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Assessing multi-scale deconstruction of wood cell wall subjected to mechanical milling for enhancing enzymatic hydrolysis

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ABSTRACT

The hierarchical structure of wood cell walls resulting from complex arrangement and distribution of the heterogeneous components is considered to impact significant impediment to enzymatic hydrolysis of cellulose for biofuels. In this work, micronized wood with significant cell wall ultrastructural deconstruction were effectively produced from ring and puck milling within 12 min. In a subsequent enzymatic hydrolysis, micronized wood resulted in increase of cellulose hydrolysability by 4–14 folds over that of starting material. The underlying mechanism towards facilitating enzymatic hydrolysis was studied through delineating the ultrastructural changes and alternation of cellulose chemistry in micronized wood cell wall using SEM, TEM, CLSM, GPC, XRD, HPLC and Simon's staining techniques. Electronic microscopy revealed distinct stages of wood cell wall deconstruction that was coincident with particle size reduction, including cell fracture and delamination, cell wall disintegration, and amorphization of cell wall fragments. Simons' staining results also indicated increasing substrate accessibility and porosity of micronized wood, likely due to the ultrastructure alternation of cell walls. GPC and XRD revealed significant decrease of cellulose degree of polymerization (DP) and crystallinity. The correlation of these factors with cellulose hydrolysability was studied and further arranged in order through principal component analysis. The major positive factors affecting hydrolysability were surface accessibility and porosity, while cellulose crystallinity and DP were the major negative factors accompanied by particle size. The established weighed order of factors behind hydrolysability provides insights of lowering cell wall structural recalcitrance by mechanical manner.

1. Introduction

Lignocellulosic biomass offers the most abundant and the cheapest carbon source on the earth with the form of biopolymers in its cell wall (Zhao et al., 2012). Due to increasing depletion of fossil resource and serious environmental issues, considerable interest has emerged in converting lignocellulosic biomass into fossil fuel alternatives to create a diverse and economically viable renewable portfolio for fuels and chemicals. One feasible biomass conversion route is the depolymerization of cell wall polysaccharides with hydrolytic enzymes, followed by fermentation of intermediate sugars to alcohols or hydrocarbons by specific microorganisms (Himmel et al., 2007; Zhao et al., 2012). However, plant cell walls have a hierarchical architecture resulted from complex interactions of these biopolymers, making the biomass difficult to deconstruct (Himmel et al., 2007). Therefore, pretreatment is recognized as a necessary step to overcome the recalcitrance and make cell wall polysaccharides more susceptible and amendable to hydrolytic action by enzymes for maximizing the release of fermentable sugars (Himmel et al., 2007).

Pretreatment has been found to improve enzymatic hydrolysis of biomass by creating changes in substrate chemistry, structure and morphology. These changes include but are not limited to crystallinity, degree of polymerization, specific surface area, and lignin distribution, which result in increasing accessibility of the cell wall polysaccharides to hydrolytic enzymes (Chundawat et al., 2011a,b; Yang et al., 2011). Several thermochemical approaches (e.g. acid, alkaline, acid sulfite pulping, organosolv pulping, steam explosion, and ionic liquid pretreatments, etc.), have made substantial progress in facilitating enzymatic hydrolysis of pretreated biomass substrates through depolymerizing and/or partial removal of the cell wall non-cellulose constitutes (Bali et al., 2015; Del Rio et al., 2011; Foston and Ragauskas, 2010; Tadesse and Luque, 2011b; Zhu et al., 2009). However, these severe chemical processes often carry high capital cost, employ chemicals and solvents, and that require recovery or treatment of liquid stream may

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lead to formation of inhibitors to down-stream microorganism metabolism to produce desired fuels or chemicals. These challenges pose significant barriers to commercializing an economically viable biomass conversion process to fuels and chemicals (Barakat et al., 2014). Mechanical milling pretreatment offers an attractive alternative to address these deficiencies by producing digestible biomass substrates with the advantages of eliminating chemicals/solvents and the formation of inhibitors typically originated from degrading and/or transforming non-cellulose components (i.e. hemicellulose and lignin) in chemical pretreatment processes (Barakat et al., 2013).

The mechanical milling process is an effective strategy to break down the robust cell wall structure of woody biomass feedstock. It has long been used to deconstruct the native structure of plant cell wall for improving substrate accessibility, which enables providing the milled wood lignin (Fujimoto et al., 2005; Goundalkar et al., 2014; Maurer and Fengel, 1992). The milling process profoundly imparts multiple length-scale structural alternations (e.g., plant, tissue, cellular and molecular levels) through disrupting supramolecular cross-links of cellulose-hemicellulose-lignin networks (Ji et al., 2016). Studies in different laboratories have demonstrated that mechanical milling facilitates enzymatic hydrolysis of various feedstock (i.e., herbaceous and woody biomass) (Barakat et al., 2014; Takahashi et al., 2013; Vaidya et al., 2016; Zakaria et al., 2014). Silva et al. (2012) has indicated that destruction of cell wall to micronize-size range is essential for high glucose yield of mechanically milled wheat straw. The improvements may occur in tandem with decreases in particle size and cellulose crystallinity in milled substrates, which are associated with increased enzyme accessibility to cell wall polysaccharides. Our prior work (Jiang et al., 2016) has demonstrated that partial breakage of softwood fiber and/or fiber bundles improves the enzymatic hydrolysis of milled softwood, resulting in limited total sugar yields of around 40%. Mechanically disintegrating the fiber cell wall into micronized fragments significantly improves the enzymatic hydrolysis, with total sugar yields of over 70%. Similarly, Ji et al. (2016) also found that mechanically fragmenting corncob samples at a cellular scale resulted in a 98.3% conversion yield of cellulose to glucose. In addition, decreasing the cellulose crystallinity during the milling process also contributes to increasing accessibility to cellulose microfibrils (Wang et al., 2014). Substantial research reveals a strong correlation between increased enzymatic hydrolysis efficiency and decreasing crystallinity in various milled feedstock (Barakat et al., 2014). Despite the demonstrated efficiency of mechanical milling pretreatment for disrupting the cell wall structure and increasing enzymatic hydrolysis yields, a clear understanding of the ultrastructural alternation and cellulose characteristics that are presumably responsible for facilitating enzymatic digestion and revealing biomass recalcitrance is still needed. On the other hand, studies on evaluating the relative importance of the different structural factors to the hydrolysability of milled biomass has not been elucidated, while most reports have only focused on correlations of hydrolysability with physicochemical characterizations.

The objective of this study is to delineate the effects of mechanical milling pretreatment on the ultrastructural properties and cell wall chemistry of micronized wood as related to the sugar release efficiency or recalcitrance properties of biomass. Specifically, the physicochemical changes resulting from the mechanical deconstruction of softwood Douglas-fir (*Pseudotsuga menziesii*) feedstock have been examined by using combined wet chemistry techniques with other analytical and imaging techniques in new ways. Transmission electron microscopy (TEM) was used to investigate the ultrastructural disintegration of the wood cell wall and, as it turned out, to reveal the increased substrate accessibility and porosity within the cell wall fragments. Confocal laser scanning microscopy (CLSM) was employed to delineate the distribution of chemical composition related to breakdown of cell walls during the milling process. Enzymatic hydrolysis experiments on micronized wood was also conducted in an effort to assess the change in the recalcitrance of milled substrates. In addition, statistically quantifying the relative importance of different structural and morphological factors on enzymatic hydrolysis efficiency provides insights into the fundamental nature of cell wall recalcitrance related to enzymatic hydrolysis efficiency of lignocellulosic biomass.

2. Materials and methods

2.1. Materials

Clean, Douglas-fir (*Pseudotsuga menziesii*) wood chips were obtained from a local company (Vaagen Brothers Lumber Inc., Colville, WA). The as-received chips were separated by a vibrating screen with 25.4-mm aperture and pre-ground into particles by a hammer mill fitted with a 3.18-mm screen. The pre-ground feedstock was subsequently conditioned to a target equilibrium moisture content of 5% (dry weight base). Before further mechanical pretreatment, the conditioned sample was stored in sealed plastic bag and the moisture content was validated using gravimetric methods according to standard protocol (Sluiter et al., 2008a).

2.2. Mechanical pretreatment process

Mechanical milling pretreatment was performed using a high-energy vibratory ring and puck mill with motor power of 1.1-kw (Rocklab Pty Ltd, New Zealand). The sample (10-g, oven-dry base) was milled in a chamber with an inner diameter of 128-mm and height of 43-mm along with a ring (78-mm inner diameter, 100-mm outside diameter, 41-mm height) and a puck (52-mm diameter and 41-mm height) as the milling media. Both the milling chamber and grinding media were made of tungsten carbide. Milling process was conducted for a total time of 2–12 min, with 2-min intervals, resulting in particles in micrometer range. Thus, the milled samples were also noted as micronized wood (or micronized particles) here.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis experiments were performed using Cellic CTec2 cellulase (15 FPU/OD g of substrate) and cellic HTec2 hemicellulase (1/9 of the cellulase amount). Digestion was carried out in 125-mL flasks with a citrate buffer (pH 4.8) at a solid loading of 2%. The flasks were settled in an incubator with a rotation speed of 180rmp at 50 °C. After digestion for 72 h, the hydrolysate was analyzed by HPAEC. Glucan and xyl/mannan conversions were defined as the percentage of glucose and xyl/mannose that was released in enzymatic hydrolysate compared to the theoretical maximum.

2.4. Composition analysis of the wood samples

The chemical composition analysis of wood material was conducted according to the two-step acid hydrolysis procedures from the NREL standard protocol (Sluiter et al., 2008b). Briefly, a 300-mg sample and 3-mL of 72% H_2SO_4 was added to a 100-mL pressure tube, and incubated at 30 °C for 1 h and stirred every 15 min. The sample was then diluted with 84-mL deionized water and autoclaved for one hour. Detection of sugars was performed with high-performance anion exchange chromatography (HPAEC) (Dionex, ICS-3000). The acid soluble lignin content was determined by UV–vis spectrophotometer (Lambda 25, PerkinElmer), while the solid residue in during acid hydrolysis process was counted as insoluble lignin. The total lignin content is the sum of acid soluble and insoluble lignin.

2.5. Structural characterization of micronized wood samples

2.5.1. Scanning electron microscopy (SEM)

Micronized wood samples were mounted on aluminum stubs using carbon tape and sputter-coated with 8-nm of gold for good conductivity