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# Water sorption in pretreated grasses as a predictor of enzymatic hydrolysis yields

Daniel L. Williams[a,b], Jacob D. Crowe[a], Rebecca G. Ong[c], David B. Hodge[a,b,d,e,*]

[a] Department of Chemical Engineering & Materials Science, Michigan State University, East Lansing, MI, USA
[b] DOE Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI, USA
[c] Department of Chemical Engineering, Michigan Technological University, Houghton, MI, USA
[d] Department Biosystems & Agricultural Engineering, Michigan State University, East Lansing, MI, USA
[e] Division of Chemical Engineering. Luleå University of Technology, SE-971 87 Luleå, Sweden

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ABSTRACT

This work investigated the impact of two alkaline pretreatments, ammonia fiber expansion (AFEX) and alkaline hydrogen peroxide (AHP) delignification performed over a range of conditions on the properties of corn stover and switchgrass. Changes in feedstock properties resulting from pretreatment were subsequently compared to enzymatic hydrolysis yields to examine the relationship between enzymatic hydrolysis and cell wall properties. The pretreatments function to increase enzymatic hydrolysis yields through different mechanisms; AFEX pretreatment through lignin relocalization and some xylan solubilization and AHP primarily through lignin solubilization. An important outcome of this work demonstrated that while changes in lignin content in AHP-delignified biomass could be clearly correlated to improved response to hydrolysis, compositional changes alone in AFEX-pretreated biomass could not explain differences in hydrolysis yields. We determined the water retention value, which characterizes the association of water with the cell wall of the pretreated biomass, can be used to predict hydrolysis yields for all pretreated biomass within this study.

## 1. Introduction

Lignocellulosic biomass can serve as an environmentally beneficial feedstock for the production of petroleum-displacing renewable biofuels and biochemicals (Souza et al., 2015). One promising route to produce liquid transportation fuels from lignocellulosic biomass utilizes thermochemical pretreatment coupled to enzymatically-catalyzed deconstruction of plant cell wall structural polysaccharides followed by microbial conversion of the sugars to metabolites such as ethanol (Qureshi et al., 2014). The pretreatment step is necessary to overcome cell wall recalcitrance, which is a consequence of the complex secondary cell wall matrix comprised of cellulose, hemicelluloses, and lignin (Himmel et al., 2007). A diverse range of pretreatments have been studied that act through a variety of chemistries and mechanisms to achieve improved polysaccharide accessibility to cellulolytic enzymes. Greater accessibility can be manifested through a number changes to the cell wall, typically involving reorganization, solubilization, and modification of lignin and hemicelluloses (Chundawat et al., 2011a; Ong et al., 2014). For dilute acid or hydrothermal pretreatments, one of the primary quantifiable outcomes is xylan removal and depolymerization (Schell et al., 2003), while lignin relocalization is also important, yet more difficult to quantify (Donohoe et al., 2008). For delignifying pretreatments such as alkaline (Stoklosa and Hodge, 2015), alkaline-oxidative (Yu et al., 2011), and organosolv (Nitsos et al., 2016), lignin removal (as well as some hemicellulose) is one of the primary outcomes, with lignin content subsequently resulting as a strong predictor of hydrolysis yields (Li et al., 2012). AFEX pretreatment has been extensively studied since the 1980s as a route for the production of cellulosic sugars and animal feed (Balan et al., 2009). While it is known that lignin and hemicellulose relocalization impact the cell wall nanoscale porosity and its susceptibility to enzymatic depolymerization (Chundawat et al., 2011b), there is no significant change in composition following AFEX pretreatment. However, like the other alkaline pretreatments, lignin content has also been negatively correlated to hydrolysis yields from AFEX-pretreated biomass (Garlock et al., 2012).

Increased enzyme accessibility following cell wall reorganization can be considered a function of surface area, surface composition and chemistry, and cell wall porosity (i.e., pore volume and pore size distribution). Furthermore, cell wall porosity is a complex function of both polymer properties and solvent environment during porosity measurement. Consequently, characterization approaches that yield

* Corresponding author at: Department of Chemical Engineering & Materials Science, Michigan State University, East Lansing, MI, USA.
*E-mail address:* hodgeda@msu.edu (D.B. Hodge).

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information about these properties can be useful for assessing the cell wall's susceptibility to enzymatic hydrolysis. Porosimetry techniques for solid porous materials such as mercury intrusion and BET porosimetry, are unsuitable for plant cell walls as these methods require dry samples and drying modified lignocellulosic fibers can result in "hornification" or irreversible collapse of pores (Fernandes Diniz et al., 2004). Nanoscale imaging techniques that have been applied to modified plant cell walls include SEM (Chinga et al., 2002), AFM (Fahlén and Salmén, 2004), and TEM tomography (Chundawat et al., 2011b), although these require careful sample preparation that preserves the pore structure. A number of methods relate water-cell wall interactions to pore and surface properties and include assessing water constraint in pores or surfaces by proton NMR (Meng et al., 2013), $T_2$ NMR relaxometry (Felby et al., 2008), freezing point depression of constrained water in plant cell walls by differential scanning calorimetry (DSC) (Park et al., 2006), and drainability of biomass fibers when subjected to centrifugation (water retention value or WRV) (Grethlein, 1985; Weise et al., 1996). Recently, we and others have investigated how quantitative metrics for cell wall-water association (e.g., WRV) can be used as a descriptor of cell wall porosity and the cell wall's response to enzymatic hydrolysis, with the potential for these techniques to be employed as high-throughput screening tools to assess biomass response to enzymatic hydrolysis. Our previous work has shown a positive, linear correlation between WRV and glucose hydrolysis yields for corn stover and switchgrass subjected to a range of alkaline-oxidative delignification and liquid hot water pretreatment conditions (Williams and Hodge, 2014) and for untreated diverse maize cultivars (Li et al., 2015).

For this work, we propose that quantifiable cell wall-water interactions, such as the WRV, may be a more useful indicator of enzymatic hydrolysis yields than any individual biomass property, such as cell wall composition, porosity and hydrophilicity, as the WRV is dependent upon both structural and compositional factors. This work expands on our previous findings to include a wider range of pretreatment chemistries and conditions. Specifically, we subjected corn stover and switchgrass to AFEX pretreatment and alkaline hydrogen peroxide (AHP) delignification at multiple conditions, with the goal of identifying the predictive capability of WRV alone or in combination with other quantifiable properties. Furthermore, select conditions were tested using DSC to find the freezing point depression of cell wall-associated water, and $T_2$ NMR relaxometry was tested to assess water constraint.

## 2. Materials and methods

### 2.1. Biomass feedstock

The untreated biomass feedstocks used in this work include switchgrass (*Panicum virgatum* L., cv. Cave-in-Rock) and corn stover (*Zea mays* L., Pioneer hybrid 36H56). The biomass was milled with a Wiley Mini-Mill (Thomas Scientific) to pass a 5 mm screen and air-dried to a moisture content of approximately 5% prior to any treatments. The structural carbohydrate and lignin composition of all materials were determined by the NREL/TP 510-42618 protocol (Sluiter et al., 2008) with minor modifications (Li et al., 2012).

### 2.2. Pretreatment

AFEX pretreatment was run in duplicate for each sample in 22 mL Parr reactors (Garlock et al., 2009). AFEX was run for each sample at two different ammonia/water combinations (1.5 g $NH_3$ and 2.0 g $H_2O$/g dry biomass or 2.0 g $NH_3$ and 0.5 g $H_2O$/g dry biomass) and four different temperatures (60, 90, 120, and 150 °C). Prior to loading the biomass in the reactors, the moisture was adjusted to the appropriate water loading and then 3 g (dry weight) of sample was loaded in the reactor. Vacuum was applied to the reactors for 20 s. For the 120 and 150 °C samples the reactors were preheated to 40 °C using an aluminum heating block. The appropriate mass of ammonia was added based on a previously determined volume-to-mass calibration using a high-pressure syringe pump (PHD 4400, Harvard Apparatus). After the ammonia was added, the reactors were heated in an aluminum heating mantle for 30 min, including the ramping time. At the end of the residence time, the reactors were vented to release the majority of the ammonia after which the biomass was unloaded and allowed to air dry in the fume hood overnight to allow the residual ammonia to evaporate. Once dry, duplicate batches were combined prior to performing subsequent experiments.

AHP delignification of corn stover and switchgrass was performed using four different conditions of $H_2O_2$ to biomass loadings, 0%, 6%, 12.5%, and 25% (g $H_2O_2$ /g biomass). Both conditions were performed in duplicate using 8 g of biomass (dry basis) at 15% (w/v). Samples were prepared in 250 mL Erlenmeyer flasks and placed in an incubator at 30 °C with shaking at 180 rpm. The flasks were sealed with parafilm to prevent evaporation but also to allow for some expansion as the pressure in the flasks increased with $O_2$ evolution. To counter the drop in pH over the course of the reaction, 5 M NaOH was added at 3, 6, and 9 h to bring the pH back up to 11.5. Delignification was stopped after 24 h by diluting the sample with 25 mL of water to 10% (w/w) solids and adjusting the pH to approximately 4.8 using concentrated sulfuric acid.

### 2.3. Enzymatic hydrolysis

AFEX pretreated biomass was loaded with water in sample flasks at 10% (w/v) solids. For both the AHP slurries mentioned previously and the AFEX pretreated biomass, 1 M citrate buffer was added to give a concentration of 50 mM buffer in the sample flasks. To prevent contamination of hydrolysate, tetracycline and cyclohexamine were added at a concentration of 10 mg/L each. An enzyme mixture of Cellic CTec2 and HTec2 (Novozymes A/S, Bagsværd, Denmark) was added in a protein mass ratio of 2:1, respectively, at an enzyme loading of 30 mg enzyme/g glucan. The protein contents of the enzymes were based on the Bradford assay (Sigma-Aldrich). Samples were then mixed by hand and placed in a shaking incubator at 50 °C and 180 rpm for either 24 or 72 h. Sugar concentrations in the hydrolysate were determined by HPLC using the method described in the NREL/TP 510-42618 protocol and converted to glucose yields based on the solids content in the reaction vessel and the untreated glucan contents for AHP samples or post-treatment glucan content for AFEX samples.

### 2.4. Water retention value

Water retention values (WRV) were determined according to a modified version of TAPPI UM 256 (TAPPI, 2015) as described in our previous work (Williams and Hodge, 2014). Briefly, the biomass samples were filter-washed using a Buchner funnel containing a 200 mesh stainless steel screen. The solids remaining after pretreatment and delignification were washed with approximately 700 mL of deionized water and vacuum-filtered to a moisture content of approximately 80%. Next, ~2.5 g of wet biomass was inserted into a spin-column (Handee Spin Column Cs4, Thermo Scientific) modified to have a 200 mesh stainless steel screen as the membrane directly under the biomass. The spin columns were then centrifuged at 900 × *g* for 15 min. The drained biomass was then weighed in an aluminum tray and placed in an oven at 105 °C for 3 h, and then weighed again. The WRV is the ratio of the mass of water remaining in the biomass after centrifuging divided by the mass of dry biomass. Samples were measured in triplicate and error bars represent standard deviations.

### 2.5. Differential scanning calorimetry

Solid residue after AHP pretreatment was washed with 500 mL of water using a Buchner funnel with a 200 mesh porous base and drained

under vacuum to a moisture content of ~80%. Approximately 15 mg of wet biomass samples was placed into DSC aluminum pans (TA Instruments, Part #900786.901 bottom and Part #900779.901 top) and then run on DSC. The measurement of freezing bound water was performed as described previously (Park et al., 2006). Briefly, sample pans were subjected to a gradient and isothermal melting regime which started by cooling the pan to −30 °C, holding for 5 min, scanning at 1 °C/min to −20 °C, and holding again for 5 min. This procedure was continued for each of the following temperatures: −15, −10, −6, −4, −2, −1.5, and −1.1 °C, and represent the melting temperature depressions corresponding to different pools of water. The amount of water at each pore size was determined by integrating the area of each peak in the thermogram and dividing that area by the specific heat of fusion for water (334 J/g) which gives the mass of water per mass of slurry at each level of freezing point depression. Samples were performed in technical duplicate.

### 2.6. NMR relaxometry

$^1$H spin-spin ($T_2$) NMR measurements were carried out in a Bruker static probe at frequencies of 300.103 MHz on a Varian 300 MHz NMR interfaced with a Dell Precision T3500 desktop running CentOS 5.6 with VnmrJ 3.2A. Spin-spin relation times were determined using a standard 2D Carr-Purcell-Meiborn-Gill (CPMG) sequence with a 5 μs (90°) $^1$H pulse, 10 μs (180°) $^1$H pulses, 16 scans, 10 s recycle delay and $\tau = 0.0002$. Eight data points were recorded with 4–1024 echoes, and data was analyzed and reported as a monocomponent exponential fit of the data (Meng et al., 2013). Technical duplicates were performed at a constant temperature (25 °C) and a sample total solids content of 25% (w/v).

## 3. Results and discussion

### 3.1. Relating pretreatment conditions to differences in hydrolysis yields and composition

Corn stover and switchgrass were subjected to either AFEX or AHP pretreatment over a range of pretreatment conditions. For AFEX pretreatment, the temperature, $NH_3$:$H_2O$ ratio, and $NH_3$ loading were varied, although $NH_3$ loading and $NH_3$:$H_2O$ ratio were not varied independently. For AHP pretreatments, the $H_2O_2$ loading was the main variable changed during the process. These pretreatment variables were previously identified as key control variables affecting pretreatment efficacy (Garlock et al., 2009; Li et al., 2012). The composition data following pretreatment (Table 1) demonstrate several clear trends. First, the two pretreatments show substantial differences in the composition of their products. For AHP, the lignin content substantially decreases with increasing oxidant loading while xylan (and glucan) are largely retained. For the AFEX pretreatment, there are minimal compositional changes in the biomass during pretreatment as there is no liquid phase to solubilize cell wall fractions following release of the ammonia. However, a nontrivial fraction of the original biomass becomes water-soluble and extractable following pretreatment and importantly, a substantial fraction of the xylan can be solubilized in water while the lignin content remains relatively unchanged (Garlock et al., 2011). Furthermore, this trend is present with increasing pretreatment temperature at both $NH_3$ loadings (alternatively $NH_3$:$H_2O$ ratios), with the amount of water-extractable xylan increases, reaching as high as 75% xylan removal for the most severe AFEX pretreatment condition (*i.e.*, corn stover for $NH_3$:$H_2O$ of 4.0 g/g and 150 °C). This substantial decrease in xylan content is comparable to that achieved during dilute acid pretreatments (Garlock et al., 2011) and is a likely an indication of cell wall reorganization, which may have implications for both cellulose accessibility and feedstock swellability. Linear relationships were plotted between glucose hydrolysis yields and feedstock lignin and xylan content after pretreatment (presented on a “per mass original biomass” basis). Lignin content was correlated with glucose hydrolysis yields (Fig. 1A) only in the AHP delignified samples ($R^2 = 0.93$; $p < 10^{-5}$), corresponding to the increase in delignification. Xylan correlations were found in Fig. 1B for both the AFEX ($NH_3$:$H_2O$ = 0.75) and AHP-delignified biomass, indicating that xylan removal can be extrapolated to increase cellulose accessibility and therefore glucose yields using multiple pretreatment processes.

In terms of the effect of pretreatment variables on hydrolysis yields, the pretreatment temperature had the greatest impact on hydrolysis yields for AFEX pretreatment, while oxidant loading had an obvious impact on AHP delignification (Fig. 2). The glucose yields trend towards a maximum in AFEX switchgrass and corn stover at 120 °C for the high ammonia loading, while continuing to increase with increasing temperature for the low ammonia loading. This supports the finding that increasing temperature also increases xylan extractability and total cell wall reorganization, which provides insight into the underlying mechanism behind the improvement in hydrolysis yields. For AHP delignification, the most severe condition (0.25 g $H_2O_2$/g biomass) resulted in maximum theoretical yields for corn stover (with slightly higher than 100% yield likely attributed to composition analysis underreporting glucan content in 0.25 g $H_2O_2$/g corn stover) and close to theoretical maximum for switchgrass (87.3% glucose yield). This condition corresponds to removal of more than two thirds of the original lignin. We have previously demonstrated that removal of more than 50% of the lignin in both hardwoods (Stoklosa and Hodge, 2015) and grasses (Li et al., 2012, 2014) can result in glucose hydrolysis yields approaching the theoretical maximum.

A final important observation is that the corn stover consistently demonstrated higher yields than the switchgrass for both pretreatments. These trends have been observed in the past for corn stover versus switchgrass using AHP (Li et al., 2012; Williams and Hodge, 2014) and AFEX pretreatment (Bals et al., 2010). Corn stover and switchgrass have nearly identical compositions (Table 1), but may have differences in the relative abundance and distribution of cell types and, at the macroscopic scale, exhibit differences in relative abundance of organs (e.g., leaf versus stem). Interestingly, when hydrolysis yield data are replotted as switchgrass versus corn stover for identical pretreatment conditions, the resulting trend is linear (Fig. 3). This result provides strong evidence that comparable pretreatment conditions yield similar responses to enzymatic hydrolysis in taxonomically similar plants (i.e., other C-4 grasses as demonstrated in this work). The implications for this are that the detailed knowledge developed around the pretreatment of a single cultivar or species from one biomass feedstock can be scaled to another, related feedstock. For example, an empirical data set relating pretreatment to hydrolysis yields for one feedstock may be able to be used to identify pretreatment requirements for another related feedstock based on only a limited number of data points.

### 3.2. Correlations between water retention value and hydrolysis yields

This work seeks to assess the potential for WRV as a predictive tool for bioenergy grasses subjected to diverse cell wall-modifying alkaline treatments (AHP delignification and AFEX pretreatment). Our previous work (Li et al., 2015; Williams and Hodge, 2014) as well as that of others (Luo and Zhu, 2011; Luo et al., 2011) has demonstrated that WRV can serve as a predictor of hydrolysis yields under certain non-limiting conditions. As an example, prior work has identified that WRV is a good indicator of cellulose accessibility after wet-pressed and heat-dried hornification has occurred, and that enzyme absorption to cellulose substrates correlates well with WRV for woody biomass (Luo and Zhu, 2011; Luo et al., 2011). It should be noted that WRV is a complex function of many properties including composition, higher order structure of the cell wall matrix (e.g., nanoscale porosity), accessible surface area, and particle size. As such, WRV can capture many of the important changes to the plant cell wall due to pretreatment that are relevant to enzymatic hydrolysis.

**Table 1**
Summary of pretreatment conditions and composition of untreated and AHP-delignified and AFEX-pretreated biomass corn stover and switchgrass.

| Untreated Feedstock | Glucan Content (%) | Xylan Content (%) | Lignin Content (%) |
|---|---|---|---|
| Corn Stover | 36.6 ± 0.7 | 24.8 ± 0.1 | 20.7 ± 0.6 |
| Switchgrass | 34.0 ± 0.3 | 25.9 ± 0.1 | 21.4 ± 0.3 |

| AFEX Pretreatment Feedstock | $NH_3$:$H_2O$ Ratio (g/g) | Temp (°C) | Glucan Content (%) | Xylan Content (%) | Lignin Content (%) |
|---|---|---|---|---|---|
| Corn Stover | 0.75 | 60 | 36.4 ± 1.7 | 19.4 ± 1.2 | 20.6 ± 0.0 |
| Corn Stover | 0.75 | 90 | 43.2 ± 0.5 | 8.9 ± 0.3 | 27.8 ± 0.5 |
| Corn Stover | 0.75 | 120 | 44.2 ± 0.8 | 10.5 ± 0.5 | 30.7 ± 0.0 |
| Corn Stover | 0.75 | 150 | 47.4 ± 0.1 | 10.3 ± 0.1 | 24.2 ± 0.2 |
| Switchgrass | 0.75 | 60 | 35.7 ± 2.3 | 22.0 ± 0.1 | 22.6 ± 0.1 |
| Switchgrass | 0.75 | 90 | 36.8 ± 1.8 | 17.8 ± 0.2 | 25.7 ± 0.6 |
| Switchgrass | 0.75 | 120 | 37.2 ± 0.6 | 18.7 ± 0.4 | 27.1 ± 1.0 |
| Switchgrass | 0.75 | 150 | 43.0 ± 0.2 | 15.5 ± 0.2 | 22.3 ± 0.1 |
| Corn Stover | 4.0 | 60 | 41.2 ± 1.3 | 14.2 ± 0.3 | 25.7 ± 0.0 |
| Corn Stover | 4.0 | 90 | 42.0 ± 1.2 | 9.1 ± 1.0 | 27.8 ± 0.2 |
| Corn Stover | 4.0 | 120 | 44.7 ± 0.1 | 12.5 ± 0.9 | 25.7 ± 0.3 |
| Corn Stover | 4.0 | 150 | 47.5 ± 1.8 | 8.1 ± 0.1 | 24.3 ± 1.3 |
| Switchgrass | 4.0 | 60 | 35.3 ± 1.6 | 20.0 ± 1.0 | 20.2 ± 0.2 |
| Switchgrass | 4.0 | 90 | 35.7 ± 0.6 | 15.8 ± 0.0 | 22.9 ± 1.2 |
| Switchgrass | 4.0 | 120 | 41.7 ± 2.6 | 15.9 ± 1.2 | 20.1 ± 0.0 |
| Switchgrass | 4.0 | 150 | 45.5 ± 0.4 | 13.7 ± 0.6 | 20.3 ± 1.1 |

| AHP Delignification Feedstock | $H_2O_2$ Loading (g/g) | Temp (°C) | Glucan Content (%) | Xylan Content (%) | Lignin Content (%) |
|---|---|---|---|---|---|
| Corn Stover | 0 | 30 | 38.7 ± 0.1 | 25.0 ± 0.3 | 18.5 ± 0.4 |
| Corn Stover | 0.06 | 30 | 40.2 ± 1.0 | 27.0 ± 0.4 | 15.8 ± 1.3 |
| Corn Stover | 0.125 | 30 | 44.6 ± 0.4 | 27.2 ± 0.4 | 11.8 ± 1.6 |
| Corn Stover | 0.25 | 30 | 53.9 ± 0.4 | 28.1 ± 0.4 | 4.6 ± 1.8 |
| Switchgrass | 0 | 30 | 36.3 ± 0.1 | 28.3 ± 0.1 | 20.2 ± 0.4 |
| Switchgrass | 0.06 | 30 | 37.3 ± 0.4 | 29.4 ± 0.1 | 18.9 ± 1.2 |
| Switchgrass | 0.125 | 30 | 39.5 ± 0.5 | 29.7 ± 0.3 | 15.5 ± 0.1 |
| Switchgrass | 0.25 | 30 | 49.5 ± 0.5 | 26.9 ± 0.2 | 9.7 ± 0.4 |

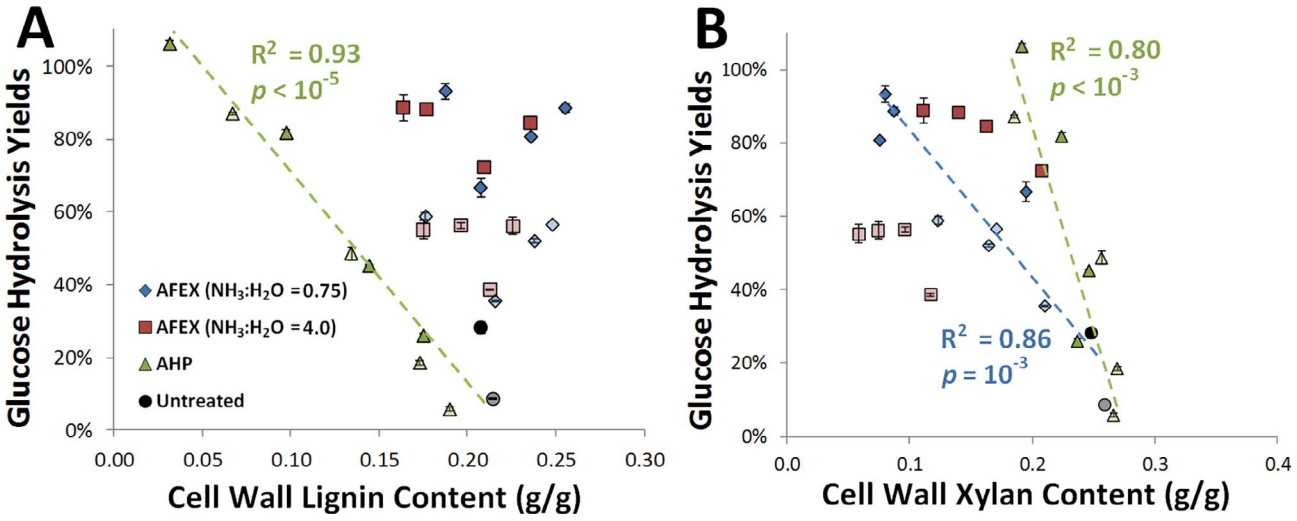


**Fig. 1.** Correlations between pretreated biomass (A) lignin and (B) xylan contents (on a per g original biomass basis) versus 72-h enzymatic hydrolysis glucose yields with significant ($p < 0.05$) correlations (green = AHP, blue = AFEX $NH_3$:$H_2O$ = 0.75) highlighted. Solid data points represent corn stover while semi-transparent data points represent switchgrass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Experimental results from this work show strong correlations between glucose hydrolysis yields and WRV for AHP-delignified and AFEX-pretreated corn stover and switchgrass over varying pretreatment conditions (Fig. 4A). Importantly, while pooled results show a positive correlation between WRV and hydrolysis yields ($R^2$ = 0.66), correlations within a single set of pretreatment conditions (i.e., AHP or AFEX at constant $NH_3$:$H_2O$ ratios) exhibited $R^2$ values above 0.96 (Fig. 4A). This result indicates for a single set of pretreatment conditions, the WRV of the pretreated material provides an excellent predictor of the hydrolysis yields. Significant differences in the impact of pretreatment on composition as well as changes to cell wall organization that impact water adsorption and swellability may account, in part, for these results. As demonstrated previously (Fig. 1), composition alone does not provide a suitable explanation for the differences in hydrolysis yields across pretreatment conditions, notably for the AFEX pretreatment. Not surprisingly, the contributions of individual cell wall component

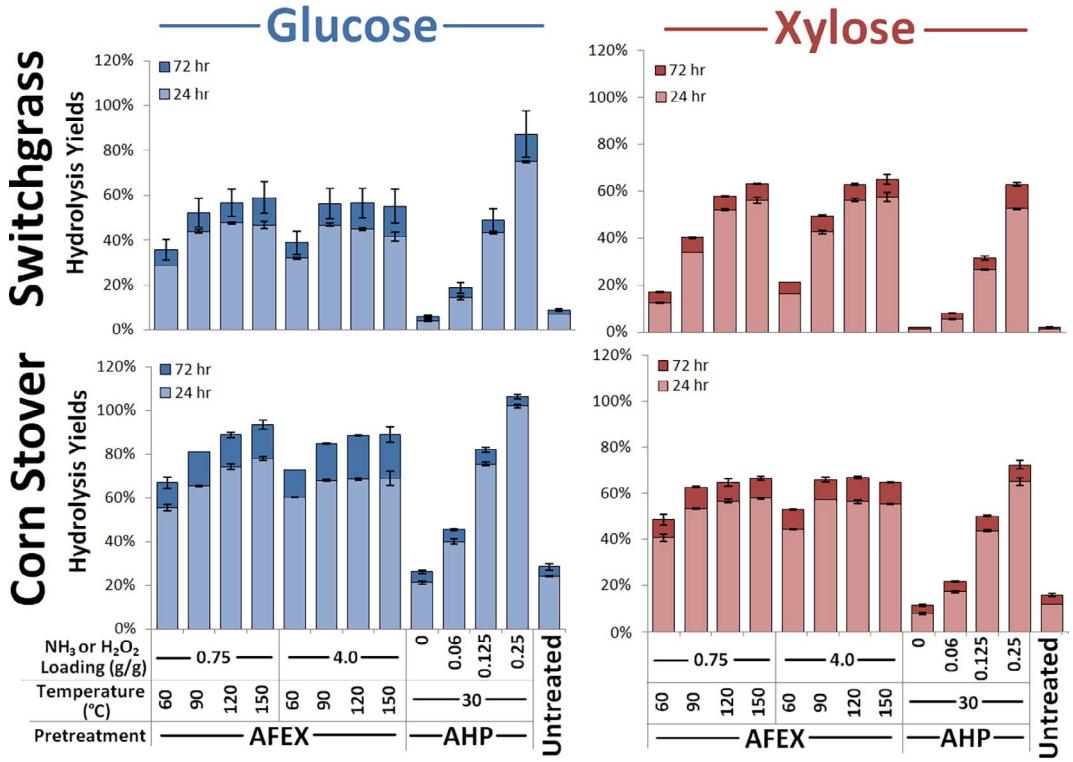


**Fig. 2.** Glucose and xylose enzymatic hydrolysis yields after 24 and 72 h for corn stover and switchgrass subjected to AFEX and AHP pretreatments conditions as indicated on the x-axis. Enzyme loading was 30 mg protein per g glucan at a 2:1 CTec2:HTec2 loading. Hydrolysis yields are calculated on a basis of per glucan and xylan in the pretreated biomass.

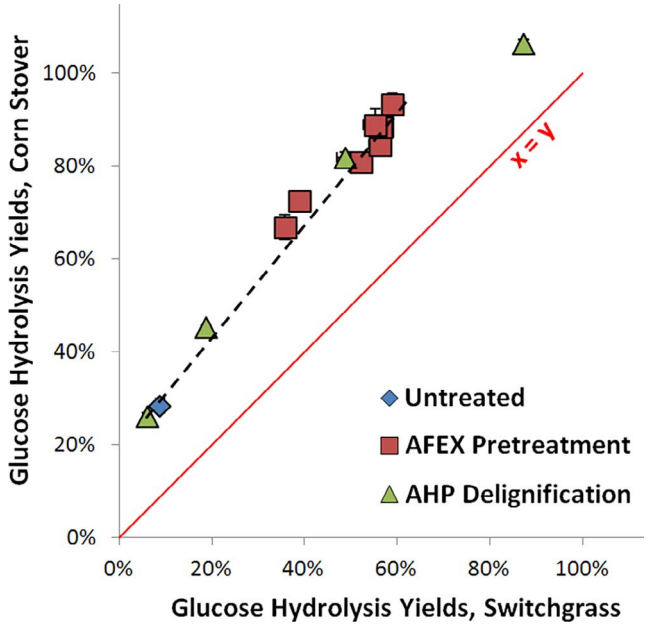


**Fig. 3.** Correlation between 72-h enzymatic hydrolysis glucose yields for corn stover and switchgrass pretreated and hydrolyzed under identical conditions.

compositions to WRV are also not strong across all pretreatments with the results for lignin (Fig. 4B) illustrating this point. Furthermore, the glucan and xylan contents exhibited no correlations with WRV for any of the pretreatment sets when plotted on a "per mass original biomass" basis (data not shown). When plotted on a "per mass biomass" basis, glucan content was positively correlated with WRV in AHP delignified biomass ($R^2$ = 0.952) and less strongly correlated to AFEX-pretreated biomass for a $NH_3$:$H_2O$ of 4.0 ($R^2$ = 0.786) samples (data not shown). In the plant cell walls of diverse agricultural and food residues, cellulose does not contribute as much to WRV as hemicelluloses and lignin (Weber et al., 1993). However, no correlation between hemicellulose content and WRV was found. This may be because other specific properties of the hemicelluloses may be more important factors that contribute to WRV, for example hydroxyl and carboxyl contents, or charged groups which are known to increase fiber swelling (Lund et al., 2012).

The results of this study likely can be extended to additional individual pretreatments for grasses, with prior work (Williams and Hodge, 2014) demonstrating linear relationships between WRV and enzymatic hydrolysis yields for liquid hot water (LHW) pretreated corn stover and switchgrass. However, structural and compositional contributions towards WRV may not be equivalent, as LHW pretreatment results in fundamentally different changes to cell wall properties. Translations of similar pretreatments to other feedstocks have not been explored yet, limiting the conclusions of this study to graminaceous feedstocks. However, it is of note that acid pretreatments in sulfite pretreated softwoods correlated with WRV and enzymatic hydrolysis yields (Weiss et al. 2016), indicating pretreatment specific response trends exist within individual pretreatments in other types of feedstocks.

### 3.3. DSC cryoporosimetry and NMR relaxometry

Pretreatment may significantly alter structural features of the cell wall, including changes to accessible surface area and accessible functional groups, which may impact cellulose accessibility and enzymatic hydrolysis by cellulolytic enzymes. To indirectly assess these changes, DSC cryoporosimetry and NMR relaxometry were utilized to quantify the impact of AHP-delignification on the nanoscale porosity and water-cell wall interaction in corn stover.

DSC cryoporosimetry is based upon quantifying heat flow endotherms associated with freezing bound water within a system to obtain a distribution of water present in different chemical states (Park et al., 2006). Unlike non-freezing bound water that encompasses the

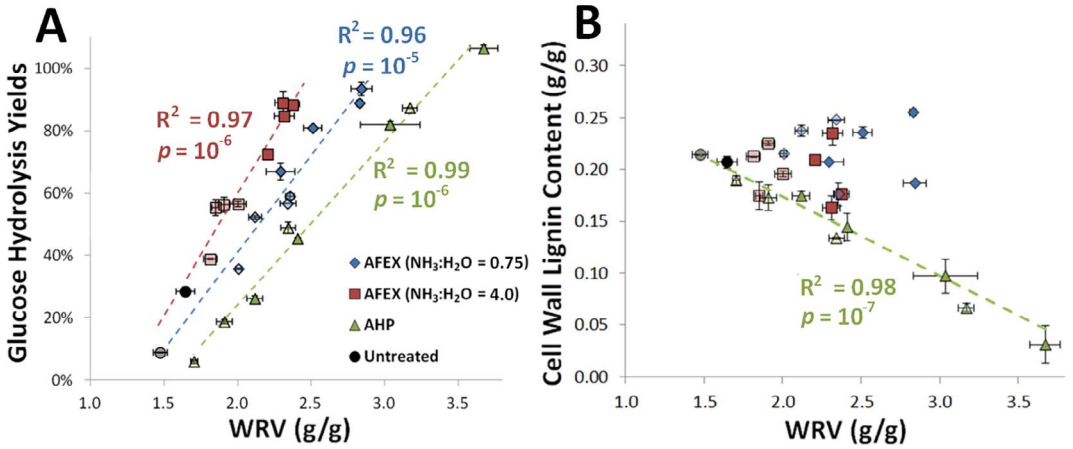


**Fig. 4.** Correlations between WRV and (A) 72-h glucose hydrolysis yields and (B) lignin content on a per mass initial biomass basis with significant ($p < 0.05$) correlations highlighted. Solid data points represent corn stover while semi-transparent data points represent switchgrass.

first one to three molecular layers of water associating on a surface, freezing-bound water is characterized by a sufficiently altered chemical environment due to proximity within a porous surface, and has the property of depressed melting temperatures compared to free water, due to the lower pressure of the curved interfaces (Jeoh et al., 2007). In addition to physical constraint of pore size, hydrostatic interactions between water and hydroxyl groups or carboxyl groups on the biomass surface also alters the chemical environment (Olsson and Salmen, 2004). Ultimately DSC cryoporosimetry quantifies a water solid-liquid phase change that is dependent on the molecular constraint of sorbed water influenced by both the pore size and chemical environment (Felby et al., 2008; Hubbe and Heitmann, 2007; Selig et al., 2012).

DSC results for AHP-delignified biomass in Fig. 5 show the cumulative bound water (freezing bound water) versus temperature for corn stover and switchgrass for the four different conditions for AHP delignification. These results demonstrate both an increase in cumulative bound water with respect to temperature step, as well as increases in total cumulative bound water depending on AHP pretreatment severity. Increases in cumulative bound water were most significant between the low and moderate delignification conditions for both corn stover and switchgrass. More subtle increases in cumulative bound water were observed between moderate and more severe delignification conditions. In addition, similar bound water distributions were observed between corn stover and switchgrass, indicating that AHP-delignification resulted in similar structural changes between the grasses.

These increases correspond with prior studies showing that delignification resulted in an increase in water retention, indicative of increased surface area or porosity, as well as an increase in carboxylic acid content, corresponding to a change in chemical environment (Li et al., 2012; Lin et al., 2016). Similar curves were observed for both severe conditions, indicating either similar pore distributions, or a saturation of quantifiable signal. One interpretation for saturation involves the diffusion of water from smaller pores into larger pores during heating, with subsequent re-freezing obscuring quantification of water melting in larger pores (Hay and Laity, 2000).

Another approach to measure physical and chemical environment involves using $T_2$ NMR relaxometry to assess water constraint. Similar to DSC, $T_2$ NMR relaxometry provides a quantifiable relationship between biomass-water interactions. $T_2$ NMR results are dependent on both the physical and chemical environment experienced by the solvent, with more solvent constraint resulting in shorter $T_2$ relaxation curves. Monocomponent exponential decay curves from CGMG $T_2$ relaxation studies for AHP-delignified corn stover (Fig. 6A) showed a general trend of increasing chemical constraint with increasing extent of delignification. Relaxometry curves were similar for low and moderate lignin removal, however become increasingly more constrained as more lignin was removed.

Increased water constraint is an indication of increased hydrogen bonding associated with biomass, and may be due to delignification increasing available hydroxyl content and carboxyl content (Williams and Hodge, 2014) or due to increased accessibility to cellulose for hydrogen bonding (Felby et al., 2008) rather than changes in surface area or particle size affecting porosity (Weiss et al., 2016). These conclusions are further supported by the correlation between $T_2$ monocomponent

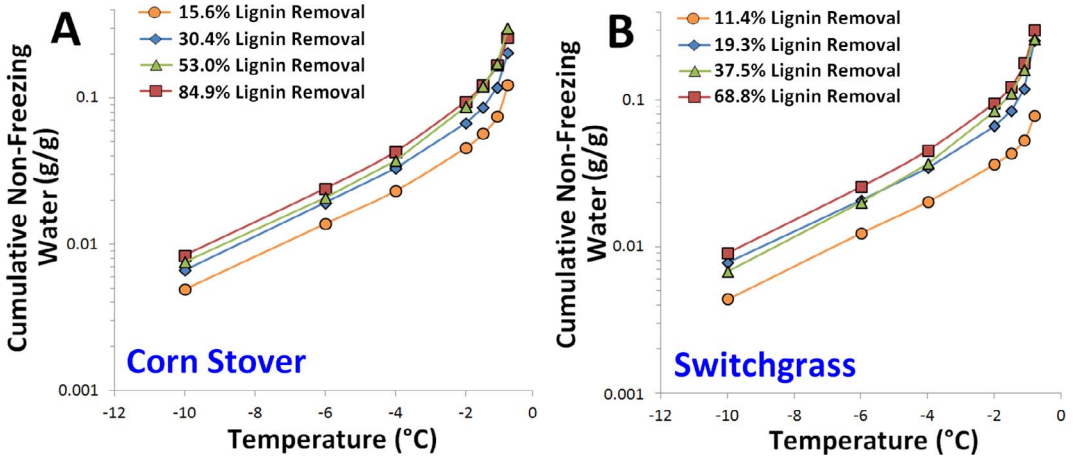


**Fig. 5.** DSC melting temperature depression endotherms for cumulative non-freezing water content (g water per g biomass) as a function of temperature for AHP-delignified (A) corn stover and (B) switchgrass.

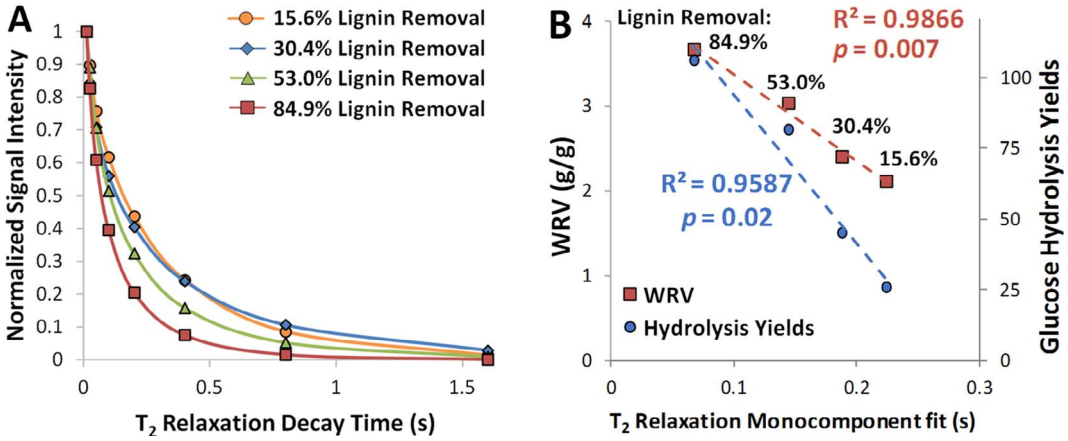


**Fig. 6.** $^{1}$H NMR relaxometry of AHP-delignified corn stover showing (A) spin-spin ($T_2$) relaxation curves and (B) correlation of monocomponent exponential fits of these relaxation curves to the corresponding WRV and glucose hydrolysis yield.

exponent fits and enzymatic hydrolysis yields (Fig. 6B), indicating that systems with more constrained water have greater cellulose accessibility and result in higher enzymatic hydrolysis yields. The linearity between WRV and $T_2$ monocomponent exponent fits (Fig. 5B) indicates that WRV and $T_2$ NMR relaxometry likely quantify the same biomass properties in AHP-delignified samples.

## 4. Conclusions

A series of AFEX pretreatment and AHP delignification conditions were performed on two graminaceous feedstocks, exhibiting diverse feedstock properties. Enzymatic hydrolysis response to pretreatment was correlated between two graminaceous feedstocks, enabling the use of one feedstock to predict hydrolysis yields of another. Composition correlations to enzymatic hydrolysis yields were observed in AHP-delignified biomass only, however WRV showed strong positive correlations with both AHP-delignification severity and AFEX pretreatment severity for each $NH_3$:$H_2O$ ratios. Finally, DSC pore size distribution analysis and $T_2$ NMR relaxometry both exhibited increases in bound water content and water association with increasing pretreatment severity.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.08.200.

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