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Carbohydrate Derived-Pseudo-Lignin Can Retard Cellulose Biological Conversion

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ABSTRACT: Dilute acid as well as water only (hydrothermal) pretreatments often lead to a significant hemicellulose loss to soluble furans and insoluble degradation products, collectively termed as chars and/or pseudo-lignin. In order to understand the factors contributing to reducing sugar yields from pretreated biomass and the possible influence of hemicellulose derived pseudo-lignin on cellulose conversion at the moderate to low enzyme loadings necessary for favorable economics, dilute acid pretreatment of Avicel cellulose alone and mixed with beechwood xylan or xylose was performed at various severities. Following pretreatment, the solids were enzymatically hydrolyzed and characterized for chemical composition and physical properties by NMR, FT-IR, and SEM imaging. It was found that hemicelluloses (xylan) derived-pseudo-lignin was formed at even moderate severities and that these insoluble degradation products can significantly retard cellulose hydrolysis. Furthermore, although low severity (CSF $\sim$ 1.94) dilute acid pretreatment of a xylan–Avicel mixture hydrolyzed most of the xylan (98%) and produced negligible amounts of pseudo-lignin, enzymatic conversion of cellulose dropped significantly (>25%) compared to cellulose pretreated alone at the same conditions. The drop in cellulose conversion was higher than realized for cellulase inhibition by xylooligomers reported previously. Plausible mechanisms are discussed to explain the observed reductions in cellulose conversions.

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KEYWORDS: pseudo-lignin; cellulose; cellulase; hydrolysis; yield

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Introduction

Abundant and renewable lignocellulosic biomass containing large amounts of structural polymeric sugars, can play a significant role in the development of energy independent and secure societies provided that this resource can be tapped efficiently and economically (Lynd et al., 1999; Wyman, 2004). To derive fuels and chemicals from cellulosic biomass, a number of process steps must be applied, with pretreatment being essential to overcome the natural resistance of most cellulosics to sugar release (Mosier et al., 2005; Sun and Cheng, 2002). Unfortunately, pretreatment is also one of the most expensive steps in the overall process. Pretreatment enhances cellulase accessibility to cellulose, and its efficacy is associated with altering the biomass structure through lignin/hemicellulose and other components dislocation/physical removal (Chundawat et al., 2011; Mosier et al., 2005). Several leading thermochemical pretreatment technologies employing acids, bases, or just water have been reported to enhance cellulosic biomass digestibility significantly (Wyman et al., 2005). Dilute acid and water-only (hydrothermal) pretreatments are often employed to enhance biomass digestibility, and steam explosion (catalyzed or uncatalyzed), liquid hot water, and flowthrough approaches have been most commonly used to pretreat biomass (Liu and Wyman, 2005; Yang and Wyman, 2004). However, although both hydrothermal and dilute acid pretreatments achieve physical removal/dislocation of hemicellulose and lignin and enhance cellulose accessibility several fold, recoveries of sugars from hemicellulose and cellulose in most cases are less than 80% and 95%, respectively (Excoffier et al., 1991; Negro et al., 2003). Monomeric forms of carbohydrates released into the aqueous phase during pretreatment can degrade into soluble compounds, for example, furans (furfural (FF)

and hydroxymethyl furfural (5-HMF)) and acids (levulinic acid (LA) and formic acid (FA)). These can further degrade to form insoluble carbon enriched compounds often termed as chars and/or pseudo-lignin (Girisuta et al., 2006; Hodge et al., 2008; Kim et al., 2011; Sannigrahi et al., 2008; Weingarten et al., 2010, 2012). It has been postulated (Li et al., 2007; Nguyen et al., 2000) and recently confirmed (Sannigrahi et al., 2011) that carbohydrates released during thermochemical pretreatments degrade to lignin-like (pseudo-lignin) compounds. However, it has never been determined whether these carbohydrate degradation products adversely affect cellulose digestion. In addition, it has been hypothesized and recently confirmed that lignin melts at temperatures above its glass transition temperature, which varies with solvent type and other conditions (Abe et al., 2010; Bouajila et al., 2006; Guigo et al., 2009), moves to outer cell walls, and redeposits back on the cellulose surface, where it greatly hampers cellulose digestibility by blocking cellulase access to the substrate and/or unproductively binding cellulase (Donohoe et al., 2008; Yang and Wyman, 2006). Selig et al. (2007) indicated that during dilute acid pretreatment, lignin derived from corn stem rind, added exogenously with filter paper, deposited on the cellulose surface in the form of globules that hampered hydrolysis rates and yields. However, it was not possible to distinguish whether these globules were from lignin or pseudo-lignin resulting from degradation of carbohydrates fraction of biomass or some combination of both.

In this study the effects of carbohydrate-derived-pseudo-lignin on cellulose saccharification were investigated, and mechanisms were hypothesized to explain the results. Due to the complex structure of cellulosic biomass, the wet chemistry method for compositional analysis (Sluiter et al., 2008b) defined by National Renewable Energy Laboratory (NREL, Golden, CO) does not distinguish between Klason lignin (K-lignin) naturally found in plants and pseudo-lignin resulting from sugars degradation during pretreatment. Therefore, to avoid the complexity often encountered with interpretation of results for real biomass, pure Avicel cellulose alone and mixed with pure xylan or monomeric xylose sugar was pretreated with dilute sulfuric acid over the range of pretreatment severities often found to give best conversion results. Following pretreatment, the solids were subjected to enzymatic hydrolysis and physical and chemical characterizations to elucidate the effects of xylan degradation products on enzymatic hydrolysis and support identification of possible mechanisms to explain the results.

Materials and Methods

Materials, Reagents, and Enzymes

Pure cellulose (Avicel® PH 101; Lot No.1316179) was purchased from FMC Biopolymer (Philadelphia, PA). Beechwood xylan (Catalog No. X-4252; Batch No. 017K0688) and xylose (99% purity; Batch No. 069K0115) were from Sigma–Aldrich (St. Louis, MO). Analysis of beechwood xylan showed it contained ~69.6 wt% xylan, 2.2% glucan, <1% arabinan, 3% acetate, 6% ash, 2.5% total lignin, and 15.7% others (Sluiter et al., 2008b). The latter might be uronic acid (~9 wt%; van Gool et al., 2012), protein, and other constituents that we could not determine. Accellerase® 1500 cellulase (Batch No.1681198062) and Multifect® xylanase (Lot No.301-04021-015) were provided by Genencor, a division of Danisco A/S (now DuPont™ Genencor® Science, Palo Alto, CA). Protein contents of cellulase and xylanase, as determined by the standard BCA method (Smith et al., 1985), were 82 and 42 mg/mL, respectively. The specific activity of Accellearse® 1500, as reported elsewhere (Alvira et al., 2011), was about 0.5 FPU/mg protein, consistent with values typically noted for commercial cellulase preparations (Berlin et al., 2006; Dien et al., 2008; Kumar and Wyman, 2009d). CBHI purified to homogeneity (single band on SDS gel, 18.5 mg/mL as determined by BCA method) from Genencor Spezyme® CP (lot 301-04075-034; 59 ± 5 FPU/mL, 123 ± 10 mg protein/mL) cellulase was prepared by Protein Labs (San Diego, CA) by following standard protocols (Palonen et al., 1999). All other laboratory reagents and chemicals used in this study were either purchased from Fisher Scientific (Pittsburgh, PA) or Sigma–Aldrich, unless stated otherwise.

Pretreatment

Dilute acid pretreatment (DAP) of Avicel PH 101 cellulose alone and mixed with xylan or with xylose was performed in a 1 L (working volume 800 mL) Hastelloy C Parr reactor (Parr Instruments, Moline, IL). The Avicel cellulose loading was 5 wt% (40 g dry basis), and xylan or xylose was added to cellulose at a 0.5:1 weight ratio, representative of the typical ratio for natural lignocellulosic biomass (Kumar et al., 2009; Sun and Cheng, 2002). Because beechwood xylan contained about 15 wt% of other compounds that could not be determined in this study, pure xylose mixed with Avicel cellulose was also pretreated as a control to evaluate any effects on cellulose reactivity that might be due to factors other than pseudo-lignin. The reactor cover was equipped with a K-type thermocouple (Model KQSS-316G-18; Omega Engineering Inc., Stamford, CT) positioned at the center of the reactor to monitor inside temperature, and a 3.5 in diameter helical shaped impeller on a two-piece shaft driven by a variable speed DC motor assembly provided mixing and heat transfer (A1750HC, Parr Instruments). A temperature controlled 4 kW fluidized sand bath was used to more rapidly heat the reactor (model SBL-2D, Techne Inc., Burlington, NJ; Lloyd and Wyman, 2005), and the heating time to reach the desired reaction temperature of between 4 and 5 min was not included in the stated reaction time. The combined severity factor was employed to estimate the overall effects of time, temperature, and acid concentration on the extent of pretreatment (Chum et al., 1990; Overend

and Chornet, 1987) and is defined as follows:

$$\text{Severity factor (SF)} = \text{Log}\, R_o; \quad R_o = t * \exp\left[\frac{T - T_{ref}}{14.75}\right]$$
$$\text{Combined severity factor (CSF)} = \text{SF} - \text{pH}$$

where t is the reaction time in min, T is the pretreatment temperature in °C, and T_{ref} is the reference temperature in °C, with an often used value of 100.

The DAP conditions were as reported in the literature for variety of biomass types (Wyman et al., 2009, 2011) and are summarized along with the corresponding combined severity factors (CSF) in Table I. Following pretreatment, the reactor was cooled to below 50°C in a room temperature water bath, and then the slurry was immediately transferred to 500 mL centrifuge bottles (Catalog No.14-375-359, Fisher Scientific) that were centrifuged for 10 min at 10,000 rpm to separate solids from the liquid in a Beckman centrifuge (Model No. J2-21, Beckman Coulter, Inc., Brea, CA). After the first centrifugation, liquid samples were collected from the supernatant for further analysis, with the rest of the supernatant discarded. Some of the unwashed solids were collected for imaging and hydrolysis experiments, and the rest was washed at least five to seven times with room temperature deionized (DI) water until the supernatant pH was close to neutral. Then, the solids were scraped off from the centrifuge bottles and stored in Ziplock® bags at 4°C for further experiments. Solids were analyzed for moisture content and composition according to NREL standard laboratory analytical procedures (LAPs; Sluiter et al., 2008a,b).

Pseudo-Lignin From Pure Xylose

Pseudo-lignin solids were formed from pure xylose in the 1 L Parr reactor with a xylose loading of 15 wt% (120 g) in a 5 wt% dilute sulfuric acid solution at 180°C for 150 min. In order to maximize the yield of pseudo-lignin, the reaction conditions were deliberately chosen to be harsher than those typically applied in dilute acid pretreatments of biomass. After 150 min of reaction at 180°C, the reactor was cooled to room temperature in a water bath. The pseudo-lignin formed in the reactor mostly settled in a cake to the bottom but also formed a sticky layer on the impeller and thermocouple probe. Pseudo-lignin samples were scraped off and washed repeatedly by vacuum filtration until the filtrate pH was neutral. The washed pseudo-lignin solids were homogenized in a coffee grinder (Model No. BCG 1000B1; KitchenAid Appliances), and analyzed for moisture content with a halogen moisture analyzer (HB43-S; Mettler Toledo, Columbus, OH).

Enzymatic Hydrolysis

Enzymatic hydrolysis of untreated Avicel cellulose and washed dilute acid pretreated (DAPt) solids were performed

Table I. Pretreatment conditions, resulting solids composition, and accountable xylose or xylan for dilute acid pretreatment of Avicel alone and mixed with xylan or xylose.

			Solids composition (%; dry mass basis)				
					Lignin		
Pretreatment conditions	CSF[a]	Substrate	C	Xyn	Acid soluble	K/pseudo[b]	Xylan or xylose accounted for[c] (%)
NA		Cellulose	98.5	0.0	0.25	0.0	NA
		Xyn	2.2[d]	69.6	1.38	1.08	
		Pseudo-lignin[e]	0.0	0.0	0.46	97.9	
140—1 wt%, 30 min[f]	1.94	Cellulose	99.0		0.27	0.0	
		C+ Xyn	99.5	<2	0.28	0.0	98
		C+ Xys	102.6		0.29	0.0	99
160—0.5 wt%, 40 min	2.38	Cellulose	100.5		0.28	0.40	ND
		C+ Xyn	100.3		0.29	0.53	
		C+ Xys	99.2		0.30	1.50	
160—1 wt%, 40 min	2.66	Cellulose	101.2		0.31	0.51	NA
		C+ Xyn	95.6		0.41	3.45	93
		C+ Xys	97.8	0.0	0.45	3.16	85
170—1 wt%, 40 min	2.95	Cellulose	93.2		0.46	3.07	NA
		C+ Xyn	89.8		0.51	8.1	79
		C+ Xys	89.1		0.55	10.6	64
180—2 wt%, 40 min	3.56	Cellulose	11.5		0.93	85.7	NA
		C+ Xyn	9.25		0.80	88.2	17
		C+ Xys	5.06		0.82	94.4	12

C, cellulose; Xyn, xylan; Xys, xylose; NA, not applicable; ND, not determined.

[a]CSF, combined severity factor that includes pretreatment time, temperature, and pH; pH values used for CSF calculations were based on H^+ concentration in the solution.

[b]Carbohydrate derived-pseudo-lignin.

[c]Recovery of xylose or xylan = 100 × (A − B)/A, where A is the initial amount of xylose or xylan (g) and B is the amount of xylan left in the solids plus the amount of xylose monomer, xylose oligomers, and xylose or xylan equivalent of furfural in the pretreatment solution (g).

[d]Glucan, collectively referred to anhydrous glucose regardless of source.

[e]Xylose derived-pseudo-lignin as described in Materials and Methods Section.

[f]Pretreatment conditions represent: reaction temperature in °C—wt% acid solution and reaction time.

at 50°C and 150 rpm in Multitron shakers (Model AJ125; Infors-HT, Laurel, MD) following a modified NREL standard protocol (Selig et al., 2008). In this case, 50 mL was used as the total reaction volume instead of the 10 mL volume specified in the NREL protocol. A solids concentration corresponding to a cellulose content of 1% (w/v) was added to 125 mL Erlenmeyer glass flasks (Catalog No. 10-041-20, Fisher Scientific) that contained 50 mM sodium citrate buffer mixed with Accellerase®1500 cellulase at protein loadings of 5 and 15 mg/g cellulose (equivalent to approx. 2.5 and 7.5 FPU/g cellulose) and 0.1% sodium azide to prevent microbial growth. The mixture was hydrolyzed in duplicates for up to 120 h, with substrate blanks without enzymes and enzyme blanks without substrate also run in parallel.

To determine the effect of pure xylose derived-pseudo-lignin on Avicel cellulose conversion, enzymatic hydrolysis was conducted at solids loadings equal to 1% (w/v) cellulose, with pseudo-lignin added to cellulose at pseudo-lignin-to-cellulose mass ratios of 0.0, 0.05, 0.15, 0.45, and 0.65. Hydrolysis was conducted for up to 120 h at 50°C with Accellerase®1500 cellulase at protein loadings of either 5 or 15 mg/g cellulose. Other conditions were the same as above.

To follow sugar release during enzymatic hydrolysis, about 1 mL of a thoroughly mixed sample was withdrawn periodically into a 2 mL microcentrifuge tube (Eppendorf PCR clean microcentrifuge tubes, Catalog No. 05-402-95, Fisher Scientific) using an Eppendorf pipette and mixed with about 15 μL of 10 wt% sulfuric acid to bring the samples pH to 1-3, Aminex HPX-87H column operating pH range. By adding acid to the samples, the sulfuric acid negative peak on the HPLC chromatogram is avoided, allowing smoother integration. To remove solids from the liquid, samples were centrifuged using Eppendorf micro-centrifuge (Model No. 5424, Fisher Scientific) at 14,600 rpm for 5 min. Then about 450 μL of clarified supernatant was transferred into a 500 μL polypropylene snap ring vial (Vendor No. 98842; Grace Davision, Deerfield, IL) and run on an HPLC along with sugars standards. Cellulose conversion for enzymatic reactions was calculated as:

$$\text{Cellulose conversion}\,(\%) = 100 \times \frac{0.90 \times (\text{Glucose}\,(\text{g}) + 1.053 \times \text{Cellobiose}\,(\text{g}))}{\text{Initial cellulose}\,(\text{g})}$$

For pretreated solids, cellulose loading was based on the amount of cellulose in the pretreated solids. The change (or drop) in cellulose conversion was calculated as:

$$\%\ \text{Change in conversion} = 100 \times \frac{(X - Y)}{X}$$

where X is the cellulose conversion for the untreated or pretreated control and Y is the cellulose conversion for untreated or pretreated cellulose mixed with xylan or xylose.

Cellulose Solubilization and Xylan/Xylose Recovery Calculations for Pretreatment

The amount of cellulose solubilized during pretreatment was calculated based on the amounts of cellobiose, glucose, hydroxymethyl furfural (HMF), and levulinic acid (LA) identified in the pretreatment liquid:

$$\text{Cellulose solubilized during pretreatment}\,(\%) = 100 \times \frac{0.9 \times (\text{Cellobiose}\,(\text{g}) \times 1.053 + \text{Glucose}\,(\text{g})) + (\text{HMF}\,(\text{g})/0.7784) + (\text{LA}\,(\text{g})/0.716)}{\text{Initial amount of cellulose}\,(\text{g})}$$

where 1.053, 0.9, 0.7784, and 0.716 are the mass conversion factors based on the stoichiometry for conversion of cellobiose to glucose, glucose to cellulose, cellulose to HMF, and cellulose to LA, respectively.

The amount of xylan/xylose recovered after pretreatment was calculated based on the amounts of xylose and furfural (FF) identified in the pretreatment liquid as follows:

$$\text{Xylan recovery}\,(\%) = 100 \times \frac{0.88 \times \text{Xylose}\,(\text{g}) + (\text{FF}\,(\text{g})/0.727)}{\text{Initial amount of xylan}\,(\text{g})}$$

$$\text{Xylose recovery}\,(\%) = 100 \times \frac{\text{Xylose}\,(\text{g}) + (\text{FF}\,(\text{g})/0.640)}{\text{Initial amount of xylose}\,(\text{g})}$$

The amount of xylose or xylan lost to pseudo-lignin can be calculated as:

$$\text{Xylan or xylose lost to pseudo-lignin} = 100 - \text{Xylan or xylose recovery}(\%)$$

where 0.727, 0.640, and 0.88 are the mass conversion factors based on the stoichiometry for conversion of xylan to furfural, xylose to furfural, and xylose to xylan, respectively. Formic acid was not included due to the low concentration detected in the liquid for pretreatment of Avicel alone and for pretreatment of Avicel mixed with xylan or xylose.

Samples Analysis

Liquid samples collected from supernatants after pretreatments and periodically collected over the course of enzymatic hydrolysis were analyzed on a Waters Alliance HPLC (Model 2695; Waters Co., Milford, MA) equipped with an auto sampler (Waters 2695) and a 2414 refractive

index (RI) detector. To separate sugars and their degradation compounds (for pretreatment liquor) via HPLC, a Bio-Rad Aminex® HPX-87H (polystyrene-divinylbenzene sulfonic acid resin packing; 300 mm × 7.8 mm; Catalog No.125-0140) column along with a micro-guard cation cartridge (Catalog No.125-0129; 30 mm × 4.6 mm; Bio-Rad Laboratories, Hercules, CA) were used. The column was heated to 65°C, with 5 mM sulfuric acid at a flow rate of 0.6 mL/min as the carrier solvent. The chromatograms were integrated, and data was imported to Microsoft Excel files using Empower® 2 software (Waters Co.).

BSA/Purified CBHI Protein Adsorption on Pseudo-Lignin

Albumin from bovine serum (BSA, 98% purity, Batch No. 078K0730, Sigma–Aldrich) and purified CBHI (Cel7A) adsorption was performed at room temperature in a 50 mM sodium citrate buffer (pH ~ 5.0). A 10 mg/mL BSA protein stock was prepared in 50 mM citrate buffer for adsorption experiments. Pure xylose derived-pseudo-lignin solids with BSA protein or CBHI were incubated overnight in 2 mL microcentrifuge vials on an end-over-end rugged rotator (Glass-Col, LLC, Terre Haute, IN) equipped with a variable speed DC motor turning at about 20 rpm. Following overnight incubation, the tubes were centrifuged in an Eppendorf microcentrifuge (Model No.5424, Fisher Scientific) at 14,600 rpm for 5 min, and the amount of free protein in the supernatant was determined by the standard BCA method (Smith et al., 1985). A fixed loading of 10 g/L pseudo-lignin was used for adsorption kinetics, with BSA or CBHI protein added at 0–2 mg/mL (0–200 mg protein/g solids). To determine the effect of pseudo-lignin loadings on the relative free protein in solution, a fixed 2 mg/mL BSA protein concentration was used, while the pseudo-lignin solids loading was varied from 0 to 100 g/L. Substrate blanks without protein and protein blanks without solids were run for both sets of experiments.

Physical and Chemical Characterizations

Untreated substrates and solids resulting after pretreatments were subjected to the chemical and physical characterizations techniques described below.

Physical Images

Images of the xylose derived-pseudo-lignin and untreated and pretreated samples of Avicel alone and of Avicel mixed with xylan or xylose were taken with a Nikon D40 6.1MP Digital SLR Camera Kit with 18–55 mm *f*/3.5–5.6G ED II AF-S DX Zoom-Nikkor Lens. Images were further processed with the Microsoft® Paint (v. 6.1) accessory.

Composition Analysis

Compositional analysis of the solids was performed following the NREL standard two-step acid hydrolysis method (Sluiter et al., 2008b). Before analysis, untreated and washed solids collected after pretreatment were dried at 60°C for several days. Because the pretreated solids formed chunks after drying, they were milled to pass through a 20 mesh (0.841 mm) screen using a Thomas Wiley® mini mill (Model No. 3383-L20, Thomas Scientific, Swedesboro, NJ). The solids (~300 mg in 3 mL of 72 wt% sulfuric acid) were subjected to primary hydrolysis at 30°C for 1 h followed by secondary hydrolysis at 121°C for 1 h in 4 wt% acid. Then, the amount of carbohydrates and acid soluble/insoluble lignin were determined following the NREL standard method (Sluiter et al., 2008b). For acid soluble lignin, the absorbance was read in 1 cm path length cuvettes at 320 nm on a UV-Vis microplate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, CA) based on an absorptivity constant of 100 L/g cm.

Solid-State ^{13}C CP/MAS NMR and FT-IR

All samples were first air dried overnight in a fume hood and then further dried in a vacuum oven at 40°C for 24 h. The samples for NMR and FT-IR were homogenized using a marble mortar and pestle. For solid-state ^{13}C CP/MAS NMR, the ground samples were packed in 4 mm zirconia rotors fitted with kel-F caps and measured at 10 KHz spinning speed. The NMR experiments were performed with a Bruker Advance-400 spectrometer operating at a ^{13}C frequency of 100.6 MHz. For FT-IR analysis, the homogenized samples were formed into pellets (without the addition of KBr) and analyzed on a Perkin Elmer Spectrum 100 FT-IR spectrometer.

Scanning Electron Microscopy (SEM)

The untreated Avicel, xylose derived-pseudo-lignin, and solid samples resulting from pretreatment were characterized using SEM after mounting dry samples on aluminum specimen stubs and sputter coating with gold. Images were acquired on a JEOL-1530 Thermally-Assisted Field Emission (TFE) Scanning Electron Microscope (SEM) at 5 kV beam accelerating voltage and various resolving powers.

Results and Discussion

Physical Images, Solids Composition, Pseudo-Lignin Formation, and Recovery of the Components

Dilute sulfuric acid pretreatment was performed on Avicel cellulose alone and mixed with beechwood xylan or xylose over a range of severities from CSF 1.94 (140°C—1 wt% acid solution, 30 min) to 3.56 (180°C—2 wt% acid solution, 40 min). Figure 1 shows the physical images of untreated Avicel cellulose, xylose derived-pseudo-lignin, and washed pretreated solids of Avicel cellulose alone and mixed with xylan or xylose. Avicel cellulose solids turned from white for untreated to brownish and then dark black as pretreatment severity was increased. The change in color from white to brownish was observed at lower severities for pretreated solids of Avicel cellulose mixed with xylan or xylose than for

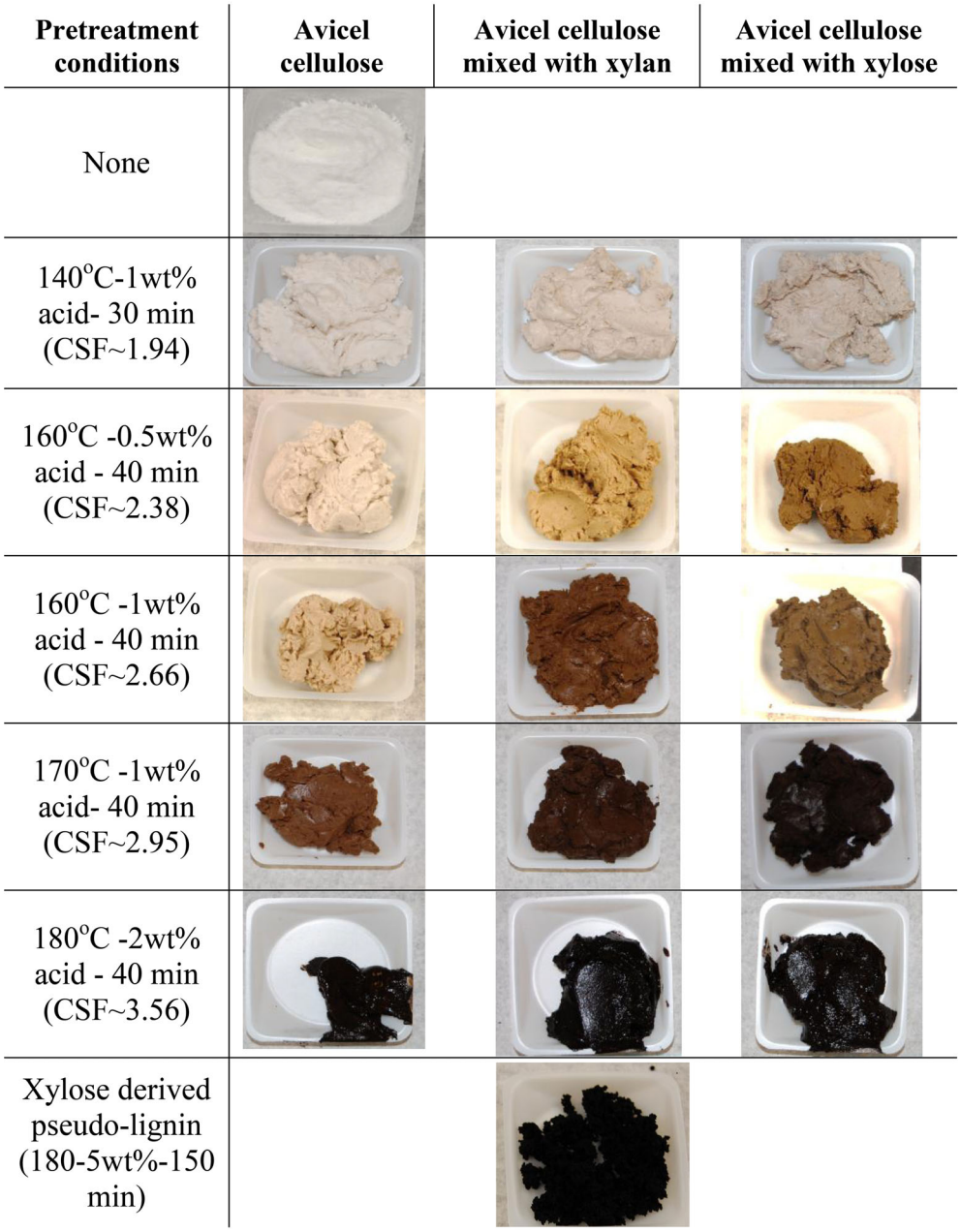

Figure 1. Pictures of untreated Avicel cellulose, xylose derived-pseudo-lignin, and dilute acid pretreated Avicel cellulose alone and mixed with xylan or xylose at the pretreatment conditions applied. CSF-combined severity factor.

cellulose alone, with the sample that had been mixed with xylose being darker. However, at the most severe pretreatment conditions (CSF ~ 3.56), pretreated solids for all three were dark black, resembling xylose derived-pseudo-lignin.

The starting substrates (Avicel cellulose, beechwod xylan, and xylose) had almost no K-lignin. Beechwood xylan contained about 70% xylan, 2.2% glucan, 6.0% ash, <1% arabinan on a dry weight basis, with the rest being uronic acids and protein, as shown elsewhere (Gray et al., 2007; van Gool et al., 2012). Solids from pretreatment of Avicel mixed with xylan or with xylose had higher amounts of pseudo-lignin than Avicel alone at all conditions (Fig. 2a). Consistent with findings by (Sannigrahi et al., 2011), the amount of ash free acid insoluble residues (AF-AIR; i.e., K-lignin as per NREL standard method) in the pretreated solids increased with pretreatment severity, with a negligible amount at CSF 1.94 and the highest amount at CSF 3.56 (95 wt%) for cellulose mixed with xylose, as shown in Figure 2a and Table I. This increase in K-lignin content resulted from carbohydrate degradation to lignin-like

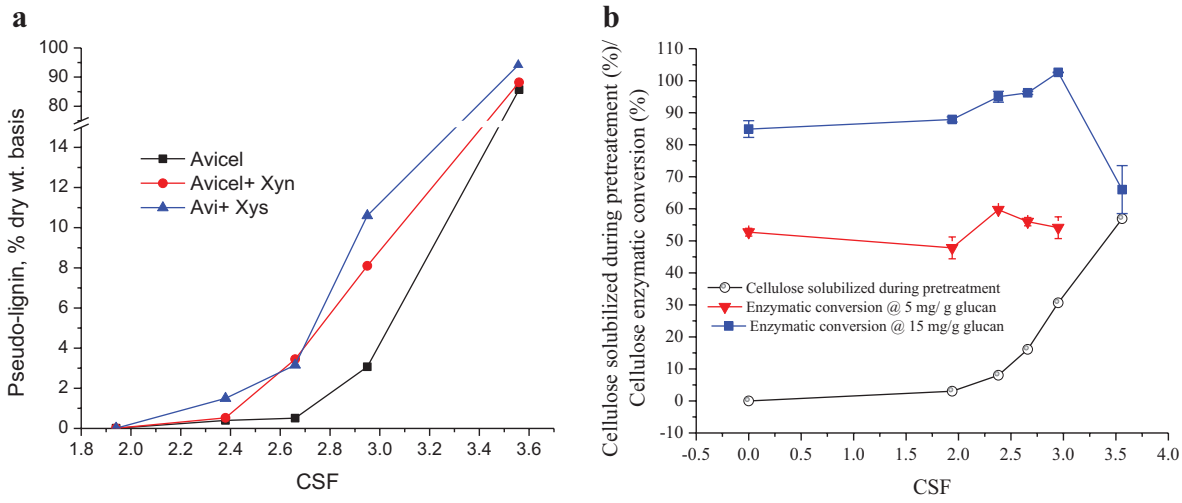

Figure 2. **a**: Pseudo-lignin (% K-lignin, dry wt. basis) in pretreated solids versus combined severity factor (CSF) for dilute acid pretreatment of Avicel cellulose alone and mixed with xylan (Xyn) or xylose (Xys). **b**: The amount of cellulose solubilized during pretreatment and cellulose digested by Accellerase® 1500 cellulase at loadings of 5 and 15 mg protein/g cellulose in the residual solids resulting from dilute acid pretreatment (DAP) of Avicel cellulose versus combined severity factor.

compounds called pseudo-lignin or char (Pan et al., 2005; Sannigrahi et al., 2011). At similar conditions, however, working with delignified poplar (holocellulose with 67.2% glucan and 21.8% xylan), Sannigrahi et al. (2011) observed a somewhat different trend in pseudo-lignin formation. For example, in our study at CSF 2.95, the amount of pseudo-lignin was about 8% (dry weight basis) for pretreatment of Avicel mixed with xylan, while Sannigrahi et al. (2011), reported about 30 wt% pseudo-lignin at CSF 2.97 for poplar holocellulose. The differences could be attributed to the substrates used and their initial compositions. For instance, the substrates for this study contained a small amount of acid soluble lignin and AF-AIR (K-lignin, as per NREL protocol), whereas their study employed poplar holocellulose with about 6.6% acid soluble lignin. In addition, cellulose in Avicel PH101 is more crystalline than in poplar and other biomass feedstocks (Kumar et al., 2009; Mittal et al., 2011), with the result that it is less prone to breakdown during dilute acid pretreatment than amorphous cellulose (Zhao et al., 2006). Therefore, it is anticipated that biomass that is less crystalline or with more free sugars, starch, pectin, more acid labile hemicelluloses, or acid soluble lignin is more likely to form higher amounts of pseudo-lignin when pretreated at high severities (Marzialetti et al., 2008; Sievers et al., 2009). Acid soluble lignin and its degradation products released during dilute acid pretreatment may also interact with sugar degradation products to increase pseudo-lignin formation (Xiang et al., 2003).

Solids resulting from pretreatment of cellulose mixed with xylan had negligible ash (data not shown) and as shown in Table I, contained almost no residual xylan except at the least severe conditions of 140°C in 1 wt% acid for 30 min (CSF 1.94, xylan content <2 wt%). The xylan/xylose accounted for shown in Table I that includes xylose left in the solids plus xylose monomers, xylose oligomers, and xylose equivalents of furfural in solution decreased drastically with pretreatment severity (from ~98% at CSF 1.94 to <15% at CSF 3.56), consistent with the well-known fragility of xylan/xylose compared to cellulose (Saeman, 1945).

Effect of Dilute Sulfuric Acid Pretreatment on Avicel Cellulose Digestibility

The amount of cellulose solubilized during pretreatment increased with pretreatment severity and was about 60% at the most severe conditions applied (CSF ~ 3.56). Because cellulose solubilization was defined to only account for the amounts of cellobiose, glucose, HMF, and LA in the pretreatment liquid, the actual loss of cellulose must be higher than shown in Figure 2b due to degradation of these compounds into pseudo-lignin and other unidentified compounds (Girisuta et al., 2006; Zhao et al., 2006). It is often speculated that the amorphous portion of cellulose is preferably solubilized by chemical and biological catalysts compared to the crystalline part, with the result that cellulose becomes more crystalline and, therefore, more recalcitrant as the amorphous fraction is more quickly broken down for prolonged times for pretreatment or enzymatic hydrolysis (Foston and Ragauskas, 2010; Sannigrahi et al., 2008). However, Figure 2b shows that the enzymatic digestion of dilute acid pretreated Avicel cellulose compared to its digestibility without pretreatment followed a different trend with increasing severities at the two cellulase protein loadings of 5 and 15 mg/g cellulose in the

Table II. Effect of dilute acid pretreatment on cellulose digestibility for Accellerase®1500 at loadings of 5 and 15 mg protein/g cellulose in pretreated solids for Avicel alone and mixed with beechwood xylan or pure xylose.

		5 mg/g cellulose		15 mg/g cellulose	
		4 h	120 h	4 h	120 h
Pretreatment conditions	Substrate	% Conv.[a] ± SD (% change[b])	% Conv. ± SD (% change)	% Conv. ± SD (% change)	% Conv. ± SD (% change)
Untreated	C	15.2 ± 0.28	58.9 ± 0.83	26.3 ± 0.28	94.5 ± 3.1
None	C+ Xyn	ND		11.0 ± 0.28 (55)[c]	75 ± 2.2 (22)
140—1 wt%, 30 min[d]	C	14.5 ± 0.2	56.5 ± 1.5	29.7 ± 0.1	94.3 ± 0.4
	C+ Xyn	13.1 ± 0.8 (9.6)	41 ± 1.0 (27.4)	25.7 ± 0.2 (13.4)	65.1 ± 0.7 (30.9)
	C+ Xys	14.1 ± 0.7 (2.7)	49.2 ± 0.3 (12.9)	28 ± 0.2 (5.7)	88 ± 0.6 (7.0)
160—0.5 wt%, 40 min	C	17.8 ± 0.1	65.2 ± 1.5	32.8 ± 0.5	98.0 ± 1.5
	C+ Xyn	17.0 ± 1.1 (4.4)	54.2 ± 1.4 (16.8)	29.1 ± 1.2 (11.5)	83.0 ± 1.3 (15.3)
	C+ Xys	17.0 ± 0.7 (4.4)	47.6 ± 0.8 (27.0)	33.3 ± 0.4 (NS)	81.1 ± 0.6 (17.2)
160—1 wt%, 40 min	C	18.2 ± 0.1	61.9 ± 2.3	32.3 ± 0.6	100.4 ± 0.4
	C+ Xyn	12.4 ± 0.3 (31.8)	48.6 ± 0.6 (21.5)	29.0 ± 0.9 (10.2)	85.6 ± 1.7 (14.7)
	C+ Xys	16.6 ± 0.4 (8.8)	52.6 ± 0.6 (15.0)	29.8 ± 0.3 (7.7)	87.2 ± 0.1 (13.1)
170—1 wt%, 40 min	C	12.3 ± 1.0	60.5 ± 4.1	32.0 ± 0.3	100.5 ± 0.8
	C+ Xyn	12.2 ± 0.8 (NS)	53.5 ± 0.5 (11.5)	27.8 ± 0.7 (13.1)	94.0 ± 0.8 (6.5)
	C+ Xys	13.2 ± 0.1 (NS)	64.8 ± 0.8 (NS)	30.1 ± 0.5 (6.0)	92.0 ± 1.1 (8.5)
180—2 wt%, 40 min	C	ND		NA	66.0 ± 7.5
	C+ Xyn				NA
	C+ Xys				36.9 ± 7.1 (44)

C, Avicel cellulose; Xyn, xylan; Xys, xylose; NA, not available; NS, not significant; ND, not determined.
[a]Cellulose conversion = 100 × (glucose [g] + 1.053 × cellobiose [g])/(1.11 × initial cellulose [g]).
[b]% change in conversion over control = 100×(% conv. for control − % conv. for solids)/% conv. for control.
[c]For this set of experiments, xylan and Avicel cellulose were physically mixed together at a ratio of 0.5, and enzymatic hydrolysis was performed.
[d]Pretreatment conditions represent: reaction temperature in °C—wt% acid solution and reaction time.

pretreated solids tested. In particular, except for the result for CSF 3.56, the 4 h initial rate and 72 h cellulose conversion either increased by ~13–20% (depending on cellulase loading) or were unchanged over the range of pretreatment severities covered, as shown in Figure 2b and Table II. Although pretreating Avicel at CSF 2.95 generated about 3 wt% pseudo-lignin, they did not appear to have a negative impact on the final cellulose conversion compared to results for untreated cellulose. However, digestion of cellulose pretreated at CSF 2.95 with a 5 mg cellulase loading was lower than for Avicel pretreated at lower severity conditions that produced less pseudo-lignin. At CSF 3.56, cellulose conversion with a 15 mg protein/g cellulose cellulase loading dropped to 66% from ~95% for untreated Avicel cellulose at the same loading, a decrease of about 30%. This drop could be attributed to reduced cellulose accessibility as a result of increased crystallinity, pseudo-lignin deposition (95% by weight) on cellulose, and/or reduced cellulase effectiveness due to non-specific binding of cellulase to pseudo-lignin.

Enzymatic Hydrolysis of Pretreated Solids

Pretreated solids were extensively washed to remove soluble compounds inhibitory to cellulase such as xylose, xylooligomers, glucose, and furfural (Kim et al., 2011; Kumar and Wyman, 2009c; Xiao et al., 2004). Washed solids from dilute acid pretreatment of Avicel cellulose alone and mixed with xylan or xylose were subjected to enzymatic hydrolysis at cellulase protein loadings of 5 and 15 mg protein/g cellulose in the pretreated solids. For comparison, untreated Avicel cellulose physically mixed with untreated beechwood xylan (cellulose to xylan weight ratio 2:1) before enzymatic hydrolysis was also enzymatically hydrolyzed at a cellulase loading of 15 mg/g cellulose at similar conditions (50 mM citrate buffer, pH 5.0, 50°C, and 150 rpm). The cellulose conversions and their standard deviations following 4 and 120 h of enzymatic hydrolysis are summarized in Table II for application of both cellulase loadings to solids prepared over a range of pretreatment severities. Also summarized as bold italics in parenthesis in Table II are percent changes in cellulose digestion of solids resulting from pretreatment of Avicel cellulose mixed with xylan or xylose compared to results for digestion of Avicel cellulose that had been pretreated by itself at the same severity.

Digestibility of Solids Prepared at Low Severity (Negligible Pseudo-Lignin)

The liquid fraction from pretreatment at CSF 1.94 contained very small amounts of carbohydrate degradation products (LA, HMF, FA, and FF, data not shown), and greater than 98% of the original xylan/xylose could be accounted for, as shown in Table I. The resulting washed solids had a small amount of xylan (<2%) and little if any AF-AIR or pseudo-lignin, as determined by the NREL method. However, surprisingly, the 4 and 120 h enzymatic digestibilities of

solids resulting from pretreatment of cellulose mixed with xylan or xylose were still considerably lower than digestion of cellulose that was pretreated alone at the same severities (Table II). In particular, cellulose conversion after 120 h of hydrolysis of solids prepared by pretreatment of cellulose mixed with xylan dropped to 41% from 56.5% for hydrolysis of cellulose alone (27.4% drop) at a cellulase protein loading of 5 mg/g cellulose and to 65% from 94.3% (31% drop) at a loading of 15 mg/g cellulose. These results were quite unexpected. Although the 6–7 wt% ash content makes it a significant constituent in commercial beechwood xylan, so little is left following pretreatment (data not shown) that it would be highly unlikely to impact hydrolysis. However, it can be hypothesized that possible cellulose acetylation due to presence of acetyl groups and methylation from 4-D-methyl glucuronic acid on the xylan backbone during pretreatment and/or residual xylan (<2 wt%) could result in less reactive cellulose, as it is well known that cellulose methylation and acetylation retards cellulase activity (Rivard et al., 1992).

Causes for cellulose reduced digestibility at low severity: cellulose acetylation. To evaluate further whether acetylation reduced cellulose conversion for solids prepared at CSF 1.94 that had negligible pseudo-lignin, Avicel cellulose at 5 wt% solids loading together with different acetic acid concentrations (acetic acid to Avicel weight ratios of 0.10, 0.25, 0.50, or 1.0) was pretreated at 140°C in 1 wt% dilute sulfuric acid for 30 min (CSF ~ 1.94). The resulting solids along with the control were enzymatically hydrolyzed at a cellulase protein loading of 15 mg/g glucan. No meaningful reduction in cellulose reactivity (data not shown) was observed, ruling it out as the cause for drop in cellulose conversion.

Causes for cellulose reduced digestibility at low severity: Cellulose methylation. To obtain representative free (methyl) glucuronic acid from xylan used in this study, the same batch of xylan at 10 wt% solid loading was acid hydrolyzed at similar conditions used for pretreatment, that is, in 1 wt% dilute sulfuric acid at 140°C for 30 min. The remaining unhydrolyzed xylan solids were separated from the liquid by vacuum filtration. The xylose solution obtained from xylan acid hydrolysis that presumably contained free (methyl) glucuronic acids was used as a (methyl) glucuronic acid source, and Avicel at 5 wt% solid loading was pretreated with this xylose solution (Avicel to xylose weight ratio ~2) at similar pretreatment conditions as were used for pretreatment of Avicel mixed with untreated xylan. The resulting solids were enzymatically hydrolyzed at a cellulase protein loading of 15 mg/g glucan in pretreated solids with other conditions being similar as described in Materials and Methods Section. The enzymatic digestion showed that methyl glucuronic acids released from the xylan backbone in pretreatment had no apparent impact on Avicel cellulose reactivity (data not shown).

Causes for cellulose reduced digestibility at low severity: Residual xylan. The washed solids resulting from pretreatment of Avicel mixed with xylan at CSF 1.94 had about 2 wt% unhydrolyzed residual xylan. Thus, to identify whether small amounts of residual xylan had a role in reduced cellulose conversion, pretreated solids for Avicel control, and Avicel mixed with xylan were hydrolyzed with cellulase (15 mg protein/g glucan) alone and cellulase supplemented with Multifect® xylanase at protein loading of 7.5 mg/g glucan (total protein loading 22.5 mg/g glucan in pretreated solids). As shown in Figure 3, adding xylanase increased the reactivity of cellulose from pretreatment of Avicel mixed with xylan to be virtually the same as for control, however, had no effect on cellulose conversion for control. It was quite surprising that such a small amount of residual, non-structural xylan had such a significant impact on cellulose digestibility, and the drop in final conversion was greater than could be accounted for by cellulase inhibition (~22%) by xylooligomers when xylan was physically mixed with Avicel (xylan to Avicel weight ratio of 0.5, corresponds to 33% xylan) before enzymatic hydrolysis, Table II. Although still unclear, this significant finding led us to hypothesize that hemicelluloses solubilize during low severity dilute acid, water-only/hydrothermal, and most possibly other thermochemical pretreatments such as soaking in aqueous ammonia (SAA) but may precipitate onto the cellulose surface upon cooling by forming strong bonds. Although evidence supports such precipitations and bond formation in pulping (Kabel et al., 2007; Köhnke et al., 2008), it has never been reported for pretreatment, and the impacts of such precipitations on cellulose enzymatic hydrolysis are not reported. This hypothesis requires further research and is beyond the scope of this study.

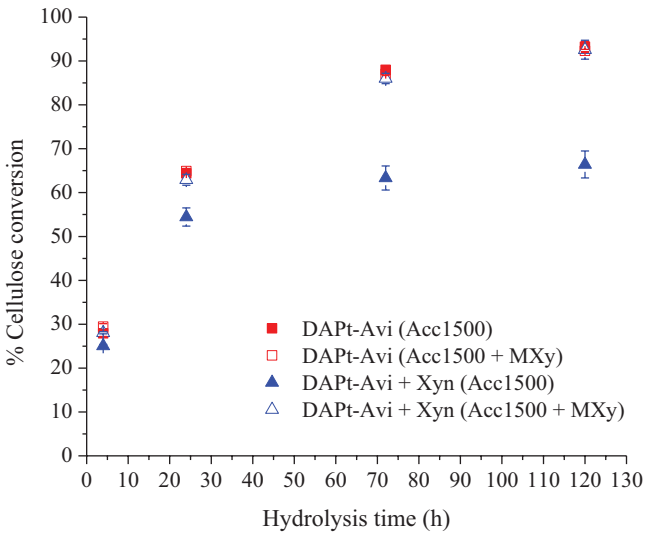

Figure 3. Effect of Multifect® xylanase (MXy) supplementation (7.5 mg protein/g glucan) of Accellerase® 1500 (Acc1500) cellulase (15 mg protein/g glucan) on cellulose conversion for washed solids from dilute acid pretreatment (140°C in 1 wt% sulfuric acid for 30 min) of Avicel by itself and mixed with xylan. DAPt- Avi—dilute acid pretreated Avicel cellulose; DAPt- Avi+ Xyn—dilute acid pretreated Avicel mixed with xylan

Digestibility of Solids Prepared at Other Severities

Although increasing pretreatment severity from 1.94 to 2.38 increased the amount of pseudo-lignin (wt% dry basis) from 0% to 0.53% for pretreatment of cellulose mixed with xylan, the conversion loss shown in Table II for CSF of 2.38 was less than for CSF 1.94: 16.8% versus 27.0% and 15.3% versus 31.0% at cellulase loading of 5 and 15 mg protein/g cellulose, respectively. However, conversion of cellulose in solids resulting from pretreatment of cellulose mixed with xylose that had more pseudo-lignin (1.5 wt%) dropped more for a CSF of 2.38 than for a CSF of 1.94 (from 65.2% to 47.6% and from 98% to 81.1% for cellulase loadings of 5 and 15 mg protein/g cellulose, respectively). For cellulose mixed with xylan, the drop in conversion for CSF 2.38 (160°C—0.5 wt%, 40 min) could be due to both pseudo-lignin and the negligible amount of residual xylan, if any, as shown earlier. However, for cellulose mixed with xylose, it appeared that the drop in conversion was exclusively due to pseudo-lignin that comprised about 1.5 wt% of solids by dry weight. For a CSF of 2.66 (160°C—1.0 wt%, 40 min), solids from pretreatment of Avicel cellulose mixed with xylan or with xylose had similar amounts of pseudo-lignin by dry weight (~3.5%). Furthermore, as shown in Table II, cellulose in both solids showed almost the same loss in conversion compared to the cellulose control prepared at the same severity (from 100% to 85.6% for cellulose plus xylan and 87.2% for cellulose and xylose at 15 mg protein/g cellulose cellulase mg/g cellulose).

Although solids from pretreatment of cellulose mixed with xylan or xylose at a CSF of 2.95 (170°C—1.0 wt%, 40 min) increased the amount of carbohydrate derived-pseudo-lignin (8–10% by dry wt.) from that at lower severities, cellulose digestibility dropped less than it did at lower severity conditions that produced less pseudo-lignin from xylose or xylan, as shown in Table II. The cause for this observation is not entirely clear but could be physiological/structural changes in pseudo-lignin formation at or beyond certain conditions/concentrations that affect cellulose hydrolysis. However, more work is needed to validate a mechanism. As shown earlier in Figure 2b, digestion of Avicel cellulose in solids pretreated at the most severe condition of CSF 3.56 dropped from 94.5% for untreated material to 66%, a 30% loss. Despite the fact that solids from pretreatment of Avicel cellulose mixed with xylan or xylose had similar amounts of pseudo-lignin as for Avicel cellulose alone, Table II shows that cellulose conversion for the former was only about 50% of the latter at a 15 mg/g cellulose cellulase loading. Thus, it appears that xylose derived-pseudo-lignin and their deposition on and/or co-existence with cellulose are more inhibitory to cellulase action than pseudo-lignin derived from six carbon sugars and/or their dehydration products (e.g., HMF and levulinic acid).

Pretreated Solids Characterizations

To determine changes in solids characteristics, pretreated solids prepared at various severities for Avicel cellulose alone and mixed with xylan or xylose were characterized by solid-state ^{13}C CP/MAS NMR, FT-IR, and SEM imaging. For comparison, untreated Avicel cellulose alone and physically mixed with xylan, xylose, and xylose-derived-pseudo-lignin were also characterized.

Solid-state ^{13}C CP/MAS NMR

The ^{13}C NMR spectra for xylose derived-pseudo-lignin and untreated and pretreated solids prepared at various severities are shown in Figure 4, with Table III showing the peak assignments for these spectra. The NMR spectra for untreated Avicel alone, Avicel mixed with xylan or xylose, and pretreated solids at most conditions were predominantly comprised of signals from the six carbons in cellulose. The NMR spectrum from xylose derived-pseudo-lignin showed broad peaks (due to spectral overlap) corresponding to aliphatic C, unsaturated C, and carbonyl (C=O) functionalities. These signals were also observed in dilute acid pretreated poplar holocellulose after pretreatment at CSF > 3.27 (Sannigrahi et al., 2011). Figure 4a shows spectra for untreated Avicel cellulose and cellulose pretreated at various severities, and spectra for dilute acid pretreated Avicel cellulose alone and mixed with xylan or xylose prepared at CSF 2.38, CSF 2.95, and CSF 3.56 are shown in Figure 4b–d, respectively. Figure 4a also presents cellulose crystallinity determined from NMR data, as discussed elsewhere (Foston and Ragauskas, 2010; Pingali et al., 2010), for untreated and pretreated Avicel cellulose solids. For solids from pretreatment at CSF 3.56, the percent crystallinity (%CrI) could not be determined due to weak signals for cellulose carbons. Figure 4a shows that there was no apparent change in cellulose structure, except at that the most severe condition (CSF 3.56) showed strong carbonyl, aromatic, and aliphatic signals. Cellulose crystallinity increased slightly with pretreatment severity (from 71.9% for untreated to about 75.8% for Avicel pretreated at CSF 2.95) and did not seem to have a negative impact on cellulose digestibility, as shown earlier in Figure 2b. Although wet chemistry compositional analysis (Table I) revealed that all pretreated solids prepared at CSF $\geq$ 2.38 had noticeable amounts of pseudo-lignin, NMR spectra only had strong signals previously attributed to pseudo-lignin for solids prepared at CSF 3.56 that had more than 80 wt% pseudo-lignin. Consistent with this, Sannigrahi et al., found that NMR spectra had strong signals attributed to pseudo-lignin only for solids prepared at CSF greater than 3.27, whereas compositional analysis revealed that solids prepared at and below CSF 3.27 also had appreciable amounts of insoluble compounds (about 30% by weight at CSF 3.27) that was measured as K-lignin. The spectra of samples prepared at CSF 3.56 were significantly different from those from other samples, with strong signals from carbonyl, aromatic, and aliphatic structures (Fig. 4d and Table III). In addition, these spectra differed from each other, probably due to changes in substrates. For instance, the methoxyl signals (56 ppm) were stronger

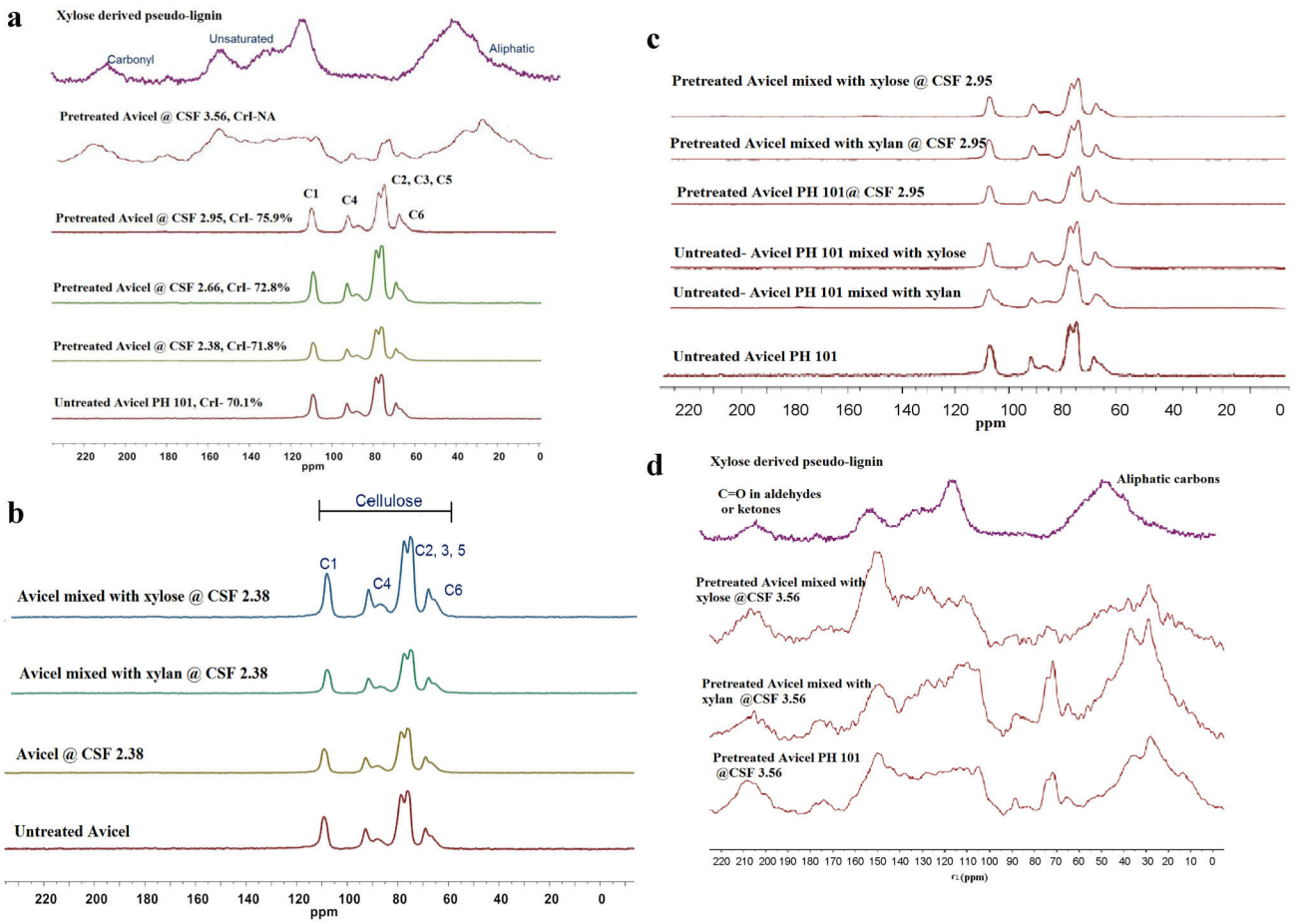

Figure 4. Solid-state ^{13}C CP/MAS NMR spectra of (**a**) untreated and dilute acid pretreated Avicel cellulose PH 101, along with crystallinity index (CrI) values calculated from NMR data, prepared at various severities, and comparison with xylose derived-pseudo-lignin; (**b**) dilute acid pretreated Avicel cellulose alone and mixed with xylan or xylose prepared at CSF 2.38; (**c**) untreated Avicel cellulose alone and physically mixed with xylan or xylose and pretreated solids prepared for these at CSF 2.95; and (**d**) dilute acid pretreated (CSF 3.56) Avicel cellulose alone and mixed with xylan or xylose. NA, not available.

for solids resulting from pretreatment of cellulose mixed with xylan or xylose than for sample cellulose alone, possibly due to large proportions of 4-*O*-methyl glucuronic acid in xylan.

Table III. Peak assignments in the NMR spectra.

Chemical shift (ppm)	Assignment
220–196	C=O in aldehydes or ketones
178–168	C=O in carboxylic acids or esters
155–142	Aromatic C–O
142–125	Aromatic C–C
125–102	Aromatic C–H
109–100	C1 of cellulose and/or C1 of xylan/xylose
92–86	The crystalline C4 of cellulose
86–79	The amorphous C4 of cellulose and/or C4 of xylan/xylose
79–68	C2, C3, and C5 of cellulose and/or C2, C3 of xylan/xylose
68–58	C6 of cellulose and/or C5 of xylan/xylose
60–55	Methoxy related to aromatic rings
50–10	Aliphatic carbons

FT-IR

FT-IR spectra of various solids used in this study are shown in Figure 5, with peak assignments presented in Table IV. Figure 5a shows FT-IR spectra for untreated Avicel cellulose alone and pretreated at various severities, and Figure 5b–d presents FT-IR spectra from pretreated solids prepared at various severities for cellulose mixed with xylan/xylose and their comparison with untreated cellulose and xylose derived-pseudo-lignin. Consistent with the NMR data, the Avicel cellulose structure remained intact at all conditions until a CSF of 3.56 and showed signals from cellulose. The pseudo-lignin sample showed a peak at 1594.7 cm^{-1} due to aromatic ring stretching that correlates to the unsaturated C signal seen in the NMR spectra and a peak at 1695.6 cm^{-1} arising from C=O stretching in ketones. However, these peaks were non-existent for pretreated samples prepared at severities below CSF 3.56. The strong peaks from cellulose and/or xylan and the weak peaks from the carbonyl and aromatic stretching regions in the spectra of these samples suggest that the carbohydrates in these

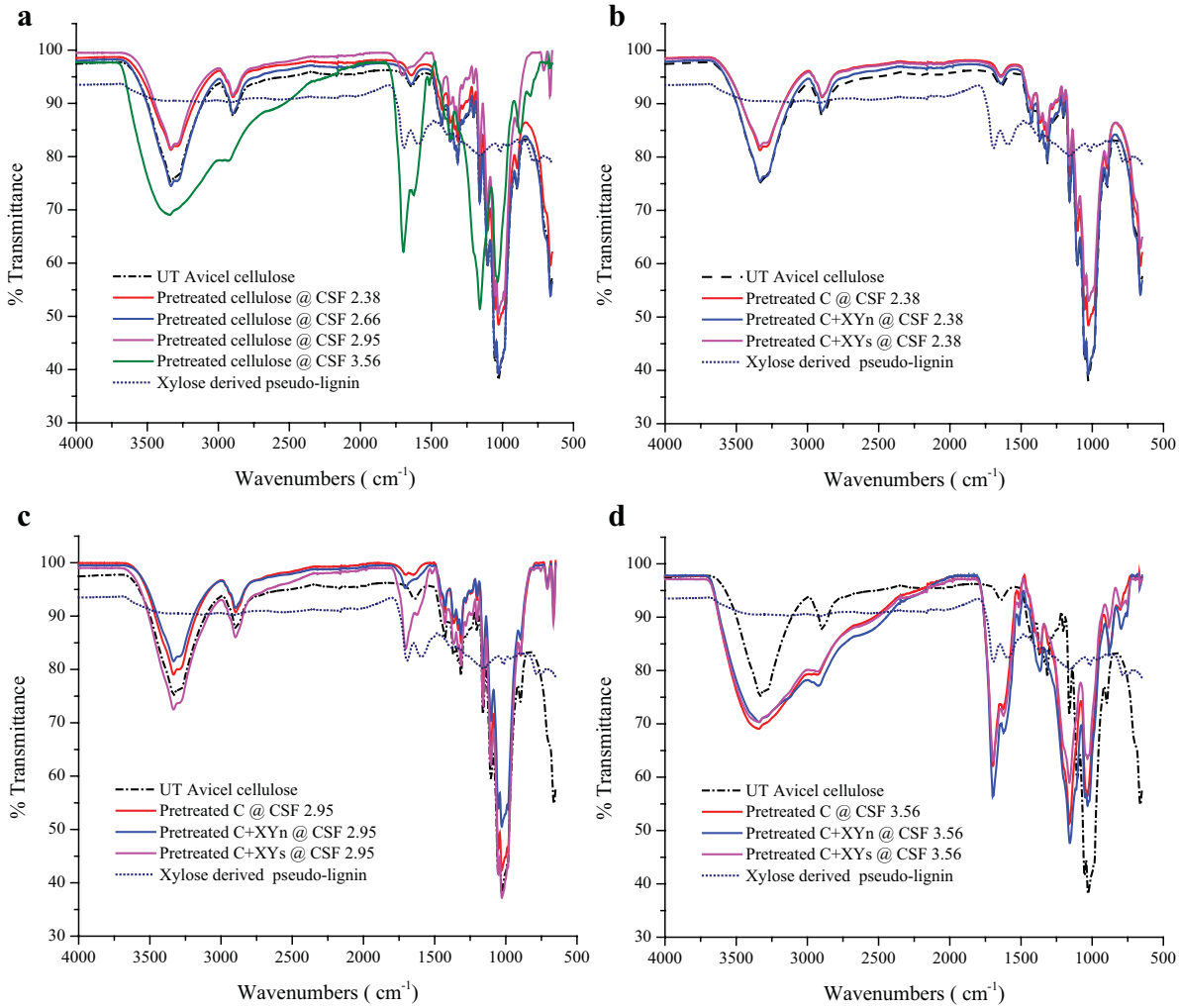

Figure 5. FT-IR spectra of untreated and dilute acid pretreated Avicel PH 101 cellulose prepared at various severities, and comparison with spectra for xylose derived-pseudo-lignin and dilute acid pretreated Avicel cellulose alone and when mixed with xylan or xylose: (**a**) untreated, (**b**) CSF 2.38, (**c**) CSF 2.95, and (**d**) CSF 3.56.

samples remained almost intact during pretreatment. However, compositional data suggested otherwise, as pretreated solids for cellulose mixed with xylan or xylose prepared at CSF 2.95 contained more than 8 wt% pseudo-lignin measured as Klason lignin. Therefore, it can be hypothesized that insoluble compounds other than those listed/identified here contribute to the positive K-lignin values in Table I. The FT-IR spectra from solids prepared at CSF 3.56 had strong peaks associated with the carbonyl and aromatic stretching regions. These FT-IR and ^{13}C CP/MAS NMR analyses of pseudo-lignin in samples for CSF 3.56 are consistent with results reported elsewhere (Sannigrahi et al., 2011).

SEM

The untreated Avicel cellulose, xylose derived pseudo-lignin, and pretreated solids were characterized using SEM. Several images were taken for each sample, with a few representative examples shown in Figure 6. Spherical droplets, which have been previously reported to be formed during pseudo-lignin formation (Sannigrahi et al., 2011), were seen in some of the samples. While the NMR and FT-IR data did not show

Table IV. Peak assignments in the FT-IR spectra.

Wavenumber (cm^{-1})	Assignment
890.2	β-Glycosidic linkages between sugar units
1054.5	C–O stretching at cellulose C-3, C-6 and C–C stretching
1162.1	Asymmetric bridge stretching of C–O–C in cellulose and hemicellulose
1314.3	$–CH_2$ wagging vibrations in cellulose and hemicellulose
1431.0	Symmetric $–CH_2$ bending in cellulose and hemicellulose
1594.7	Aromatic ring stretching (lignin)
1696.0	C=O stretching in unconjugated ketones
3342.91	O–H stretching in alcohols or phenols

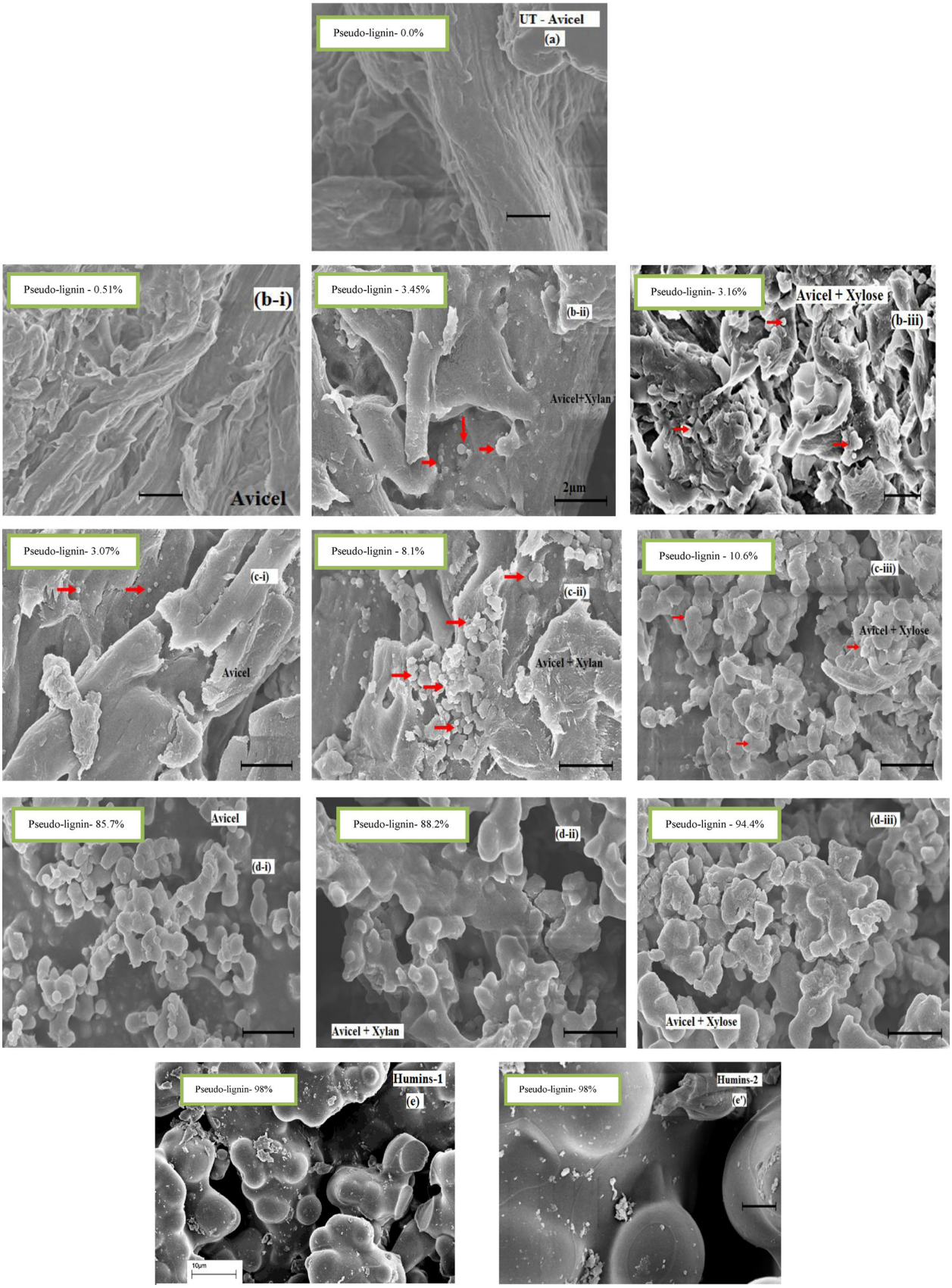

Figure 6. SEM images of (**a**) untreated Avicel cellulose (magnification 20k×) and of pretreated solids at (**b**) CSF 2.66 (magnification 20k×), (**c**) 2.95 (magnification 20k×), and (**d**) 3.56 (magnification 20k×). (**e**) and (**e′**) xylose derived-pseudo-lignin at 5k× and 20k× magnifications. Marker (**i**) designates solids from pretreatment of Avicel cellulose alone, (**ii**) from pretreatment of cellulose mixed with xylan, and (**iii**) cellulose mixed with xylose. For example, figure notation 6c-iii is for pretreated solids prepared at CSF 2.95 of cellulose mixed with xylose. Scale bar length = 2 µm, unless otherwise noted.

evidence of the formation of lignin-like materials for solids prepared at CSF less than 3.56, a few spherical droplets can be seen in the SEM images for solids pretreated even at CSF 2.66 (Fig. 6b) and even more for solids from pretreatment at CSF 2.95. More of these droplets were evident on the surface of the pretreated solids of cellulose mixed with xylan or xylose compared to cellulose pretreated alone at the same severities. However, unlike solids pretreated at high severities, where droplets seemed plentiful, as noted by arrows in Figure 6c and d (CSF 2.95 and 3.56, respectively), the occurrence of such drops were not widespread for severities less than 2.95, suggesting that these conditions were not severe enough to form significant amounts of solid degradation products, in agreement with the compositional data in Table I. The xylose-derived-pseudo-lignin sample shown in Figure 6e and e′ was entirely made up of pseudo-lignin spheres as were the solids prepared at higher severity shown in Figure 6c and d. These results add evidence that pseudo-lignin is formed from acid catalyzed degradation of carbohydrates that deposit on the surface and may cause the observed drop in cellulose conversion. It should also be noted that the pretreated solids before physical characterizations such as SEM were ground. However, to identify if pseudo-lignin form a film on cellulose surface, which is not possible to detect with the characterization techniques used in this study, advanced imaging and analytical tools should be applied in the future.

Plausible Mechanisms of Hydrolysis Retardation by Pseudo-Lignin

Reduced Cellulase Effectiveness Through Inhibition/Unproductive Binding

Lignin is known to unproductively bind cellulase and inhibit cellulase action (Kumar and Wyman, 2009b; Yang and Wyman, 2006). In an earlier study, we showed that addition of lignin derived from pretreated poplar prepared by leading pretreatment technologies to a hydrolysis mixture can significantly affect Avicel cellulose conversion thorough irreversible binding (Kumar and Wyman, 2009a). However, the effect varied with pretreatment type and method applied to isolate lignin from pretreated biomass (i.e., enzymatic vs. acidic hydrolysis). Similar to lignin, pseudo-lignin derived from hemicellulose/carbohydrates may also inhibit and/or bind cellulase unproductively and limit enzyme effectiveness. To evaluate whether pseudo-lignin affect cellulase effectiveness and bind cellulase unproductively, Avicel cellulose was hydrolyzed in the presence of pure xylose derived-pseudo-lignin (See Materials and Methods Section). The 120 h digestibility data in Figure 7a and b for cellulase loadings of 5 and 15 mg protein/g cellulose, respectively, show that the presence of xylose derived-pseudo-lignin did not have a major impact on the initial cellulose conversion. However, the effect became more pronounced as hydrolysis time was extended, with the extent depending on cellulase and pseudo-lignin loadings and the final cellulose digestibility, as indicated by the 120 h yields reported in Table V. It is interesting to note that even a small amount of pseudo-lignin (5 wt% of cellulose) had a noticeable negative impact on cellulose conversion at both cellulase loadings (21% and 9% reductions in 120 h conversion at 5 and 15 mg/g cellulose enzyme loadings, respectively). Furthermore, Table V shows that conversion seemed to decrease with pseudo-lignin loading but reached an asymptote. The cause (s) for such behavior is not clear at this point. Another interesting point was that at the highest pseudo-lignin loading (pseudo-lignin to cellulose ratio of 0.65) and for cellulase loading of 15 mg/g glucan, the decrease in cellulose conversion was almost comparable to the decrease in cellulose conversion with xylan (~22%; Table V). For the latter, however, the decrease in initial rate was much higher than for pseudo-lignin (55% vs. 5%) and was due to strong cellulase inhibition by xylooligomers, as shown elsewhere (Kumar and Wyman, 2009c). Adsorption of BSA and purified CBHI on pseudo-lignin in Figure 8a revealed that pseudo-lignin binds protein unproductively, while Figure 8b shows the relative amount of free protein in solution decreased as the pseudo-lignin loading was increased. Thus, it can be concluded that pseudo-lignin binds protein unproductively and can make less cellulase available for action on cellulose.

Reduced Cellulose Accessibility

Chemical and physical characterizations and enzymatic hydrolysis data showed that xylan degrades into insoluble compounds which can significantly impede cellulose digestibility. Although NMR and FT-IR characterizations did not show strong evidence of pseudo-lignin formation at low severities, SEM imaging, and compositional analysis revealed that pseudo-lignin formed even at low severities and deposited on the surface in spheres and/or co-existed with cellulose. Deposition of pseudo-lignin on the cellulose surface would directly affect its accessibility by blocking surface binding sites, and may be another possible cause for lower cellulose digestion.

Conclusions

Avicel cellulose alone and mixed with xylan or xylose was pretreated in dilute sulfuric acid over a range of conditions typically applied to cellulosic biomass to understand what effects xylan derived-pseudo-lignin might have on cellulose digestibility. For Avicel cellulose alone, dilute acid pretreatment solubilized a significant amount of cellulose and increased crystallinity by few points (from 70.1% to 75.9%) but did not affect cellulose digestibility negatively until the combined severity factor CSF was increased to 3.56, at which point a large fraction of pseudo-lignin appeared to be formed from cellulose and xylan/xylose degradation. However, pseudo-lignin formed from xylan/xylose degradation even at severities lower than often applied in dilute

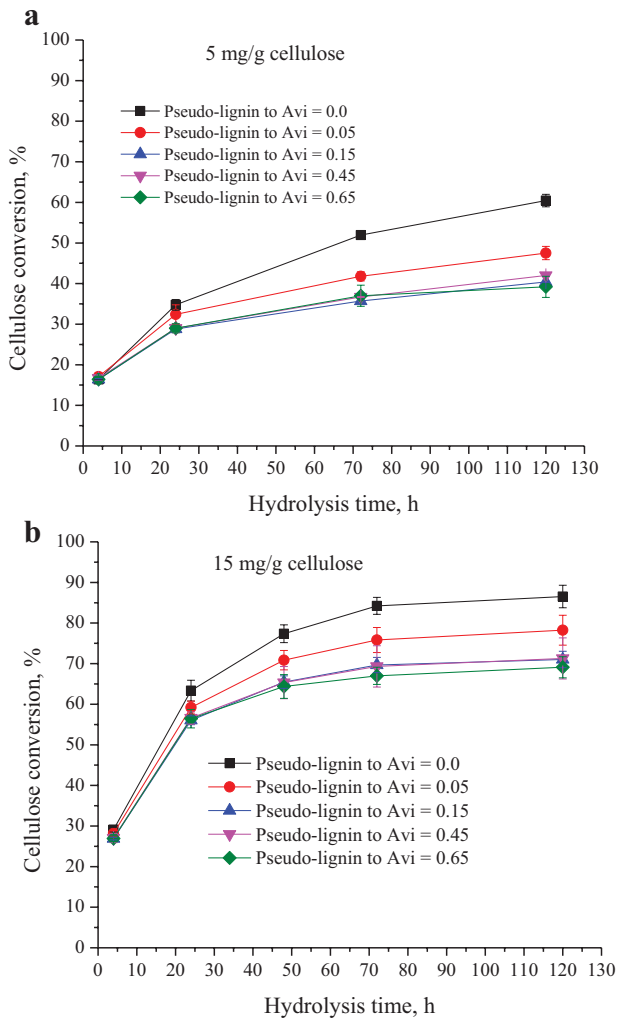

Figure 7. Effects of various levels of exogenously added pseudo-lignin on cellulose conversion at cellulase protein loadings of (**a**) 5 mg/g cellulose and (**b**) 15 mg/g cellulose.

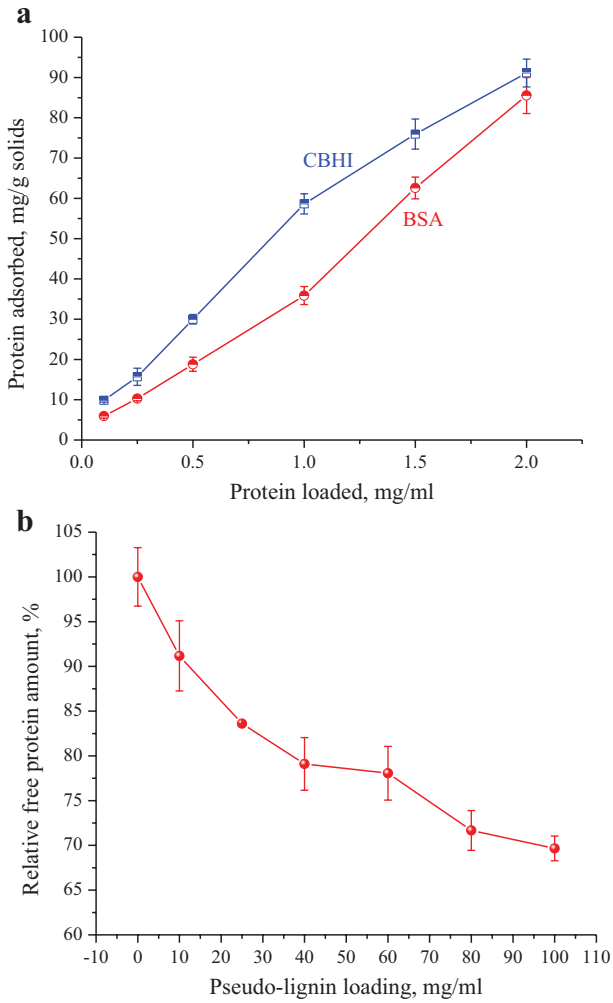

Figure 8. **a**: BSA and purified CBHI protein adsorption on xylose derived-pseudo-lignin (mg/g solids) at a pseudo-lignin solids loading of 10 g/L. **b**: Effect of pseudo-lignin loading on relative amount of free BSA protein in solution for a BSA loading of 2 mg/mL.

Table V. Percentage decrease[a] in cellulose conversion compared to Avicel cellulose control versus hydrolysis time for addition of xylan and various amounts of pseudo-lignin at enzyme loadings of 5 and 15 mg cellulase/g cellulose.

	5 mg cellulase/g cellulose				15 mg cellulase/g cellulose			
Substrate	4 h	24 h	72 h	120 h	4 h	24 h	72 h	120 h
C + pseudo-lignin-1	−4.4	6.8	19.6	21.4	3.2	6.6	10.0	9.6
C + pseudo-lignin-2	0.1	17.1	31.2	33.2	7.6	11.5	17.3	17.9
C + pseudo-lignin-3	−1.8	16.7	29.4	30.6	7.2	10.6	17.7	17.6
C + pseudo-lignin-4	0.1	16.7	28.7	35.1	7.5	10.9	20.4	20.1
C + xylan[b]	Not determined				55.0	33.4	24.0	22.0

C, Avicel cellulose; Pseudo-lignin-1, pseudo-lignin to cellulose weight ratio = 0.05; Pseudo-lignin 2, pseudo-lignin to cellulose weight ratio = 0.15; Pseudo-lignin-3, pseudo-lignin to cellulose weight ratio = 0.45; Pseudo-lignin-4, pseudo-lignin to cellulose weight ratio = 0.65.

[a]% decrease = 100×[1 − (Reference sample yield (%)/Control yield (%))].

[b]Xylan to cellulose weight ratio was 0.5.

acid pretreatment and affected cellulose hydrolysis through reduced cellulase effectiveness and/or cellulose accessibility. The impact of pseudo-lignin was magnified at lower enzyme loadings that would be commercially appropriate. Therefore, pretreatment effectiveness would be improved by avoiding sugar degradation that can lead to pseudo-lignin formation, particularly for subsequent application of lower enzyme loadings. Furthermore, it was noted that even a small amount of residual (non-structural) xylan, possibly precipitated on cellulose surface, significantly retard cellulose digestibility. However, this finding needs further research to understand hemicellulose (xylan) precipitation, its mechanism(s), and its effects on cellulose conversion.

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