Hydroxyl Availability in Aspen Wood After Dilute Acid Pretreatment and Enzymatic Saccharification

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The production of cellulosic biofuels often leaves behind solid residues, which can be converted to useful co-products *via* chemical modification and processing. The objective of this study was to examine the changes in hydroxyl accessibility of a hardwood after the extraction of fermentable sugars (saccharification). Saccharification was performed on milled and dilute-acid pretreated aspen wood and resulted in a glucan-to-glucose conversion of 91%. The unhydrolyzed (solid) fraction was then analyzed for hydroxyl availability using an acetylation method, and the data were related to information of accessible pore volume evaluated using nitrogen adsorption. Different pore volumes were also created by oven-, air-, or freeze-drying of the samples. The results showed that more hydroxyls are available if the physical accessibility (pore volume) of a given substrate is better preserved. Upon saccharification, the accessible hydroxyls were reduced by at least half of that in untreated wood, while the specific pore volume increased 10 times. This finding suggests that future strategies for utilizing saccharification residues for co-products should tap the increased porosity and lower polarity of the substrate.

Keywords: Biomass; Biofuels; Biochemical conversion; Residue; Co-products; Pore volume; Hydroxyl number; Chemical modification; Acetylation

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INTRODUCTION

The production of cellulosic biofuels leaves behind solid residues, which can be regarded as a new class of industrial feedstock. The biochemical route for biofuel production from lignocellulose involves two initial steps, in which the cell wall structure is "loosened" through pretreatment, and then fermentable sugars are released *via* enzymatic hydrolysis of polysaccharides to monosaccharides. Dilute acid is a promising, ubiquitous pretreatment option, as it promotes high glucose yields in subsequent saccharification (Brodeur *et al*. 2011). The solid waste stream from this conversion option typically consists of unhydrolyzed, lignin-rich residue, which can potentially be converted to valuable co-products. Co-products from residuals are an emerging focus to enable biofuels viability, as well as provide novel stand-alone products (DOE 2015).

The usual approach to converting lignin-rich biomass residues is by extracting the lignin for further chemical processing (*e.g.*, Jin *et al.* 2010). Converting the residues without this pre-purification step has the potential to increase cost efficiency (Zhang *et al.* 2013), but its success depends on the accessibility and availability of reactive sites in the substrate. Hydroxyl groups are the most abundant reactive sites in lignocellulosic materials. One possible route to convert the saccharification residues would be to utilize their reactive

hydroxyl groups, for example, in the reaction with isocyanates to produce polyurethane-based materials. Rials *et al.* (2001) demonstrated, through the use of wood pulp fiber, that the accessibility of hydroxyl functionality to the polyol/isocyanate resin had a strong influence on the mechanical performance of the resulting polyurethane composites. Moving further upstream, the saccharification residues could also be modified by reacting with the hydroxyls to incorporate functional groups that are desirable for subsequent reactions. One such example is acetylation, which is a common technique for improving biomass properties, for instance, compatibility with hydrophobic polymers in wood-plastic composites (Özmen *et al.* 2013) and absorption of oil spills (Sun *et al.* 2002). In acetylation of wood, the importance of accessibility was evidenced by the faster initial rate of reaction when the treatment was performed on the sample previously solvent-exchanged to maintain an open micropore structure as opposed to that oven-dried in which the micropores collapsed (Hill *et al.* 2004). Therefore, knowledge of accessibility and hydroxyl availability is expected to contribute to the efficiency of chemical modification and conversion of the saccharification residues.

The objective of this study was to examine changes in the hydroxyl availability of hardwood biomass upon dilute-acid pretreatment and subsequent enzymatic hydrolysis. Many published studies (*e.g.* Thompson *et al*. 1992) have characterized the accessibility (specific surface area, pore volume, *etc*.) of (pretreated) biomass to infer the ease of enzyme access in subsequent saccharification with the intent to relate the findings to sugar yield. However, the solid residues after sugar extraction have not been well studied for accessibility and hydroxyl availability. To assess the hydroxyl availability, we opted to use a wet chemistry approach – acetylation. Acetylation involves substitution of one hydroxyl group by one acetyl group. This reaction has been adopted in standard methods (*e.g.*, ASTM E222-10 (2010)) to determine the hydroxyl content of a material. The present work employed the standard method to probe open accessible hydroxyls.

Although the adopted ASTM method by itself does not distinguish the type of hydroxyls and the cell wall polymer (cellulose, hemicelluloses, or lignin) to which they belong, the biomass of interest in our study is saccharification residues whose hydroxyls are expected to primarily come from lignin. Hemicelluloses are either mostly removed during the (widely practiced) dilute acid pretreatment (*e.g.*, Schilling *et al*. (2009)) or subsequently hydrolyzed, together with the accessible cellulose, to fermentable sugars through the action of (different) enzymes. Focusing on lignin, it is known to contain aliphatic hydroxyls (primary and secondary) and phenolic hydroxyls, which exhibit different reactivities (Pu and Ragauskas 2005); such distinctions are beyond the scope of our pragmatic study. We instead discuss our findings in the context of total hydroxyls accessible in the substrates. Indeed, for reactions that are diffusion limited (acetylation of wood is an example), the different reactivities of various hydroxyls (reactive sites) do not dictate the reaction rate of the substrate when compared to the time needed (influenced by substrate morphology) for the reagent to travel to the sites (Hill and Hillier 1999).

EXPERIMENTAL

Materials

The biomass source for this study was quaking aspen (*Populus tremuloides*), which is a fast-growing tree species used as a pulpwood source in the upper Midwest region of the United States. It is genetically related to hybrid poplar, which is currently grown as an energy crop. Aspen wood from a single chipped tree was air-dried and Wiley-milled to

pass through a 40-mesh screen. A portion of the resulting particles was then treated with dilute acid (0.5% v/v H_2SO_4) for 2 h at 170 °C at a wood-to-liquor ratio of 1:6. Saccharification was then performed on a portion of the pretreated material at 50 °C using Celluclast® 1.5 L (endoglucanase and exoglucanase from *Trichoderma reesei*) and Novozyme 188 (β-glucosidase of *Aspergillus niger*), based on NREL-specified loadings (Brown and Torget 1995). The resulting glucan-to-glucose conversion was 91%, accounting for some hydration during hydrolysis. The solid (unhydrolyzed) fraction was recovered by filtration, rinsed with distilled water until pH-neutral, and dried for further analysis. The lignin content of the air-dried solid was determined following TAPPI T222 OM-06 (2006), while carbohydrates were separated and quantified (as peak area) using high-performance liquid chromatography (1200 series HPLC, Agilent Technologies, MN, USA) as detailed in Schilling *et al*. (2009).

Methods

As preliminary tests, four different drying methods were used to prepare samples (170-mesh size) with various degrees of pore collapse to be used in examining the hydroxyl availability. The first two of these drying methods involved sublimation, otherwise known as freeze-drying, utilizing either slow pre-freezing with one day in a freezer, or rapid pre-freezing using liquid nitrogen. Both types of freeze-dried samples had (oven-dry basis) moisture contents (average ± standard deviation) of 3.0 ± 0.5%. The third method was evaporation by air drying under a fume hood, and this resulted in sample moisture contents (MCs) of 8.0 ± 0.6%. The air-dried samples were further dried over lithium chloride (as a desiccant) to a 4.8 ± 0.6% moisture content in an attempt to attain a MC close to that of freeze-dried samples. A fourth drying method, oven drying (105 °C), was also performed to induce the highest degree of pore collapse in the sample.

In testing the hydroxyl availability (ASTM E222-10), the residual water in variously dried samples was kept to a minimum by using a sample size of 0.5 g (oven-dry weight equivalent), which is below the (permitted) amount estimated using the equation in the standard method for water-containing samples. The samples were reacted at 98 ± 2 °C for four hours with 50 mL of acetylation reagent containing acetic anhydride and pyridine (127:1000 volumetric ratio). Fifty milliliters of reagent, instead of the specified 20 mL, was used to ensure sufficient and uniform reagent coverage of the sample. At the end of the reaction, the excess (unconsumed) reagent was hydrolyzed with water (ice), and the acetic acid produced was titrated with a standard sodium hydroxide (NaOH) solution (*B* in mL). The same reaction and titration procedure were conducted for the blank, *i.e*., acetylation reagent containing no solid sample in it. The blank at the end of the reaction had a higher content of unconsumed acetic anhydride, and so the amount of NaOH titrant (*A* in mL) consumed was larger than that for the sample. The difference was used to calculate the amount of hydroxyls reacted in (or available for) the acetylation reaction:

$$Hydroxyl\ availability\left(\frac{mmol\ OH}{g\ sample}\right)=\frac{\left[(A-B);\ mL\right]\left(Normality\ of\ NaOH;\ {}^{mol\ OH}\!/_{1000mL}\right)}{Mass\ of\ sample;g} \quad (1)$$

The reacted hydroxyls were quantified using the term "hydroxyl number," which refers to the hydroxyl availability in the sample relative to that in the potassium hydroxide (KOH) used as the reference compound:

$$Hydroxyl\ number\left(\frac{equiv\ mg\ KOH}{g\ sample}\right) = hydroxyl\ avail.\left(\frac{mmol\ OH}{g\ sample}\right)\ x\ 56.1\left(\frac{g\ KOH}{mol\ OH}\right) \quad (2)$$

To illustrate the expression of a hydroxyl number, a value of 200 would mean that the hydroxyls available in one gram (oven-dry weight equivalent) of sample are as many as the hydroxyls in 200 mg of potassium hydroxide.

To better understand the hydroxyl availability data, the study included pore volume in the analyses to examine the influence of physical accessibility. The pore volume data were obtained from nitrogen adsorption by BET measurement according to the Brunauer-Emmett-Teller theory (Brunauer *et al*. 1938). The BET measurements (5 g for each analysis) were performed without replication because of sample limitation. The sample was loaded into a Micromeritics Tristar II 3020 V1.03 (Micromeritics, Norcross, GA) to first outgas for 16 h under vacuum at room temperature. Then, nitrogen adsorption and desorption isotherms were obtained at -196 °C to calculate the specific surface area and pore volume using, respectively, the BET and Barrett-Joyner-Halenda (BJH) methods (Barrett *et al*. 1951).

RESULTS AND DISCUSSION

The first step in using an acetylation reagent for probing accessible hydroxyls is to examine the ability of the method to distinguish the physical accessibility of the substrate. To determine this, four types of drying were carried out on acid-pretreated samples before hydroxyl number and BET measurements. The small sample size, aimed at avoiding interference of water in acetylation, resulted in a substantial variability of the hydroxyl numbers (Table 1). Despite the variability, noticeable differences were evident, with the average hydroxyl numbers ranging from 132 (oven-dried) to 539 (freeze-dried).

Table 1. Hydroxyl Availability (from acetylation) and Physical Accessibility (from BET measurements) of Dilute Acid-Treated Biomass Dried until Constant Weight under Different Conditions

Sample	Hydroxyl number	BET surface area (m^2/g)	Cumulative pore volume (cm^3/g)
Oven-dried at 105 °C	132 (23)	1.87	0.0094
Air-dried under fume hood followed by desiccant-dried over lithium chloride	341 (70)	2.09	0.010
Freeze-dried #1: pre-freezing (1 day) in freezer	503 (67)	2.00	0.011
Freeze-dried #2: pre-freezing in liquid nitrogen	539 (56)	1.98	0.011

*Note: Standard deviations for hydroxyl number measurements (five replicates) are shown in parentheses

To determine whether the interference of residual water was negligible for the data in Table 1, a practical control was run to examine how the results would be shifted after accounting for the influence of water. The approach was to obtain for Eq. 1 a more representative, water-containing blank (acetylation reagent) that would possibly cancel out the influence of water in the sample when their titration values were subtracted (*A'-B*). The practical control was prepared by adding in the acetylation reagent (50 mL) 15 mg and 25

mg of water to respectively simulate the amount of water present in the 0.5 gram freeze-dried (3.0 % MC) and air-dried (5.0% MC) samples. The mixtures (no solid sample in it) were heated (98 ± 2 °C) for four hours, as in the main experiment, after which the unconsumed acetic anhydride was hydrolyzed with water (ice) and titrated with NaOH. The titration values (*A'*) for the blank that initially contained 15 mg and 25 mg of water, respectively, averaged (4-6 replicates) 0.2 and 0.5 mL lower than the actual blank (*A*; no water in it). These lower values (*A'* mL), if used instead of *A* in Eq. 1, would result (after going through Eq 2.) in lower hydroxyl numbers. Specifically, the difference in values between *A* and *A'* would be magnified 56.1 times in this study (sample mass was 0.5 g; titrant normality was 0.5; *B* remained the same) to result in calculated hydroxyl numbers that are 11 (freeze-dried sample) and 28 (air-dried sample) in values lower than the results shown in Table 1. In other words, the hydroxyl numbers reported in Table 1 were somewhat "over-estimated," but the extent was negligible when compared to the standard deviations (±56 and ±70) of the "unadjusted" data. Shifting focus to the solid sample in acetylation reagent, it seems intuitive to be concerned about the presence of water (from non-oven dry solid) because it could, through partial hydrolysis to acetic acid, reduce the amount of acetic anhydride for reacting with the solid. This hydrolytic reaction involves one mole each of acetic anhydride and water and produces two moles of acetic acid (Rowell 1990). In the present study, the hydrolysis of acetic anhydride via reaction with water was negligible, with up to 1.4 mmol of acetic anhydride expectedly consumed by 25 mg of water (1.4 mmol; highest amount in this study), producing a small amount of acetic acid (2.8 mmol or 170 mg). In this regard, the work of Rowell (1990) revealed that the weight percent gain (indicative of the amount of incorporated acetyl groups) of the acetylated wood was only seriously affected beyond an acetic acid content of 30% in the acetylation reagent. Based on the aforementioned, it is judged that the presence of residual water in this study did not affect the hydroxyl data and interpretation.

Table 1 also shows an ascending order, similar to the case of hydroxyl number, for specific pore volume in samples that were oven-, air-, and freeze-dried. This is the inverse of the expected order for cell wall pore collapse, which is known to be highest after oven-drying, followed by air-, then freeze-drying. A linear relationship between the hydroxyl number and the pore volume (Fig. 1) was evident (R^2 = 0.979).

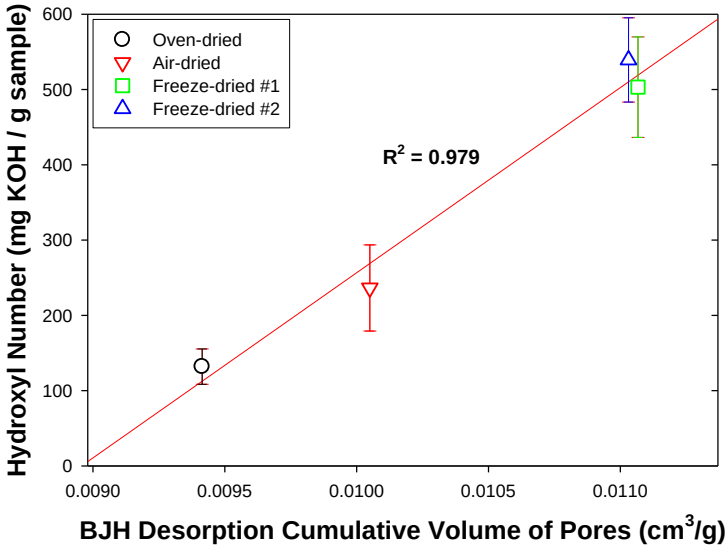

Fig. 1. Hydroxyl number *versus* cumulative pore volume (from BJH desorption) of dilute acid-treated biomass

The linear fit in Figure 1 suggests that the increased hydroxyl availability is related to a larger accessible pore volume. The figure also shows that both freeze drying approaches resulted in similar outcomes, so only the second approach, pre-freezing with liquid nitrogen, was used for later freeze drying.

The next endeavor was to assess the meaningfulness of absolute values in the measured hydroxyl availability. This was addressed by comparing the measured value against published theoretical estimates of native wood. The average hydroxyl number of the freeze-dried aspen wood was 597 (Fig. 2), which translates, based on Eq. 2, to 10.6 mmol of hydroxyls per gram of sample dry mass. This hydroxyl availability was lower than 14.8 mmol/g or 19.8 mmol/g, which were calculated, respectively, for Scots pinewood (softwood) and beechwood (hardwood) by assuming that all hydroxyls are accessible (Hill 2006). The calculated hydroxyl availability would be lower, thus closer to the measured value, when taking into account inaccessible hydroxyls in the crystalline region of cellulose. Using Scots pine as an example, the calculated hydroxyl availability is decreased to a value of 8.6 mmol/g when 65% of cellulose hydroxyls are excluded, *i.e*., assuming a cellulose crystallinity of 65% (Hill 2006). The measured value is therefore within a reasonable range for the theoretical hydroxyl availability of wood. Within this range, nevertheless, the exact amount of hydroxyls is difficult to ascertain, as the standard test involves the use of pyridine, which according to the literature (Mantanis *et al*. 1995) has the potential to disrupt cellulose crystallinity, exposing inaccessible hydroxyls. However, strong swelling agents such as pyridine and dimethylformamide are commonly used as reaction media in chemical modifications of lignocellulose. Thus the hydroxyl availability in this study can be regarded as a practical measure of what would be expected of a substrate subjected to selective drying in modifications of a similar nature.

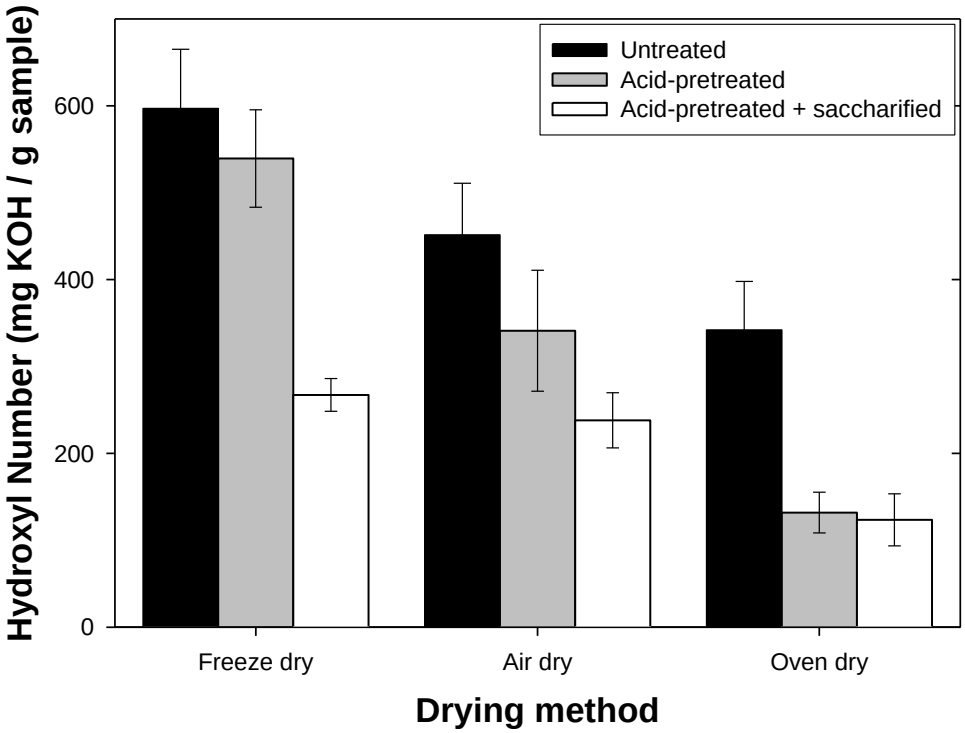

Fig. 2. Hydroxyl numbers of untreated, dilute acid-pretreated, and dilute acid-pretreated followed by enzymatically saccharified biomass analyzed after freeze-, air-, or oven-drying
*Note: Air-dried samples were further conditioned over LiCl before hydroxyl number analysis

The effect of the acid pretreatment can be seen in Table 2. Upon acid treatment, xylan was the main component removed, and the resulting increase in pore volume and specific surface area is expected for pretreatments that promote enzyme accessibility and glucan digestibility. Xylan contains two hydroxyls for each pyranose unit, so its removal resulted in a decreased hydroxyl number (Fig. 2). For freeze-dried samples, the subtle decrease in the hydroxyl number from 597 ± 68 to 539 ± 56 (Fig. 2) does not seem to correspond to the large removal of xylan. To explain this observation, we postulate that the hydroxyl loss was partially offset by a gain in accessible cellulose hydroxyls as a result of crystallinity disruption. One possible cause of crystallinity disruption is the dilute acid pretreatment. However, the literature shows that there are no crystallinity changes detected for isolated cellulose from poplar wood treated with dilute acid, based on the results of nuclear magnetic resonance spectroscopy (Sun *et al*. 2014). The other possible cause is the penetrating or disrupting effects of pyridine, as discussed earlier, during the hydroxyl number determination. One would argue that if this is possible, then both untreated and acid-treated samples should be affected to a similar extent. However, given that both samples were freeze-dried before the hydroxyl number analysis, the possibility of crystallinity disruption was higher in acid-treated samples, because their cell wall becomes more accessible than those of untreated samples, whose cumulative pore volume is one-third of the former (Table 2).

Table 2. Chemical Composition (without Normalization) and Physical Accessibility of Untreated, Dilute Acid-Pretreated, and Dilute Acid-Pretreated followed by Enzymatically Saccharified Biomass

Sample	Lignin (%)	Glucan (%)	Xylan (%)	Mannan (%)	BET surface area (m^2/g)	Cumulative pore volume (cm^3/g)
Untreated	20.6	42.5	17.0	2.7	1.22	0.0033
Dilute acid-pretreated	35.8	53.5	0.7	0.4	1.96	0.0093
Dilute acid-pretreated and enzymatically-saccharified	61.3	9.8	0.0	1.3	5.49	0.0320

*Note: BET samples were freeze-dried before analysis

Differing from freeze-dried samples, the hydroxyl number reduction upon acid pretreatment was more pronounced in the oven-dried samples, followed by the air-dried samples (Fig. 2). These samples showed cell wall collapse, which hindered the penetration of disrupting agents such as pyridine; therefore, within the framework of earlier postulation, there was less interference from crystallinity disruption. As a result, they exhibited a more distinctive reduction in hydroxyls compared with that of freeze-dried samples as a response to acid pretreatment or xylan removal.

After saccharification, the accessible hydroxyls had been reduced to at least half of that seen in untreated wood, among freeze-, air-, or oven-dried samples (Fig. 2). The available hydroxyls of the residue were reduced even though (as shown in Table 2) its specific pore volume, in freeze-dried samples, had increased 10 times (from 0.0033 to 0.0320 cm^3/g) and the BET surface area increased 5 times (from 1.22 to 5.49 m^2/g). The depleted hydroxyl availability was attributable to the considerable loss of glucan (residual glucan was ~10 wt.% of solid residue; Table 2). This finding suggests that reagents could

access saccharification residues of higher porosity more easily to possibly shorten the reaction time during chemical modification or conversion. The larger pore volume also means that larger molecules could be introduced to the substrate to effectively increase its presence by reacting with only a small number of hydroxyl sites. From a different perspective, the lower hydroxyl availability will favor modifications aimed at masking the reactivity of the substrate.

CONCLUSIONS

1. The sensitivity of the acetylation method was established for probing changes in the substrate hydroxyl availability induced by drying (pore collapse), dilute acid pretreatment (xylan removal), and saccharification (glucan removal).
2. More hydroxyls are available if the pore volume of a given substrate is better preserved. On the other hand, fewer hydroxyls and larger pore volumes are available after xylan and glucan are removed from the substrate.
3. This study demonstrated that saccharification residues will have improved accessibility for modifying reagents, but fewer hydroxyl-specific reactive sites than what is typical for native biomass substrates. This information will be useful in deciding strategies for utilizing saccharification residues, for example, by tapping their increased porosity and lower polarity.

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