., Huang, P., Feng, S., Chen, P., Zheng, Y., 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.
Jiang, J., Wang, J., Zhang, X., Wolcott, M., 2017. Assessing multi-scale deconstruction of wood cell wall subjected to mechanical milling for enhancing enzymatic hydrolysis. Ind. Crop. Prod. 109, 498–508.
Jin, W., Chen, L., Hu, M., Sun, D., Li, A., Li, Y., Hu, Z., Zhou, S., Tu, Y., Xia, T., Wang, Y., Xie, G., Li, Y., Bai, B., Peng, L., 2016. Tween-80 is effective for enhancing steam-exploded biomass enzymatic saccharification and ethanol production by specifically lessening cellulase absorption with lignin in common reed. Appl. Energy 175, 82–90.
Kan, Q., Wu, W., Yu, W., Zhang, J., Xu, J., Rengel, Z., Chen, L., Cui, X., Chen, Q., 2016. Nitrate reductase-mediated NO production enhances Cd accumulation in Panax notoginseng roots by affecting root cell wall properties. J. Plant Physiol. 193, 64–70.
Kevrešan, S., Kiršek, S., Kandrač, J., Petrović, N., Kelemen, D., 2003. Dynamics of cadmium distribution in the intercellular space and inside cells in soybean roots, stems and leaves. Biol. Plant. 46, 85–88.
Kumar, U., Bandyopadhyay, M., 2006. Sorption of cadmium from aqueous solution using pretreated rice husk. Bioresour. Technol. 97, 104–109.
Lee, W.C., Kuan, W.C., 2015. *Miscanthus* as cellulosic biomass for bioethanol production. Biotechnol. J. 10, 840–854.
Lewandowski, I., CliftonBrown, J., Scurlock, J., Huisman, W., 2000. *Miscanthus*: european experience with a novel energy crop. Biomass Bioenergy 19, 209–227.
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., Xie, G., Huang, J., Zhang, R., Li, Y., Zhang, M., Wang, Y., Li, A., Li, X., Xia, T., Qu, C., Hu, F., Ragauskas, A.J., Peng, L., 2017. OsCESA9 conserved-site mutation leads to largely enhanced plant lodging resistance and biomass enzymatic saccharification by reducing cellulose DP and crystallinity in rice. Plant Biotechnol. J. 15, 1093–1104.
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., 2015. 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. 13, 514–525.
Li, M., Feng, S., Wu, L., Li, Y., Fan, C., Zhang, R., Zou, W., Tu, Y., Jing, H.C., Li, S., Peng, L., 2014a. 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, X., Liao, H., Fan, C., Hu, H., Li, Y., Li, J., Yi, Z., Cai, X., Peng, L., Tu, Y., 2016. Distinct geographical distribution of the *Miscanthus* accessions with varied biomass enzymatic saccharification. PLoS ONE 11, e0160026.
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., 2014b. The minor wall-networks between monolignols and interlinked-phenolics predominantly affect biomass enzymatic digestibility in *Miscanthus*. PLoS ONE 9, e105115.
Ok, Y.S., Usman, A.R., Lee, S.S., Abd El-Azeem, S.A., Choi, B., Hashimoto, Y., Yang, J.E., 2011. Effects of rapeseed residue on lead and cadmium availability and uptake by rice plants in heavy metal contaminated paddy soil. Chemosphere 85, 677.
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.
Pinto, A.P., Mota, A.M., Varennes, A.D., Pinto, F.C., 2004. Influence of organic matter on the uptake of cadmium, zinc, copper and iron by sorghum plants. Sci. Total Environ. 326, 239.
Puri, V.P., 1984. Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification. Biotechnol. Bioenerg. 26, 1219–1222.
Sang, T., Zhu, W., 2011. China's bioenergy potential. GCB. Bioenergy 3, 79–90.
Santos, M.S.S., Pedro, C.A., Gonçalves, S.C., Ferreira, S.M.F., 2015. Phytoremediation of cadmium by the facultative halophyte plant *Bolboschoenus maritimus* (L.) Palla, at different salinities. Environ. Sci. Pollut. R 22, 15598–15609.
Scott, F., Venturini, F., Aroca, G., Conejeros, R., 2013. Selection of process alternatives for lignocellulosic bioethanol production using a MILP approach. Bioresour. Technol. 148, 525–534.
Si, S., Chen, Y., Fan, C., Hu, H., Li, Y., Huang, J., Liao, H., Hao, B., Li, Q., Peng, L., Tu, Y., 2015. Lignin extraction distinctively enhances biomass enzymatic saccharification in hemicelluloses-rich *Miscanthus* species under various alkali and acid pretreatments. Bioresour. Technol. 183, 248–254.
Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignin in biomass. Labor. Anal. Proced. 1617, 1–16.
Sun, J., Cui, J., Luo, C., Gao, L., Chen, Y., Shen, Z., 2013. Contribution of cell walls, nonprotein thiols, and organic acids to cadmium resistance in two cabbage varieties. Arch. Environ. Con. Toxicol. 64, 243–252.

Sun, S., Li, M., Zuo, J.H., Jiang, W.S., Liu, D.H., 2015. Cadmium effects on mineral accumulation, antioxidant defence system and gas exchange in cucumber. Zemdirbyste-Agric. 102, 193–200.
Walker, G.M., 2004. Metals in yeast fermentation processes. Adv. Appl. Microbiol. 54, 197–230.
Wan Ngah, W.S., Hanafiah, M.A., 2008. Removal of heavy metal ions from wastewater by chemically modified plant wastes as adsorbents: a review. Bioresour. Technol. 99, 3935–3948.
Wang, Y., Fan, C., Hu, H., Li, Y., Sun, D., Wang, Y., Peng, L., 2016. Genetic modification of plant cell walls to enhance biomass yield and biofuel production in bioenergy crops. Biotechnol. Adv. 34, 997–1017.
Wang, Y., Huang, J., Li, Y., Xiong, K., Wang, Y., Li, F., Liu, M., Wu, Z., Tu, Y., Peng, L., 2015. Ammonium oxalate-extractable uronic acids positively affect biomass enzymatic digestibility by reducing lignocellulose crystallinity in *Miscanthus*. Bioresour. Technol. 196, 391–398.
White, J., Munns, D.J., 2013. Inhibitory effect of common elements towards yeast growth. J. I. Brewing 57, 175–179.
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, 1–14.
Wysocki, R., Tamas, M.J., 2010. How Saccharomyces cerevisiae copes with toxic metals and metalloids. Fems. Microbiol. Rev. 34, 925–951.
Xiong, J., An, L., Lu, H., Zhu, C., 2009. Exogenous nitric oxide enhances cadmium tolerance of rice by increasing pectin and hemicellulose contents in root cell wall. Planta 230, 755–765.
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.
Zahoor, Sun, D., Li, Y., Wang, J., Tu, Y., Wang, Y., Hu, Z., Zhou, S., Wang, L., Xie, G., Huang, J., Alam, A., Peng, L., 2017a. Biomass saccharification is largely enhanced by altering wall polymer features and reducing silicon accumulation in rice cultivars harvested from nitrogen fertilizer supply. Bioresour. Technol. 243, 957–965.
Zahoor, Tu, Y., Wang, L., Xia, T., Sun, D., Zhou, S., Wang, Y., Li, Y., Zhang, H., Zhang, T., Madadi, M., Peng, L., 2017b. Mild chemical pretreatments are sufficient for complete saccharification of steam-exploded residues and high ethanol production in desirable wheat accessions. Bioresour. Technol. 243, 319–326.
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.
Zhu, X.F., Wang, Z.W., Dong, F., Lei, G.J., Shi, Y.Z., Li, G.X., Zheng, S.J., 2013. Exogenous auxin alleviates cadmium toxicity in Arabidopsis thaliana by stimulating synthesis of hemicellulose 1 and increasing the cadmium fixation capacity of root cell walls. J. Hazard. Mater. 263, 398–403.