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Effects of drying-induced fiber hornification on enzymatic saccharification of lignocelluloses☆

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ABSTRACT

This study investigated the effect of fiber hornification during drying on lignocellulosic substrate enzymatic saccharification. Two chemically pretreated wood substrates and one commercial bleached kraft hardwood pulp were used. Heat drying at 105 and 150 °C and air drying at 50% RH and 23.8 °C for different durations were applied to produce substrate with various degrees of hornification. It was found that substrate enzymatic digestibilities (SEDs) of hornified substrates made from the same never-dried sample correlate very well to an easily measurable parameter, water retention value (WRV), and can be fitted by a Boltzmann function. The hornification-produced SED reduction at a given degree of hornification as the percentage of the total SED reduction when the substrate is completely hornified depends on two parameters. The first is $\overline{WRV}$, which is primarily a function of the effective enzyme molecule size, and Δ, which is related to the substrate pore size distribution shape. The low values of SED_{CH}, SED of a completely hornified substrate, obtained from curve fittings for the three sets of samples studied, suggest that enzyme accessibility to cellulose is mainly through the pores in the cell wall rather than substrate external surface. The SEDs of hornified substrates were found to correlate to Simons' staining measurements well. A new parameter was proposed to better correlate enzyme accessibility to cellulose using the two-color Simons' staining technique.

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1. Introduction

Waste paper is a potential biomass feedstock for the future biobased economy. In 2008, about 26 million tons of post-consumer paper and paperboard was landfilled in the United States, and an additional 29 million tons was exported (American Forest and Paper Association, Washington, DC, 2009, http://www.paperrecycles.org/stat_pages/recovery_rate.html). Only about 18 million tons was recycled to produce paper products within the United States. About 50 million tons of waste paper is therefore potentially available each year, counting exported waste paper. This waste paper is from mixed office waste (MOW), primarily consisting of bleached softwood and hardwood pulps, having very low lignin content of 5% and very high cellulose content of about 70%, and old corrugated containers (OCC), made of unbleached softwood chemical pulps that also have a low lignin content of about 10–15% and a high cellulose content of 50%. Therefore, this waste paper is an attractive feedstock for cellulosic ethanol production. This use of waste paper would have added benefit of reducing unnecessary landfilling and ultimately protecting the environment.

Several studies have been reported on producing ethanol from waste paper through enzymatic saccharification and fermentation. A few pretreatment methods were examined [1–3] and enzyme assay screening studies were also conducted [4,5] to enhance enzymatic cellulose saccharification of waste paper. Process integration for ethanol production from waste paper was also carried out [6]. However, few studies placed great emphasis on two fundamental issues unique to bioconversion of waste paper to ethanol: (1) the composition of waste paper and (2) the effect of fiber hornification caused by drying in the paper production process on enzymatic hydrolysis of waste paper cellulose. With the adoption of co-mingling practice in collecting recyclable waste by many municipalities in the United States to reduce collection cost, the collected waste paper becomes a very complex mixture that consists of different grades of paper, such as ONP (old newsprint paper), OCC, MOW, etc., from both softwoods and hardwoods. The significant variability (from bale to bale) of this mixture made it

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difficult to design proper pretreatment processes and conditions to achieve high cellulose saccharification efficiency. Drying-induced fiber hornification has been well studied [7–9], including some recent works [10–12]. It was suggested that the low enzymatic saccharification efficiency of waste paper was due to changes in fiber structure through drying, which reduced enzyme accessibility to cellulose [13]. However, limited data were reported that demonstrated the reduced substrate enzyme digestibility (SED) due to reduced cellulase accessibility to the substrate upon drying evidenced by a Simon Staining method [14,15]. Furthermore, the degrees of fiber hornification were not quantitatively reported and the effects of hornifications were not isolated from other contributing factors. Only a limited number (4) samples were studied with a narrow range of degree of hornification and therefore SED in these studies. The effect of fiber hornification on enzymatic hydrolysis of cellulose was never quantified to substantiate this argument. Careful studies to eliminate factors other than drying-induced hornification on enzymatic cellulose saccharification are required to obtain a complete and fundamental understanding of this issue.

This study focuses on understanding the effect of fiber hornification during drying in paper manufacturing on enzymatic cellulose saccharification. Hornification refers to the irreversible loss of water binding ability upon drying of celluloses [7,9]. Hornification is a consequence of the irreversible change of cell wall structure through the drying–rewetting process [16]. Cellulose fibrils are tightly packed through hydrogen bonding upon drying. The hydrogen bonding causes the shrinkage and loss of pores in the cell wall. When rewetting, this packed cell wall structure swells and some of the hydrogen bonds are broken, which leads to a pattern of internal fibrillation. But rewetting can only partially reopen the tightly packed cell wall structure due to the irreversible nature of hornification. This was verified by the reduction in the measured amount of accessible water in the cellulose pores upon drying–rewetting [8,9]. In this study, we will carefully design a drying and rewetting procedure to isolate the effect of fiber hornification on enzymatic hydrolysis of hornified fibers. We will quantify fiber hornification using substrate water retention value (WRV), an easily measurable parameter, according to its original definition of fiber hornification [7,9]. We will then correlate hornified substrate enzymatic cellulose saccharifcation efficiency to WRV. WRV has been found to correlate to substrate specific surface [17] and used to effectively correlate the rate of enzymatic hydrolysis of swollen fibers [18]. The objective of this study is to provide quantitative data to substantiate the effect of fiber hornification on the loss of enzyme accessibility to cellulose to improve the fundamental understanding of drying on enzymatic hydrolysis of waste paper for ethanol production.

2. Experimental

2.1. Materials

Three types of never-dried (ND) fibrous samples were used. A SPORL (sulfite pretreatment to overcome recalcitrance of lignocellulose [19,20])-pretreated lodgepole pine solid substrate (PLPS) after drying was used to simulate OCC. Commercial wet-bleached eucalyptus pulp (BEP) donated by Kimberly Clark (Appleton, Wisconsin) was used to simulate MOW through drying. A SPORL-pretreated aspen solid substrate (PASS) was also used as a reference to examine the effect of hornification on enzymatic hydrolysis of real lignocellulosic substrate. The SPORL pretreatment has demonstrated robust performance for efficient cellulosic ethanol production from woody biomass [21]. Lodgepole pine trees were harvested from the Canyon Lakes Ranger District of the Arapaho-Roosevelt National Forest, Colorado. The log was chipped at the USDA Forest Service, Forest Products Laboratory. Aspen wood chips were donated by Wisconsin Rapids pulp mill of New Page Corporation (Wisconsin Rapids, Wisconsin). The screened wood chips with sizes between 6 and 38 mm were used for chemical pretreatment by SPORL (Fig. 1) in a 1-L wood pulping digester as described elsewhere [21]. The pretreatments were conducted at 180 °C for 20 min and 170 °C for 25 min for lodgepole pine and aspen, respectively. The sulfuric acid and sodium bisulfite charges on oven dry (od) wood were 0% and 8% for lodgepole pine and 1.1% and 3% for aspen, respectively. Liquid to wood ratio (L/W) was fixed at 3 for all pretreatments. Pretreated wood chips were disk milled at 2570 rpm using D2-B505 disk plates (Andritz, Inc., Springfield, Ohio) with a disk plate gap of 1.0 mm and solids-loading of 10%. Detailed description of wood chip pretreatment and disk milling for substrate production can be found in our previous studies [19,21]. The size-reduced solid (substrate) was directly dewatered by pressing using a canvas bag to a solids content of about 30%. At last, the total yields of lodgepole pine and aspen were 65% and 61%, respectively. The resultant substrate from lodgepole pine is similar to neutral sulfite pulp [22] for producing corrugated containers.

Accellerase 1500 was donated by Genencor (Palo Alto, California). Direct blue (DB, Pontamine Fast Sky Blue 6BX) and direct orange (DO, Pontamine Fast Orange 6RN) dyes were purchased from Pylam Products Co., Inc. (Garden City, New York). Ultrafiltration membranes with 100 k (molecular weight cutoff) and nylon membranes with 0.45-μm pore size and 47-mm diameter were obtained from Millipore (Millipore, Bedford, Massachusetts) and Cole-Parmer (Vernon Hills, Illinois), respectively. Sodium acetate, acetic acid, sulfuric acid, and sodium bisulfite were used as received from Sigma–Aldrich (St. Louis, Missouri). Other chemicals in analytical grade were purchased from Fisher Scientific (Hanover Park, Illinois).

2.2. Handsheet making, drying, and rewetting/disintegrating

All three never-dried substrates described above need to go through a drying and rewetting process to study the effect of fiber hornification on enzymatic cellulose hydrolysis. Due to inter- and intra-fiber hydrogen bonding, fibers/fiber bundles in the wet substrate can easily form very hard and strong fiber flocks of size varying from several millimeters to several centimeters upon drying. The resultant dried substrate becomes fiber flocks of different sizes. The enzymatic hydrolysis results obtained using this form of dried material are strongly affected by the formation of fiber flocks and the flock size distribution due to their effects on enzyme accessible surface area in addition to fiber hornification. Strong mechanical shearing through re-pulping can break the fiber flocks but can also significantly reduce fiber length and diameter and therefore increase enzyme accessible surface area to cellulose. Furthermore, the shearing process can act as pulp beating to partially reverse hornification [8,9,13]. Ideally, one needs to dry individual fibers/fiber bundles in a manner that separates other factors from pure fiber hornification due to fiber drying and its effect on enzyme accessibility to cellulose and therefore enzymatic cellulose hydrolysis. This issue was not clearly addressed in the literature [13,14]. Therefore the reported cellulose hydrolysis data can be attributed to many factors other than fiber hornification. A special experimental procedure was followed in this study (Fig. 1). Each wet substrate was gently disintegrated using a disintegrator (Model 73-06-01, TMI, Ronkonkoma, New York, USA) for 5000 revolutions at 312 rpm with a 5% consistency at room temperature. This low disintegrating speed was chosen based on a set of experiments that showed the SEDs of the resultant pulps were not affected by the disintegrating speed used. The pulp was then diluted to a solid consistency of 1% in the 20-L mixing tank of a handsheet making system. The diluted pulp suspension was used to make handsheets according to TAPPI Standard Method T205 sp-95 [23] using a standard laboratory handsheet mold. The basis weight of the handhseets was varied from 40 to 100 g/m^2. SEM images of cross section of handsheets showed about 5–10 fiber layers in the sheet thickness direction for the sheets with basis weight of 40 g/m^2 (Fig. 2).

Both air drying and heat drying of the handsheets were used to study the effects of drying processes on fiber hornification. Air drying (AD) was conducted by simply hanging the handsheets in a temperature (23.8 °C) and relative humidity (RH = 50%) controlled room. Heat drying was conducted at two temperatures of 105 °C (LHD) and 150 °C (HHD) by laying a handsheet on a heated plate of the dryer (Model A-310, Adirondack Machine Corp., Glens Falls, New York). Drying time was varied from 10 s to 60 min to obtain different degrees of hornification of the handsheets. After drying, the moisture contents of the sheets were measured and the samples were stored in plastic bags for enzymatic hydrolysis and Simons' staining.

The rewetting of the dried handhseets through proper disintegrating is a necessary step to prepare substrates for enzymatic hydrolysis. Two pieces (total about 1.4 g in od weight) of dried handsheet were disintegrated in 1-L deionized water after 10,000 revolutions at 312 rpm using a disintegrator (Model 73-06-01, TMI, Ronkonkoma, New York, USA). Complete disintegration to individual fibers is not necessary for effective enzymatic hydrolysis, as will be demonstrated in this study. The wet weight of substrate was recorded after filtering by a nylon membrane with pore size of 0.45 μm. This handsheet making, drying, and rewetting/disintegrating experimental procedure ensured the uniform drying of a fairly constant number of fiber layers to result in a relatively uniform dried substrate without the formation of large flocks in comparison to the resultant substrate from direct drying wet substrate. The procedure also resembles the papermaking process so the results are applicable to enzymatic cellulose saccharification of waste paper.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis was conducted at 2% substrate solids (w/v) in 50 mM acetate buffer, pH 4.8, with 50 ppm tetracycline as antibiotic. Accellerase 1500 of Genencor (Palo Alto, California), a complex-enzyme including cellulase and β-

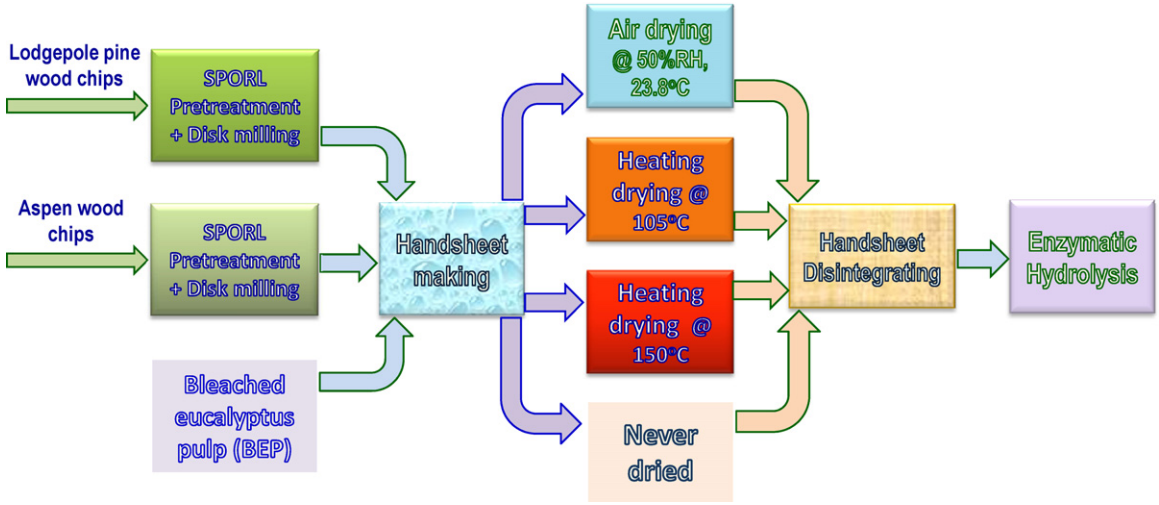

Fig. 1. Schematic process flow diagram of the experiments for isolating fiber hornification on enzymatic cellulose saccharification.

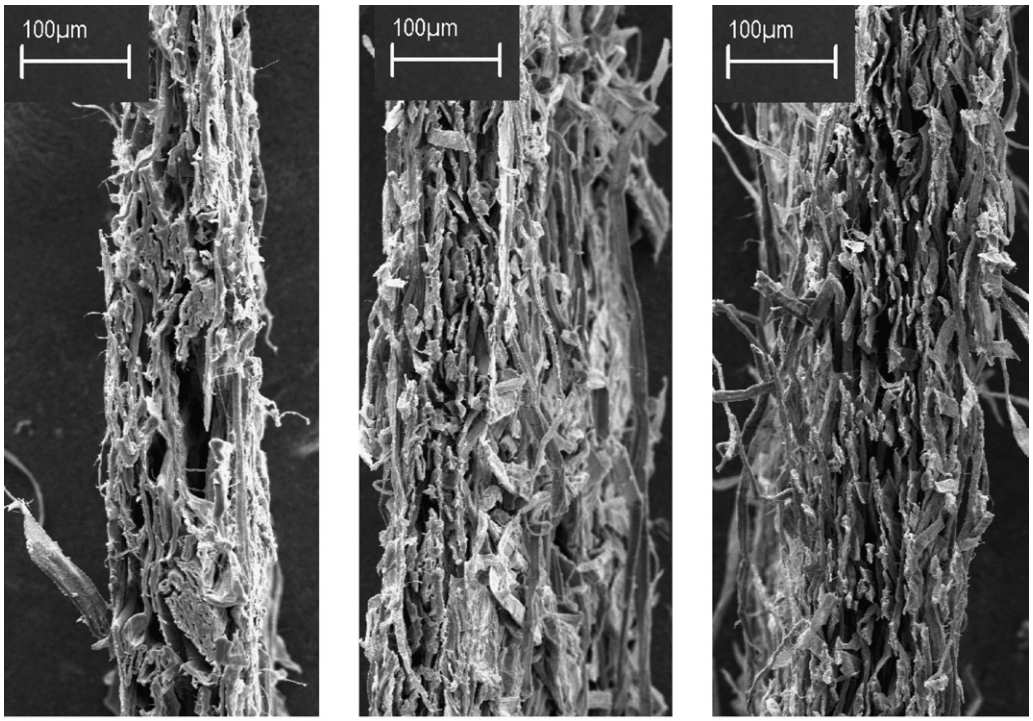

Fig. 2. SEM image of the cross section of a dried handsheet (40 g/m^2) that shows the number of fiber layers in the out-of-plan paper direction.

glucosidase, was used at a loading of 0.24 mL/g od substrate, or about 10 FPU/g substrate for enzymatic hydrolysis. The solid substrate suspension (100 mL) was incubated on a shaker (Thermo Fisher Scientific, Model 4450, Waltham, Massachusetts) at 50 °C and 200 rpm. Hydrolysate was sampled periodically, and glucose concentration was determined using a commercial glucose analyzer (YSI 2700S, YSI Inc., Yellow Springs, Ohio).

2.4. Determination of substrate chemical composition

The main chemical compositions of the three never-dried solid samples were analyzed by the Analytical Chemistry and Microscopy Laboratory (ACML) of the USDA Forest Service, Forest Products Laboratory, using an improved high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method [24]. The Klason lignin content was measured gravimetrically after washing and drying the solid residue from the acid hydrolysis. Ash contents were measured according to TAPPI Standard Method T211 om-93 [23]. Results are listed in Table 1.

Table 1
Chemical compositions of the three wood samples used in this study.[a]

Component	PLPS (%)	PASS (%)	BEP (%)
Ash	Nd	0.03	0.10
Klason lignin	32.00	24.32	1.20
Arabinan	Nd	Nd	0.01
Galactan	0.05	Nd	0.01
Rhamnan	0.10	Nd	0.10
Glucan	53.09	70.80	81.91
Xylan	2.94	2.03	5.70
Mannan	1.75	0.21	0.02
Total yield	89.8	97.4	89.05

[a] Nd, not determined.

Table 2
Results of experimental repeatability tests using a never-dried SPORL-pretreated lodgepole pine sample (PLPS).

Run no.	Sheet basis weight (g/m^2)	SED (%)	WRV (%)	Simons' staining measurements	
				A_{DB} (mg/g od substrate)	A_{DO} (mg/g od substrate)
1	34.83	55.77	148.8	18.13	51.61
2	35.50	54.42	140.8	19.12	52.35
3	35.17	53.57	144.5	17.10	50.38
Mean	35.17	54.59	144.7	18.12	51.44
STD[a]	0.34	1.11	4.1	1.01	1.00
RSTD[b] (%)	0.97	2.04	2.8	5.56	1.94

[a] STD, standard deviation.
[b] RSTD, relative standard deviation.

2.5. Measurements of water retention value (WRV)

Fiber hornification was originally quantified by percentage reduction in water retention value (WRV) [7]. Rigorously, it should be quantified by percentage reduction in fiber saturation point (FSP), the amount of water retained in the fiber cell wall (all pores, excluding the water in the gross capillary, such as lumen) of unit mass of fibers [25]. Because the measurement of FSP involves a very time-consuming procedure, such as solute/polymer exclusion technique [25–27], we use WRV, an easily measurable parameter, to quantify fiber hornification in this study. WRV is a good estimate of FSP as it can correlate to the amount of water in cell wall pores [28] or the volume of pores [29]. WRV was measured following Scandinavian test method SCAN-C 62:00 [30]. Approximately 1 g (od) handsheet was disintegrated in 1 L deionized water for 10,000 revolutions at 312 rpm and then soaked for 2 h. The resulting suspension was carefully filtered using a nylon membrane with pore size of 0.45 μm. The filter cake was then removed and added with deionized water to make a suspension of about 10% solids. The suspension was wrapped in a nylon screen with mesh opening of 100 μm (Cole-Parmer, Vernon Hills, Illinois). The wrap was placed in a centrifuge tube with support to leave space for water accumulation at the bottom of the tube and centrifuged at 3000 × *g* for 15 min in a laboratory centrifuge (Thermo Fisher Scientific, Sorvall Legend 40/40R, Waltham, Massachusetts). The wet centrifuged sample was first weighed, then oven dried at 105 °C overnight and weighed again. The WRV is simply the percentage of retained water (weight change of the substrate before and after drying) of the dried substrate, i.e.

$$\text{WRV}\ (\%) = \frac{w_{\text{wet}} - w_{\text{dried}}}{w_{\text{dried}}} \times 100 \quad (1)$$

where w_{wet} and w_{dried} are the wet and dry weights of the substrate, respectively.

2.6. Simons' staining method

The Simons' staining method [31] can determine the amount of dye adsorbed by the cell wall through the pores of a lignocellulosic substrate as a way to represent the amount of substrate pore surface accessible to the dye [14,15]. When different dyes with different molecular sizes are used, the distribution of substrate pore size/surface can be determined. The determined distribution of substrate pore size can be used as a measure for enzyme accessibility to substrate cellulose [32,15] A two-color method [15] is used in the present study to verify the use of WRV for characterization of the reduction of enzyme accessibility due to hornification. The low-molecular-weight fraction of the direct orange (DO) dye has similar affinity to cellulose as the direct blue (DB) dye [33]. Therefore, the DO dye is filtered by a 100 k cut off membrane using an ultrafiltration apparatus (Amicon, Beverly, Massachusetts) driven by pressurized nitrogen at 2 atm pressure. Dried paper was first disintegrated as described previously. Approximately 100 mg (od) pulp was put into each of a set of 5 test tubes (20 mL, 5 mm inner diameter). Different amounts of 10 g/L concentration of dye solution, ranging from 0.25 to 3 mL, were added to each tube. This was used to obtain the dye adsorption isotherm. The dye solution was a mixture of DO and DB with a 1:1 ratio. Then 1 mL 0.3 M phosphate buffer, containing 0.3 mM NaCl, was added to each tube to make a 10-mL solution using deionized water. The dye solutions were then incubated at 70 °C for 6 h and centrifuged at 10,000 rpm for 5 min. The supernatant dye solution was measured by a UV–Vis spectrophotometer (U-3010, HITACHI Ltd., Tokyo, Japan). The amounts of adsorptions of DO and DB by the cell wall pores, A_{DO} and A_{DB}, can be calculated from the initial amounts of dye applied and the amounts measured in the supernatant solution.

3. Results and discussions

3.1. Experimental repeatability

Triplicate experiments of the entire experimental process were conducted using a never-dried SPORL-pretreated lodgepole pine substrate (PLPS) to evaluate the repeatability of the experiments. The results indicate the excellent repeatability in controlling the handsheet basis weight and measuring substrate enzymatic digestibility (SED), water retention value (WRV), and dye absorption using the Simons' staining method (Table 2). The SED is defined as the percentage of substrate glucan enzymatically hydrolyzed to glucose. The relative standard deviations (RSTDs) of the triplicate runs for all the listed parameters were less than 3%, except for adsorption of the direct blue dye (DB) of about 5.6%.

3.2. Effect of handsheet basis weight on substrate enzymatic digestibility (SED)

Enzymatic cellulose saccharification of a dried sheet can be affected by factors other than fiber hornification through drying, such as fiber flock formation, as discussed previously. To demonstrate the effectiveness of the present procedure (handsheet making, drying, and rewetting) (Fig. 1) for eliminating the effect of factors other than fiber hornification, such as fiber flock formation, on substrate enzymatic digestibility (SED), enzymatic hydrolysis experiments were carried out using dried handsheets (HHD, heat drying at 150 °C for 4 min) of different basis weights or thicknesses but from the same original never-dried substrate. The results clearly indicate that SED of dried sheets was not affected by basis weight/sheet thickness within certain basis weight ranges (Fig. 3). The limits of the basis weight ranges were 60 and at least 100 g/m^2 for the tested sheets made from the SPORL-pretreated lodgepole pine substrate (PLPS) and the bleached eucalyptus pulp (BEP), respectively. The relative standard deviations (RSTDs) of the data points within these two limits are only 0.79% and 1.06% for BEP and PLPS, respectively, both within the measurement error of 2% based on repeatability experiments (Table 2). This suggests that the sizes of the formed fiber flocks are too small to affect enzyme accessibility to cellulose. This also suggests that the method of

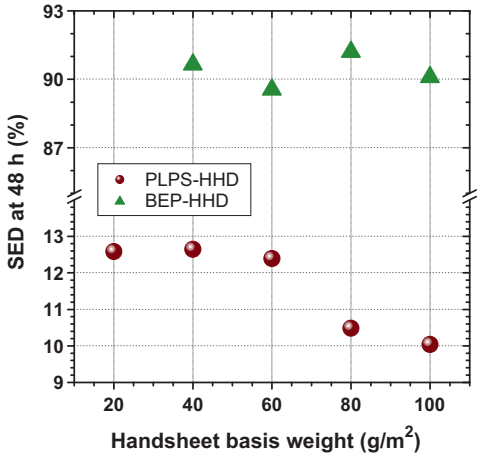

Fig. 3. Effect of handsheet basis weight on enzymatic digestibility (SED) of dried and rewetted substrates.

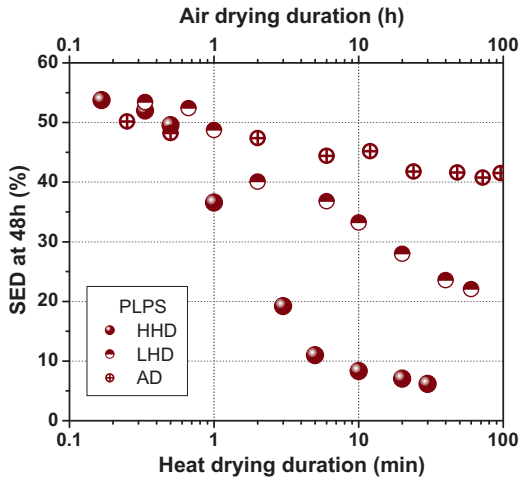

Fig. 4. Effect of drying method and time on enzymatic digestibility (SED) of dried and rewetted SPORL-pretreated lodgepole pine substrates (PLPS).

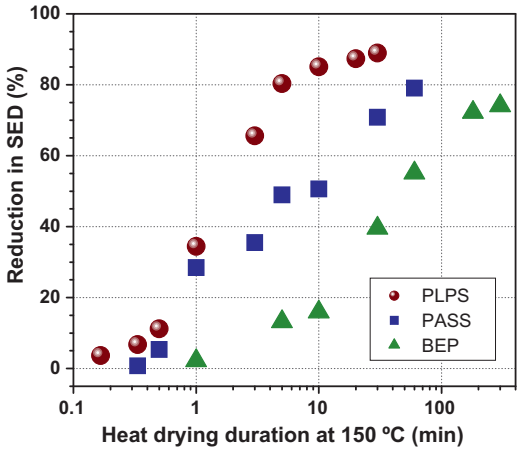

Fig. 5. Comparison of substrate susceptibilities to hornification upon drying at 150 °C for different periods of time on hornified substrate enzymatic digestibility (SED).

gently disintegrating dried handsheets for a short period of time (described in Section 2) did not reverse hornification to affect the resultant substrate enzymatic saccharification. The difference in the limit of basis weight between BEP and PLPS is probably due to the differences in fiber morphology and therefore handsheet formation (fiber flocculation potential), chemical composition, fiber cell wall structure (porosity) associated with enzyme accessibility to cellulose, etc. Basis weight of 40 g/m^2 was therefore selected for all handsheets in the following experiments based on the results in Fig. 3.

3.3. Effects of drying on SED during the process of drying

Both drying method and drying duration affect the extent of drying and therefore fiber hornification and enzymatic cellulose saccharifcation as a result. Heat drying has a much greater effect on dried substrate enzymatic digestibility than air drying does (Fig. 4). The higher the heat drying temperature is, the greater the effect (Fig. 4). Increasing drying time also enhances the degree of fiber hornification and therefore its effect on SED for all drying methods (Fig. 4). For sheets produced from PLPS, SED was reduced from 54% to about 40% after 100 h air drying (Fig. 4), to about 25% and 6% after 30 min heat drying at 105 °C and 150 °C, respectively (Fig. 4).

Different never-dried substrates have different susceptibilities to drying due to the difference in cell wall structure or pore size distribution, which can result in differences in hornification and its effect on SEDs even under the same drying conditions. The time-dependent reduction in SED demonstrates that PLPS is most susceptible to drying, with the largest reduction in SED when dried for the same duration at 150 °C compared to PASS and BEP (Fig. 5). The maximal reduction in SED was about 90% and reached only after 20 min of drying for PLPS. The maximal reduction in SED was slightly below 80% and reached after 5 h drying for BEP. This difference is probably due to the difference in substrate chemical composition, morphology, fiber cell wall structure (pore size distribution and porosity), and the potential and susceptibility of pore shrinkage upon drying, all of which affected enzyme accessibility to cellulose. The lignin contents of BEP and PLPS are about 1% and 32%, respectively (Table 1). Extensive delignification opens fiber pores of BEP, which requires stronger hornification than PLPS to produce equal effects on reduction in enzyme accessibility.

3.4. Characterization of SED reduction upon drying using WRV

It is conceivable to characterize the reduction in SED of dried and rewet substrate due to hornification—irreversible loss of water in fiber cell wall—using water retention value (WRV). This is because WRV is a good measure of the amount of water in the cell wall. It was found that the SEDs of rewetted substrates produced from the same initial never-dried (ND) sample using different drying methods and for different durations fall on a single curve (Fig. 6). It should be pointed out that the lignin becomes metastable to deformation when the substrate is heated to 150 °C that is higher than the lignin glass transition temperature [34]. Statistical analysis indicates that there are differences between the two data sets for HHD and LHD of PLPS, suggesting lignin softening may have some effect on SED. However, very similar results were obtained when the measured SED data sets of HHD and LHD were fitted using the following Boltzmann function,

$$y = A_2 + \frac{A_1 - A_2}{1 + \exp[(x - \bar{x}) \cdot \Delta]} \quad (2)$$

The data in Fig. 6 clearly indicate SED reduction was slow initially as WRV decreases or pore water is lost through drying. Then SED decreases very rapidly as WRV further decreases. Finally SED reduction slows and achieves an asymptotic value. This asymptotic

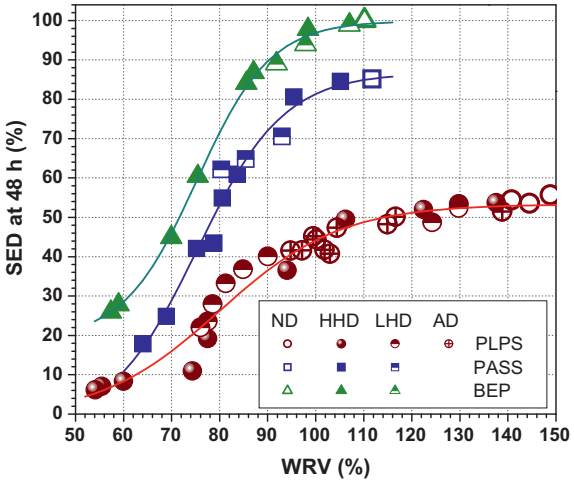

Fig. 6. Nonlinear fittings of enzymatic digestibility (SED) of dried and rewetted substrates to a Boltzmann function using substrate water retention value (WRV).

Table 3
Results of nonlinear curve fittings of hornified substrate enzymatic digestibilities (SEDs) using Boltzmann Eq. (3) for the three never-dried samples studied.[a]

Never-dried sample label	Measured SED_{ND} (%)	SED_{ND} (%)	SED_{CH} (%)	$\overline{WRV}$ (%)	Δ	Relative fitting error[b]
PLPS	54.6	53.3 (53.2)	−0.9 (0)	80.0 (80.4)	0.0795 (0.0816)	0.0744 (0.0713)
PASS	86.2	86.7 (86.3)	−2.7 (0)	75.4 (76.0)	0.1117 (0.1166)	0.0457 (0.0444)
BEP	100.0	99.9	18.8	75.3	0.1308	0.0131

[a] Values in parentheses are from fittings by forcing $SED_{CH} = 0$.

[b] Relative fitting error = $\sum_1^N \left[\sqrt{[y_{i,\text{measured}} - y_{i,\text{predicted}}]^2} / y_{i,\text{predicted}} \right] / N$.

value, represented by A_1 in Eq. (2), is obtained when the substrate is completely hornified, i.e., all cell wall pores are not accessible to enzymes (very small WRV) and the only accessible surfaces to enzymes are the substrate external surfaces. A_1 can be denoted as SED_{CH}. The SED of the initial never-dried sample is represented by A_2 in Eq. (2) and can be denoted as SED_{ND}. The WRV that corresponds to the rapid reduction in SED (vs WRV) is $\bar{x}$ and can be denoted as $\overline{WRV}$. The lines in Fig. 6 are calculated SED according to Eq. (2) using nonlinear fitted parameters (Table 3). The behavior of SED shown in Fig. 6 can be explained by the fact that the size/diameter of cell wall pores has a broad distribution [27,32,35]. Drying produces shrinkage of all pores to different degrees due to hydrogen bonding. However, small pores whose sizes/diameters are smaller than the size of the enzyme molecule, including those bottle-necked pores [26], will not contribute to SED reduction upon drying, simply because these pores are not accessible to enzyme molecules anyway. It is the shrinkage of large pores that reduces enzyme accessibility to substrate cellulose resulting in SED reduction. Most large pores have some tolerance to initial drying that produced a small degree of shrinkage and are still accessible to enzyme molecules, because their initial sizes are larger than the size of enzyme molecule. Further drying eliminates this tolerance for many large pores as the shrinkages of these pores become significant, which causes significant numbers of large pores to become inaccessible. Significant and rapid reductions in SED result. As the substrate becomes severely hornified, drying only further shrinks the sizes of the remaining pores but does not affect enzyme accessibility to most of these pores as they are already small enough to be inaccessible to enzyme molecules.

Eq. (2) can be rearranged to the following form with the substitution of the new notations SED_{ND}, SED_{CH}, and $\overline{WRV}$ discussed above to further understand the effect of fiber hornification on enzymatic saccharifcation of lignocellulose,

$$\delta_{\text{SED-H}} = \frac{SED_{ND} - SED}{SED_{ND} - SED_{CH}} = \frac{1}{1 + \exp[(WRV - \overline{WRV} \cdot \Delta)]} \quad (3)$$

where $\delta_{\text{SED-H}}$ can be considered as SED reduction due to hornification as a fraction of the total SED reduction caused by hornification when the never dried sample is completely hornified. $\delta_{\text{SED-H}}$ is different from SED reduction shown in Fig. 5, which is simply the percentage of reduction in SED relative to the SED of the never-dried sample. $\overline{WRV}$ is a characteristic value that represents a hornification state at which rapid reduction in SED occurs due to a great number of large pores are shrinking to a size equal to the effective size of the enzyme molecule. Therefore, $\overline{WRV}$ should be primarily dependent on the effective enzyme molecular size. The larger the enzyme molecule, the greater the $\overline{WRV}$ should be. For the present study, the same cellulase enzyme in the commercial Accellerase 1500 complex was used, therefore $\overline{WRV}$ obtained from different samples should be close to a constant. The nonlinear fitting of the three data sets obtained using PLPS, PASS, and BEP confirmed this argument (Table 3). The differences in $\overline{WRV}$ among these three sets of data are less than 6%. This 6% difference can well be due to the error in nonlinear data fitting. The difference between the two hardwood samples, PASS and BEP, is only about 1%. The parameter Δ should be the characteristics of the cell wall structure, specifically the shape of the pore volume distribution curve of the never-dried sample. The SED_{ND} obtained from nonlinear fitting was found to be very close to the measured SED of the never-dried substrate for each of the three samples studied (Table 3). The nonlinear fitting produced very small negative values of SED_{CH}, −0.9 and −2.7, for the two pretreated samples PLPS and PASS, respectively. This suggests that all the cell wall pores are completely inaccessible to enzymes and therefore produced no enzymatic saccharification when PLPS and PASS are completely hornified, i.e., $SED_{CH} = 0$. The negative values are errors produced in fitting (refitted results are listed in parentheses in Table 3 by forcing $SED_{CH} = 0$). The fitted value of SED_{CH} was 18.8 for BEP, suggesting enzymatic cellulose saccharification of bleached eucalyptus pulp can prevail to certain degree (18.8%) even if the pulp is completely hornified. The near zero lignin content of the BEP sample (Table 1) certainly made it possible for the enzymes access cellulose from the fiber external surface to produce a certain degree of saccharification even when it was completely hornified.

The SED_{CH} value has significant implications to understanding enzyme accessibility to cellulose of lignocellulosic substrates. The low values of SED_{CH} presented above suggest that enzyme access to cellulose is mainly through the pores in the cell wall rather than through the substrate external surfaces. Even for a fully bleached kraft pulp with close to zero lignin content, the accessible external surface area only contributes less than 20% of the enzymatic cellulose saccharification. The measured SEDs for the three substrates (PLPS, PASS, BEP) dried at 150 °C for 60 min (the most severe drying studied in this work) are 6%, 10%, and 16%, respectively, indirectly confirmed this argument and the value of SED_{CH}. For typical lignocellulosic substrates (e.g., PLPS, PASS) with very high lignin contents of about 25% or higher (Table 1), enzyme accessibility to cellulose through substrate external surfaces is close to zero. This argument is partially supported by our recent study that showed physical size reduction of wood substrate had no effect on enzymatic cellulose saccharification when the substrate size is reduced to a certain degree [19].

3.5. SED reduction due to hornification evaluated using Simons' staining

The two-color Simons' staining method can provide only semi-quantitative information about the distribution of fiber pore surface. The measured adsorption of the DO dye is mainly contributed by the large pores, whereas the adsorption of the DB dye is due to the small pores. The combined adsorption of A_{DO} and A_{DB} was correlated to SED, but the correlation was not satisfactory. We believe this is because the sum of A_{DO} and A_{DB} represents only the total pore surface area, not the enzyme-accessible pore surface area. When a substrate experiences hornification, pores in the cell wall shrink, as discussed previously. The shrinkage not only reduces the total pore surface but also alters the pore size distribution, which reflects the susceptibility of fiber to hornification. This susceptibility should vary with the drying method as well as the never-dried substrate itself. Therefore, we proposed to use the following parameter $A_{DO}(A_{DO}/A_{DB})$ to correlate SED. (A_{DO}/A_{DB}) is a

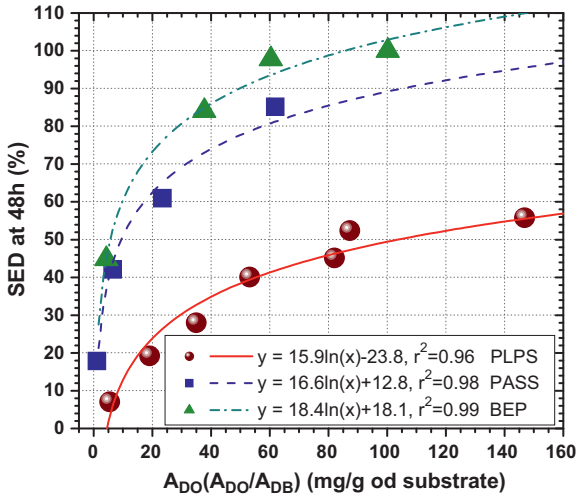

Fig. 7. Correlations of enzymatic digestibility (SED) of dried and rewetted substrates to an absorption parameter, $A_{DO}(A_{DO}/A_{DB})$, from Simons' staining measurements.

correction factor for the shape of the pore size distribution curve contributing to the enzyme-accessible surface area. A_{DO} represents the surface area of the large pores that are more likely accessible to enzymes rather than all the pores. When SED is plotted using this new parameter, each data set corresponding to each of the three never-dried samples follows the same functionality though do not fall onto the same curve (Fig. 7). This same functionality is much less certain when SED were plotted using $(A_{DO} + A_{DB})$ or (A_{DO}/A_{DB}) alone (not shown), suggesting the improvement of the proposed new parameter over the ones used in the literature.

The proposed parameter, $A_{DO}(A_{DO}/A_{DB})$, also shows better correlation with water retention value (WRV) than other parameters. It was found that the measured WRV and $A_{DO}(A_{DO}/A_{DB})$ for all the substrates produced from the three never-dried samples fall onto a universal line and can be fitted by the following linear equation:

$$\mathrm{WRV} = 64.7 + 0.563A_{DO}\left[\left(\frac{A_{DO}}{A_{DB}}\right)\right] \quad (4)$$

with $r^2 = 0.84$. This indicates that both WRV and Simons' staining method can be effective for measuring enzyme accessibility. However, WRV measurement is much easier.

4. Conclusions

This study was a fairly comprehensive investigation of one of the most pertinent and fundamental issue related to cellulosic ethanol production from waste paper: the effect of fiber hornification upon drying on lignocellulosic substrate enzymatic saccharification. The study designed a special procedure for substrate drying, rewetting, and disintegrating to isolate the effect of fiber hornification on enzymatic saccharification of lignocelluloses. This effect is dependent on the cell wall physical and chemical structure of the never-dried material, the susceptibility of the never-dried materials to drying, the drying method, and drying duration. The water retention value (WRV), an easily measurable parameter used to quantify hornification, was found to correlate very well with substrate enzymatic digestibility (SED) of hornified substrates made from the same never-dried sample. This correlation can be fitted by a Boltzmann function. For a given degree of hornification, the hornification-produced reduction in SED as the percentage of the total SED reduction when the substrate is completely hornified, $\delta_{SED\text{-}H}$, depends on a characteristic $\overline{WRV}$ that is primarily a function of the effective enzyme molecular size and a substrate pore size distribution shape parameter Δ. The low values of SED_{CH}, SED of a completely hornified substrate, obtained from curve fitting of each data set corresponding to each of the three never-dried samples, suggests that enzyme accessibility is mainly contributed by the pores in the cell wall rather than substrate external surfaces. Study using two-color Simons' staining method reveals similar results. A new parameter was proposed to better correlate enzyme accessibility to cellulose using the two-color Simons' staining technique. This study also demonstrates that fiber hornification can be used as an effective means to study enzyme accessibility to cellulose through varying the cell wall pore structure.

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