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

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HIGHLIGHTS

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

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ABSTRACT

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

1. Introduction

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

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

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

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

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

2. Methods

2.1. Plant materials

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

2.2. Plant cell wall fractionation

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

2.3. Total hexoses and pentoses assay

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

2.4. Total lignin analysis

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

2.5. Cellulose crystalline index (CrI) detection

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