Biomass properties from different *Miscanthus* species

Chenchen Liu\(^1\), Liang Xiao\(^2\), Jianxiong Jiang\(^2\), Wangxia Wang\(^3\), Feng Gu\(^3\), Dongliang Song\(^1\), Zili Yi\(^2\), Yongcan Jin\(^3\) & Laigeng Li\(^1\)

\(^1\)Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Rd, Shanghai, 200032, China

\(^2\)Department of Biotechnology, Hunan Agricultural University, Hunan Changsha, 410128, China

\(^3\)Jiangsu Provincial Key Lab of Pulp and Paper Science and Technology, Nanjing Forestry University, Nanjing, 210037, China

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**Correspondence**
Laigeng Li, Key Laboratory of Synthetic Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Rd, Shanghai, 200032, China.
Tel: 86-21-54924151; Fax: 86-21-54924015; E-mail: lgli@sibs.ac.cn

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### Abstract

*Miscanthus*, a peculiar genus originating from East Asia, has been considered a promising type of gramineous plant for the bioenergy industry. In this study, four major *Miscanthus* species widely distributed in China, *Miscanthus sinensis*, *Miscanthus floridulus*, *Miscanthus sacchariflorus*, and *Miscanthus lutarioriparius* were assessed for their biomass production, chemical composition, and saccharification efficiency. Results show that the annual dry biomass yields of *M. sinensis*, *M. floridulus*, *M. sacchariflorus*, and *M. lutarioriparius* averaged 16.7, 22.5, 16.7, and 32.0 t ha\(^{-1}\) over 3 years, respectively. *M. sinensis* and *M. floridulus* have similar chemical compositions, but different from *M. sacchariflorus* and *M. lutarioriparius*. The efficiencies of enzymatic saccharification were assayed after pretreatment with dilute acid and green liquor, respectively. The *M. sinensis* and *M. floridulus* biomass displayed higher saccharification efficiency in the case of dilute acid pretreatment, while the *M. sacchariflorus* and *M. lutarioriparius* biomass showed higher efficiency following the green liquor pretreatment. Furthermore, a rapid estimation model for predicting *Miscanthus* biomass saccharification efficiency was established on the basis of near-infrared reflectance spectrometry analysis.

### Introduction

*Miscanthus* Andersson (Poaceae), a genus of perennial C4 plants, includes 14–20 species and has recently been considered a potential energy crop (Sun and Cheng 2002; Melligan et al. 2011; Haverty et al. 2012). China is a genetic center of diverse *Miscanthus* germplasm, of which four *Miscanthus* species, *Miscanthus sinensis* (Ms), *Miscanthus floridulus* (Mf), *Miscanthus sacchariflorus* (Mc), and *Miscanthus lutarioriparius* (Ml), are most widely distributed. So far, several *Miscanthus* species have been studied for possible production of second generation biofuels in a number of laboratories (Tayebi et al. 2001; Xi and Jeżowski 2004; Chung and Kim 2012). It appears that different *Miscanthus* species display various growth adaptions, biomass productivity, and biomass characteristics, which would be important factors for *Miscanthus* breeding and biofuel refining.

Chemical composition and conversion efficiency of lignocellulosic biomass are key factors affecting biofuel production, and thus characterizing biomass properties from various species is essential and would provide needed information for bio-processing as well as bioenergy crop improvement. Lignocellulosic properties including fiber length, cellulose and lignin content have been previously analyzed in *Miscanthus* biomass (Huang et al. 2008; Fan et al. 2010). *M. x giganteus* has been broadly cultivated and...
studied for biomass production in Europe (Pyter et al. 2009; Heaton et al. 2010). Many studies of M. x giganteus have concerned the procedures of pretreatment and enzymatic hydrolysis, and chemical composition prediction models using near-infrared (NIR) reflectance spectrometry (Le Ngoc Huyen et al. 2010; Haverty et al. 2012; Hayes 2012). However, the biomass quality and enzymatic hydrolyzing efficiency of other Miscanthus species, particularly those widely grown in China, are not well characterized.

Lignocellulosic properties affect the processing and efficiency of biofuel conversion. Usually, biofuel production from lignocellulosic biomass includes a process of pretreatment, polysaccharide hydrolysis, and fermentation. In this study, Ms, Mf, Mc, and Ml were compared in terms of their biomass productivity. They were analyzed for their biomass chemical composition, examined for their reaction with different pretreatments, and evaluated on their enzymatic saccharification efficiency. In addition, a fast assessment of enzymatic hydrolyzing efficiency was established for Miscanthus with the relationship of chemical analysis with NIR reflectance spectrometry.

Materials and Methods

Miscanthus biomasses production

Rhizomes of Miscanthus genotypes were collected from different regions of China and planted in a Miscanthus nursery of Hunan Agricultural University in May 2007. The research farm site has a subtropical climate with a mean annual rainfall of 1360 mm and temperature of 17.2°C. Each plot was composed of 10 individuals which were planted in a 1.5 × 0.75 m. No fertilizer was applied and the plants were only treated with pesticide and irrigated, if necessary, during the growing season. The aboveground biomass was hand harvested from late December to early January each year.

Sample preparation

The samples of Miscanthus biomass were air dried, and then crushed and ground with a knife mill. The ground powder was passed through 35-mesh screens and dried in an oven as described in (Hames et al. 2008). Then, the samples were scanned for NIR spectrometry and used for dilute acid pretreatment. Air-dried samples were cut to a length of 3–5 cm and directly subjected to green liquor pretreatment.

Analysis of biomass chemical components

Benzene–alcohol extractive, polysaccharide, and ash content were measured according to the protocol developed by (Sjöström and Alen 1999). Lignin content was determined as described by (Sluiter et al. 2008).

Dilute acid pretreatment and enzymatic hydrolysis

Dried ground sample (1 g) was added to 10 mL of dilute sulfuric acid at different concentrations (1.5, 2.0, 3.0, and 4.0%) in a 150-mL conical flask and soaked for 1 h at room temperature, and then heated to 121°C for 30 min in an autoclave. After cooling to ambient temperature, 40 mL of distilled water and 3 mL of 1 mol/L sodium citrate buffer were added to each flask. The pH was adjusted to around 4.6 with calcium carbonate and the flask was set down for 30 min to reach pH equilibrium. Then, the pH was accurately adjusted to 4.8 using 1 mol/L citric acid and 1 mol/L sodium citrate. Enzyme cellulase of 20 FPUI/g was added to each sample and then the volume was adjusted to 60 mL for saccharification at 50°C, 180 rpm for 48 h. Sugars in hydrolysate were analyzed using an High-performance liquid chromatography (HPLC) method for sugar type analysis and dinitrosalicylic acid (DNS) method for total sugar yield determination. Cellulase activity was determined using the standard procedures of the US National Renewable Energy Lab (Adney and Baker 1996).

Green liquor pretreatment and enzymatic hydrolysis

Green liquor pretreatment was carried out in a ten-bomb lab scale pulping system with oil bath. Green liquor solution was prepared by mixing Na2S and Na2CO3 with a sulfidity of 20% (Gu et al. 2012). The total titratable alkali (TTA) charge as Na2O on oven dried biomass was at 4, 8, 12, 16, and 20%. The biomass samples (80 g) were each treated in a 1 L vessel. The solid-to-solution ratio for green liquor was 1:6 (g/mL). The vessel was incubated at 80°C for 30 min, and then heated to 140°C at a speed of 2°C/min, and kept for 1 h. Then the pulp was washed with distilled water. After the pulp was centrifuged to remove water, it was placed in airtight containers to reach moisture equilibrium and ground with a disc refiner (300 mm in diameter, Kumagai riki kogyo co., ltd., Nerima-Ku, Tokyo, Japan) at 3000 rpm, and centrifuged again to remove water. Part of the ground pulp was dried for chemical analysis and the remainder was used for enzymatic hydrolysis. Enzymatic hydrolysis was performed in 150-mL conical flasks. Water-balanced ground pulp equivalent to 2 g of dried biomass was loaded into a flask and with 40 mL of 0.1 mol/L acetate buffer at pH 4.8 and enzyme Cellic CTe2 (Novezyme, Denmark) at 20 FPUI/g of pulp. Then, the conical flask was incubated at 50°C, 180 rpm for 48 h. After enzymatic hydrolysis, the enzymatic hydrolysate was tested for sugar type and total yield.
Fermentation analysis
Fermentation of biomass hydrolysate was carried out in 50-mL anaerobic jars. The 40-mL enzymatic hydrolysate and 2-mL *S. cerevisiae* suspension culture (at an OD of 10.0) were loaded for ethanol production at 30°C with agitation at 150 rpm. The jars were sampled at 0, 2, 4, 6, 8, and 10 h time intervals to measure glucose and ethanol concentration using HPLC (Yang et al. 2012) and Gas chromatography (Hou and Li 2011) analysis.

NIR data collection and processing
Near-infrared spectra were collected from 172 samples of *Miscanthus* species with one XDS Rapid Content Analyzer NIR spectrophotometer (Foss Analytical, Denmark). The NIR spectrum for each sample was collected three times with reloading of samples after each spectrum collection. The reflectance spectrum covered a range from 400 to 2500 nm, and each spectrum collection was an average of 32 scans. The spectrum data were processed using WinISI 4.0, and the MPLS (Modified Partial Least-Squares) regression method was applied in modeling. Four combinations including No Scatter Treatment with first or second derivative and standard normal variate and detrend (SNVD) with first or second derivative were examined in establishing and verifying the models establishment and verification. The models generated by the four combinations were evaluated by SECV (standard error of cross-validation) and 1-VR (statistic 1-variance ratio) in developing the model, and the R-square (RSQ) multiple correlation coefficients and SEP(C) (corrected standard error of prediction) in verifying them. In addition, 35 samples were collected and analyzed. The results were used to verify the prediction models. The combination of SNVD with second derivative showed the best results, generating the highest 1-VR (0.932) and lowest SECV (0.008) values, as well as the highest RSQ (0.927) and lowest SEP(C) (0.010) values.

Results

**Miscanthus biomass production**
Four *Miscanthus* species, Ms, Mf, Mc, and Ml, were grown for comparison at a research farm in Hunan Province, China. The aboveground biomass was harvested from late December to early January each year. After the plots were established for 2 years, Ms, Mf, Mc, and Ml annual dry biomass yields averaged 16.7, 22.5, 16.7, and 32.0 t ha⁻¹ over 3 years, respectively. This productivity is similar to that reported in the European *Miscanthus* Improvement project, which obtained annual *Miscanthus* yields of 15–25 t ha⁻¹ from central Germany (lat. N 50°) to southern Italy (lat. N 37°) (Lewandowski, 1998). The four species in our trials showed significant differences in biomass production. The biomass yield of Ml was almost double that of Ms, indicating genetic potential for improvement of *Miscanthus* biomass yield.

**Biomass composition analysis**
The four *Miscanthus* species displayed different plant phenotypes (Fig. 1) with distinct leaf and straw compositions for the aboveground biomass. As shown in Table 1, the ratio of leaf to straw biomass varied for the four species. The leaf/straw proportion was higher in Ms and Mf than in Mc and Ml. This difference suggests the plant structure of *Miscanthus* species could have an impact on agronomic practices and feedstock characteristics when commercial *Miscanthus* production is established. The chemical composition of Ms and Mf was also different from Mc and Ml (Table 2). The content of Klasson lignin in Mc and Ml (about 21–22%) was higher than that in Ms and Mf (about 18%). Glucan and xylan are two major polysaccharides in biomass. The glucan content in Mc and Ml (about 39–44%) is higher than that in Ms and Mf (about 35%). Xylan content was lower in Ms (17.5%), but not significantly different from the other three species (19.5–19.9%). However, the araban content in Mc and Ml (1.9–2.0%) is lower than in Ms and Mf (2.7–3.0%). Furthermore, the ash percentage was high (6.0%) in Ms and low (3.1%) in Ml. Overall, Ms and Mf biomass showed similar chemical compositions, such as relatively low lignin and glucan content, while Mc and Ml shared similarities with high lignin and glucan content in chemical composition.

**Dilute acid pretreatment and saccharification**
*Miscanthus* biomass was treated with dilute acid (H₂SO₄) in a series of concentrations: 1.5, 2.0, 3.0, and 4.0% (Fig. 2). The yield of biomass residues decreased slightly as the acid pretreatment concentration increased. However, feedstock from Ms and Mf reacted differently than those from Ml and Mc. The total biomass residue yields for Mc and Ml after pretreatment were much higher than that for Ms and Mf. The four species showed different ratios of biomass retention after dilute H₂SO₄ pretreatment, indicating the feedstock from the four *Miscanthus* species displayed different reactivity during pretreatment, which may be related to their chemical composition.

The concentration of two types of major polysaccharides, glucan and xylan, was determined after dilute acid pretreatment (Table 3). The dilute acid pretreatment had different effects on the two polysaccharides. In general,
H\textsubscript{2}SO\textsubscript{4} had a stronger effect reducing xylan retention than glucan retention. More than 90% of glucan was recovered, while 30% or less of xylan was preserved after pretreatment. In the four species, Ms and Mf had higher glucan retention than Mc and Ml.

After dilute acid pretreatment, the recovered residues were hydrolyzed for saccharification. After 48 h of enzymatic hydrolysis, the percentage of total sugar

\begin{table}[h]
\centering
\begin{tabular}{lcccc}
\hline
Plant component & Species & Ms & Mf & Mc & Ml \\
\hline
Leaf & 57.4 & 63.7 & 28.6 & 10.8 \\
Straw & 42.6 & 36.3 & 71.4 & 89.2 \\
\hline
\end{tabular}
\caption{Ratio of leaf and straw biomass for four Miscanthus species (% dry weight).}
\end{table}

Figure 1. Plant phenotypes of four Miscanthus species. The photos were taken in September after the four Miscanthus species have fully developed. Bar =1 m.
released was highest in Mf (>40% after 4% H₂SO₄ pretreatment), followed by Ms, Mc, and Ml (Fig. 3). The lower levels of sugar released in Mc and Ml could be due to higher lignin content in their biomass composition. The four *Miscanthus* species showed two types of chemical compositions. Under the experimental conditions, the recovery retention and saccharification efficiency were correlated with the biomass composition, indicating the biomass compositions in *Miscanthus* affected biomass conversion efficiency. These results suggest that the biomass chemical composition be an important trait in *Miscanthus* cultivar improvement.

Ethanol fermentation was performed with the hydrolysate after enzymatic saccharification of the acid treated biomass. After 6 h of fermentation, about 98% of the glucose was consumed. The conversion efficiency of glucose to ethanol was calculated as 40.9, 41.0, 43.3, and 39.6% for hydrolysate from Ms, Mf, Mc, and Ml, respectively. There was no significant difference in the conversion efficiency of the hydrolysate glucose among the four *Miscanthus* species.

### Green liquor pretreatment and saccharification

The *Miscanthus* feedstocks were pretreated with green liquor in a series of TTA concentrations: 4, 8, 12, 16, and 16%.

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Table 2. Biomass chemical components in four species (% dry weight ± SD).

| Species | Klason Lignin | Acid-soluble Lignin | Glucan | Xylan | Araban | Ash | SiO₂ in Ash |
|---------|---------------|---------------------|--------|-------|--------|----|-------------|
| Ms      | 18.1 ± 0.3    | 2.6 ± 0             | 35.2 ± 0.1 | 17.5 ± 0.2 | 3.0 ± 0.2 | 6.0 ± 0.1 | 3.4 ± 0   |
| Mf      | 17.8 ± 0.2    | 3.0 ± 0             | 34.8 ± 0.4 | 19.5 ± 0.2 | 2.7 ± 0.1 | 4.5 ± 0.1 | 2.2 ± 0   |
| Mc      | 22.4 ± 0.1    | 2.1 ± 0             | 38.8 ± 0.2 | 19.5 ± 0.6 | 1.9 ± 0.1 | 4.1 ± 0.1 | 3.3 ± 0   |
| Ml      | 21.2 ± 0.2    | 2.0 ± 0             | 43.9 ± 0.4 | 19.9 ± 0.3 | 2.0 ± 0.2 | 3.1 ± 0.1 | 1.9 ± 0   |

Figure 2. Biomass residues remaining after dilute acid pretreatment.

Table 3. Retention of polysaccharides after dilute acid pretreatment (% mean ± SD).

| Polysaccharide type | Pretreatment H₂SO₄% | Species |
|---------------------|---------------------|--------|
| Glucan              |                     | Ms     | Mf     | Mc     | Ml     |
| 1.5                 | 99.0 ± 0.4          | 98.0 ± 0.3 | 91.3 ± 0.5 | 92.9 ± 1.1 |
| 2.0                 | 98.4 ± 0.3          | 97.5 ± 0.4 | 91.0 ± 0.4 | 92.6 ± 1.6 |
| 3.0                 | 97.7 ± 0.4          | 96.6 ± 0.4 | 90.4 ± 1.4 | 91.3 ± 0.8 |
| 4.0                 | 97.1 ± 0.4          | 96.0 ± 0.4 | 90.1 ± 1.5 | 90.7 ± 1.4 |
| Xylan               |                     | Ms     | Mf     | Mc     | Ml     |
| 1.5                 | 26.9 ± 2.3          | 30.1 ± 0.9 | 28.0 ± 0.4 | 29.8 ± 3.0 |
| 2.0                 | 26.8 ± 4.3          | 28.4 ± 1.3 | 25.9 ± 1.0 | 28.1 ± 4.2 |
| 3.0                 | 26.6 ± 2.8          | 23.1 ± 1.3 | 20.7 ± 0.6 | 22.5 ± 1.6 |
| 4.0                 | 26.3 ± 4.0          | 22.3 ± 1.6 | 19.0 ± 1.1 | 20.1 ± 0.9 |
20%. Biomass recovery after green liquor pretreatment was negatively related to TTA concentration up to 16% (Fig. 4). However, the recovery ratio was little changed when TTA concentration was further increased from 16% to 20%. In the four species, over 65% of lignin was removed in the pretreatment with 20% TTA. The lignin removal was also negatively correlated with the biomass recovery ratio. Meanwhile, the four Miscanthus species displayed different biomass recovery and a degree of lignin removal after green liquor pretreatment. Figure 5 indicates that lignin removal had a different effect on biomass recovery in the four species. At the same percentage of lignin removal, MI and MC had higher biomass recovery ratio than Ms and Mf. This may be due to different lignin content in their biomass components. This study suggests that dilute acid and green liquor pretreatment methods have different effects and the two methods may be applied conditionally depending on the chemical composition of the biomass feedstock being processed.

After pretreatment with green liquor, feedstock was subjected to enzymatic hydrolysis for 48 h. The sugar released analysis showed sugar yields were enhanced as TTA concentration increased to 16% and then leveled off at 20%, except MI which continued to increase (Fig. 6).

It is interesting to note that the level of sugar released in MI biomass increased much more dramatically than for other species. Different from the dilute acid pretreatment, the green liquor pretreatment resulted in a higher ratio of sugar released from the biomass of MI and MC than from Ms and Mf. Due to higher lignin content in MI and MC, lignin removal during the green liquor pretreatment would improve the sugar release efficiency from MI and MC biomass. Thus, green liquor pretreatment may be more beneficial to the saccharification process for biomass with higher lignin content.

Establishment of NIR methods for rapid estimation of enzymatic hydrolysis

Miscanthus species differ in their biomass chemical composition, which affects conversion efficiency. We applied NIR spectrometry to develop a quick evaluation method for the hydrolysis efficiency of Miscanthus biomass. NIR spectral information for 172 samples of different Miscanthus species or varieties was collected (see Materials and Methods). The samples were pretreated with 1.5% sulfuric acid and enzymatic hydrolysis was carried out. MPLS regression analysis was performed to establish the relationship between NIR spectral information and biomass sugar released after hydrolysis. In the process of model development, a series of statistical analyses were applied, including the combination of derivative and SNVD treatments. The prediction model established with a second derivative and SNVD analysis showed a RSQ of 0.966, SECV of 0.010, 1-VR of 0.932, and an SEC (standard error of calibration) of 0.008, indicating high accuracy for this model. To further verify the model, 35 additional samples collected from various sources of Miscanthus biomass were tested and compared to the data predicted by the model. As shown in Figure 7, the prediction reached a RSQ of 0.927 and SEP(C) of 0.010, suggesting the
model was highly feasible for quickly predicting the enzymatic digestibility of Miscanthus biomass.

Discussion and conclusion

Miscanthus is a promising bioenergy crop with high biomass productivity and hardiness. Ms, Mf, Mc, and Ml are four major Miscanthus species widely grown in China. Here, we report a comparative characterization of their biomass productivity and composition response to pretreatments and saccharification efficiencies. The four species displayed different plant structure compositions, biomass productivity, and chemical characteristics. Under the experimental conditions, Ml had the highest biomass yield, reaching 32.0 t ha\(^{-1}\) of aboveground biomass annually, almost double that of Ms and Mc. Pretreatment is a major step in biomass bioprocessing. A number of biomass pretreatment methods have been reported (Galbe and Zacchi 2007). In our study, we examined two methods to treat Miscanthus biomass, dilute acid pretreatment and green liquor pretreatment. In general, the two pretreatment methods have different effects on biomass. Dilute acid pretreatment produces microholes mainly through the hydrolysis of hemicellulose, which facilitates the release of xylan by allowing enzymes to access polysaccharides (Fry et al. 2000). Green liquor pretreatment is an alkali method which serves primarily to remove lignin from biomass by facilitating the enzymatic hydrolysis of polysaccharides (Jin et al. 2010). Previously, a detailed comparison of the efficacy of the two pretreatment methods for different biomass species was not well documented. When dilute acid pretreatment was applied to the four Miscanthus species, Ms and Mf showed higher levels of sugar released. On the other hand, when green liquor pretreatment was applied, Ml displayed a higher level of sugar released. This reflects the different biomass chemical compositions of the four Miscanthus species.

Biomass from Mc and Ml likely contain higher glucan polysaccharides as well as lignin polymers that can be more easily removed with green liquor solution. Assessing the chemical composition of biomass allows for the optimization of pretreatment conditions and consequently leads to improved conversion efficiency.

Lignocellulosic biomass varies enormously in chemical composition. Critical factors such as lignin and hemicellulose content affect pretreatment efficacy and subsequent enzymatic hydrolysis. Currently, the efficiency of lignocellulosic biomass conversion into biofuel is still a major technological bottleneck in the biorefining process. Therefore, development of convenient and fast methods to measure the chemical composition of biomass is vitally important for the targeting, selecting, and engineering of energy plants. Methods such as Fourier transform infrared (FT-IR) and NIR spectroscopy have been reported (Sanderson et al. 1996; Allison et al. 2009; Hou and Li 2011). This study demonstrates that NIR can be used to accurately evaluate the hydrolysis efficiency of various Miscanthus species. Establishment of a quick Miscanthus biomass evaluation system provides a convenient tool for improvement of Miscanthus through traditional plant breeding and genetic engineering.

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Conflict of Interest

None declared.

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