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Three lignocellulose features that distinctively affect biomass enzymatic
digestibility under NaOH and H$_2$SO$_4$ pretreatments in Miscanthus

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Abstract

In this study, total 80 typical Miscanthus accessions were examined with diverse
lignocellulose features, including cellulose crystallinity (CrI), degree of
polymerization (DP), and mole number (MN). Correlation analysis revealed that the
crude cellulose CrI and MN, as well as crystalline cellulose DP, displayed
significantly negative influence on biomass enzymatic digestibility under
pretreatments with NaOH or H$_2$SO$_4$ at three concentrations. By contrast, the
comparative analysis of two Miscanthus samples with similar cellulose contents
showed that crude cellulose DP and crystalline cellulose MN were positive factors on
biomass saccharification, indicating cross effects among the cellulose levels and the three cellulose features. The results can provide insights into mechanism of the lignocellulose enzymatic digestion, and also suggest potential approaches for genetic engineering of bioenergy crops.

**Key words:** Miscanthus; Crystallinity index (CrI); Degree of polymerization (DP); Mole number (MN), Biomass enzymatic digestibility

**1. Introduction**

Energy crisis and global warming have led to an intensive demand for renewable and sustainable fuels. Lignocellulose is the most abundant biomass and represents a major source of carbon for biofuels and other chemical products (Himmel et al., 2007). Over the past years, a great deal of effort has been exerted to increase lignocellulose conversion rate toward high biofuels. However, due to plant cell wall recalcitrance, such goal remains difficult and costly for biomass processes that include three major steps: physical/chemical pretreatment, enzymatic degradation, and yeast fermentation (Ragauskas et al., 2006). Therefore, understanding lignocellulosic features and their effects on biomass conversion is scientifically important for the development of bioenergy products (Reddy and Yang, 2005).

Cellulose is a long chain of glucose molecules linked to one another primarily by glycosidic bonds (Arioli et al., 1998). Cellulose makes up about 30% of the dry mass of primary cell walls and up to 40% of the secondary cell walls (Fry, S.C., 1988). The
hydrogen bonds between different layers of polysaccharides and the van der Waals forces between the parallel chains contribute to the crystalline structure of cellulose. The cellulose crystalline regions alternate with amorphous regions (Bansal et al., 2010; Park et al., 2010). Cellulose microfibrils are insoluble cable-like structures typically composed of about 36 hydrogen-bonded glucan chains, each containing between 500 and 14000 β-1,4-linked glucose molecules (Mohnen et al, 2008). Various models of microfibril substructures have been proposed to account for amorphous and crystalline regions in native cellulose (Haigler, C.H., 1987).

Crystallinity index (CrI) has been used to describe the relative amount of crystalline material in cellulose (Segal et al., 1959). The techniques used for determining the cellulose degree of crystallinity include X-ray powder diffraction, solid-state 13C nuclear magnetic resonance, density measurements, and Fourier transform Raman spectroscopy (Bansal et al., 2010). Highly crystalline cellulose is less accessible to cellulase attack than amorphous cellulose. Thus, the crystallinity of cellulose negatively affects the biomass enzymatic hydrolysis efficiency (Chang and Holtzapple, 2000; Laureano-Perez et al., 2005). However, in most studies, researchers utilized the substrates of relatively pure cellulose to establish the correlation between crystallinity and enzymatic hydrolysis rate, which most likely do not represent the heterogeneous lignocellulosic substrate obtained from biomass pretreatment and sequential enzymatic hydrolysis.

Degree of polymerization (DP) is also an important cellulose feature for biomass enzymatic hydrolysis (Yang et al., 2011). Currently, two techniques are commonly
used to measure cellulose DP, namely, viscometry and gel-permeation chromatography (GPC) methods (Hallac and Ragauskas, 2011). The viscometry technique is more popular in determining lignocellulosic samples (Kumar et al., 2009). Poplar and aspen have a cellulose DP of 3500 and 4500, respectively, (Hallac and Ragauskas, 2011). Agricultural residues have cellulose DP ranging from 1800 to 4000 (Kumar, 2009). Several reports have indicated that DP values correlate inversely with biomass digestibility. The reduction in cellulose DP significantly improves biomass enzymatic hydrolysis due to the increase in the number of cellulose chain-reducing ends (Pan et al, 2008; Puri, V.P., 1984; Zhang and Lynd, 2004). However, other reports have suggested that DP does not noticeably influence the efficiency of lignocellulose hydrolysis (Sinitsyn et al., 1991).

Aside from CrI and DP, mole number (MN) of cellulose is another important parameter accounting for both cellulose content and DP. Principally, cellulose MN can be calculated subjective to dividing cellulose content per unit length/weight by mol wt of cellulose (Kokubo et al., 1991). However, very little is yet known about cellulose MN impact on biomass enzymatic saccharification. In addition, other biomass structural features may also affect biomass enzymatic digestibility, such as accessible biomass surface area, biomass particle size, and physical distribution of lignin and hemicelluloses, as well as their association with cellulose (Zhu et al., 2008). Cross effects between structural features may also occur during pretreatment, and the change in one structural feature may lead to changes in the others (Hallac and Ragauskas, 2011).
*Miscanthus* is a typical C4 perennial grass species that has great potential as a leading sustainable energy crop (Lewandowski et al., 2003). *Miscanthus* originates from East Asia; each species covers different ecological and regional types, contributing to a diverse germplasm resource, and more than 1400 natural *Miscanthus* accessions have been collected in previous research (Xie and Peng, 2011, Huang et al., 2012). In an attempt to interpret the relationship between biomass digestibilities with cellulose features, 80 representative *Miscanthus* accessions were analyzed in this study. These accessions exhibited a diversity of cell wall composition, biomass enzymatic digestibility, and cellulose structural features characterized as CrI, DP and MN. The distinctive effects of cellulose CrI, DP, and MN on biomass saccharification in *Miscanthus* were then detected in experimental and statistical anlysis.

**2. Materials and Methods**

**2.1. Materials**

The *Miscanthus* samples were typically selected from *Miscanthus* natural germplasm accessions collected in China in 2007. The mature stem tissues of *Miscanthus* samples harvested from Hunan experimental field in 2009 season were dried at 50 °C after treated at 105 °C for 5 min. The dried tissues were ground through a 40 mesh sieve and stored in a dry container until use (Huang et al., 2012, Xu et al., 2012). The collected samples were derived from 5-10 individual mature stem tissues and the ground powders were mixed well prior to use for cell wall fractionation and composition analysis, biomass pretreatments and enzymatic hydrolysis as described below.

**2.2. Determination of biomass digestibility**
2.2.1. Physical and chemical pretreatments

H$_2$SO$_4$ pretreatment: The ground biomass powder (0.5 g) was added with 10 mL H$_2$SO$_4$ at three concentrations (0.25%, 1%, 4%, v/v), respectively. The sample tube was sealed well and heated at 121 °C for 20 min in autoclave (15 psi). Then, the sample was shaken at 150 rpm for 2 h at 50 °C, and centrifuged at 3,000 g for 5 min. The remaining pellet was washed three times with 10 mL distilled water, and all supernatants were combined for sugar analysis. The remaining residue was collected for enzymatic hydrolysis, and the sample added only with 10 mL distilled water was shaken for 2 h at 50 °C as control. All experiments were carried out in biological triplicate (Xu et al., 2012).

NaOH pretreatment: The ground biomass powder (0.5 g) was supplemented with 10 mL NaOH at three concentrations (0.5%, 1%, 4%, w/v), respectively. The sample tube was shaken at 150 rpm for 2 h at 50 °C, and centrifuged at 3,000 g for 5 min. The remaining pellet was washed three times with 10 mL distilled water, and all supernatants were combined for sugar analysis. The remaining residue was collected for enzymatic hydrolysis, and the sample with 10 mL distilled water only was shaken for 2 h at 50 °C as control. All experiments were conducted in biological triplicate (Xu et al., 2012).

2.2.2 Enzymatic hydrolysis

The remaining residues from various pretreatments were washed 2 times with 10 mL distilled water, and once with 10 mL mixed-cellulases reaction buffer (0.2 M acetic acid-sodium acetate, pH 4.8). The washed residues were added with 10 mL (2 g/L) mixed-cellulases (containing $\beta$-glucanase $\geq 6 \times 10^4$ U) and cellulase $\geq 600$ U and xylanase $\geq 10 \times 10^4$ U from Imperial Jade Bio-technology Co., Ltd) at 0.16% (w/w) concentration for H$_2$SO$_4$ and NaOH pretreated samples. During the enzymatic hydrolysis, the samples were shaking under 150 rpm at 50 °C for 48 h. After
centrifugation at 3,000 g for 10 min, the supernatants were collected for determining amounts of pentoses and hexoses released from enzymatic hydrolysis. The sample with 10 mL reaction buffer only was shaken for 48 h at 50 °C as control. All experiments were performed in biological triplicate (Xu et al., 2012).

2.2.3 Colorimetric assay of total hexoses and pentoses

UV–VIS Spectrometer (V-1100D, Shanghai MAPADA Instruments Co., Ltd. Shanghai, China) was applied for total hexoses and pentoses analysis. Hexoses were detected using the anthrone/H$_2$SO$_4$ method (Fry, S.C., 1988), and pentoses were assayed by the orcinol/HCl method (Dische, Z., 1962). Anthrone was purchased from Sigma-Aldrich Co. LLC., and ferric chloride and orcinol were obtained from Sinopharm Chemical Reagent Co., Ltd. The standard curves for hexoses and pentoses were drawn using D-glucose and D-xylose as standards (purchased from Sinopharm Chemical Reagent Co., Ltd.) respectively. Total sugar yield from pretreatment and enzymatic hydrolysis was subject to the sum total of hexoses and pentoses. Considering the high pentoses level can affect the absorbance reading at 620 nm for hexoses content by anthrone/H$_2$SO$_4$ method, the deduction from pentoses reading at 660 nm was carried out for final hexoses calculation. A series of xylose concentrations were analyzed for plotting the standard curve referred for the deduction, which was verified by GC-MS analysis. All experiments were carried out in biological triplicate.

2.3 Plant cell wall fractionation

The procedures of plant cell wall fractionation were previously described by Peng
et al (2000) and Xu et al (2012) with minor modification as listed in Figure S1. In this study, the ground biomass powder was defined as raw material. As shown in Figure S1, the raw material was extracted with phosphate buffer, chloroform-methanol and DMSO. The remaining residue was suspended in 0.5% (w/v) ammonium oxalate and heated for 1 h in a boiling water bath, and the supernatants were combined as total pectin. The remaining pellet was suspended in 4 M KOH containing 1.0 mg/mL sodium borohydride for 1 h at 25 °C, and the combined supernatant was neutralized, dialyzed and lyophilized as hemicelluloses. The non-KOH-extractable residue defined as crude cellulose, was further extracted with acetic-nitric acids for 1 h at 100 °C and the remaining pellet was termed as crystalline cellulose. All experiments were conducted in biological triplicate.

2.4 Assay of crude cellulose and crystalline cellulose enzymatic digestion

The crude cellulose and crystalline cellulose samples obtained as described in Figure S1 were treated with 10 mL mixed-cellulases at two concentrations (12 U/mL and 1200 U/mL), respectively. The samples were shaken under 150 rpm at 50 °C for 48 h. The supernatants were collected every 12 h for determining pentoses and hexoses yields. All experiments were carried out in biological triplicate.

2.5 Detection of cellulose crystallinity (CrI)

X-ray diffraction (XRD) method was previously described by Xie et al (2012) for detection of cellulose crystallinity index (CrI) using Rigaku-D/MAX instrument (Ultima III, Japan). The raw biomass powder was laid on the glass sample holder (35
× 50 × 5 mm) and detected under plateau conditions. Ni-filtered Cu Kα radiation (λ = 0.154056 nm) generated at voltage of 40 kV and current of 18 mA, and scanned at speed of 0.0197°/s from 10° to 45°. The crystallinity index (CrI) was estimated using the intensity of the 200 peak (I_{200}, \theta = 22.5°) and the intensity at the minimum between the 200 and 110 peaks (I_{am}, \theta = 18.5°) as the follow: CrI = 100 \times \frac{(I_{200} - I_{am})}{I_{200}} (Segal et al., 1959). 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 Determination of degree of polymerization (DP) of cellulose

Degree of polymerization was measured by the viscosity method (Puri V.P., 1984; Kumar and Kothari, 1999) according to the equation: DP^0.905 = 0.75 [\eta]. And [\eta] is the intrinsic viscosity of the solution. All experiments were performed at 25±0.5 °C using an Ubbelohde viscosity meter and cupriethylenediamine hydroxide (Cuen) as the solvent. The intrinsic viscosity was calculated by interpolation using the USP table (USP, 2002) that lists the predetermined values of the product of intrinsic viscosity and concentration. The [\eta]C, for cellulose samples exhibiting relative viscosity (\eta_{rel}) values between 1.1 and 9.9. \eta_{rel}, was calculated using the equation: \eta_{rel} = \frac{t}{t_0}, where t and t_0 are the efflux times for the cellulose solution and Cuen (blank) solvent, respectively. All experiments were performed in triplicate.

2.7 Determination of cellulose DP by GPC

Cellulose samples (15 mg) were dried overnight under vacuum at 40 °C and were placed in separate test tubes equipped with micro stir bars. Anhydrous pyridine (4.00
mL) and phenyl isocynate (0.50 mL) were added sequentially via syringe. The test tubes were capped with Teflon-lined caps and were placed in an oil bath at 70 °C stirring for 48 h. Methanol (1.00 mL) was added to quench any remaining phenyl isocynate. The mixture of each test tube was then poured into a 7:3 methanol/water (100 mL) to promote precipitation of derivatized cellulose. The solids were collected by centrifuge and were purified through centrifuge by washing with methanol/water (1 × 50 mL) and water (2 × 50 mL). The derivatized cellulose samples were dried overnight under vacuum at 40 °C.

Prior to GPC analysis, the derivatized cellulose samples were dissolved in THF (1 mg/mL), filtered through a 0.45 µm filter and placed in a 2 mL auto-sampler vial. The molecular weight distributions of the cellulose tricarbanilate samples were analyzed by Agilent GPC SECurity 1200 system equipped with four Waters Styragel columns (HR0.5, HR2, HR4, HR6), Agilent refractive index (RI) detector and Agilent UV detector (270 nm). THF was used as the mobile phase (1.0 mL/min) and the injection volume was 30 µL. A calibration curve was constructed based on 12 narrow polystyrene standards ranging in molecular weight from $1.2 \times 10^3$ to $3.6 \times 10^6$ g/mol. Data collection and processing were performed by Polymer Standards Service WinGPC Unity software (Build 6807). Number-average and Weight-average molecular weights ($M_n$ and $M_w$) were calculated by the software relative to the universal polystyrene calibration curve. Number-average degree of polymerization ($DP_n$) and weight-average degree of polymerization ($DP_w$) for the cellulose samples were obtained by dividing $M_n$ and $M_w$ by 519 g/mol, which is the molecular weight of
the tricarbanilated cellulose repeating unit.

2.8 Calculation of cellulose mole number (MN)

Cellulose MN was calculated subjective to cellulose content per 0.1 g divided by mole weight of cellulose. Cellulose mole weight was calculated based on cellulose glucosyl mole weight multiplied by DP of cellulose.

2.9 Statistical calculation of correlation coefficients

Superior Performance Software Systems software package (SPSS 17.0, Inc., Chicago, IL) was applied for the statistical analyses. Correlative analysis was conducted by using Spearman’s rank correlation analysis at the two-sided 0.05 level of significance (* $p < 0.05$, ** $p < 0.01$). The variation and regression analysis are developed using Origin 8.0 software (Microcal Software, Northampton, MA) for the best fit curve from the experimental data. This analysis used the average values calculated from all original determinations for a given trait pair.

3. Results and discussion

3.1. Diversity of cell wall composition and variation of biomass digestibility in Miscanthus

A total of 80 representative Miscanthus samples were selected from more than 1000 Miscanthus germplasm accessions gathered from a nation-wide collection in China (Xie and Peng, 2011; Huang et al, 2012). A diverse cell wall composition was determined in the selected Miscanthus accessions. The cellulose content ranged from 19.7% to 38.5% of dry matter, hemicelluloses from 14.3% to 25.6%, and lignin from 19.28% to 31.10% (Figure 1A and Table S1). One quarter of the Miscanthus samples displayed cellulose contents of approximately 24%, whereas less than 2% of the
samples exhibited cellulose levels of approximately 38%. Hence, the selected 80 Miscanthus accessions presented a large population of biomass samples available for correlation analysis between lignocellulose features and biomass saccharification.

As previously described (Huang et al, 2012; Xu et al, 2012), biomass digestibility or saccharification was defined by accounting either the hexose yield (hexose/cellulose) released from hydrolysis by a crude cellulase mixture of lignocellulose after pretreatment, or the total sugar yield (hexose and pentose/dry weight) from both pretreatment and enzymatic hydrolysis. In the current study, the selected Miscanthus accessions showed a great variation of biomass digestibility under pretreatments with NaOH and H2SO4 at three concentrations (0.5%, 0.25%, 1%, and 4%) (Figure 1B, Figure S2). The biomass digestibility variation can reach 2 to 4 times, and the NaOH pretreatments can result in relatively higher biomass saccharification than that of H2SO4. The diversity of lignocellulose and biomass digestibility can therefore provide the possibility for analysis of the effects of cellulose features on biomass saccharification.

3.2. Diversity of cellulose features in Miscanthus

Cellulose CrI, DP, and MN are important parameters on cellulose structural features (Zhu et al., 2008). Three types of Miscanthus biomass materials were used for the detection of cellulose features, including raw materials, and crude and crystalline celluloses (Figures 2 and S1). Each cellulose feature was measured in the two types of biomass materials. Due to a wide range of cellulose levels (Figure 2A),
the selected Miscanthus accessions exhibited a large variation for each cellulose feature. The CrI values in the raw materials ranged from 23.51% to 59.93%, whereas those in the crude cellulose varied from 40.09% to 64.62% (Figure 2B). The crude cellulose exhibited CrI values much higher than that of the raw materials in the Miscanthus accessions (Table 1) due to 4 M KOH extraction (Figure S1), which was consistent with the recent report about the increased CrI from NaOH catalyzed steam explosion for enhancing biomass enzymatic digestibility in Eucalyptus grandis (Park et al, 2012).

A wide variation of crude- and crystalline cellulose DP was also observed in the selected Miscanthus accessions by using viscometry technique (Figure 2C, Table 1). The crude cellulose DP ranged from 1100 to 2056, whereas that of crystalline cellulose varied from 149 to 385. The average crystalline cellulose DP was sevenfold lower than that of crude cellulose due to the mixed-acid extraction (Figure S1). Currently, the two most common techniques used for cellulose DP measurements are viscometry and gel-permeation chromatography (GPC) (Hallac and Ragauskas, 2011). Viscometry and GPC techniques were compared by measuring the crystalline cellulose DP of 17 randomly selected Miscanthus samples (Table S2); both methods exhibited a significantly positive correlation at $R^2 = 0.68$ and $p < 0.05$ (Figure S3). Thus, the viscometry method can be used to determine the crude and crystalline cellulose DP.

Cellulose MN was calculated based on the cellulose content (per 0.1 g), divided by the molecular weight of cellulose. The diversity of MN was also observed in crude
and crystalline celluloses in *Miscanthus* accessions (Figure 2D, Table 1). By contrast, the average crystalline MN was much higher than that of crude cellulose. Although they were distinct in the three types of biomass materials, the cellulose CrI, DP, and MN values largely varied in *Miscanthus* accessions. Thus, a correlation analysis could be performed using a large population of *Miscanthus* samples, which led to finding out the distinct effects of the three cellulose features on biomass saccharification.

3.3. Negative effects of the cellulose level and features on biomass digestibility

A correlation analysis was performed to ascertain the distinct impacts of cellulose level and features on biomass digestibility (Figure 3). In general, *Miscanthus* accessions exhibited a negative correlation between cellulose content/features and the hexose yields released from enzymatic hydrolysis after pretreatments with NaOH and H₂SO₄ at three concentrations. CrI is customarily detected using raw biomass materials, and has briefly been reported as a negative factor on biomass digestibility (Zhang and Lynd, 2004). Although recent report indicated that cellulose CrI can be negatively affected by hemicelluloses level and positively affected by lignin and cellulose contents, the mechanism remains unclear (Xu et al., 2012). In the present study, the two CrI (raw materials and crude cellulose) values were highly correlated with biomass digestibility (Figure 3A). Despite that the correlation R² values of the crude cellulose CrI ranging from 0.1429 to 0.3316 were not much high (Figure S4), the correlation coefficients reached the significant levels at p < 0.01. Furthermore, the crude cellulose contained less than 20% of the total hemicellulose and lignin (Table
S1), but its enzymatic hydrolysis was positively correlated with that of the biomass residues released from various physical (steam explosion) and chemical (NaOH and H₂SO₄) pretreatments at $p < 0.01$ (Figure S5). Hence, the crude cellulose could be a favorable substrate for cellulose CrI characterization.

With respect to cellulose DP, the Miscanthus accessions did not show any significant correlation in terms of crude cellulose, but displayed a significantly negative effect of crystalline cellulose on biomass saccharification under various pretreatments (Figures 3B and S6), indicating that the lengths (DP) of crystalline regions in the cellulose chains can be the major recalcitrance factor on sugar release. The results were consistent with the recent report that the lower cellulose DP of Alamo switchgrass contributed to a greater cellulose-to-glucose yield (Hu et al, 2011). It also suggested that the reduced cellulose DP may provide more reducing ends for enzymatic hydrolysis (Zhang and Lynd, 2004).

Although the raw biomass CrI and crystalline cellulose DP have been briefly reported in terms of their influences on biomass digestion (Pan et al., 2008; Puri, V.P., 1984; Zhang and Lynd, 2004), little is known about the cellulose MN impact on biomass digestion. In this study, a significantly negative correlation was observed between crude cellulose MN and hexoses yield released from enzymatic hydrolysis after pretreatments with three concentrations of NaOH or H₂SO₄ (Figures 3B and S7), suggesting that the crude cellulose MN could be the important parameter accounting for biomass saccharification rates in Miscanthus. In addition, despite the correlation coefficients at insignificant levels ($p > 0.05$), the crude cellulose DP and crystalline
cellulose MN all displayed a positive tendency with the biomass enzymatic digestion after NaOH and H$_2$SO$_4$ pretreatments at three concentrations (Figure 3B), suggesting that the two features may also be applicable for prediction of biomass saccharification in some cases.

3.4. Correlation among cellulose content and three cellulose features

Although cellulose CrI, DP, and MN are distinctive cellulose features, they all can account for biomass enzymatic digestibility under various chemical pretreatments. In the current study, a correlation analysis was performed to determine the relationships among cellulose CrI, DP, and MN in different types of biomass materials in Miscanthus accessions (Table 2). The cellulose CrI was positively correlated with crystalline cellulose DP at $p < 0.01$, indicating that the length of crystalline regions in the cellulose chains is a factor that could determine lignocellulose crystallinity. As expected, the DP was negatively correlated with the MN in both crude and crystalline celluloses of Miscanthus accessions at $p < 0.01$. The CrI of crude cellulose showed a negative correlation with the MN of crystalline cellulose at $p < 0.05$. Thus, either the CrI or the MN of the crude cellulose exhibited the three significant correlations with other features, suggesting that crude cellulose should be the optimal material accounting for the lignocellulose features that significantly affect biomass saccharification upon various chemical pretreatments.

Furthermore, the cellulose contents were positively correlated with the cellulose CrI and MN values at $p < 0.05$ or 0.01, but showed non-correlation with cellulose DP
(Table 2). The positive correlation between cellulose content and CrI may be due to the increased cellulose level that can relatively enhance the crystalline cellulose proportion. Since cellulose MN was calculated subject to cellulose content divided by DP, it presented positive and negative relationship with cellulose content and DP, respectively. Therefore, the relationships among cellulose content and cellulose CrI, DP and MN can be estimated using three types of biomass materials, and the correlation analysis among three cellulose features could account for their cross effects on biomass enzymatic digestion.

3.5. Dual effects of the cellulose features in representative Miscanthus samples

Based on the previous correlation analysis, two representative samples (Msi52 and Msa02) were selected from the 80 Miscanthus accessions for further characterization of cellulose feature influences on biomass digestibility (Figure 4). Msi52 exhibited about 1- to 2-fold higher biomass saccharification rate than that of Msa02 released from both pretreatments and sequential enzymatic hydrolysis with NaOH and H₂SO₄ at three concentrations (Figure S8). Using the crude cellulose as substrate, the Msi52 sample displayed a remarkably higher hexose yield (% cellulose) than Msa02 during the time course of mixed-cellulase hydrolysis at 12 U/mL concentration (Figure 4A), However, the Msi52 and Msa02 samples did not show any significant difference in the enzymatic digestion of the crystalline cellulose substrates, suggesting that the crystalline cellulose property was much altered due to extremely chemical extractions.
As expected, both Msi52 and Msa02 can release sugars at a similar rate from the mixed-cellulase hydrolysis of crude and crystalline cellulose substrates under extremely high enzyme concentration at 1200 U/mL (Figures 4B). Notably, the crude cellulose substrates maintained a 95% hexose yield under high enzyme concentration, whereas the crystalline cellulose only reached less than 80%, confirming that the crude cellulose was the optimal substrate for cellulase hydrolysis analysis.

Despite of the similar crude and crystalline cellulose levels in the two representative samples (Figure 5A), Msi52 appeared to have distinct cellulose features from Msa02. For instance, Msi52 significantly exhibited much lower values of cellulose structural features than that of Msa02, including CrI and MN of crude cellulose and DP of crystalline cellulose (Figure 5B). Based on the comparative analysis between Msi52 and Msa02 samples, therefore, the crude cellulose CrI and MN and crystalline cellulose DP exhibited negative impact on biomass saccharification, consistent with the correlation analysis of the 80 Miscanthus germplasm samples (Figure 3). By contrast, due to similar cellulose contents between Msi52 and Msa02 samples (Figure 5A), their crude cellulose DP and crystalline cellulose MN displayed a positive effect on biomass digestion at $p < 0.01$ (Figure 5C), which was not completely consistent with the correlation analysis of the 80 Miscanthus samples that could not reach the significant level at $p > 0.05$ (Figures 3B).

As described above, however, the crude cellulose DP and crystalline cellulose MN of 80 Miscanthus samples all showed the positive tendencies with the biomass enzymatic digestibility upon various chemical pretreatments (Figure 3B), and their
coefficients at insignificant levels ($p > 0.05$) may be due to the most *Miscanthus* samples with different crude- and crystalline cellulose contents (Figures 1A and 2A). Provided the cellulose contents were not significantly altered, the crude cellulose DP and crystalline cellulose MN could be the positive factors significantly affecting biomass enzymatic digestibility in *Miscanthus*. Therefore, cellulose MN is the parameter that specifically reflects the cross effects of cellulose content and cellulose features, and the cross effects could lead to dual impacts of cellulose features on biomass enzymatic digestibility under various chemical pretreatments.

In addition, the Msi52 and Msa02 samples did not show any difference in crystalline cellulose CrI values (Figure 5C), which was in agreement with the enzymatic hydrolysis of their crystalline cellulose substrates (Figures 4).

### 5. Conclusions

Using a large population of *Miscanthus* germplasm accessions, three cellulose features (CrI, DP, and MN) were distinctively characterized as negative factors on lignocellulose enzymatic digestibility upon various chemical pretreatments. Based on comparative analysis of the two representative *Miscanthus* samples with similar cellulose levels, the crude cellulose DP and crystalline cellulose MN were determined as the positive factors significantly affecting biomass saccharification. The results can not only provide insights into the biomass enzymatic hydrolysis, but also suggest the potential of plant cell wall modification for bioenergy crop breeding in *Miscanthus* and beyond.
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References


carbohydrates in Arabidopsis root cell walls shows that three radial swelling loci are specifically involved in cellulose production. Planta 211, 406-414.


Figure Legends

Fig. 1 Variation of cell wall composition and biomass digestibility of Miscanthus accessions (n=80). (A) Cell wall composition (% dry matter); (B) Hexoses yield (% cellulose) released from enzymatic hydrolysis after pretreatments with 1% NaOH and 1% H$_2$SO$_4$.

Fig. 2 Diversity of cellulose features of Miscanthus accessions. (A) Cellulose content;
(B) Lignoellulose CrI; (C) Lignocellulose DP; (D) Lignocellulose MN.

**Fig. 3** Correlation analysis among cellulose content, three features and hexose yields released from enzymatic hydrolysis after pretreatments with NaOH or H$_2$SO$_4$ at three concentrations (n=80). (A) Cellulose content and CrI; (C) cellulose DP and MN. * and **: A significant at $p < 0.05$ and 0.01, respectively.

**Fig. 4** Enzymatic hydrolysis of crude cellulose and crystalline cellulose in Msi52 and Msa02 samples. (A) Hexoses yield released from mixed-cellulase hydrolysis of the crude- and crystalline cellulose at 12 U/mL concentration; (B) Hexoses yield released from mixed-cellulase hydrolysis of the crude- and crystalline cellulose at 1200 U/mL concentration. The bar indicated SD (n=3).

**Fig. 5** Analysis of cellulose content and futures in Msi52 and Msa02 samples. (A) Contents of crude- and crystalline cellulose; (B) Left: CrI of crude cellulose, Middle: DP of crystalline cellulose, Right: MN of crude cellulose; (C) Left: CrI of crystalline cellulose, Middle: DP of crude cellulose, Right: MN of crystalline cellulose; * and **: A significant difference by t-test at $p < 0.05$ and 0.01, respectively (n=3).
Table 1. Variation of cellulose features in *Miscanthus* accessions (n=80)

<table>
<thead>
<tr>
<th>Materials</th>
<th>Cellulose features</th>
<th>CrI</th>
<th>DP</th>
<th>MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw material</td>
<td></td>
<td>46.4(^a)</td>
<td>ND(^c)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(23.5~59.9)(^b)</td>
<td></td>
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</tr>
<tr>
<td>Crude cellulose</td>
<td></td>
<td>54.6</td>
<td>1502</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(40.1~64.6)</td>
<td>(1100~2056)</td>
<td>(73.6~180.6)</td>
</tr>
<tr>
<td>Crystalline cellulose</td>
<td></td>
<td>ND</td>
<td>210</td>
<td>741</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(149~385)</td>
<td>(415.5~1155.7)</td>
</tr>
</tbody>
</table>

\(^a\) Mean value, \(^b\) Minimum and maximum values; \(^c\) Not detectable.
Table 2. Correlation coefficients among cellulose content and three cellulose features in *Miscanthus* (n=80)

<table>
<thead>
<tr>
<th></th>
<th>Crl Raw-</th>
<th>Crude-</th>
<th>DP Crude-</th>
<th>Crude-</th>
<th>Crystalline-</th>
<th>MN Crude-</th>
<th>Crude-</th>
<th>Crystalline-</th>
<th>Content Crude-</th>
<th>Crude-</th>
<th>Crystalline-</th>
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<tbody>
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<td>Crl</td>
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</tr>
<tr>
<td>Raw-</td>
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</tr>
<tr>
<td>Crude-</td>
<td>.501**</td>
<td>1.000</td>
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<td></td>
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<tr>
<td>Crude-</td>
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<tr>
<td>Crystalline-</td>
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<td>.487**</td>
<td>-0.173</td>
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</tr>
<tr>
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<tr>
<td>Crude-</td>
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<td>.248*</td>
<td>-.667**</td>
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<tr>
<td>Crystalline-</td>
<td>-0.105</td>
<td>-.227*</td>
<td>0.094</td>
<td>-.757**</td>
<td>.328**</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Crude-</td>
<td>.378**</td>
<td>.268*</td>
<td>-0.064</td>
<td>0.062</td>
<td>.757**</td>
<td>.549**</td>
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<td>.306**</td>
<td>-0.049</td>
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<td>.730**</td>
<td>.528**</td>
<td>.974**</td>
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* and ** significant at $p < 0.05$ and 0.01, respectively.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Highlights

(1) Crude- and crystalline cellulose features were distinct on biomass saccharification

(2) Crude cellulose CrI/MN and crystalline DP negatively affect biomass digestibility

(3) Crude cellulose DP and crystalline MN were positive factors in some cases

(4) Cross effects occur among cellulose level and three cellulose features

(5) Suggest the potential to genetically engineering of bioenergy crops