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Fluorescence techniques can reveal cell wall organization and predict saccharification in pretreated wood biomass

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ABSTRACT

The conversion of cell wall biomass to sugar for fermentation to ethanol requires chemical or physical pretreatments to disrupt the recalcitrant plant cell walls and to make the cellulose accessible to cellulolytic enzymes. Multiscale study of biomass deconstruction gives access to key insights into the cell wall and lignin changes induced by pretreatment. Few studies have compared the effect of pretreatment of different biomass species on the cell wall accessibility.

Considering representative softwood (pine) and hardwood (poplar) biomass, we studied the impact of two pretreatments on enzymatic saccharification and cell wall structure and accessibility. Lignin fluorescence properties were investigated by measuring fluorescence lifetime in addition to chemical analysis, and the accessibility of biomass was assessed using fluorescent probes consisting of rhodamine labeled polyethylene glycol (PEG) molecules ranging from 10 to 40 kDa. Hot water treatment and chlorite delignification altered chemical structure and fluorescence lifetime, which was positively correlated with glucose conversion and negatively correlated with lignin and β-O-4′ contents. Imaging distribution of the probes indicated that chlorite pretreatment resulted in a more uniform distribution of probe in the cell wall compared to hot water treatment. The interaction between cell wall and fluorescent PEG probes was evaluated using Förster Resonance Energy Transfer (FRET) and fluorescence microscopy. The FRET efficiency showed a high negative correlation with the probe size and was greatly increased by chlorite delignification, reflecting increased accessibility to the probe and interaction. Thus the accessibility and interactions of small probes in pretreated biomass could be a relevant indicator of potential for saccharification, whereas fluorescence lifetime provides a new criteria for assessing relevant cell wall structural modifications related to enzymatic conversion of lignocellulosic biomass.

1. Introduction

There is increasing interest in the use of plant biomass for the production of sustainable liquid fuels and chemicals to replace fossil carbon, due to declining supply, and to mitigate increasing atmospheric CO_2 (Menon and Rao, 2012). Plant cell wall biomass consists of a framework of cellulose surrounded by a matrix of complex polymers including hemicellulose and lignin. These polymers are interconnected by non-covalent and covalent linkages resulting in a complex structural and chemical network (Burton et al., 2010) with physical and chemical barriers limiting cellulase enzyme penetration and progression (Paës et al., 2017), leading to decreased enzyme activity (Arantes and Saddler, 2011; Zhao et al., 2012). Cellulase activity is limited by the crystallinity of cellulose and its restricted accessibility inside the cell wall matrix of hemicelluloses and lignin (Ding et al., 2012). In addition to being a structural obstacle, lignin can give rise to non-productive enzyme binding, so it is considered to be the main factor responsible for lignocellulose recalcitrance (Vaidya et al., 2014; Zeng et al., 2014). The conversion of cell wall biomass to monosaccharides for fermentation into ethanol thus requires chemical or physical pretreatments to disrupt the plant cell walls and increase porosity in

Abbreviations: FLIM, fluorescence lifetime imaging microscopy; FRET, Förster (or Fluorescence) resonance energy transfer; MW, molecular weight; RhPEG1, rhodamine labeled polyethylene glycol of 1 kDa; RhPEG3.4, rhodamine labeled polyethylene glycol of 3.4 kDa; RhPEG10, rhodamine labeled polyethylene glycol of 10 kDa; RhPEG40, rhodamine labeled polyethylene glycol of 40 kDa

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order to make the cellulose accessible to cellulolytic enzymes while minimizing the formation of by-products (Silveira et al., 2015).

A wide variety of pretreatments has been proposed and each has advantages and limitations (Agbor et al., 2011; Singh et al., 2015). Some pretreatments improve the accessibility of cellulose by removal of matrix components such as lignin (delignification associated with pulping) and hemicelluloses (hot water treatment, steam-explosion or acid treatments). Removal of lignin alone is usually insufficient to optimize sugar yield because hemicelluloses are covalently linked to lignin and/or closely interact with the surface of elementary cellulose, thus forming a physical and chemical barrier that limits enzymatic accessibility and cellulose conversion yields (Ding et al., 2012; Meng et al., 2015; Penttilä et al., 2013; Trajano et al., 2013). Improvement of cellulose conversion is related to the severity and the type of pretreatment and depends on the biomass species, due to variation in the content and composition of lignin and hemicellulose (Arantes and Saddler, 2011; Asada et al., 2015; Singh et al., 2015). For instance, hardwoods and softwoods differ in terms of lignin composition (mixed syringyl-guaiacyl vs guaiacyl) and hemicellulose composition (xylan vs mannan) (Ralph et al., 2004; Scheller and Ulvskov, 2010). These differences contribute to the effectiveness of biomass pretreatment, with xylans being readily extractable by hot water whilst mannans require more intensive treatment (Meng et al., 2015; Nitsos et al., 2016). Syringyl-rich hardwood lignins are more easily chemically degraded than guaiacyl-type softwood lignin due to a lower degree of condensation, a less complex structure, smaller polymer size and higher content of β-ethers (Mottiar et al., 2016; Ralph et al., 2004; Studer et al., 2011).

Apart from chemical and structural characterization, microscopy has been an effective tool for the study of biomass pretreatment at multiscale levels (Donohoe and Resch, 2015). Light microscopy and microspectrometry combining UV, infrared and Raman analysis have provided critical information on cell wall chemical changes caused by pretreatments and enzyme hydrolysis (Belmokhtar et al., 2013; Chundawat et al., 2011; Ma et al., 2014; Singh et al., 2015). Among microscopy approaches, confocal fluorescence microscopy is widely used to visualize biomass deconstruction, and gives key insights into cell wall and lignin changes induced by pretreatment (Singh et al., 2015). For example, confocal fluorescence spectroscopy and fluorescence lifetime imaging (FLIM) were used to characterize lignin modification after pretreatments in sugar cane bagasse and eucalyptus fiber (Coletta et al., 2013) and in poplar, wheat straw and miscanthus (Auxenfans et al., 2017b; Zeng et al., 2015). Confocal microscopy is also a fast method to provide a spatial and temporal visualization of fluorescently labeled enzyme and to characterize enzyme-substrate interactions within biomass (Ding et al., 2012; Donaldson and Vaidya, 2017; Luterbacher et al., 2013; Moran-Mirabal, 2013; Thygesen et al., 2011). Confocal fluorescence microscopy can also reveal cell wall modification and cellulose porosity by determining the distribution and mobility of fluorescent probes that vary in molecular weight (MW) or affinity towards cell wall polymers (Donaldson et al., 2015; Moran-Mirabal, 2013; Paës, 2014; Paës et al., 2017; Yang et al., 2013).

Few studies have compared the effect of pretreatment of different biomass species on the accessibility of the cell wall. Donaldson et al. (2014) have shown that rhodamine labeled polyethylene glycol (PEG) penetrates steam-exploded wood. The fluorescent PEGs can then be localized by direct imaging of the rhodamine dye and their strength of interaction can be evaluated by measuring the Förster (or Fluorescence) Resonance Energy Transfer (FRET) between rhodamine and cell wall lignins at the molecular level.

In this work, we use a set of fluorescence microscopy techniques to study the effects of two different pretreatments (chlorite delignification and hot water extraction) on cell wall accessibility in poplar (hardwood) and pine (softwood) using rhodamine labeled PEGs of various sizes as probes. Chlorite delignification reduces lignin content whilst hot water extraction mainly reduces hemicellulose content; both treatments induce alteration of the residual lignin structure (Kumar et al., 2013; Ma et al., 2014; Paës et al., 2017), thus allowing comparison of cell wall accessibility in hardwood and softwood biomass. In particular, interactions of probes with pretreated biomass was assayed by FRET measurements, while FLIM was used to assess cell wall modifications. Such complementary microscopic techniques provide new insights for cell wall accessibility which cannot be obtained by porosity measurements.

2. Materials and methods

2.1. Sample preparation

Small wood blocks (3–4 mm width × 2 cm long) were isolated from the basal region of 3-year-old short rotation poplar coppice (Orléans, France) and from mature pine wood (Rotorua, New Zealand) which were subsequently dried at 40 °C for 2 days. Pretreatments were performed as previously described (Paës et al., 2017). Hot water treatment was performed for 1 h at 170 °C using mineralization reactors equipped with Teflon tubes (PARR, USA). Sodium chlorite-acetic acid delignification treatment (Wise et al., 1946) was performed on 1 g poplar samples using acetic acid and sodium chlorite at 70 °C for 1 h and the reaction was repeated 5 times. Untreated control samples were also obtained after water washing at 4 °C (1 h). After pretreatment, samples were washed several times with deionized water until the pH of the wash was about 6.0. Then samples were dried at 40 °C in an air forced oven for 2 days. One sample fraction was ground to 200 µm size prior to chemical analysis and enzymatic saccharification, and the other fraction was sectioned with a sledge microtome at a thickness of 30 µm for confocal fluorescence microscopy.

2.2. Chemical analysis and enzymatic saccharification

The sugar monomer composition was determined using a two-step sulfuric acid hydrolysis (Seaman et al., 1954) followed by high-performance anion-exchange chromatography (HPAEC-PAD) with 2-deoxy-D-ribose as internal standard (Paës et al., 2017). Lignin content was quantified using a spectrophotometric method after acetyl bromide dissolution of the lignocelluloses (Iiyama and Wallis, 1990) and determination of the monomer composition of the alkyl aryl ether lignin structures was achieved by thioacidolysis as previously described (Belmokhtar et al., 2013; Lapierre et al., 1986).

Enzymatic saccharification was performed on the ground samples (2% w/v) using a commercial cellulase preparation, Cellic ® CTec2 kindly provided by Novozymes A/S (Bagsværd, Denmark), in 10 mL sodium citrate buffer (0.1 M; pH 5.0) for 48 h at 50 °C and 200 rpm using an enzyme loading of 40 FPU/g-glucan. The reaction mixtures were pre-incubated for 1 h at 50 °C and 200 rpm. Enzymatic hydrolysis was initiated by addition of the cellulase solution and stopped after 72 h by heating for 15 min at 100 °C. Control experiments without enzyme were also carried out. The amount of glucose released was determined using high-performance anion-exchange chromatography (HPAEC-PAD). Conversion yields were expressed as a percentage of initial glucose amount (Auxenfans et al., 2017a).

2.3. Confocal fluorescence microscopy

Samples of untreated control and pretreated pine and poplar wood were sectioned in the transverse plane with a sledge microtome at a thickness of 30 µm. Sections were used for fluorescence spectroscopy, FLIM, and FRET measurements. Fluorescence imaging was performed using a Leica SP5 II confocal microscope at 1024 × 1024 pixel resolution with a 63× glycerol immersion lens. FLIM measurements were performed on the untreated control and pretreated pine and poplar wood sections mounted in 50% glycerol in 10 mM phosphate buffer at pH 7 (Donaldson and Radotic, 2013) using a Zeiss LSM710 multiphoton