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Alkaline Peroxide Delignification of Corn Stover

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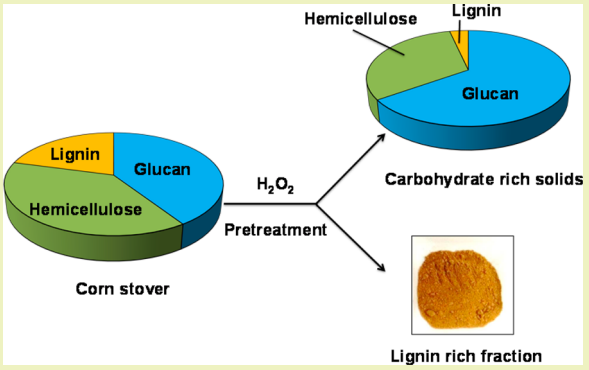
Supporting Information

ABSTRACT: Selective biomass fractionation into carbohydrates and lignin is a key challenge in the conversion of lignocellulosic biomass to fuels and chemicals. In the present study, alkaline hydrogen peroxide (AHP) pretreatment was investigated to fractionate lignin from polysaccharides in corn stover (CS), with a particular emphasis on the fate of the lignin for subsequent valorization. The influence of peroxide loading on delignification during AHP pretreatment was examined over the range of 30−500 mg H_2O_2/g dry CS at 50 °C for 3 h. Mass balances were conducted on the solid and liquid fractions generated after pretreatment for each of the three primary components, lignin, hemicellulose, and cellulose. AHP pretreatment at 250 mg H_2O_2/g dry CS resulted in the pretreated solids with more than 80% delignification consequently enriching the carbohydrate fraction to >90%. Two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy of the AHP pretreated residue shows that, under high peroxide loadings (>250 mg H_2O_2/g dry CS), most of the side chain structures were oxidized and the aryl-ether bonds in lignin were partially cleaved, resulting in significant delignification of the pretreated residues. Gel permeation chromatography (GPC) analysis shows that AHP pretreatment effectively depolymerizes CS lignin into low molecular weight (LMW) lignin fragments in the aqueous fraction. Imaging of AHP pretreated residues shows a more granular texture and a clear lamellar pattern in secondary walls, indicative of layers of varying lignin removal or relocalization. Enzymatic hydrolysis of this pretreated residue at 20 mg/g of glucan resulted in 90% and 80% yields of glucose and xylose, respectively, after 120 h. Overall, AHP pretreatment is able to selectively remove more than 80% of the lignin from biomass in a form that has potential for downstream valorization processes and enriches the solid pulp into a highly digestible material.



KEYWORDS: *Pretreatment, Lignin, Alkaline peroxide, Delignification*

INTRODUCTION

Thermochemical pretreatment of lignocellulose has long been studied as a means to make biomass polysaccharides more amenable to enzymatic and microbial deconstruction,[1−4] with the ultimate objective of producing sugars to be upgraded to biofuels and chemicals. The primary objective in pretreatment has traditionally focused on maximizing soluble sugar yields, with lignin being typically slated for combustion to provide heat and power to the biorefinery.[5−7] This scenario has meant that the effect of pretreatment on lignin chemistry and structure is often not a key consideration in pretreatment process development. More recently, however, the concept of lignin valorization in the biorefinery has witnessed a re-emergence[8−10] as a primary driver toward economic biofuels production, especially for the viable production of drop-in hydrocarbon biofuels.[11] This resurgence in lignin valorization research has prompted detailed re-evaluations of pretreatment strategies toward selective biomass fractionation processes, the purpose of which is to isolate both lignin and sugars in high yields and in forms amenable for downstream conversions. This requires an understanding of how pretreatment chemistry and processing conditions impact both polysaccharides and lignin structure.[12−14]

Alkaline pretreatment has been used extensively to separate lignin and alter the structure of biomass to make it more amenable to enzymatic hydrolysis. The main benefits of employing alkaline pretreatment are (1) selective separation of lignin from carbohydrates; (2) reduction of overall biomass recalcitrance by disrupting the lignocellulose matrix; and (3) limiting sugar losses via degradation reactions.[3,5] Alkaline pretreatment methods that have been investigated include sodium hydroxide,[15] lime,[16,17] alkaline hydrogen peroxide

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(AHP),[18−20] anhydrous ammonia,[14,21] and ammonia fiber expansion (AFEX).[22,23]

Among alkaline treatments, hydrogen peroxide has been used extensively in the pulp and paper industry to bleach mechanical and chemical pulps and enhance the pulp brightness.[24] Traditionally, hydrogen peroxide has been used as a non-delignifying agent with the addition of stabilizers, such as sodium silicate for peroxide stability.[25] Under alkaline conditions (pH 11.5), hydrogen peroxide dissociates to form the hydroperoxyl anion, which is responsible for the oxidation of chromophores in lignin through the cleavage of side chains.[26,27] In the absence of the stabilizers and in the presence of heavy-metal ions, hydrogen peroxide decomposes to highly reactive hydroxyl and superoxide anion radicals, which are known to be powerful oxidants and are responsible for delignification via depolymerization of lignin to yield low molecular weight water-soluble products.[24,26] Unfortunately, these radicals, along with delignification, also result in cellulose depolymerization, which is an undesirable reaction in achieving efficient lignocellulose fractionation. Thus, an optimization of operational variables, i.e., peroxide loading, residence time, and temperature, is required to achieve maximum delignification and carbohydrate retention.

Several researchers have contributed to the development of AHP pretreatment for enhancing the enzymatic saccharification of lignocellulosic feedstocks.[18−20,28−30] For example, Hodge and co-workers reported a high glucose yield of 95% from enzymatic hydrolysis of AHP-treated corn stover (CS) at 10% solids with a modest protein loading (15 mg/g of glucan).[20] Gould and co-workers reported obtaining glucose in near-quantitative yields after enzymatic hydrolysis of AHP-treated crop residues.[18,31] These reports and most others focused on enhancing the monomeric sugar yields via enzymatic saccharification of AHP-treated lignocellulosic feedstocks with less attention to date on the fate of solubilized lignin obtained under AHP pretreatment.[13,19,20,28−30,32]

In this work, AHP pretreatment is employed to fractionate CS into carbohydrates and lignin. The main objective is to investigate and identify the optimum operating conditions required to achieve efficient delignification while minimizing the solubilization of carbohydrates present in CS, which mirrors our efforts in alkaline pretreatment using NaOH.[15,33] AHP pretreatment was performed on raw CS in 2 L shake flasks at a range of operating conditions (peroxide loading, reaction time, temperature, and solids loading).[18−20] Mass balances are conducted on the solid and liquid fractions generated after pretreatment for each of the three primary components (lignin, hemicellulose, and cellulose), and the yield of the individual biomass components remaining in the pretreated CS is determined. In addition, to evaluate the potential utility of the solubilized lignin for fuels or chemicals, characterization is performed using liquid chromatography (LC) techniques, e.g., liquid chromatography/refractive index detection (LC/RID), liquid chromatography/mass spectrometry (LC/MS), and gel permeation chromatography/diode array detection (GPC/DAD), and by using heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy.

RESULTS AND DISCUSSION

Mass Solubilization. Figure 1A shows the total mass solubilized during AHP pretreatment of CS conducted at 50 °C. As expected, AHP pretreatment results in increased mass removal with increased peroxide loading. At the lowest peroxide loading of 30 mg H_2O_2/g dry CS, a mass removal of 12% was obtained, whereas a mass removal of 49% was obtained at the highest peroxide loading examined here (500 mg H_2O_2/g dry CS). The amount of mass removed from CS in this work is similar to those reported previously for AHP pretreatment of wheat straw,[18,32] bagasse,[34] and CS.[19] These data also suggest that mass removal increases linearly with the peroxide loading, resulting in up to 30% mass removal at 125 mg H_2O_2/g dry CS. After that, a significant increase in mass removal, of up to 45%, was obtained at 250 mg H_2O_2/g dry CS. However, by further raising the peroxide loading by 2-fold, to 500 mg H_2O_2/g dry CS, only a modest increase in mass removal (5%) was obtained, indicating that the effect of AHP pretreatment on mass solubilzation is almost complete at a peroxide loading beyond 250 mg H_2O_2/g dry CS, under the conditions studied here.

Solid Yield and Composition of Residual Solids. The residual dry solids yield and compositional analysis data of the residual pretreated solids obtained with AHP pretreatment conducted at 50 °C at increasing peroxide loading are provided in Table S1 in the Supporting Information. The data indicate that, as the peroxide loading increases from 30 mg H_2O_2/g dry CS to 500 mg H_2O_2/g dry CS, the solids yield decreases progressively from 88% to 51%. The effects of pretreatment conducted at 50 °C on the composition of the pretreated solid residue, as a function of peroxide loading, are shown in Figure 1B. The compositional analysis of the raw CS is shown for reference, which contains 36.3% ± 2.3% glucan, 34.2% ± 4.0% hemicelluloses (26.4% xylan, 5.3% arabinan, and 2.5% acetyl), 18.5 ± 1.6% lignin, and 11.3% others (5.5% ash and 5.8% water extractives).

As shown in Figure 1B, the glucan content of pretreated CS increases with increasing peroxide loading, reaching 65.2% at a peroxide loading of 500 mg H_2O_2/g dry CS from 36.3% raw CS. The hemicellulose content of pretreated CS also increases with increasing peroxide loading, reaching 35.1% at a peroxide loading of 250 mg H_2O_2/g dry CS, whereas, upon increasing the peroxide loading further, to 500 mg H_2O_2/g dry CS, it decreases to 31.4%. In contrast, the percentage lignin content of the peroxide pretreated CS decreases progressively with increased peroxide loading to 3.5% at a peroxide loading of 500 mg H_2O_2/g dry CS from 18.5% raw CS. Clearly, as the peroxide loading is increased, the percentage carbohydrate content increases to 96.6% at a peroxide loading of 500 mg H_2O_2/g dry CS from 70.5% raw CS, because of the selective removal of lignin, extractives, and hemicellulose (to some extent) and enrichment of the cellulose content present in the pretreated CS.

In addition to the peroxide loading, the effect of pretreatment temperature on the composition of the pretreated CS during AHP pretreatment was also evaluated at peroxide loadings of 60 and 90 mg H_2O_2/g dry CS (see Figure S1 in the Supporting Information). However, no noticeable effect of pretreatment temperature on the composition of the pretreated CS is observed during AHP pretreatment.

Component Yield and Mass Balance. The effects of peroxide loading on the solubilization of lignin, hemicellulose, and cellulose for pretreatment at 50 °C are shown in Figure 1C. Both lignin and hemicellulose removal increase with increasing peroxide loading, which is consistent with previous work.[18,19] At a peroxide loading of 90 mg H_2O_2/g dry CS, cellulose and hemicellulose removal are 8% and 27%, respectively, while lignin removal is 42%, whereas, at a peroxide loading of 125 mg

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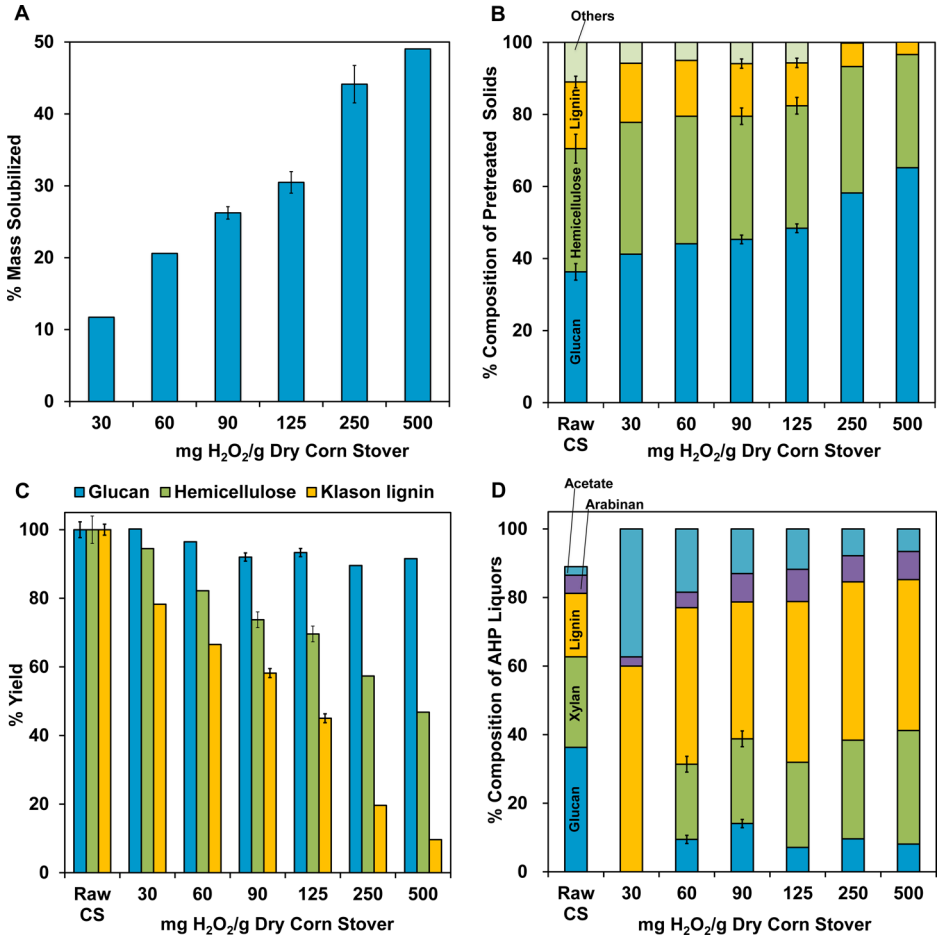

Figure 1. (A) Percentage of total mass solubilized, as a function of peroxide loading during AHP pretreatment of whole corn stover (CS) conducted at 50 °C at 10% solids loadings. (B) Composition of the untreated (Raw) and AHP-pretreated CS. (C) Percentage yields of lignin, hemicellulose, and cellulose remaining in the pretreated solids. (D) Composition of CS solubilized in liquors (w/w, excluding water). The composition of the liquor is calculated by difference from the measured composition of untreated CS and pretreated CS. The error bars show the standard deviation for the analyses conducted in triplicate.

H_2O_2/g dry CS, cellulose and hemicellulose removal were 7% and 30%, respectively, while lignin removal was 55%. Upon further increasing the peroxide loading to 250 and 500 mg H_2O_2/g dry CS, a significant increase in hemicellulose and lignin removal is observed. For example, a lignin removal of 80%−90% was achieved at 250−500 mg H_2O_2/g dry CS. However, under the same conditions, up to 50% hemicellulose and <10% cellulose solubilization was also observed. Selig et al.[19] conducted AHP pretreatment of CS under similar conditions (50 °C, 3 h, 1% (w/w) H_2O_2, 4% solids loading) and observed 56.3% and 17.6% removal of lignin and xylan, respectively. Correia et al.[35] conducted AHP pretreatment of cashew apple bagasse at 35 °C for 24 h with 4.3% (v/v) H_2O_2 and observed 65% mass removal and 80% delignification. Qi et al.[36] performed AHP pretreatment of wheat straw in a two-step method by initially treating straw with 1.5% (w/v) NaOH for 6 h, followed by a 6 h treatment with 0.3% (v/v) H_2O_2, both at 50 °C on a shaker (150 rpm) resulting in 20.1% removal of hemicelluloses and 65.9% delignification.

Lignin Removed during AHP Pretreatment. The pretreated liquors are enriched in solubilized lignin, hemicellulose, and extractives. For efficient utilization of these liquors, a detailed understanding of the yield and characterization of the solubilized products is essential. However, depending on the pretreatment conditions employed, these liquors consist of a myriad of organic compounds. In addition to chemical heterogeneity, these liquors are also sensitive to pH changes.[15,33] The chemical complexity, coupled with the pH sensitivity of these liquors, poses a serious challenge, with regard to their characterization, especially in the isolation and quantification of individual compounds. Nevertheless, to evaluate the utility of the AHP-pretreated liquors for the production of value-added products, the constituents of these liquors are estimated using the mass solubilization data (Figure 1A) and the composition of the untreated and pretreated CS (Figure 1B). In addition, qualitative analyses of the soluble compounds present in the liquors were performed using liquid chromatography techniques (e.g., LC/RID, LC/MS, and GPC/DAD).

Composition of Mass Solubilized in Liquors. The composition percentages of various biomass components solubilized in the liquors, obtained as a function of peroxide loading, are presented in Figure 1D. By comparing the composition of the liquors (dry basis) with the composition of the raw CS, one can infer that mostly lignin and acetate are solubilized at low peroxide loading, possibly due to the solubilization of easily accessible lignin present in the middle lamella of cell walls and liberation of the acetyl groups present on the xylan backbone, because of cleavage of the ester bonds under the alkaline conditions in AHP pretreatment.[37] It has

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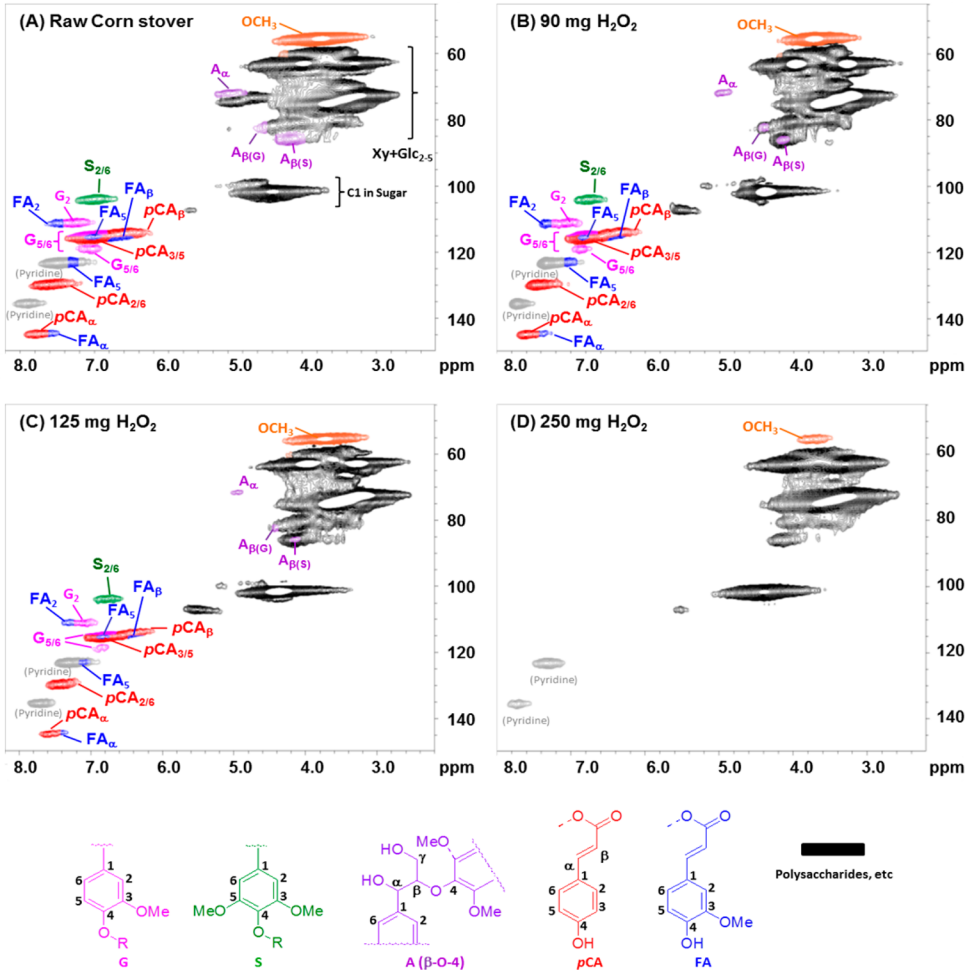

Figure 2. HSQC NMR spectra of alkaline peroxide pretreated CS: (A) raw CS, (B) 90 mg H_2O_2/g dry CS, (C) 125 mg H_2O_2/g dry CS, and (D) 250 mg H_2O_2/g dry CS. [Legend: G, guaiacyl; S, syringyl; A, β-O-4/β-aryl ether; *p*CA, *p*-coumarate; and FA, ferulate.]

been reported in previous studies that, under alkaline conditions, AHP pretreatment results in the formation of organic acids including lactic, formic, succinic, and acetic acids from sugar degradation reaction.[38] In addition, monomeric aromatic compounds, such as vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, and 4-hydroxybenzaldehyde, are also reported to be formed from alkaline wet oxidation of wheat straw.[39]

Solids Determination in AHP Pretreatment Liquors. The quantification of the solids content (soluble and insoluble materials) present in the liquors obtained from AHP pretreatment of CS conducted at multiple H_2O_2 loadings was performed and are shown in Figure S2 in the Supporting Information. The results indicate that the theoretical values of the solids content increases linearly as the peroxide loading increases, reaching up to 1.2 wt % at a peroxide loading of 500 mg H_2O_2/g dry CS. The values determined by the TAPPI and neutralization and centrifugation (NC) methods follow a similar trend but are generally lower, when compared to the theoretical values. A plausible explanation for obtaining lower solids content values could be the possible loss of the volatile compounds present in the liquor during drying. Also note that the difference in the solids content values determined by the NC method and the theoretical values is significantly larger for the liquors obtained at a high peroxide loading of 250−500 mg H_2O_2/g dry CS. Moreover, from Figure S2, it can also be inferred that the lignin solubility factor, which is obtained by dividing the yield of soluble solids by the amount of insoluble solids obtained from the NC method, increases from 9 to 67 at 250 mg H_2O_2/g dry CS and then decreases to 43 at 500 mg H_2O_2/g dry CS loading, which suggests that, at pH 7, the majority of solids present in the liquor remains soluble under the AHP pretreatment conditions studied here.

HPLC Analysis of Liquors. The HPLC analysis of these liquor samples (presented in Table S2) revealed that glucose and acetic acid were present in the 0.5−0.8 g/L range, which is expected, based on the glucan and acetate removal, according to the mass balance presented for AHP pretreatment (see Figure 3, presented later in this work). However, xylose and arabinose were present at an order-of-magnitude-lower concentration than expected at 2.5 and 0.7 g/L, respectively, based on the amount of xylan and arabinose solubilized during pretreatment. This observation is not surprising, since it is known that, under AHP pretreatment conditions, hemicellulose undergoes degradation reactions to form organic acids, including lactic acid, formic acid, succinic acid, and acetic acid.[38] However, not all of these products could be detected under the standard HPLC protocol utilized for sugar analysis; therefore, the liquor samples were analyzed using LC/MS for the detection of lignin degradation products.

LC/MS Analysis of Liquors. Monomeric aromatic compounds in the soluble lignin fraction obtained after AHP

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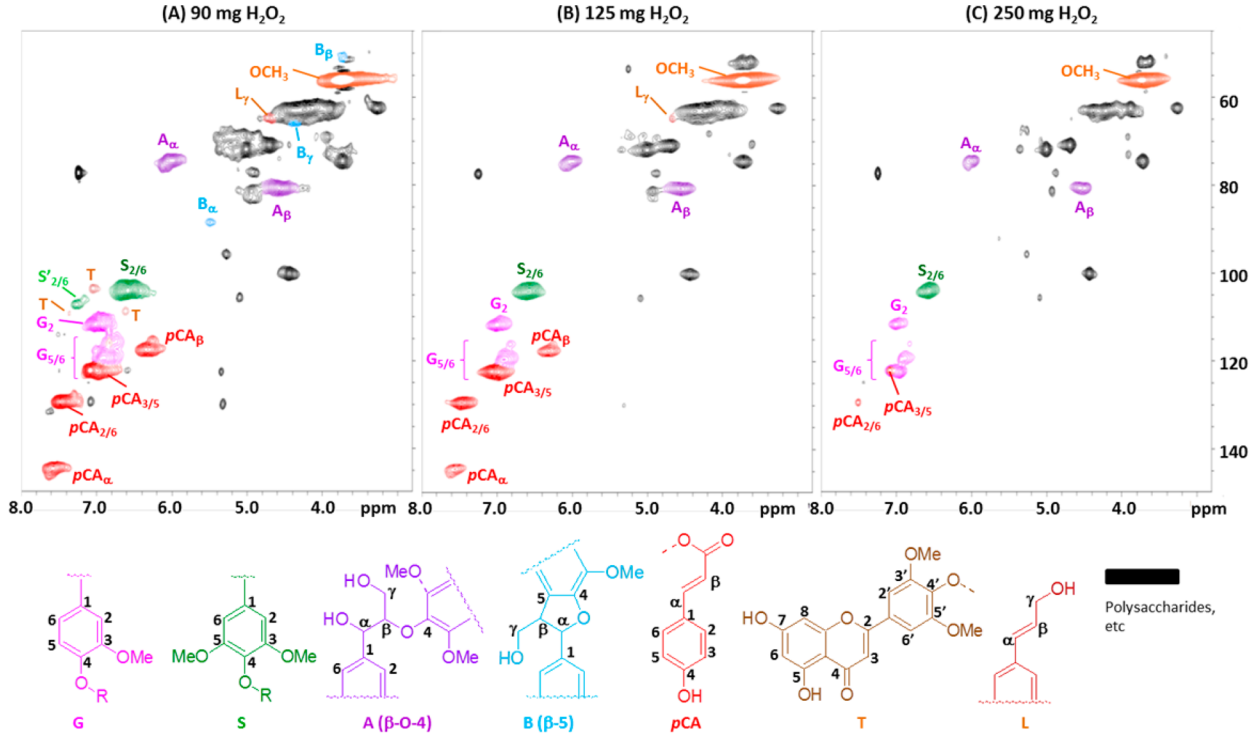

Figure 3. HSQC NMR spectra of aqueous soluble fractions after alkaline peroxide pretreatment with (A) 90 mg H_2O_2, (B) 125 mg H_2O_2, and (C) 250 mg H_2O_2. [Legend: G, guaiacyl; S, syringyl; A, β-O-4/β-aryl ether; B, β-5/phenylcoumaran; *p*CA, *p*-coumarate; T, tricin; and L, cinnamyl alcohol end.]

pretreatment were identified from tandem mass spectroscopy (MS/MS) experiments. Fragment ions from each eluting compound peak were induced from collision energies and manually interpreted. Compounds for which authentic standards were available were compared for designation accuracy. The resulting identifications are presented in Table S3 in the Supporting Information. Analytes consisting of vanillic acid, *p*-coumaric acid, ferulic acid, syringic acid, and 4-hydroxybenzaldehyde appeared to be the base constituents for the majority of compounds released from AHP pretreatment. This indicates that AHP pretreatment of CS correlates with aromatics released from wheat straw with alkaline wet oxidation.[39]

2D NMR of Insoluble Solids and Soluble Lignin. To obtain structural information on insoluble and soluble fractions obtained after AHP pretreatment, both fractions were analyzed by HSQC NMR spectroscopy. In the whole CS (Figure 2A), the β-O-4 unit is the most abundant interunit linkage, with other linkages, such as resinol and phenylcoumaran present in small amounts. Ferulate and *p*-coumarate units are present, but no peaks for the *p*-hydroxyphenyl unit were present. Anomeric peaks from both cellulose and hemicellulose also appear at δ_C/δ_H 95−105/3.6−5.0 and 57−100/2.8−5.2 ppm, respectively. In AHP-pretreated CS obtained with 90 mg H_2O_2/g dry CS (Figure 2B), β-O-4 units decrease while other ferulate and *p*-coumarate units do not change significantly, indicating that only side chain structures of lignin were oxidatively degraded, whereas conjugated double bonds on the aromatic rings do not react with the hydroxyl radical generated under mild peroxide concentrations. Hemicellulose peaks at δ_C/δ_H 70−75/4.4−5.2 diminished due to hemicellulose losses under AHP pretreatment, as confirmed by compositional analysis data (Figure 1C). In AHP-pretreated CS obtained with 125 mg H_2O_2/g dry CS (Figure 2C), increased reduction in β-O-4 units is observed, compared to the other aromatic peaks in ferulate and *p*-coumarate units, whereas, in AHP-pretreated CS obtained with 250 mg H_2O_2/g dry CS (Figure 2D), both β-O-4 unit as well as lignin aromatic peaks disappeared, suggesting that, under high peroxide concentration, most of the side-chain structures were oxidized and the aryl-ether bonds are dissociated. The lignin content in the solid residue obtained with 250 mg H_2O_2/g dry CS is 5−8 wt % (Table S1), indicating that ∼20% of the initial CS lignin still remains in the solid. However, no lignin peaks in the gel-state HSQC spectrum were detected. The most plausible explanation for this observation is that some low molecular weight (LMW) lignin moieties present in close association with hemicellulose in the untreated CS were selectively removed into the aqueous soluble fraction after AHP pretreatment, leaving high molecular weight (HMW) lignin in the solid residue with altered structure and limited functionalities that are required for gel formation after reaction with d_6-DMSO/d_5-pyridine.

2D-HSQC NMR was also performed to characterize the interunit linkages and functionalities of the lignin solubilized into the aqueous phase. HSQC spectra of the acetylated soluble lignin fractions are shown in Figure 3. In the soluble fraction obtained with 90 mg H_2O_2/g dry CS (Figure 3A), large peaks for the β-O-4 unit are detected, as well as small peaks for phenylcoumaran (β-5) units. *p*-Coumarate peaks appeared while ferulate was not detected, although both units were detected in the corresponding AHP-pretreated CS solids obtained with 90 mg H_2O_2/g dry CS, implying that *p*-coumarate is more easily liberated under alkaline conditions in AHP pretreatment than ferulate. The presence of oxidized syringyl units and a small amount of tricin are also observed. In the soluble fraction obtained with 125 mg H_2O_2/g dry CS (Figure 3B), a slight decrease in the β-O-4 unit was observed.

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Both phenylcoumaran (β-5) and tricin units also disappear, potentially due to cleavage of ether linkages in these units. The peaks for guaiacyl and *p*-coumarate units also decrease slightly, whereas the peak for oxidized syringyl units are absent, likely due to complete oxidation of the side-chain structure to syringaldehyde or syringic acid. In the soluble fraction obtained with 250 mg H_2O_2/g dry CS (Figure 3C), β-O-4, syringyl, and guaiacyl units remain, although the amount of *p*-coumarate decreases dramatically, likely due to the oxidative losses. Hemicellulose peaks decrease in the soluble fractions with increased peroxide concentration, which are likely ring-opened to small carboxylic acids.[38] Overall, the HSQC data of the insoluble and soluble fractions show that AHP pretreatment selectively solubilizes lignin, along with hemicellulose at higher peroxide loadings (>125 mg H_2O_2/g dry CS).

GPC Analysis of Soluble Lignin Removed during AHP Pretreatment. GPC was conducted on the freeze-dried soluble fractions obtained after AHP pretreatment of CS at various peroxide loadings to obtain the molecular weight (MW) distribution. The MW distributions of the lignin (which is the primary biomass component that absorbs in the ultraviolet (UV) range) in the soluble fractions and the ball-milled lignin are shown in Figure 4, and their weight-average molecular

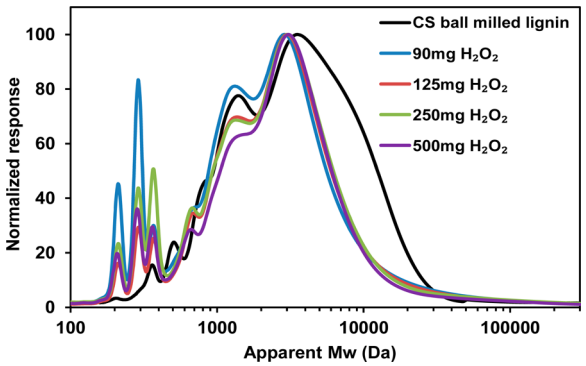

Figure 4. GPC chromatograms of CS ball-milled lignin and lignin solubilized, as a function of peroxide loading during AHP pretreatment of whole CS conducted at 50 °C at 10% solids loadings.

weight (M_w) and polydispersity (PD) results are given in Table 1. The GPC profile in the control sample (ball-milled lignin)

Table 1. Impact of AHP Pretreatment on the Molecular Weight of Extracted Lignin

H_2O_2 loading (mg/g dry CS)	number-average molecular weight, M_n	weight-average molecular weight, M_w	polydispersity, PD
CS ball-milled lignin	1700	6400	3.8
90	890	5600	6.2
125	1200	6000	5.0
250	1000	5900	5.9
500	1100	5600	5.0

exhibits a minor peak between ~200−600 Da, which is attributed to monomeric and LMW species, followed by a broad peak up to 40 000 Da, representing HMW lignin. The soluble lignin fraction obtained after AHP pretreatment with 90 mg H_2O_2/g dry CS (Figure 4) exhibits the lowest M_w value, with a large monomer peak (M_w < 450 Da). The soluble lignin fractions obtained at high peroxide loadings (125−500 mg H_2O_2/g dry CS) exhibit a trend roughly similar to that of 90 mg H_2O_2 extracted lignin with a difference of reduction in the peak at 1000−2000 Da with increasing peroxide loading, which corroborate the 2D NMR results that more ether and ester bonds were cleaved at increased peroxide loadings. A reduction in the number-average MW (M_n) by one-half and an increase in the polydispersity (PD) (Table 1) show that AHP pretreatment effectively depolymerizes HMW lignin in CS into LMW lignin fragments in the aqueous fraction, which could be valorized to value-added products.[40−42]

Multiscale Microscopic Characterization of AHP-Pretreated Solids. Surface examination of the pretreated CS particles revealed a pattern of increasing bleaching with increasing H_2O_2 loading (Figures 5a−5e). The 500 mg H_2O_2/g dry CS-treated samples appear nearly completely white (Figure 5e). The other trend revealed by stereoscope examination is particle fragmentation and decreasing particle size. At the two higher H_2O_2 loadings (250 and 500 mg H_2O_2/g dry CS), there is an abundance of fine particles (arrows features in Figures 5d and 5e) and the large appearing particles are actually clusters of many much smaller particles (marked by asterisks in Figures 5d and 5e).

Using confocal scanning laser microcopy (CSLM), the untreated cell walls displayed bright, relatively uniform staining (Figure 5f). At the lower H_2O_2 pretreatment concentrations (90 and 125 mg H_2O_2/g dry CS), the walls are less bright than untreated (indicative of lignin loss) and show evidence of some delamination within secondary cell walls (features marked by arrows in Figures 5g and 5h). At the higher H_2O_2 loadings, the wall staining is much less bright, but fairly uniform, lacking the evidence of discrete delamination in the S1 and S2 layers of the secondary wall. However, these samples also display an abundance of cell disjoining at the middle lamella (Figure 5i, j arrows) which is consistent with our earlier findings where removal of the lignin present in the middle lamella resulted in less-recalcitrant plant cell walls with enhanced susceptibility for xylan hydrolysis during pretreatment.[43]

By transmission electron microcopy (TEM), the untreated secondary cell walls appear uniformly dense and finely textured. In the samples obtained with lower H_2O_2 loadings (90 and 125 mg H_2O_2/g dry CS), the secondary walls display a more granular texture and a clear lamellar pattern indicative of layers of varying lignin removal or relocalization (features marked by arrows in Figures 5l and 5m). The 250 mg and 500 mg H_2O_2/g dry CS-pretreated samples display a uniform electron density without the layered pattern of the lower pretreatment severities. They are also much less densely stained (indicative of lignin loss) than the untreated secondary cell walls and show evidence of multiple delaminations (features marked by arrows in Figures 5n and 5o). The particle, tissue, and cell wall scale all provide evidence for a surprising change in the physical impact of AHP pretreatment between the H_2O_2 loadings of 125 and 250 mg H_2O_2/g dry CS. At loadings of 250 mg H_2O_2/g dry CS and above, the particle size is more reduced, yielding an abundance of fines; the cells within vascular tissues become separated at the middle lamella, and the secondary walls show a uniform loss of lignin and develop delaminations.

Glycome Profiling of AHP-Pretreated Solids. We conducted glycome profiling[44] on untreated CS and AHP-pretreated residues to delineate the pretreatment-induced alterations in the compositional and structural cell wall features, and extractabilities of most major noncellulosic matrix glycan components in these residues (see Figure S3 in the Supporting

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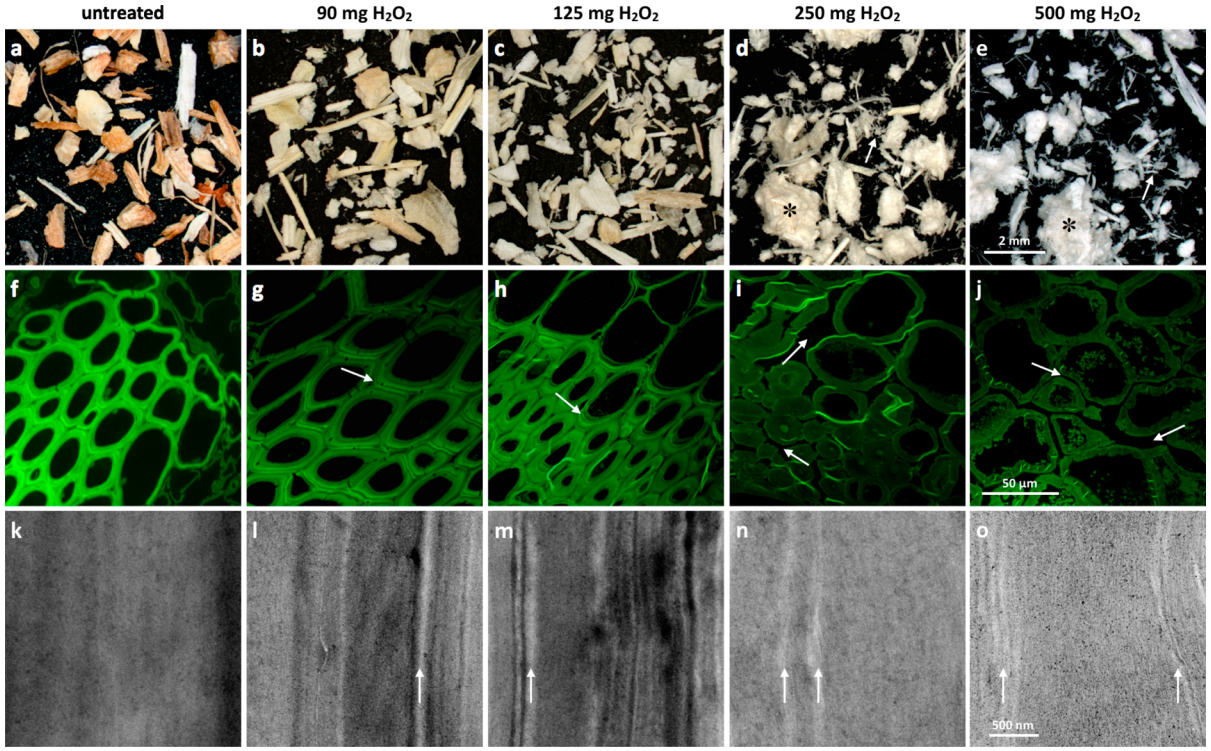

Figure 5. (a–e) Stereoscope micrographs, (f–j) confocal scanning laser micrographs, and (k–o) transmission electron micrographs of AHP-pretreated CS treated with varying H_2O_2 loadings.

Information). Detailed cell wall characteristics, as revealed by glycome profiling, have been reported for the untreated CS in this work.[14] As expected, significant changes were observed in the glycome profiles of AHP-pretreated CS residues (Figure S3). Overall, cell wall extractabilities, based on the amounts of carbohydrate materials released, follow an identical pattern in all samples analyzed with 1 M KOH extract releasing the highest amounts of carbohydrate materials (see bar graphs at the top of Figure S4 in the Supporting Information). However, there are variations in the amounts recovered in 4 M KOH extractions, wherein 90 and 125 mg H_2O_2/g dry CS residues show higher amounts (bar graphs, top of Figure S4).

Generally, all AHP-pretreated residues exhibit obvious changes in the glycome profiles, the clearest of which is the increased extractability of xylan epitopes including unsubstituted homoxylan epitopes and substituted xylan epitopes. This is indicated by the significantly enhanced abundance of these xylan epitopes in the least harsh extracts, such as oxalate and carbonate extracts. Note that even the oxalate extract from the lowest regime of pretreatment, 60 mg H_2O_2/g dry CS residues, also exhibits the presence of xylan epitopes that are recognized by xylan-4, xylan-5, and xylan-7 groups of mAbs. An increase in the extraction of xylan epitopes by the least harsh extracts of AHP-pretreated CS corroborates the recent work of Hodge and co-workers.[45] The abundance of almost all xylan epitope classes (unsubstituted and substitutes xylan epitopes) were significantly higher in the carbonate extracts from all pretreated residues, irrespective of the regimes applied, compared to the untreated residues. Such pretreatment-induced enhanced extractability of noncellulosic matrix glycans was also evident in the case of pectic backbone and pectic arabinogalactan epitopes. For instance, invariably, oxalate and carbonate extracts from all pretreated residues exhibit a higher abundance of RG-I backbone epitopes (recognized by the increased binding of RG-I backbone group of mAbs). Enhanced extractability of homogalacturonan backbone epitopes were also noted in oxalate extracts from all pretreated residues. Such a pattern of enhanced extractability was also evident in the case of pectic-arabinogalactan epitopes in pretreated residues (indicated by the increased binding of RG-Ic, RG-I/AG, and various AG groups of mAbs). Overall, the above results show significantly enhanced extractability of hemicellulosic glycans, from AHP-pretreated residues in the least harsh extracts, such as oxalate and carbonate, highlighting the effectiveness of AHP pretreatment in reducing biomass recalcitrance and facilitating separation of lignin from carbohydrate fraction.

Enzymatic Hydrolysis of AHP Pretreated Solids. The glucose and xylose yields obtained after enzymatic hydrolysis of AHP pretreated CS are shown in Figure 6. The extent of glucan and xylan conversion was calculated based on the release of cellobiose, glucose, and xylose during hydrolysis. As expected, the untreated CS showed poor susceptibility toward enzymatic hydrolysis, with 23% and 17% glucose and xylose yields, respectively. Conversely, the effect of AHP pretreatment on enzymatic hydrolysis is clearly observed with higher glucose and xylose yields obtained at increased peroxide loading. For example, the maximum glucose and xylose yields of 69% and 57%, respectively, were obtained for CS pretreated with 125 mg H_2O_2/g dry CS. Furthermore, there is a significant increase in both the rate and extent of glucan and xylan conversion of pretreated CS obtained at peroxide loadings of 250 and 500 mg H_2O_2/g dry CS. For CS pretreated with 250 and 500 mg H_2O_2/ g dry CS, a high glucose yield of 72% and 88%, respectively, was obtained in just 4 h which plateau at 98% and 88%, respectively, at 16 h (Figure 6A). A higher xylose yield of 80% was obtained for CS pretreated with 250 mg H_2O_2/g dry CS, compared to a xylose yield of 70% for CS pretreated with 500 mg H_2O_2/g dry CS in 16 h (see Figure 6B).

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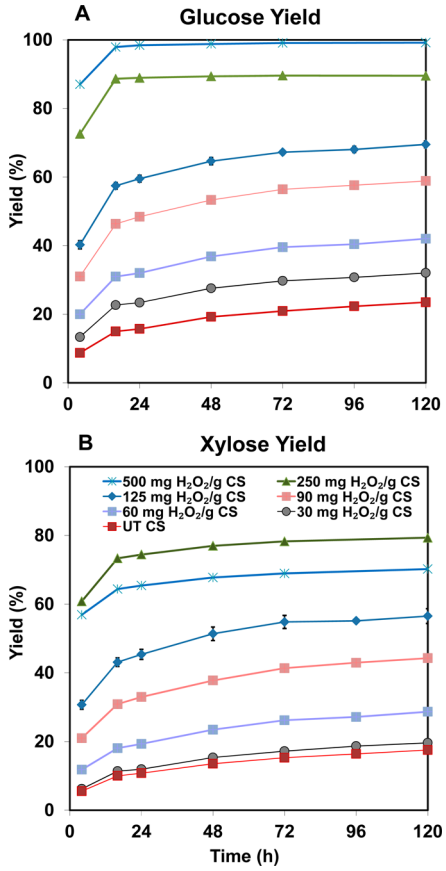

Figure 6. (A) Glucose and (B) xylose yields obtained after enzymatic hydrolysis of AHP-pretreated CS treated with varying H_2O_2 loadings. Enzymatic hydrolysis was performed at 1% solids with 20 mg (16 mg CTec3 + 4 mg HTec3)/g glucan. The error bars show the standard deviation for the analyses conducted in triplicate.

A significant increase in the glucan and xylan hydrolysis obtained for CS pretreated at peroxide loadings of >250 mg H_2O_2/g dry CS indicates that, under these loadings, substantial changes occur in the composition and structure of pretreated CS. This can be inferred from Figure 1A, where a significant increase in mass removal is observed above 250 mg H_2O_2/g of dry CS; as a consequence, considerable changes in the composition of AHP-pretreated residues is observed. In Figure 1B, the main difference observed between the CS pretreated at 125 and 250 mg H_2O_2/g dry CS is that, in the latter, all the extractives were solubilized, resulting in a noticeable increase in cellulose content and delignification. Similarly, in Figure 1C, it can be observed that, at a peroxide loading of 125 mg, the lignin removal was 55%, which increased to 80% and 90% at the peroxide loadings of 250 and 500 mg H_2O_2/g dry CS, respectively. Moreover, as revealed in Figure 5, noticeable structural changes in the pretreated CS occur with increased peroxide loadings, especially at the peroxide loadings of 250 mg H_2O_2/g dry CS and above, where the particle size is reduced, yielding an abundance of fines, the cells within vascular tissues become separated at the middle lamella, and the secondary walls show a uniform loss of lignin and develop delaminations. These changes in the compositional and structural features (at high peroxide loadings of >250 mg H_2O_2/g dry CS) of the pretreated CS render it highly susceptible to enzymatic hydrolysis. This is in agreement with the results reported by Gould and co-workers,[18] in which delignification of agricultural residues was most efficient at the peroxide loading of at least 0.25 g H_2O_2/g biomass. It is important to note that, upon increasing the peroxide loading further to 500 mg H_2O_2/g dry CS, an increase in glucan conversion is accompanied by a reduction in the xylan conversion. One plausible explanation for this observation could be the reduced accessibility of cellulose microfibrils, because of the localized cell-wall collapse phenomena driven by the migration and extrusion of the solubilized lignin,[46] especially due to significant delignification (~90%) of the pretreated residue obtained with CS pretreated with 500 mg H_2O_2/g of dry CS.

The high rate and extent of enzymatic hydrolysis obtained in this work with increased peroxide loading are in accordance to the results reported in the literature.[19,20,26] Both the rate and extent of enzymatic hydrolysis of biomass are considered to be influenced by the presence of hemicellulose and lignin and have a tendency to increase with an increased level of removal of both hemicellulose and lignin.[47,48] Both hemicellulose and lignin act as a physical barrier to cellulose, thus restricting cellulase accessibility. Moreover, it is well-known that non-specific binding of cellulases to lignin results in the loss of cellulase activity, decreasing the overall yields of cellulose hydrolysis.[49−52] As shown in Figure 1C, a significant amount of lignin is removed with increased peroxide loading, thereby resulting in carbohydrate-enriched residue. This is evident in Figure S4A in the Supporting Information, where the carbohydrate content in the pretreated residue increases linearly with increased delignification, reaching up to 96% carbohydrate at 90% delignification for the pretreatment with 500 mg H_2O_2/g of dry CS. Because of the increasing carbohydrate content of AHP-pretreated CS residues obtained at increasing peroxide loadings, glucan and xylan conversions were expected to increase with increased delignification. These trends are clearly shown in Figure S4B in the Supporting Information. Both glucan and xylan conversions exhibit a strong linear correlation with lignin removal, reaching 90% and 80% conversion for glucan and xylan, respectively, within 72 h at a lignin removal of >80%.

From the data reported above, it is evident that high lignin removal and enzymatic hydrolysis conversions of up to 100% and >80% of the glucan and xylan, respectively, are realized at peroxide loadings >250 mg H_2O_2/g dry CS. However, the relatively higher cost of hydrogen peroxidem compared to other alkaline catalysts, e.g., NaOH coupled with high peroxide loadings would put an additional cost burden on the economic feasibility of such a process. Therefore, utilization of the AHP process industrially would require finding opportunities to both recycle the catalyst and valorization of lignin streams to value-added products in high yields.[40−42]

CONCLUSIONS

Alkaline hydrogen peroxide (AHP) pretreatment of corn stover (CS) has been investigated over the range of 30−500 mg H_2O_2/g dry CS at 50 °C for 3 h, with a particular emphasis on the fate of lignin. Lignin removal increased with increasing peroxide loading and H_2O_2 loading of 250 mg/g dry CS resulted in the removal of more than 80% lignin with >90% carbohydrates remaining in the pretreated residual solids. Two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy of the AHP-pretreated residue shows that, under high peroxide loadings (>250 mg H_2O_2/g dry CS), most of the side-chain structures were oxidized and the main aryl-ether bonds in lignin were absent, given the significant delignification of the

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pretreated residues. Gel permeation chromatography (GPC) analysis suggests that AHP pretreatment effectively depolymerizes CS lignin into low molecular weight (LMW) lignin fragments in the aqueous fraction. Imaging of AHP-pretreated residues shows, especially at the 250 mg H_2O_2/g dry CS loading and above, that the particle size is more reduced, yielding an abundance of fines, the cells within vascular tissues become separated at the middle lamella, and the secondary walls show a uniform loss of lignin and develop delaminations. Enzymatic hydrolysis of this pretreated residue with Ctec3 and Htec3 (20 mg/g glucan) resulted in 90% and 80% yields of glucose and xylose, respectively, after a digestion period of 120 h.

Overall, the results of this work show that AHP pretreatment was able to selectively delignify CS and obtain highly digestible carbohydrate-rich pretreated residues while promoting the depolymerization and subsequent solubilzation of a large portion of lignin in the aqueous fraction, which could potentially be valorized and aid the overall economics of the lignocellulosic biorefinery.[11,53]

EXPERIMENTAL SECTION

Alkaline Hydrogen Peroxide (AHP) Pretreatment. AHP pretreatment was performed on raw corn stover (CS) in a 2 L shake flask in a shake incubator for 3 h maintained at 50 °C. A 30% (w/w) stock solution of hydrogen peroxide was added in the range of 30−500 mg H_2O_2/g dry CS, followed by the addition of deionized (DI) water to obtain a solids loading of 10% (w/v). The pH of the suspension was adjusted to 11.5 ± 0.2 by adding 5 M NaOH with additional NaOH being added during the course of the pretreatment to maintain the pH at 11.5 ± 0.2. After 3 h, the pretreated suspension was filtered through a coarse felt, and the solids fraction was thoroughly washed with DI water to neutral pH and stored at 4 °C. In addition to investigate the effect of hydrogen peroxide loading on lignin solubilization, the effect of temperature was also investigated by varying the pretreatment temperature in the range of 25−80 °C at two peroxide loadings (i.e., 60 and 90 mg H_2O_2/g dry CS). To determine the structural carbohydrates and lignin components of the pretreated solids, the compositional analysis was conducted according to standard NREL Laboratory Analytical Procedures (LAPs).[54,55]

Total Solids Determination in AHP-Pretreated Liquors. The carbohydrate content of the wash fraction was measured according to the NREL LAPs.[56] The solids content (soluble and insoluble materials) present in the liquors obtained from AHP pretreatment of CS conducted at multiple H_2O_2 loadings was determined. Two different methods—namely, neutralization and centrifugation (NC) and a conventional method[57]—were employed to determine the total solids present in the liquors and were compared to the theoretical yield, which was determined by the difference of the initial mass of CS and the mass of pretreated residue obtained after pretreatment. In the NC method, 25 g of the liquor was first neutralized, followed by centrifugation at 8500 rpm to separate insoluble material. Both soluble and insoluble fractions obtained after centrifugation were freeze-dried to determine the total solids of the liquor. These values were corrected for the sodium sulfate salt generated by neutralization of the liquor. In the conventional method,[57] the total solids were determined by drying 25 g of liquor in a crucible containing high-silica sand in a 105 °C oven overnight. These values were corrected for the amount of NaOH added during AHP pretreatment.

X-ray Diffraction. The crystallinity indexes (CrI) of both untreated and AHP-pretreated CS samples were measured by X-ray diffraction (XRD). using a Rigaku (Tokyo, Japan) Ultima IV diffractometer with Cu Kα radiation having a wavelength $\lambda(K\alpha_1)$ = 0.15406 nm generated at 40 kV and 44 mA. The diffraction intensities of freeze-dried samples placed on a quartz substrate were measured in the range of 8°−42° 2θ, using a step size of 0.02° at a rate of 2°/min. The CrI of the cellulose samples were calculated according to the method described by Segal et al.[58]

Imaging. *Stereomicroscopy.* Whole pieces of milled, untreated, and AHP-pretreated CS were examined without further processing. Images were viewed using a Nikon SMZ1500 stereomicroscope and captured with a Nikon DS-Fi1 CCD camera operated by a Nikon Digital Sight system (Nikon Instruments, Melville, NY). Fiji (ImageJ) was used to adjust the brightness and white balance images.

Microwave Processing. Untreated and pretreated CS tissue was processed using microwave processing as described previously.[46] Briefly, samples were fixed 2 × 6 min (with variable power) in 2.5% gluteraldehyde buffered in 0.1 M sodium cacodylate buffer (EMS, Hatfield, PA) under vacuum. The samples were dehydrated by treating with increasing concentrations of ethanol and heating in a specially outfitted microwave oven for 1 min each dilution (i.e., 15%, 30%, 60%, 90%, and 3 × 100% ethanol). After dehydration, the samples were infiltrated with LR White resin (EMS, Hatfield, PA) by incubating at room temperature for several hours to overnight in increasing concentrations of resin (15%, 30%, 60%, 90%, 3 × 100% resin, diluted in ethanol). The samples were transferred to capsules and the resin polymerized by heating to 60 °C overnight.

Confocal Scanning Laser Microscopy (CSLM). LR White embedded samples were sectioned to 300 nm with a Diatome diamond knife on a Leica EM UTC ultramicrotome (Leica, Wetzlar, Germany). Semi-thin sectioned samples were positioned on glass microscope slides and stained with 0.1% acriflavine. Images were captured using a 40× 1.4NA Plan Fluor lens on a Nikon C1 Plus microscope (Nikon, Tokyo, Japan), equipped with the Nikon C1 confocal system. Samples were excited with a 488 nm laser and fluorescence detected at 530 nm. A *Z*-axis stack of images was collected and then projected onto a single plane.

Transmission Electron Microscopy (TEM). LR White embedded samples were sectioned to 60 nm with Diatome diamond knife on a Leica EM UTC ultramicrotome (Leica, Wetzlar, Germany). Thin sections were collected on 0.5% Formvar coated slot grids (SPI Supplies, West Chester, PA). Grids were post-stained for 6 min with 2% aqueous uranyl acetate and 3 min with 1% $KMnO_4$. Images were taken with a 4 megapixel Gatan UltraScan 1000 camera (Gatan, Pleasanton, CA) on a FEI Tecnai G2 20 Twin 200 kV LaB_6 TEM (FEI, Hillsboro, OR).

HSQC NMR. Heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectra were acquired for both untreated and AHP-pretreated CS samples. Each sample (0.2 g) was ball-milled by a planetary ball mill, using a Retsch PM100 mill fitted with a 50 mL ZrO_2 grinding jar and 10 × 10 mm ball bearings, set at 600 rpm. The ball-milled sample (80 mg) was suspended into 0.5 mL of d_6-DMSO/d_5-pyridine (4:1, v/v) in a NMR tube. The mixture was sonicated for 5 h until gel became homogeneous.[59] Spectra were acquired at 40 °C on a Bruker 400 MHz spectrometer, using a BBO probe with a Z gradient. Spectra were acquired with 1024 points and a sweep width of 15 ppm in the F2 (^{1}H) dimension and 512 points and 220 ppm of sweep width in the F1 (^{13}C) dimension. DMSO peak was used as an internal reference (δ_H 2.5, δ_C 39.51 ppm). Peak assignment was performed according to the literature.[21,59,60] Aqueous soluble fraction (100 mg) after AHP pretreatment contained a large amount of salt after neutralization, which interferes in the gel formation of lignin with d_6-DMSO/d_5-pyridine, thus preventing NMR signals of reasonable quality. Therefore, the soluble fraction was first acetylated in a mixture of acetic acid and pyridine (1:1, v/v, 1 mL) at 40 °C for 24 h. The acetylated sample was extracted with $CDCl_3$ (2 mL), and the extracts were analyzed by HSQC under the same conditions. Tetramethylsilane (TMS) was used as a reference for acetylated samples.

Gel Permeation Chromatography (GPC) Analysis. To determine the molecular weight distribution of the soluble lignin in the liquors obtained after AHP pretreatment, 30 mg of freeze-dried samples were derivatized and analyzed by HPLC as described previously. Briefly, samples were acetylated in a mixture of pyridine (0.5 mL) and acetic anhydride (0.5 mL) at 40 °C for 24 h with stirring. The reaction was terminated by the addition of methanol (0.2 mL). The acetylation

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solvents were then evaporated from the samples at 40 °C under a stream of nitrogen gas. The samples were further dried in a vacuum oven at 40 °C overnight. A final drying was performed under vacuum (1 Torr) at room temperature for 1 h. The dried, acetylated lignin samples were dissolved in tetrahydrofuran (THF) (Baker HPLC grade). The dissolved samples were filtered (0.45 μm nylon membrane syringe filters) before GPC analysis. The acetylated samples were completely soluble in THF. Gel permeation chromatography (GPC) analysis was performed using an Agilent HPLC with 3 GPC columns (Polymer Laboratories, 300 × 7.5 mm) packed with polystyrene−divinylbenzene copolymer gel (10 μm beads) having nominal pore diameters of 10^4, 10^3, and 50 Å. The eluent was THF and the flow rate 1.0 mL/min. The HPLC was attached to a diode array detector measuring absorbance at 260 nm (bandwidth 80 nm). Retention time was converted to molecular weight (M_w) by applying a calibration curve established using polystyrene standards of known molecular weight (from 1 × 10^6 to 580 Da) plus toluene (92 Da).

Glycome Profiling Analyses. Cell wall materials (AIR) were prepared from various biomass residues and glycome profiling of these AIR preps was performed using the methods described earlier.[61] Glycome profiling analyses, in brief, involved generation of sequential cell wall extracts using increasingly harsh reagents (ammonium oxalate, sodium carbonate, 1 M KOH, 4 M KOH, chlorite, and 4 M KOHPC), as explained in the Methods section. These extracts were subsequently screened with a comprehensive collection of cell wall glycan directed mAbs monitoring epitope abundances of most major noncellulosic cell wall glcyans.[61] The collection of mAbs were obtained from laboratory stocks (CCRC, JIM, and MAC series) at the Complex Carbohydrate Research Center (available through CarboSource Services; http://www.carbosource.net) or from BioSupplies (Australia) (BG1, LAMP).

Enzymatic Hydrolysis. Enzymatic hydrolysis of untreated and AHP-pretreated CS was performed in duplicate in 125 mL Erlenmeyer shake flasks at 1% solids loading, 50 °C, and 130 rpm for 120 h. CTec3 was added at 16 mg protein per gram of glucan to evaluate the initial hydrolysis rate and the extent of maximum conversion of glucan after 120 h. CTec3 was supplemented with HTec3 at 4 mg protein per gram of glucan to reduce the physical barrier of hemicelluloses and enhance cellulase accessibility to cellulose. The total volume of the saccharification slurries after adding enzymes and 50 mM citrate buffer (pH 4.8) was 50 mL. To determine the progress of cellulose conversion, a 1 mL aliquot of the well-mixed slurries was taken at predetermined time points, starting with 4, 16, and 24 h. Thereafter, samples were removed every 24 h until 120 h.

Sugar Analysis. The digestion samples taken at each time point were immediately filtered through a 0.2 μm filter and then refrigerated until subjected to monomeric sugar analysis. The monomeric sugar yield (glucose and xylose) and cellobiose were measured by HPLC/RID using a HPX-87H 7.8 × 300 mm i.d., 9 μm column (BioRad, Hercules, CA) with an isocratic flow of 0.01 N H_2SO_4 at 0.6 mL/min for a total run time of 27 min using standard protocols.[56] Standards and samples were injected onto the column at a volume of 20 μL, while the temperature of the column and detector were maintained at 55 °C. Sugar standards used to construct calibration curves were purchased from Absolute Standards (Hamden, CT).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.7b01424.

Figures S1−S5 describe the composition analysis of the pretreated solids, shown as a function of pretreatment temperature at two peroxide loadings (i.e., 60 and 90 mg H_2O_2/g CS); total solids determined in alkaline pretreated liquors, as a function of peroxide loading; glycome profiling analyses of untreated and various AHP-pretreated CS residues; effect of lignin removal on the composition of AHP pretreated CS and its susceptibility to enzymatic hydrolysis during AHP pretreatment; X-ray diffractograms showing the effects of AHP pretreatment at increased peroxide loading. Tables S1−S4 give data for the compositional analysis of pretreated CS obtained after AHP pretreatment conducted at 50 °C at various peroxide loadings; sugar analysis of AHP-pretreated liquors with HPLC/RID; identifications of compounds in AHP-pretreated liquors (125 mg H_2O_2/g dry CS) using LC/MS; and cellulose crystallinity of untreated and AHP-pretreated CS, as a function of peroxide loading (PDF)

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Notes

The authors declare no competing financial interest.

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