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Fibre size does not appear to influence the ease of enzymatic hydrolysis of organosolv-pretreated softwoods

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ABSTRACT

To determine the effect of fibre size on enzymatic hydrolysis, organosolv-pretreated lodgepole pine was size-fractionated into six substrates ranging in average size from 0.20 to 3.4 mm. Other than the fines fraction (<0.2 mm) which contained most of the lignin, the fractionated substrates were more readily hydrolyzed than the original substrate with nearly complete hydrolysis after 72 h at 5 FPU g^{-1} cellulose. Surprisingly, fibre size was found to have little influence on enzymatic hydrolysis likely due to similarities in the substrates' chemical composition, accessible surface area, cellulose crystallinity and degree of polymerization. To determine the influence of the fines on enzymatic hydrolysis, their content was artificially increased (from 8.9% to 55.4%) however; this did not have a noticeable effect. These results show that within the range of fibre sizes tested, other substrate characteristics likely play a more significant role in the ease of hydrolysis of pretreated substrates.

1. Introduction

Lignocellulosic biomass is an ideal source of sugars for fermentation to liquid fuels such as ethanol due to their renewable nature, potential to reduce greenhouse gas emissions and lack of competition with food production (Somerville et al., 2010). However, an ongoing challenge to the effective use of biomass is its inherent resistance to degradation by microbial enzymes. It is believed that the recalcitrance of biomass is partly governed by a variety of physico-chemical properties spanning several orders of magnitude from the macroscopic to the molecular scale (Chundawat et al., 2011). Some of these properties include the heterogeneity of cell wall constituents and the low accessibility of cellulose to cellulolytic enzymes and water due to strong interchain hydrogen bonding and hydrophobic interactions between cellulose sheets (Himmel et al., 2007). As a result, a pretreatment step is typically required to increase the accessibility of cellulose to cellulolytic enzymes (Chandra et al., 2007).

Although there have been significant improvements in the bioconversion of less recalcitrant biomass feedstocks such as agricultural residues and hardwoods, bioconversion of softwoods such as lodgepole pine has been considerably more challenging typically requiring higher enzyme loadings and longer incubation times to achieve effective saccharification (Várnai et al., 2010). However, due to its abundance in many parts of the world, such as Canada, Russia and Scandinavia, it is likely that softwood biomass will play a significant role in future biorefinery applications. Among the pretreatment options being explored, processes that solubilise lignin such as organosolv and SPORL (sulphite pretreatment to overcome recalcitrance of lignocellulose) can effectively process softwoods to generate substrates that are amenable to cellulolytic hydrolysis (Zhu et al., 2009; Del Rio et al., 2010). The main advantages of the organosolv process include a relatively pure lignin fraction that has the potential for co-product development, and a cellulose-rich solid fraction that can be readily hydrolyzed. Although organosolv-pretreatment can significantly reduce the recalcitrance of softwood-derived cellulose, moderate to high enzyme loadings (10–40 FPU g^{-1} cellulose) are still usually required to obtain rapid and complete enzymatic hydrolysis. Possible reasons for the remaining recalcitrance of pretreated substrates include the location and amount of residual lignin and hemicellulose, the crystallinity, swelling, and degree of polymerization of cellulose itself affecting the substrate's accessibility to cellulases as well as irreversible adsorption of cellulases to residual lignin (Nakagame et al., 2010; Pan et al., 2008).

In addition to the roles that cellulose swelling and crystallinity might have on the substrate's susceptibility to cellulolytic enzymes, gross fibre characteristics such as length, width and size distribution (i.e. external surface area) have been shown to influence enzymatic hydrolysis (Pan et al., 2008; Del Rio et al., 2010; Yeh et al., 2010). However, past studies that have tried to correlate these properties to the substrate's ease of enzymatic hydrolysis have often given inconclusive results, likely due to the methods

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used to change one of the substrates characteristics also influencing other substrate characteristics. For example, increasing the pretreatment severity to reduce fibre length also reduces the cellulose degree of polymerization (DP, cellulose chain length), which has also been associated with biomass recalcitrance (Chang and Holtzapple, 2000). Similarly, trying to reduce initial size via milling also decreases cellulose crystallinity (Yeh et al., 2010). Thus it is difficult to ascertain how each of these substrate characteristics independently influences recalcitrance.

In the work reported here, organosolv-pretreated lodgepole pine was size-fractionated to investigate the effect that initial fibre size, and the concomitant changes in other characteristics such as crystallinity and degree of polymerization, might have on the enzymatic hydrolysis of substrates that have been generated under the same conditions.

2. Methods

2.1. Organosolv-pretreatment

A 200 g (oven-dried weight equivalent) batch of never-dried gray phase mountain pine beetle-killed lodgepole pine chips (generously provided by FP innovations-PAPRICAN) was pretreated on a custom-built, four-vessel (2 L each) rotating digester (Aurora Products Ltd., Savona, BC, Canada) using the "centre point" conditions described by Pan et al. (2008). After cooling to room temperature in a water bath, the pretreated substrate and spent liquor were processed and stored until further use as described by Del Rio et al. (2010).

2.2. Fibre size fractionation

Fibre size fractionation was carried out in a Bauer-McNett Fibre classifier fitted with 14-, 28-, 48-, 100-, and 200-mesh screens (corresponding to sieve openings of 1.19, 0.60, 0.30, 0.15, and 0.074 mm, respectively). The fraction retained by a given screen was termed R*X* where *X* refers to the mesh size (i.e. the fraction retained by the 14-mesh screen is R14, by the 28-mesh screen R28, etc.). The fraction that passed through all the screens was termed the P200 fraction and was obtained by collecting the water/P200 mixture that is usually sent to waste into a large container, allowed to settle for 7 days, decanted and filtered through a 400-mesh screen. The filtrate was recirculated three times to minimize the loss of material. The fractionated pulp samples were stored at 4 °C for further analysis.

2.3. Analytical methods

The moisture content of the pretreated and fractionated substrates were determined from the mass loss after drying to constant weight at 105 °C in a convection oven. The Klason lignin content of the substrates was determined according to TAPPI standard method T-222 om-98. The hydrolysate was retained for determination of monosaccharide composition and acid-soluble lignin content. Acid soluble lignin was determined as described by Del Rio et al. (2010). Monosaccharides were determined with a DX-3000 HPLC system (Dionex, Sunnyvale, CA), equipped with an anion exchange column (Dionex CarboPac PA1), with fucose as the internal standard as described by Del Rio et al. (2010).

2.4. Substrate characterization

The fibre length distribution of the substrates was measured using a Fibre Quality Analyzer (FQA, LDA02, OpTest Equipment, Inc., Hawkesbury, On, Canada) as previously described (Pan et al., 2008; Del Rio et al., 2010). All FQA measurements were the average of 10,000 fibres. The FQA measures the arithmetic (L_n) and length-weighted (L_w) average fibre lengths, the fines (fibres ranging in length from 0.07 to 0.2 mm) content (%fines-n and %fines-w, respectively), and the average fibre width.

The substrates were delignified with sodium chlorite at room temperature prior to viscosity measurements according to the Pulp and Paper Technical Association of Canada (PAPTAC) Useful Method G10.U. The viscosity of substrate solutions containing 0.06%, 0.1%, 0.125%, and 0.5% (w/v) delignified substrates in 0.5 M cupriethylenediamine was measured on a capillary viscometer (Cannon Ubbelohde Viscometer, Cannon Instrument Co., State College, PA) according to the guidelines found in TAPPI standard method T230 om-99. The intrinsic viscosity of each substrate was calculated as described by Lapierre et al. (2006). The viscosity average cellulose degree of polymerization (DPv) was calculated from the intrinsic viscosity as described by Van Heiningen et al. (2004). All viscosity measurements were performed in triplicate. Simon's stain of the fractionated pulp samples was performed according to the procedure by Chandra et al. (2008a).

Cellulose crystallinity was examined using a Spectrum One Fourier-transform infrared spectrometer (FT-IR) with a PIKE MIRacle™ single bounce attenuated total reflectance (ATR) accessory (Perkin Elmer, Wellesley, MA) as previously described (Del Rio et al., 2011).

2.5. Enzymatic hydrolysis

Commercial cellulases (Spezyme CP) and β-glucosidase (Novozym 188) were provided by Genencor International Inc. (a Danisco division, Rochester, NY) and Novozymes (Franklington, NC), respectively. Cellulase and β-glucosidase activities were determined according to established protocols (Wood and Bhat, 1988) and are expressed in terms of filter paper units (FPU) and international units (IUs), respectively.

Batch hydrolysis was conducted at 2% (w/v) solids content in 50 mM acetate buffer, pH 4.8, with 0.004% tetracycline and 0.003% cycloheximide, to prevent microbial contamination. The cellulase to β-glucosidase ratio was 1:2 FPU:IU at enzyme loadings of 5 FPU g^{-1} cellulose. The reaction mixture (50 mL) was incubated at 150 rpm, 50 °C, in a rotary shaker and sampled periodically for glucose determination over a total of 72 h. Glucose was quantified by HPLC. Hydrolysis data are averages of triplicate experiments.

2.6. Generation of substrates enriched in small fibres (<0.2 mm)

To obtain a set of substrate enriched in small fibres or "fines", the unfractionated organosolv-pretreated substrate was subjected to a primary enzymatic hydrolysis as described above with the exception that cellulase loadings were 5, 10, 20, and 25 FPU g^{-1} cellulose and incubated for 4 h. After the primary hydrolysis, the substrates were cooled down to 4 °C, filtered under suction, and the filtrate recirculated to minimize the loss of small fibres. Cellulases adsorbed to the prehydrolyzed substrates were removed via the addition of proteases as described by Nakagame et al. (2010) followed by re-suspending and washing the substrates under suction three times with 100 mL distilled H_2O. After the primary hydrolysis, the prehydrolyzed substrates were delignified with sodium chlorite and their chemical composition and fibre length distributions were determined. The primary hydrolysis and delignification was followed by a secondary hydrolysis using a cellulase loading of 2 FPU g^{-1} cellulose. A control substrate was generated as described above but without the addition of cellulases during the primary hydrolysis.