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Enzymatic hydrolysis of hemicelluloses from *Miscanthus* to monosaccharides or xylo-oligosaccharides by recombinant hemicellulases

Hailong Li[a,b], Yong Xue[a], Jinlian Wu[a], Hongli Wu[a], Guanghui Qin[a], Chengguo Li[a], Jiajia Ding[a], Jian Liu[a,*], Lihui Gan[a], Minnan Long[a,*]

[a] College of Energy, Xiamen University, Xiamen 361102, PR China
[b] Key Laboratory of Renewable Energy, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou 510640, PR China

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ABSTRACT

Hemicelluloses isolated from holocellulose (MHOC) from *Miscanthus* were characterized by RP-HPLC-UV, FT-IR, and NMR. The hemicelluloses and recombinant hemicellulases, including endo-β-1,4-xylanases (HoXyn11A and AnXyn10C), β-xylosidases (AnXln3D), and α-L-arabinofuranosidases (AnAxh62A), as well as their interaction mechanisms were investigated by enzymatic hydrolysis. AnXyn10C released shorter end products than HoXyn11A from isolated hemicelluloses (IHEC). AnAxh62A was able to release all single-substituted α-L-arabinofuranosyl residues from IHEC. AnXyn10C and HoXyn11A were able to directly act on MHOC, whereas AnAxh62A and AnXln3D did not. The combination of HoXyn11A and AnAxh62A produced the highest xylo-oligosaccharides (XOS) yield from IHEC, whereas AnXyn10C alone produced the highest XOS yield from MHOC. The combination of HoXyn11A, AnAxh62A, and AnXln3D achieved the highest xylose yield from IHEC, whereas the combination of AnXyn10C, AnAxh62A, and AnXln3D achieved the highest xylose yield from MHOC. This study contributes to the development of efficient enzyme cocktails for the bioconversion of hemicelluloses from *Miscanthus* into monosaccharides and XOS.

1. Introduction

Among various sources of lignocellulosic biomass, *Miscanthus* is a dedicated energy crop that has drawn considerable attention in the biorefinery industry for the production of biofuels and value-added chemicals because it can be cultivated with high biomass yield, high carbohydrate content, and low nutrient requirements (Heaton et al., 2008; Li et al., 2012b). Although the utilization of *Miscanthus* for the production of fuels and chemicals has been widely studied (Le Ngoc Huyen et al., 2010; Li et al., 2013a, 2014b; Liu et al., 2013; Sorensen et al., 2008; Vanderghem et al., 2012), the structure of hemicelluloses in *Miscanthus* is poorly characterized. Hemicelluloses in grasses consist mainly of xylan, which interlocks cellulose microfibrils through hydrogen bonds and cross-links polyphenolic lignin to form lignin-carbohydrate complexes (McCann and Carpita, 2008). Xylan is characterized by a linear β-1,4-linked backbone of xylosyl residues substituted by 4-*O*-methylglucuronic acid or arabinose (Faik, 2010). Recently, it has been reported that xylan appear to play a role in the enzymatic digestion of lignocellulosic biomass (Himmel et al., 2007; Jeoh et al., 2007; Kumar and Wyman, 2009; Qing et al., 2010; Yang and Wyman, 2004; Zhang et al., 2012; Zhang and Viikari, 2012). Xylan has been described as restricting cellulase access to cellulose by forming a defensive structure for protecting cellulose (Himmel et al., 2007; Jeoh et al., 2007; Kumar and Wyman, 2009). Furthermore, xylan and xylooligmers significantly inhibited the hydrolysis of cellulose by cellulases (Qing et al., 2010; Zhang et al., 2012; Zhang and Viikari, 2012). Several studies showed a direct relationship between hemicellulose removal and cellulose digestibility (Lv et al., 2013; Yang and Wyman, 2004; Yang et al., 2013). Nevertheless, xylo-oligosaccharides (XOS) and arabinoxylo-oligosaccharides (AXOS), the intermediate products of hemicellulose hydrolysis, are value-added food ingredients that can be produced at industrial scale by enzymatic hydrolysis of arabinoxylan-containing lignocellulosic biomass (Rantanen et al., 2007; Riviere et al., 2013; Saha, 2003). The efficient enzymatic hydrolysis of hemicelluloses into monosaccharides or XOS at the lowest possible enzyme loading is of great importance for the lignocellulosic bioenergy and biorefinery industry.

* Corresponding authors.
E-mail addresses: jianliu@xmu.edu.cn (J. Liu), longmn@xmu.edu.cn (M. Long).

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The hydrolysis of *Miscanthus* hemicelluloses by crude enzymes from *Aspergillus niger*, *Hypocrea orientalis*, and *Trichoderma reesei* were previously compared (Li et al., 2014b). The enzymes from *A. niger* showed a high efficiency in producing monosaccharides, whereas the enzymes from *T. reesei* and *H. orientalis* were more preferable for obtaining XOS (Li et al., 2014b). However, considering the complex structure of hemicelluloses in *Miscanthus* and the wide range of glycosyl hydrolases (GH) in crude enzymes, the interaction mechanisms between hemicelluloses and hemicellulases were difficult to study using uncharacterized hemicelluloses and crude enzymes. Determination of the exact chemical structure of hemicelluloses and the investigation of the interactions between hemicelluloses and hemicellulases could facilitate the construction of minimum enzymes cocktails for the bioconversion of specified feedstocks into monosaccharides or XOS.

In our previous study, hemicellulases including endo-β-1,4-xylanases (HoXyn11A and AnXyn10C), β-xylosidases (AnXln3D), and α-L-arabinofuranosidases (AnAxh62A) from *H. orientalis* and *A. niger* were heterologously expressed at a high level and were well characterized (Li et al., 2014a). In this study, hemicelluloses were isolated from *Miscanthus* holocellulose (MHOC) by aqueous alkali. The structure of isolated hemicelluloses (IHEC) was characterized by reverse-phase high-performance liquid chromatography with ultraviolet detection (RP-HPLC-UV), Fourier transform infrared spectroscopy (FT-IR), and nuclear magnetic resonance (NMR). The end products released from the IHEC by the action of HoXyn11A, AnXyn10C, AnXln3D, and AnAxh62A were analyzed by thin-layer chromatography (TLC), RP-HPLC-UV, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Furthermore, the synergistic hydrolysis of MHOC and IHEC by these recombinant enzymes was investigated.

2. Materials and methods

2.1. Materials

Miscanthus floridulus was harvested at a local site in Zhangzhou (Fujian, China). The composition of the extractive-free *M. floridulus* was cellulose 38.22%, hemicellulose 24.05%, lignin 25.87%, and ash 5.54%. Recombinant hemicellulases including HoXyn11A (1136 IU/mg), AnXyn10C (785 IU/mg), AnXln3D (58.7 IU/mg), and AnAxh62A (177 IU/mg) were prepared as described previously (Li et al., 2014a). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was obtained from Acros Organics (Geel, Belgium). Glucose (Glu), xylose (Xyl), and arabinose (Ara) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Wheat arabinoxylan (WAX), xylohexaose (X_6), xylopentaose (X_5), xylotetraose (X_4), xylotriose (X_3) and xylobiose (X_2) were obtained from Megazyme (Bray, Ireland). Oat spelt xylan (OSX) was purchased from Sigma–Aldrich (USA).

2.2. Preparation of holocellulose and extraction of hemicelluloses

Miscanthus was first dried in an oven at 45 °C, and then smashed into powder (40–60 mesh) for use. Fig. 1 describes the scheme for the preparation of holocellulose and extraction of hemicelluloses from *Miscanthus* (Whistler et al., 1948). The powder was dewaxed with 2:1 (v/v) toluene-ethanol in a Soxhlet apparatus for 6 h. The dewaxed powder was delignified with 0.6 g/g dry matter of sodium chlorite and 0.6 mL/g of dry matter of acetic acid with a solid to liquid ratio of 1:32 (g/mL) at 70 °C for 4 h as described by Kumar et al. (2013). The residue, holocellulose, was subsequently washed by distilled water and 70% ethanol, and then dried in an oven at 45 °C for 16 h. The obtained holocellulose was extracted by 10% KOH solution with a solid to liquid ratio of 1:25 (g/mL) at 25 °C for 16 h. The filtrate was adjusted to pH 5.5 with acetic acid and concentrated to 100 mL. Then, three volumes of ethanol were added and incubated at 4 °C for 12 h, and the precipitate was recovered by centrifugation (4000 rpm, 20 min). After three washes with 70% ethanol, the precipitate was freeze-dried.

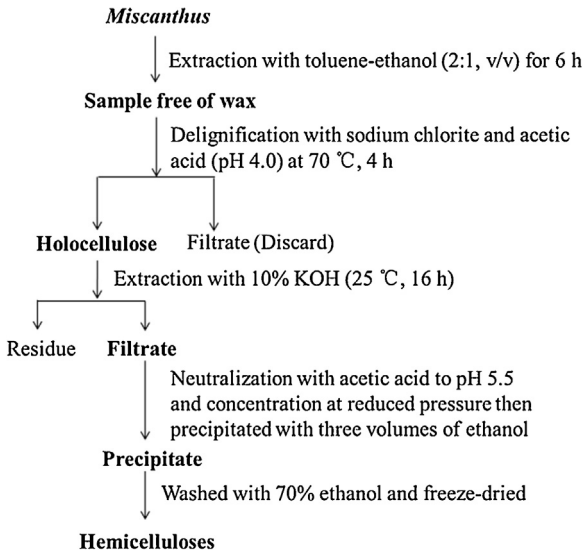

Fig. 1. Scheme for isolation of hemicelluloses from *Miscanthus*.

2.3. Structural characterization

The monosaccharide composition of MHOC and IHEC was determined by RP-HPLC-UV. The MHOC samples were first hydrolyzed by 72% H_2SO_4 at 30 °C for 1 h. Then, the pre-hydrolyzed MHOC samples and the IHEC samples were hydrolyzed with 4% H_2SO_4 at 121 °C for 1 h and then neutralized for monosaccharide composition analysis. The uronic acid (Uro) content was determined by the meta-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973). The FT-IR spectra were recorded on an FT-IR spectrophotometer (Nicolet 330) using a KBr disk containing 1% finely ground samples over the wavenumbers of 4000–400 cm^{-1} at a resolution of 2 cm^{-1}. To investigate the interactions between AnAxh62A and IHEC, AnAxh62A (0.55 mg/g substrate) was incubated with 20 mL of 0.2% IHEC in sodium citrate buffer (50 mM, pH 5.0) at 50 °C for 24 h. The reaction mixture was boiled for 10 min to inactivate the enzyme. Then, the reaction mixture was dialyzed against water to remove released arabinose and lyophilized. The solution-state ^1H NMR spectra were recorded on an AVANCE III NMR spectrometer at 600 MHz using 8 mg of samples (IHEC, AnAxh62A-pretreated IHEC, Oat spelt xylan) in 0.5 mL of D_2O. The ^{13}C NMR spectra were obtained on an AVANCE III spectrometer at 600 MHz using 40 mg of IHEC in 0.5 mL of D_2O. The ^{13}C NMR spectra were recorded at 25 °C after 10000 scans. The heteronuclear single quantum coherence (HSQC) NMR experiment was conducted with 10 mg of sample dissolved in 0.5 mL D_2O after 128 scans. The number of collected complex points was 1024 for the ^1H dimension with a recycle delay of 5 s. The number of transients for the HSQC spectra was 128, and 256 time increments were always recorded in the ^{13}C dimension. The $^1J_{C\text{-}H}$ used was 146 Hz.

2.4. Enzymatic hydrolysis

The IHEC (20 mg/mL) was individually or sequentially incubated with HoXyn11A (0.26 mg/g substrate), AnXyn10C (0.38 mg/g substrate), AnXln3D (0.21 mg/g substrate), and AnAxh62A (0.06 mg/g substrate) in sodium citrate buffer (50 mM, pH 5.0) containing