

Chemical Changes during Anaerobic Decomposition of Hardwood, Softwood, and Old Newsprint under Mesophilic and Thermophilic Conditions

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S Supporting Information

ABSTRACT: The anaerobic decomposition of plant biomass is an important aspect of global organic carbon cycling. While the anaerobic metabolism of cellulose and hemicelluloses to methane and carbon dioxide are well-understood, evidence for the initial stages of lignin decomposition is fragmentary. The objective of this study was to look for evidence of chemical transformations of lignin in woody tissues [hardwood (HW), softwood (SW), and old newsprint (ONP)] after anaerobic decomposition using Klason and acid-soluble lignin, CuO oxidation, and 2D NMR. Tests were conducted under mesophilic and thermophilic conditions, and lignin associations with structural carbohydrates are retained. For HW and ONP, the carbon losses could be attributed to cellulose and hemicelluloses, while carbon loss in SW was attributable to an uncharacterized fraction (e.g., extractives etc.). The 2D NMR and chemical degradation methods revealed slight reductions in β -O-4 linkages for HW and ONP, with no depolymerization of lignin in any substrate.

KEYWORDS: anaerobic decomposition, CuO oxidation, lignin, HSQC, NMR

INTRODUCTION

Woody tissues make up about 75% of terrestrial plant biomass, which in turn is estimated to represent 0.95×10^{18} g, or 29% of the active global organic carbon reservoir.¹ As plant tissues are composed primarily of lignocellulosic material, the study of lignocellulose decomposition is essential to understanding carbon turnover in the environment. Plant biomass is made up primarily of three biopolymers: cellulose, hemicelluloses, and lignin. While both cellulose and hemicelluloses are readily converted to methane and carbon dioxide during anaerobic decomposition, lignin is generally considered preserved.² Information on the chemical changes in lignocellulose during anaerobic decomposition is important toward understanding the fate and reactivity of lignocellulose in anaerobic environments such as landfills, which are estimated to receive about 149 million metric tons of municipal solid waste (MSW) annually in the U.S.³ Lignocellulose in MSW takes the form of paper products, wood, food, and yard waste. The storage of carbon in landfills due to the recalcitrance of lignocellulose has been reported.^{4–6} Furthermore, lignocellulosic materials from MSW represent viable feedstock for production of energy and valuable chemicals.

The anaerobic metabolism of cellulose and hemicelluloses in both mesophilic and thermophilic environments is well-documented.^{2,7–9} However, because of its complexity, the anaerobic metabolism of the lignin polymer is not as well-understood. Studies on different lignin-derived monomers,¹⁰ oligomers,¹¹ lignin isolates,^{7,12} and methoxyl substituents¹³ have provided the foundation of our understanding of the

anaerobic decomposition behavior of lignin and lignin-derived compounds. These studies suggest that the limiting factor to the decomposition of lignin is the initial sequence of steps where the lignin is cleaved to more degradable fractions. Evidence showed that one of the initial steps in the anaerobic decomposition of lignin-like compounds is the demethylation of aromatic methoxyl groups and eventually ring cleavage where a benzoyl CoA intermediate is transformed to acetyl CoA.^{14,15} Several approaches have been employed to study lignin degradation by bacteria, including lignin isolation and isotopic labeling.¹⁶ The widely accepted lignin preparation representative of native lignin is milled wood lignin (MWL).¹⁷ However, this lignin preparation only accounts for up to 30% of total lignin¹⁸ and suffers bias from fractionation during solvent extraction with dioxane/water.¹⁹ In addition, this preparation is not entirely free of carbohydrates, thus complicating the relationship between measured CH₄ generation and lignin decomposition. Moreover, results from decomposition studies using lignin isolates do not represent the actual behavior of lignin in its native form during decomposition of lignocellulose. Lignin isotopic labeling by growing plants fed with ¹⁴C-labeled precursors such as phenylalanine to form [¹⁴C-lignin] lignocellulose has been limited to twigs and soft/nonwoody tissues as opposed to mature wood, and this method is subject

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to ^{14}C -protein contamination.²⁰ Advances in high resolution nuclear magnetic resonance spectroscopy (NMR) and the development of methodologies to completely solubilize the entire plant cell wall²¹ make it possible to observe structural transformations of lignocellulosic components. For example, this approach has been previously employed to look for evidence of lignin decomposition by brown rot fungi²² and in the structural characterization of thermochemically treated plant biomass.²³

The objective of this study was to look for evidence of chemical changes in lignin during anaerobic decomposition of different woody plant materials under mesophilic and thermophilic conditions. To our knowledge, this is the first time that high resolution 2D NMR of completely dissolved cell walls has been used to look for evidence of chemical transformations during anaerobic decomposition of lignocellulosic materials in their native state (i.e., when the natural complex associations between cellulose, hemicelluloses, and lignin are retained).

MATERIALS AND METHODS

Experimental Design. Anaerobic decomposition experiments were conducted with lignocellulosic materials (HW, hardwood *Quercus rubra*; SW, softwood *Pinus taeda*; and ONP, old newsprint Washington Post) at mesophilic (37 °C) and thermophilic (55 °C) temperatures. ONP was selected to represent a pulp and paper product in which the lignin remains in the product. Copy paper was selected as a positive control because it contains primarily cellulose and very little lignin.

Each material was incubated under conditions optimal for anaerobic decomposition in 160 mL serum bottles containing an inoculum and biological growth medium. Decomposition was allowed to proceed until no significant methane production was measured so as to allow the bioavailable substrates to be consumed. In order of preference, lignin degradation will not proceed unless readily degradable substrates such as cellulose and hemicelluloses have been depleted. The incubation period for different materials ranged from 467 days to 585 days. At the completion of incubation, the solid residue was recovered by drying the entire contents of the bottle at 50 °C. The solid residue was subjected to the wet chemical and spectroscopic analyses described below.

Sample Collection and Preparation. Mature wood samples (oven-dried at 105 °C for 48 h and stored at room temperature) were obtained from the collection of Dr. Ilona Peszlen of the Department of Forest Biomaterials at NC State University. The copy paper used in the experiments is a commercially available white paper. The ONP was represented by the Washington Post and was collected in August 2011. Samples were dried at 50 °C, then ground to pass a 60-mesh screen (0.251 mm opening) in a Wiley mill, and then redried at 50 °C to constant weight. This temperature is well below the predicted glass transition temperature of lignin.²⁴ Samples were stored in Mason jars at room temperature until used.

Preparation of Anaerobically Decomposed Materials. The goal of the preparation of decomposed materials was to generate solids for subsequent analysis with negligible background lignin, cellulose, and hemicelluloses. Anaerobically decomposed materials were prepared following the biochemical methane potential (BMP) test with some modifications.⁹ Test material weighing 2 g was incubated in a 160 mL serum bottle with 80 mL of reduced medium and 20 mL of inoculum according to Wang et al.⁹ The bottle was capped with a butyl rubber stopper and then sealed with an aluminum crimp. The head space was flushed with a mixture of CO_2/N_2 (20:80, v/v). Flushing was done for about 10 min at a flow rate of at least 100 mL/min. This was sufficient to replace the headspace gases with CO_2/N_2 (20:80, v/v). The BMP inoculum described in Wang et al.⁹ is rich in humic materials that could interfere with lignin analyses. Thus, it was

necessary to prepare mesophilic and thermophilic inocula with negligible background lignin.

The mesophilic inoculum has been maintained on ground (<1 mm) residential MSW in our laboratory for over 15 years at 37 °C. The thermophilic inoculum was obtained from a thermophilic anaerobic sludge digester (Carrboro, NC) operated at 55 °C and was collected in March 2011. Serial transfer (1:1, v/v) of the inoculum was done every 2 weeks into a fresh mixture of reduced medium with copy paper as the growth substrate until no significant lignin was detected by CuO analysis, a process that required about 14 transfers over 7 months. The copy paper was initially tested for methane potential (~250 mL of CH_4/g) to ensure that the paper does not contain inhibitory substances. Copy paper used in the experiments contained less than 1% Klason lignin. The viability of the inoculum was monitored by measuring gas production.⁹

Analytical Methods. CuO Digestion and HPLC Detection. A sample containing 2–5 mg of C equivalent was placed in a 25 mL Teflon vial with 0.5 g of CuO powder, 0.1 g of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, and 5 mL of 2 M NaOH (O_2 -free), sealed, and locked under a N_2 headspace in an anaerobic hood. Digestion was conducted in a furnace at 150 °C for 3 h. After digestion and cooling, the liquid portion of the digestate was separated by centrifugation and then acidified ($\text{pH} \leq 2$). An aliquot of this solution was diluted as necessary to obtain a concentration within the calibration range (0.05–25 μM for each phenolic monomer) and was analyzed using a LC-20AT high performance liquid chromatograph (HPLC) equipped with a degasser (DGU-20AS) and a SPD 20A photodiode array detector (PDA) (Shimadzu, Canby, OR). Chromatographic analyses were conducted using a gradient program at 55 °C with a Kinetex 2.6u C18 100A column (Phenomenex, Torrance, CA) as described previously.^{25,26}

Peak integration for each lignin phenol (LP) was done at its respective maximum wavelength using LC solutions software (Shimadzu, Canby, OR). The 11 LP liberated during CuO oxidation that were analyzed are as follows with the corresponding chemical structure number and are illustrated in Figure 1: [4-hydroxybenzaldehyde (1); 4-hydroxyacetophenone (2); 4-hydroxybenzoic acid (3); 4-hydroxy-3-methoxybenzaldehyde (vanillin, 4); 4-hydroxy-3-methoxyacetophenone (acetovanillone, 5); 4-hydroxy-3-methoxybenzoic acid (vanillic acid, 6); 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde, 7); 3,5-dimethoxy-4-hydroxyacetophenone (acetosyringone, 8); 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid, 9); 4-hydroxycinnamic acid (*p*-coumaric acid, 10); 4-hydroxy-3-methoxycinnamic acid (ferulic acid, 11)]. All HPLC samples and standard solutions were filtered through 0.2 μm Millex Teflon filter (Millipore, Billerica, MA) prior to injection. Aqueous solutions were prepared using filtered (0.2 μm) and UV-treated deionized water. Standard LPs were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity available.

Cellulose, Hemicelluloses, Klason, and Acid-Soluble Lignin. A ~1 g sample was extracted with 140 mL of toluene/ethanol (2:1, v/v) and then dried. A known weight (~0.1–0.3 g) of ground sample was subjected to a two-stage acid hydrolysis.^{27,28} Sugars (arabinose, galactose, glucose, mannose, and xylose) liberated from acid digestion were then analyzed by HPLC using an ICS 2500 pulsed electrochemical detector (Dionex, Sunnyvale, CA).^{27,28} Anhydro correction was done to convert glucose to cellulose and the other sugars to hemicelluloses. Klason lignin was measured from the solids remaining after acid hydrolysis as the weight loss on ignition at 550 °C for 2 h. Acid-soluble lignin was determined from UV absorbance at 205 nm (extinction coefficient of 110) of the filtered (0.45 μm) acid hydrolysate from the second stage digestion.^{19,29}

Total Organic Carbon (TOC). Total organic carbon (TOC) was measured with a PE 2400 CHN analyzer (PerkinElmer, Akron, OH). All samples were acid washed (1 M HCl) to eliminate inorganic carbon prior to analysis.³⁰

Methoxyl Group Determination. The lignin methoxyl group content of the samples was determined by gas chromatography as developed previously with some modifications.³¹ Briefly, a 10 mg sample was digested with 0.5 mL of 57% HI in a Tekmar 22 mL Teflon septum-sealed headspace vial (Teledyne, Thousand Oaks, CA) at 130 °C for 35 min. The digestate was allowed to cool to room

1	<i>p</i> -hydroxybenzaldehyde 4-hydroxybenzaldehyde	
2	<i>p</i> -hydroxyacetophenone 4'-Hydroxyacetophenone	
3	<i>p</i> -hydroxybenzoic acid	
4	Vanillin 4-Hydroxy-3-methoxybenzaldehyde	
5	Acetovanillone 4'-Hydroxy-3'-methoxyacetophenone	
6	Vanillic Acid 4-Hydroxy-3-methoxybenzoic acid	
7	Syringaldehyde 4-Hydroxy-3,5-dimethoxybenzaldehyde	
8	Acetosyringone 4'-Hydroxy-3',5'-dimethoxyacetophenone	
9	Syringic Acid 4-Hydroxy-3,5-dimethoxybenzoic acid	
10	<i>p</i> -coumaric acid <i>trans</i> -4-Hydroxycinnamic acid	
11	Ferulic acid <i>trans</i> -4-Hydroxy-3-methoxycinnamic acid	

Figure 1. Suites of lignin phenols (LP) liberated during CuO oxidation of lignin.⁴⁵

temperature and neutralized by addition of 0.5 mL of 6 M NaOH. The headspace was analyzed for methyl iodide using an 7890A GC (Agilent, Santa Clara, CA) equipped with a model 7000 headspace auto sampler (Teledyne, Thousand Oaks, CA), capillary column (30 m × 0.25 mm × 0.1 μm), and flame ionization detector (FID) at 250 °C. The carrier gas was 3.8 mL of He/min. Headspace gas concentration was determined from an external calibration curve constructed by the full evaporation technique, injecting 0.1 to 10 μL of pure methyl iodide as previously described.³¹

Cell Wall Dissolution. Complete dissolution of plant cell walls for subsequent high resolution solution state nuclear magnetic resonance (NMR) spectroscopy has been described.^{21,32,33} A ground sample (<60 mesh) weighing 2 g was ball-milled in toluene using alumina fortified porcelain jars charged with zirconia grinding medium (6.4 × 6.4 mm) in a 0.4 L jar mill (Cole-Parmer, Vernon Hills, IL) rotating at 30 rpm under a N₂ headspace.^{34,35} Optimal charging of 45–55% (v/v) was used as per the manufacturer's instructions. A ball-milling experiment showed that complete dissolution is possible after 14–28 days depending on the sample. Studies have shown that ball-milling results in only a minor increase in detectable cleaved lignin structures, and ball-milling in toluene is recommended as it serves to regulate temperature and acts as a radical scavenger.^{34,35} After ball-milling, the sample suspended in toluene was centrifuged at 2850 rpm for 15 min. The toluene layer was aspirated and the remaining solvent was removed by evaporation at 50 °C under vacuum.

A ball-milled sample weighing 600 mg was dissolved in 10 mL of dimethyl sulfoxide (DMSO) and 5 mL of *N*-methylimidazole. A clear amber color solution was produced after stirring for at least 4 h. The solution was acetylated in situ by addition of 3 mL of acetic anhydride and stirred for 1.5 h, after which a dark brown solution was formed. The acetylated cell wall solution was precipitated in 2 L of deionized water, stirred overnight, recovered by vacuum filtration (0.2 μm nylon filter), washed with 250 mL of DI water, and then dried under vacuum (50 °C). The yield of acetylated cell wall was about 136% of the initial weight of material, which is typical for sample acetylation.²¹ After drying, 90 mg of acetylated cell wall was dissolved in 0.75 mL of DMSO-*d*₆ (Cambridge Isotope Laboratories, Tewksbury, MA) and then transferred to a 5 mm NMR tube for analysis.

Nuclear Magnetic Resonance (NMR) Spectroscopy. One-bond ¹H–¹³C heteronuclear single quantum coherence (HSQC) NMR spectra were obtained at the Bio-NMR facility of the Department of Molecular and Structural Biochemistry, NC State University, using an Avance III 700 MHz spectrometer (Bruker, The Woodlands, TX) equipped with QNP cryoprobe implementing the Bruker's hsqcetg-psisp2.2 pulse sequence program at 30 °C. HSQC is a 2D NMR

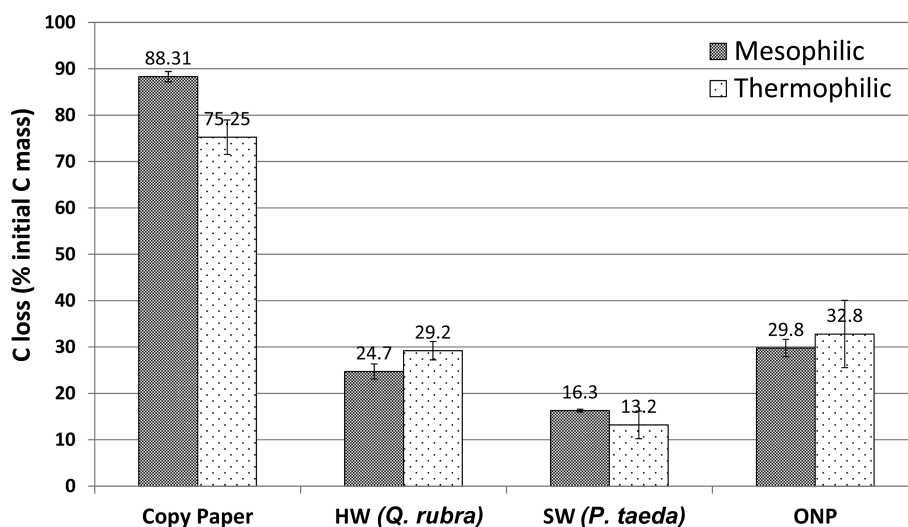


Figure 2. Carbon loss during anaerobic decomposition of different lignocellulosic materials under mesophilic and thermophilic conditions. HW, hardwood; SW, softwood; ONP, old newsprint. Error bar represents ± sd (*n* = 3).

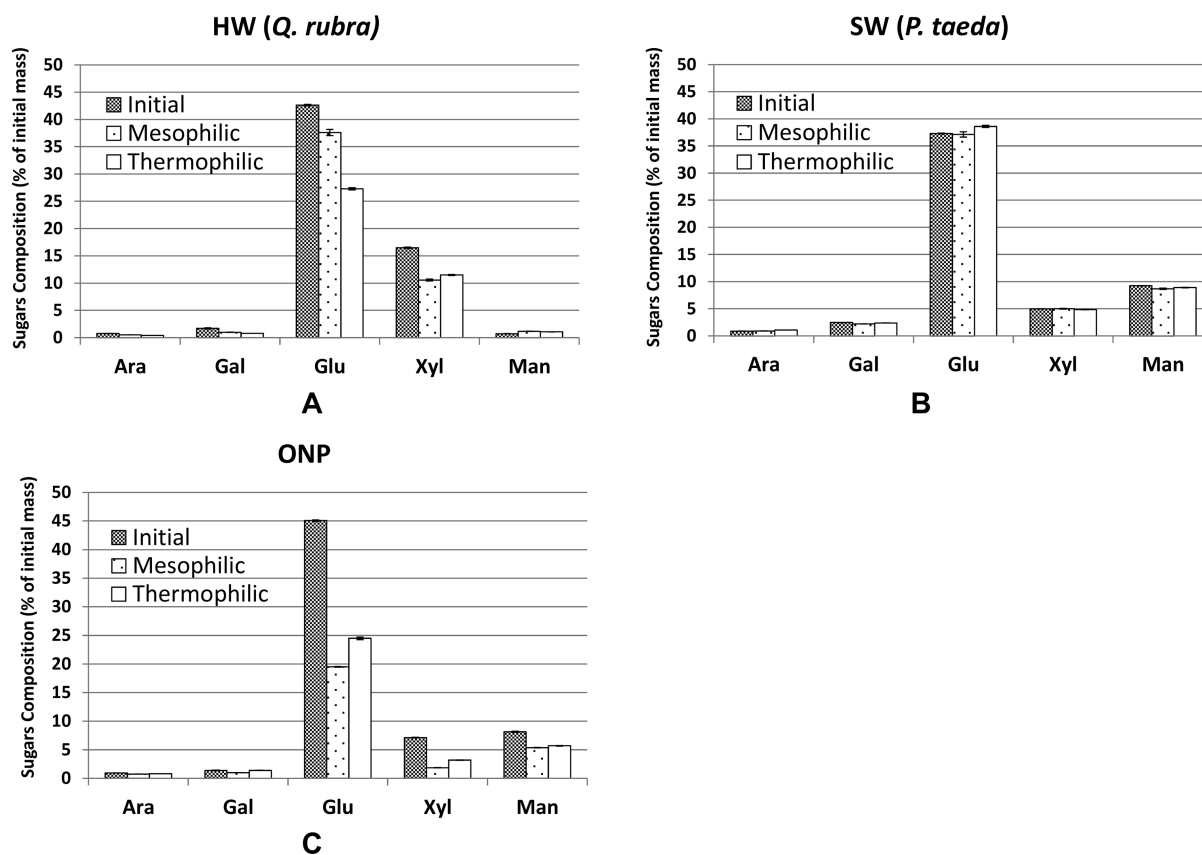


Figure 3. Changes in composition of sugars during decomposition under mesophilic and thermophilic conditions normalized to the initial mass of the sample. Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glu, glucose. Error bar represents $\pm$ sd ($n = 3$).

experiment which creates cross peaks that correlate the hydrogen in a molecule with its directly attached carbon.³⁶ The F_2 acquisition time for these NMR experiments were about 8 h with about 1 h for F_1 acquisition time. The DMSO- d_6 peak ($\delta_H/\delta_C = 2.49$ ppm/39.5 ppm) was used for internal calibration reference. Data processing and integration were done using Topspin 3.2. No attempts were made to conduct NMR experiments using nonacetylated samples. Acetylation of the whole cell wall does result in the loss of information on the natural acetates found on mannan and xylan.

NMR Quantitation. Peak assignment was performed using the lignin NMR database³⁷ and comparison with previously published lignin spectra for different lignin preparations.^{33,38–41} Calculations to obtain absolute quantitation of different substructures were done as follows (eq 1) using the methoxyl group content obtained from wet chemical analyses as a reference.²²

$$C_{\text{linkage}} \left[\frac{\text{mmol}}{\text{g of sample}} \right] = 2D_{\text{linkage}} \times \frac{C_{-\text{OCH}_3} \left[\frac{\text{mmol}}{\text{g of sample}} \right]}{2D_{-\text{OCH}_3}} \quad (1)$$

where C_{linkage} [mmol/(g of sample)] = concentration of a given linkage or substructure; $2D_{\text{linkage}}$ = HSQC volume integral of a given linkage or substructure; $C_{-\text{OCH}_3}$ [mmol/(g of sample)] = concentration of methoxyl group in the sample as determined by wet chemistry; $2D_{-\text{OCH}_3}$ = HSQC volume integral of the methoxyl group. Since the intensity of the HSQC regions are dependent on a coupling constant, comparisons of the different substructures were done on the basis of their $H\alpha-C\alpha$ correlations having a similar chemical environment. The resinol volume was divided by 2 as two correlations are involved in each linkage unit. To quantitate the C_9 units of lignin, the volume integral for $H_{2/6}-C_{2/6}$ correlations of the HSQC spectra was used for *p*-hydroxyphenyl (H) and syringyl (S) units while H_2-C_2 was used for guaiacyl (G) units. The volume integral of H_2-C_2 correlation of G units was logically doubled because it involves a single correlation.³²

The different lignin substructures were normalized to C_9 units assuming a molecular weight of 220 mg/mmol and 198 mg/mmol for HW and SW, respectively. The molecular weight of ONP was assumed to be equal to that of SW.

RESULTS AND DISCUSSION

Carbon Loss. Variation in carbon loss from anaerobic decomposition of different lignocellulosic materials representative of MSW components was observed (Figure 2). The high carbon losses in the copy paper positive control demonstrate the viability and the suitability of the anaerobic inocula to anaerobically convert substrate to methane. While methane yields were not measured, the gas composition data over the entire incubation period showed that the gas produced was methane and carbon dioxide. The carbon losses observed in HW and ONP were comparable, while the lowest carbon loss was recorded in SW. The carbon losses presented in Figure 2 are higher than previously reported using laboratory-scale decomposition experiments.^{42,43} The differences in carbon loss are perhaps a result of the smaller particle size and lower solids to liquid ratio in BMP tests conducted here relative to reactor studies designed to measure methane yields under simulated landfill conditions in which materials were shredded (3×4 cm) and had a higher solids to liquid ratio.

The inoculum used in this study was enriched on copy paper which does not contain significant amounts of lignin. This may have promoted changes in the relative abundance of different microbial communities in the culture. However, the carbon losses measured in this study for lignocellulosic substrates are comparable to values reported previously in tests with an inoculum enriched on MSW that would contain lignocellulosic

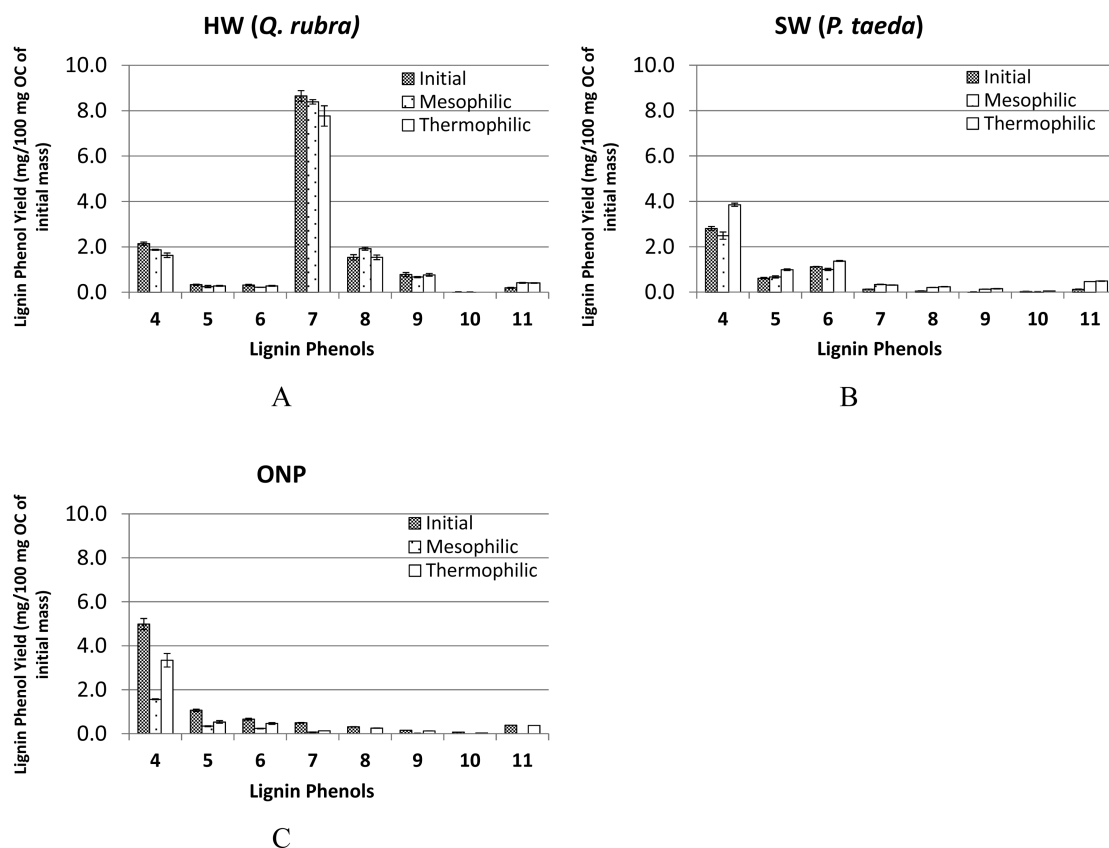


Figure 4. Changes in CuO lignin composition during anaerobic decomposition under mesophilic and thermophilic conditions normalized to the initial mass of the sample. Error bar represents $\pm$ sd ($n = 3$).

substrates.^{9,42,43} Thus, the use of a lignin-free inoculum did not influence the extent of decomposition and the measured C conversions. This suggests that all of the readily degradable substrates have been depleted or that the accessibility to the readily degradable substrates is limited by the lignin in which the polysaccharides are encased, putting pressure on microorganisms to utilize more recalcitrant substrates such as lignin.

Structural Carbohydrates. The plant cell wall is composed of the structural carbohydrates cellulose and hemicelluloses, which account for the majority of its dry mass. Cellulose is a homopolymer of glucose linked together by β (1 $\rightarrow$ 4) glycosidic linkages. The linear structure of cellulose makes up its crystalline tertiary structure, which is the building block of cellulose microfibril. Hemicelluloses are heteropolymers of different sugar residues, the composition of which varies according to tissue type. Evidence has shown that hemicelluloses link cellulose microfibrils and lignin together, making up the cell wall lignocellulose composite.⁴⁴ The plant cell wall's rigidity and chemical reactivity is attributed to these intricate and complex associations between lignin and structural carbohydrates.

The changes in sugar residues after anaerobic decomposition of different lignocellulosic materials could provide insights on the reactivity and the preferential attack of anaerobic microorganisms to the different cell wall carbohydrates (Figure 3). For HW, the major sugar residues are glucose from cellulose and xylose from xylan, with xylan being a major component in angiosperms. About 12% and 36% of the initial glucose and about 36% and 30% of the initial xylose were lost after mesophilic and thermophilic anaerobic decomposition, respectively.

No significant change in sugars was observed in SW (Figure 3). This result is surprising because we observed relatively small but significant C losses in SW for both mesophilic and thermophilic decomposition (Figure 2). The result of the sugars analysis suggests that the methane and carbon dioxide mineralization in softwood did not come from cellulose and hemicelluloses.

In contrast, in ONP, a SW mechanical pulp based on CuO oxidation products (Figure 4), the glucose loss was 57% and 46%, while mannose loss was about 34% and 30% for mesophilic and thermophilic decomposition, respectively. Mannose sugar residue comes from glucomannan, which is a major component of SW hemicelluloses. The data suggests that the conversion of cell wall sugars is highly correlated to bioavailability rather than the differences in the reactivity of individual sugars.

While ONP was derived from SW pulp, its decomposition behavior is significantly different from the SW that was tested. Significant carbon loss from cellulose and hemicelluloses was recorded in ONP. This demonstrates the effect of mechanical pulping as well as the role of lignin on the accessibility/bioavailability of cellulose and hemicelluloses in SW. Mechanical pulping could involve mechanical, thermal, and some chemical processes, resulting in chemical modifications of lignin leading to disruption of the S2 layer and releasing the cellulose fiber. This process is different from Wiley milling used during sample preparation, which is a mechanical process of particle size reduction.

Lignin CuO Oxidation Products. The CuO oxidation products of initial and anaerobically decomposed samples are presented in Figure 4. For HW, the sum of 8 LP (Figure 1), A_8

$[\Lambda_8 = \text{LP } 4 + \text{LP } 5 + \text{LP } 6 + \text{LP } 7 + \text{LP } 8 + \text{LP } 9 + \text{LP } 10 + \text{LP } 11, \text{ mg}/100 \text{ mg of organic carbon (OC)}]$, after mesophilic and thermophilic decomposition is not significantly different when compared with the initial material ($14.0 \pm 0.6 \text{ mg}/100 \text{ mg of OC}$). The Λ_8 is a measure of total releasable CuO lignin monomers normalized to OC and could indicate degradation/repolymerization of lignin during decomposition. The *p*-hydroxy phenols (LP 1, 2, and 3 in Figure 1) could have nonlignin sources such as amino acids tyrosine and phenylalanine and therefore were excluded from the sum of LP. Similarly, for HW, the acid (LP 6) to aldehyde (LP 4) ratios of vanillyl phenols (Ad/Al)_V of the initial material (0.15) and after mesophilic (0.12) and thermophilic (0.17) decomposition are not significantly different ($p > 0.05$) from each other. The (Ad/Al)_V is a measure of the extent of degradation of lignin. The result suggests that HW lignin was not significantly degraded after anaerobic decomposition.

For SW, the Λ_8 of mesophilic decomposed substrate ($5.3 \pm 0.3 \text{ mg}/100 \text{ mg of OC}$) is not significantly different when compared with the initial material ($4.9 \pm 0.2 \text{ mg}/100 \text{ mg of OC}$). The Λ_8 increase in thermophilic ($7.5 \pm 0.2 \text{ mg}/100 \text{ mg of OC}$) is anomalous, and there is no indication that it is a result of decomposition as indicated by the constant (Ad/Al)_V of 0.4 for SW initial material, after mesophilic and after thermophilic decomposition.

The CuO oxidation products of lignin can also be used as a biomarker to fingerprint the plant tissue source of lignin in an unknown sample.⁴⁵ The ratio of S ($S = \text{LP } 7 + \text{LP } 8 + \text{LP } 9$) to vanillyl (V) ($V = \text{LP } 4 + \text{LP } 5 + \text{LP } 6$) (S/V) has been used to discriminate between angiosperm (HW, leaves, and grasses) and gymnosperm (SW and needles) tissues while the ratio of cinnamyl (C) ($C = \text{LP } 10 + \text{LP } 11$) to V phenols (C/V) has been used to discriminate between woody (HW and SW) and nonwoody (leaves, grasses, and needles) tissues.⁴⁵ The low C/V and low S/V ratios indicate that the source of lignin in ONP is softwood.

While the lignin CuO oxidation products of ONP are similar to those of SW, their decomposition behaviors are different. Significant loss of VAL phenols was recorded in ONP for both mesophilic and thermophilic decomposition. The (Ad/Al)_V of fresh ONP (0.13) is not significantly different from those of materials decomposed under mesophilic (0.15) and thermophilic (0.14) conditions ($p > 0.05$). This indicates that the lignin polymer has not been significantly depolymerized during decomposition. The loss of VAL without a corresponding increase in (Ad/Al)_V is an indication that no significant lignin cleavage occurred during anaerobic decomposition of ONP. This result suggests that reaction of VAL is limited to side chain oxidation without destruction of the aromatic structure.

Lignin Methoxyl Group. Losses in lignin methoxyl group were observed after anaerobic decomposition (Figure 5). The C_9 normalized methoxyl group contents of initial materials were 1.74, 1.00, and 0.54 mmol $-\text{OCH}_3/\text{mmol}$ of C_9 unit for HW, SW, and ONP, respectively. As SW is dominated by G units, with minute amounts of S units,^{44,46} it contains one methoxyl group per C_9 unit. Similarly, since HW is a combination of both S (that contains two methoxyl groups per C_9 unit) and G units, the methoxyl group content per C_9 unit is between one and two. The methoxyl group content of ONP confirms the SW character of this material having one methoxyl group per C_9 unit.

After anaerobic decomposition, the loss of methoxyl group was 23% and 13% for HW, and 35% and 20% for ONP, for

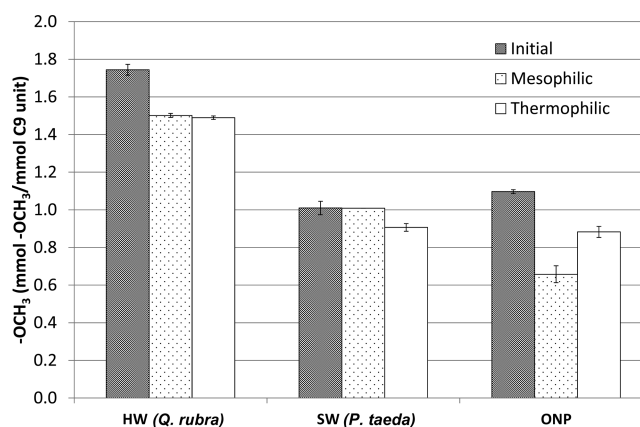


Figure 5. Changes in lignin methoxyl group composition during decomposition under mesophilic and thermophilic conditions normalized to the initial mass of the sample. Error bar represents $\pm$ sd ($n = 3$).

mesophilic and thermophilic decomposition, respectively. The loss in the methoxyl group content of SW for both mesophilic and thermophilic decomposition was not statistically significant ($p > 0.05$). The loss of methoxyl group content in ONP is statistically similar between mesophilic and thermophilic conditions. In contrast, the methoxyl group loss in HW was significantly greater under mesophilic conditions ($p < 0.05$).

Evidence of the demethoxylation of lignin-derived molecules under anaerobic conditions has been reported previously where ^{14}C -labeled aromatic $-\text{OCH}_3$ was metabolized to CO_2 and acetates.¹³ The results of this study support the notion that one of the first steps in the metabolism of aromatic molecules is the removal of aromatic substituents such as methoxyl groups,^{14,15} which in turn can be metabolized by acetogens.¹³ Perhaps because of the complexity of SW lignin polymer, these initial steps in SW lignin decomposition are more difficult compared to other lignocellulosic substrates.

Klason and Acid Soluble Lignin. The Klason and acid-soluble lignin (ASL) contents of initial and anaerobically decomposed samples are presented in Figure 6. While Klason lignin is operationally defined as the volatile fraction of the acid-insoluble material remaining after acid hydrolysis, it is widely used to measure the total lignin of a sample.^{19,44}

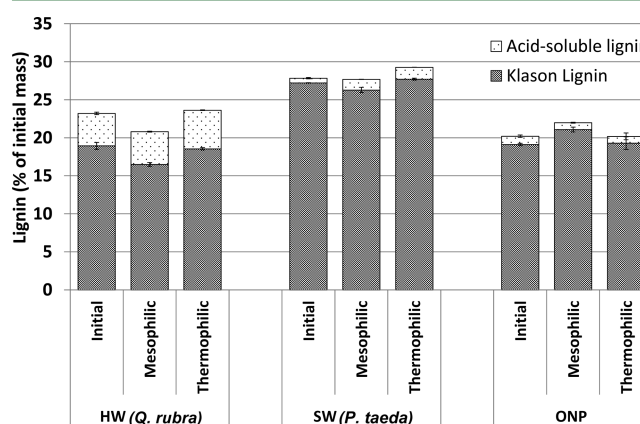


Figure 6. Changes in Klason and acid-soluble lignin composition during decomposition under mesophilic and thermophilic conditions normalized to the initial mass of the sample. Error bar represents $\pm$ sd ($n = 3$).

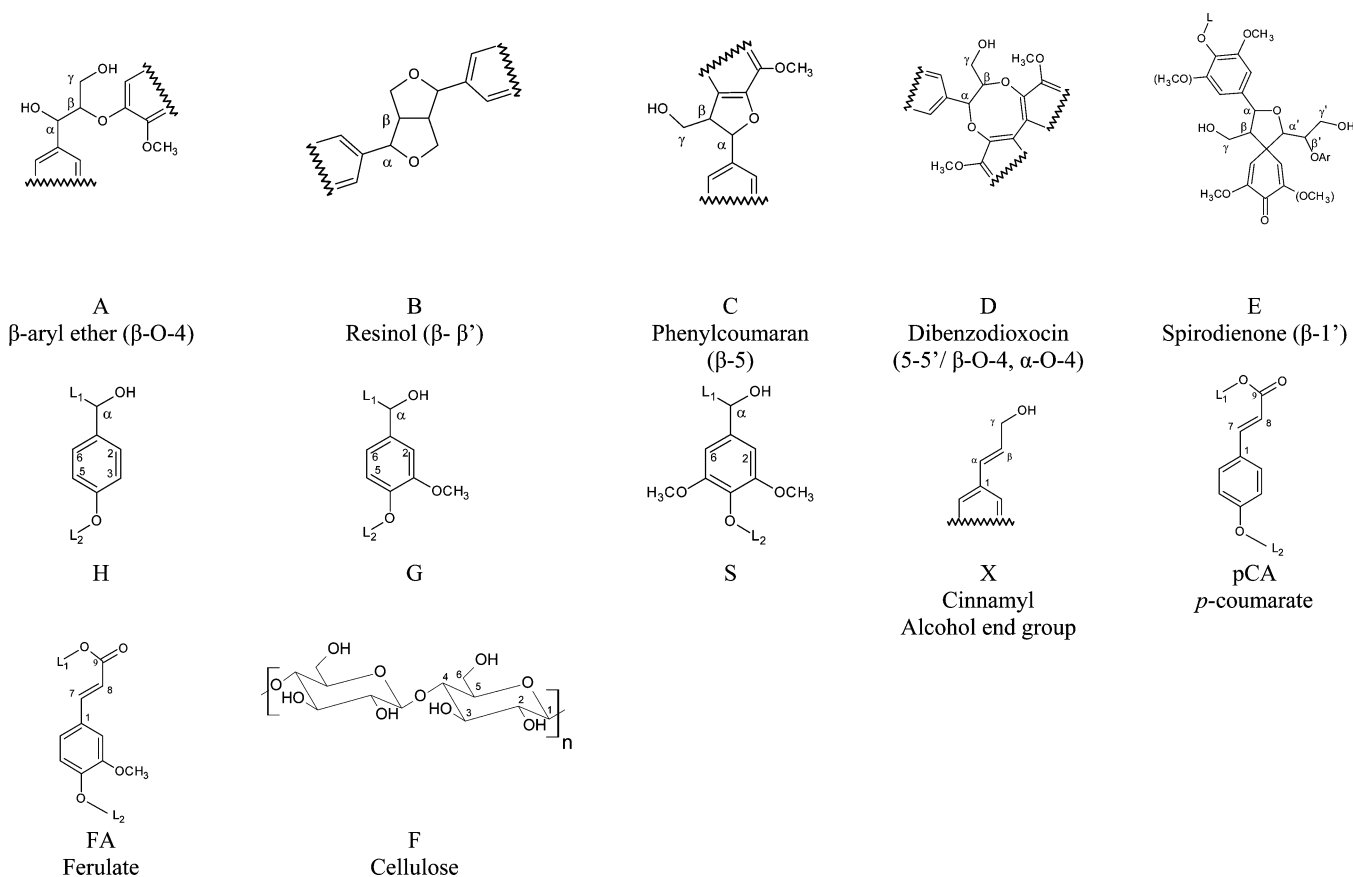


Figure 7. Linkages and substructures in lignin and lignocellulose. L, lignin polymer.

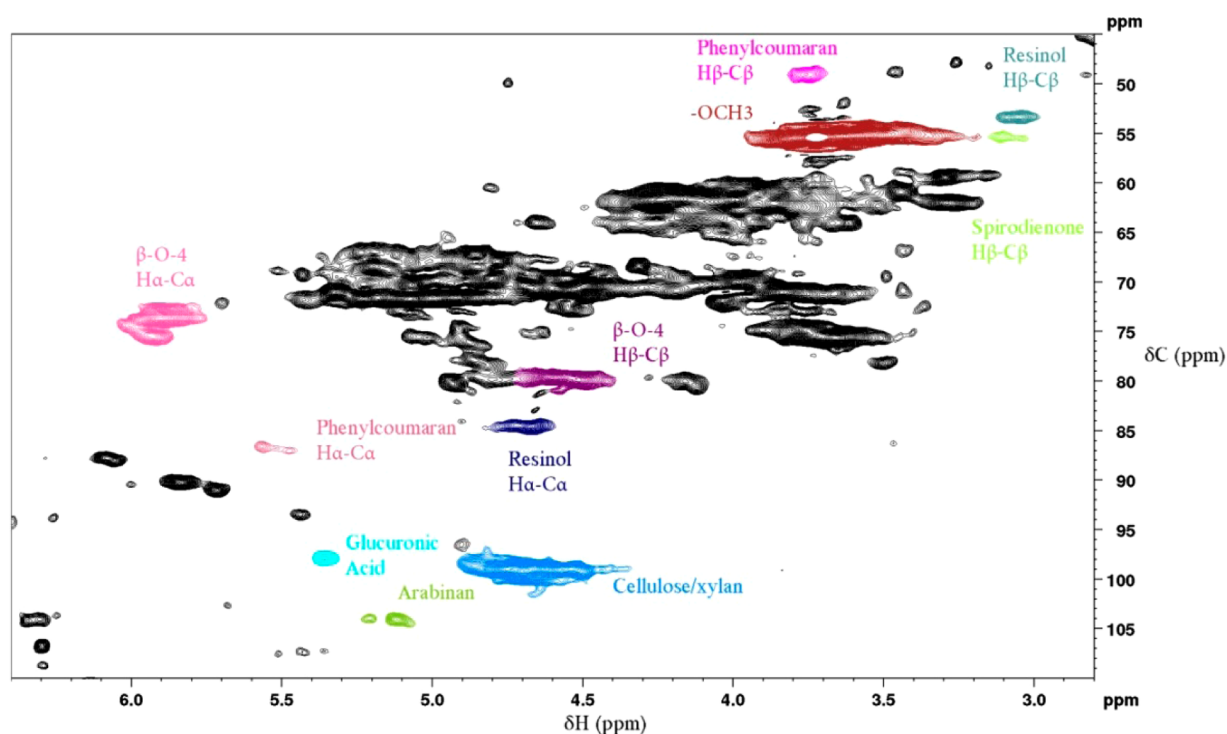
The loss of HW Klason lignin under mesophilic conditions (13%) was significant ($p < 0.05$) while no significant difference was observed under thermophilic conditions. In contrast, losses of HW ASL were significant only under thermophilic conditions. In some tissues such as in nonwoody angiosperms, the amount of ASL can be significant. Moreover, the amount of ASL could also be an indicator of the extent of decomposition as some lignin can be broken down to lower molecular weight fractions and then to soluble fractions rendering them more amenable to anaerobic decomposition. There was no significant change in either Klason lignin or ASL in ONP.

There were no significant losses in either Klason lignin or ASL for SW. As noted above, anaerobic decomposition of SW resulted in a small but significant carbon loss. It is interesting to note, however, that, based on chemical analyses, the carbon loss recorded in SW was not due to the cellulose, hemicelluloses, or lignin but from other components. To evaluate whether this observation is plausible, the amount of carbon that can be attributed to these major lignocellulosic components was estimated assuming empirical formulas for cellulose, hemicelluloses, and lignin as $(C_6H_{10}O_5)_n$, $(C_5H_8O_4)_n$, and $C_{(9)}H_{(7.92)}O_{(2.40)}(OCH_3)_{(0.92)}$, respectively. Based on the empirical formulas and their corresponding compositions, about 85% of the carbon in SW can be attributed to cellulose, hemicelluloses, and lignin. Thus, the uncharacterized cell components of SW (e.g., proteins, tannins, extractives, etc.), amounting to about 15% of the total carbon (estimated by difference), in theory could explain the observed carbon loss. This possibility is supported by the result of extractives in the softwood sample which was determined to be $17 \pm 0.73\%$ of the initial mass.

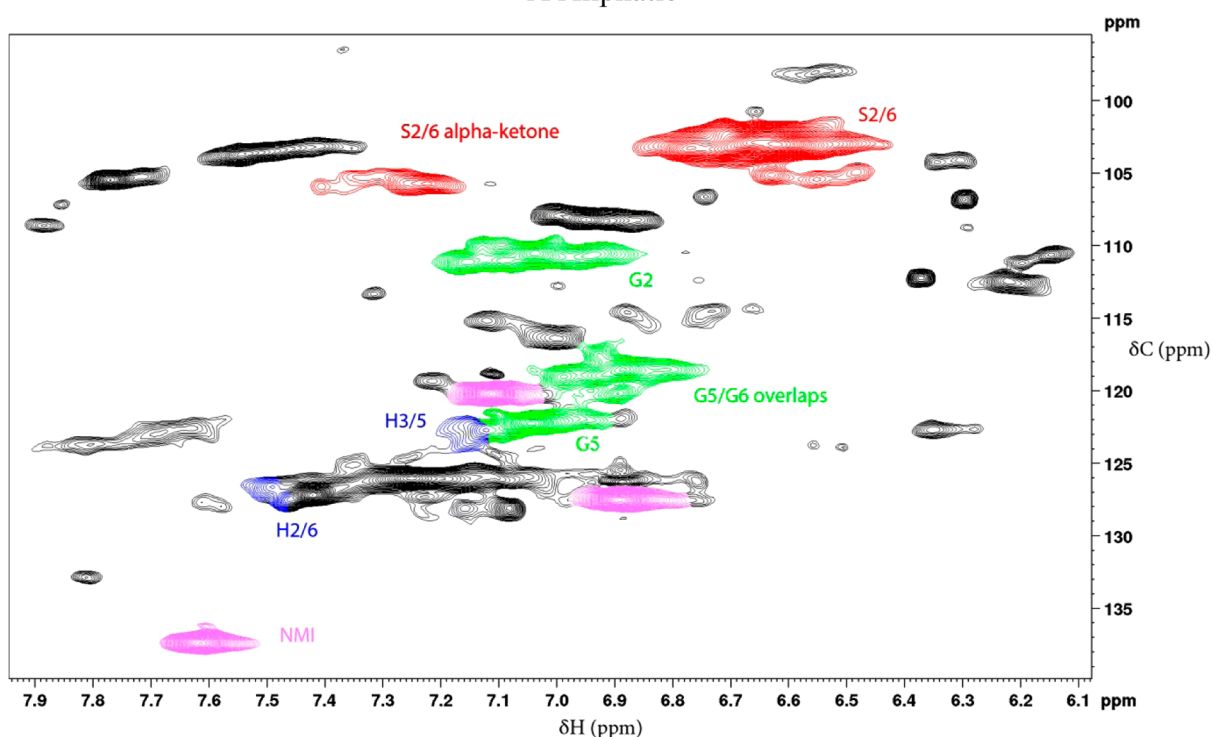
It is well-documented that SW lignin is dominated by G units. This lignin unit is more complex because of more branching as a result of the availability of the 5-position in lignin phenolic units to cross-link, making the SW lignin polymer more difficult to depolymerize.^{19,44} Moreover, the complex structure of SW lignin hinders microbial enzyme access to structural carbohydrates, resulting in minor mineralization of cellulose, hemicelluloses, and lignin.

Lignin Substructures. Decomposition of lignocellulose components may not necessarily result in mass loss due to mineralization to CH_4 and CO_2 . The ability of lignin to depolymerize and repolymerize makes the study of the transformation of lignin complicated.⁴⁷ In this section, evidence of lignin transformations was examined using HSQC NMR spectroscopy. The different lignin and carbohydrates substructures are illustrated in Figure 7. Figures 8 to 10 show the aliphatic and the aromatic regions of the HSQC spectra of different test materials. The spectra for initial and decomposed materials are quite similar, and the only difference was the intensities of different contours. As such, only the spectra of the initial material are presented. The difference between the initial and decomposed material is reflected in the volume integrations of different contours corresponding to different lignin substructures as summarized in Table 1. The HSQC spectra were color referenced for easy comparison.

Quantitative comparisons of the absolute amount of different substructures normalized to C_9 unit are presented in Table 1 for different materials (initial material, and after mesophilic and thermophilic decomposition). The relative abundance of different lignin substructures has been reported for different types of tissues. The majority of inter C_9 unit linkages in lignin



A Aliphatic



B Aromatic

Figure 8. HSQC spectra of HW (*Quercus rubra*): (A) aliphatic region; (B) aromatic region.

are β -O-4, followed by significantly smaller and varying amounts of resinol, phenylcoumaran, spirodienone, and dibenzodioxocin structures (Figure 7). For example, in white birch (*Betula pendula* Roth) MWL, the relative abundance of lignin inter C₉ unit linkages is about 69% β -O-4, 17% resinol, 3% phenylcoumaran, and 4% spirodienone estimated from 2D HSQC spectra.⁴⁸

The HSQC spectrum of the HW aliphatic region (Figure 8A) illustrates the well-resolved peak corresponding to the Ha–Ca ($\delta_H/\delta_C = 5.91$ ppm/74.3 ppm) correlation in the β -O-4 linkage with distinct separation between the *threo* and *erythro* diastereomers. Figure 8A also shows the intense methoxyl group peak ($\delta_H/\delta_C = 3.60$ ppm/55.3 ppm). Three of the major inter C₉ unit linkages in lignin were quantified as presented in

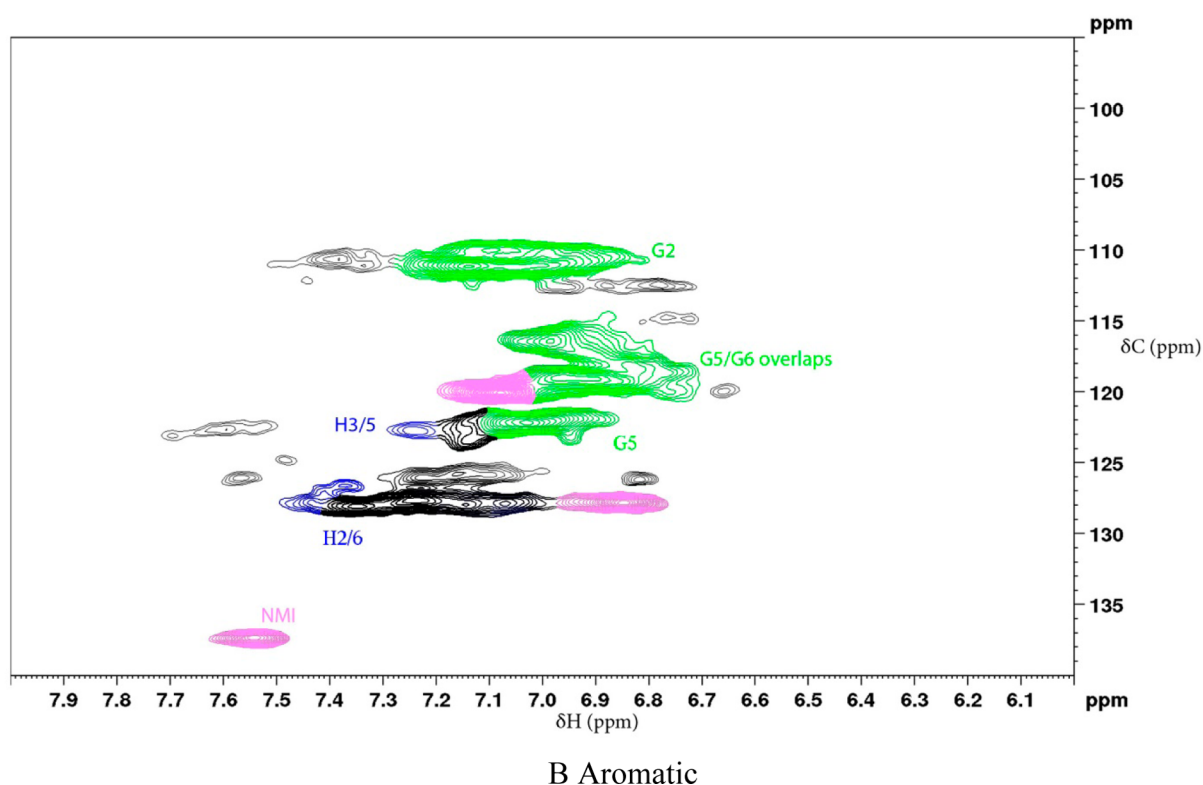
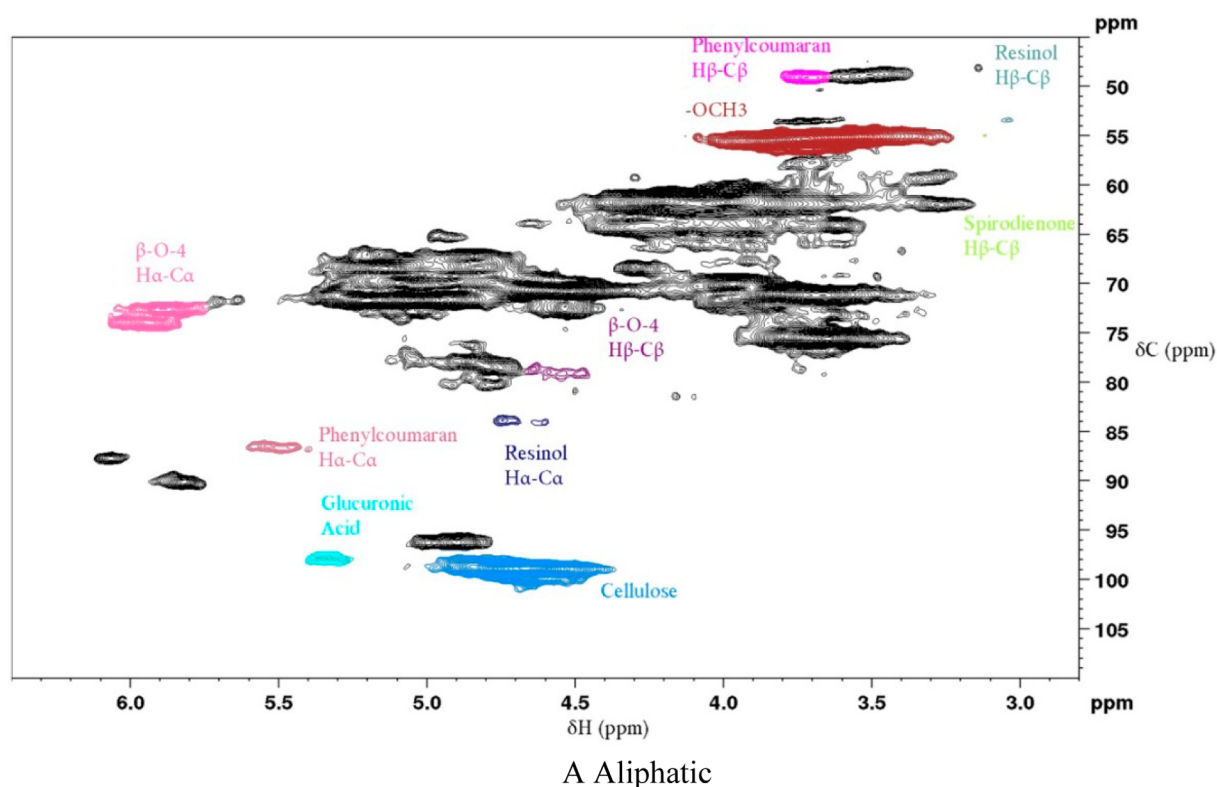


Figure 9. HSQC spectra of SW (*Pinus taeda*): (A) aliphatic region; (B) aromatic region.

Table 1. While other lignin substructures have been identified, their quantities were not calculated because the relative amounts are small and are subject to high uncertainty. In addition to lignin substructures, Figures 8–10 show the different structures found in cellulose and hemicelluloses. The

black regions in the aliphatic regions of HSQC spectra are unidentified overlapping peaks primarily due to carbohydrates.

In the aromatic regions (Figure 8B), the intense $H_{2/6}-C_{2/6}$ ($\delta_H/\delta_C = 6.65 \text{ ppm}/102.7 \text{ ppm}$) contour region is due to S units, which are the major C_9 unit in angiosperms such as HW. In a majority of the HSQC spectra, *N*-methylimidazole

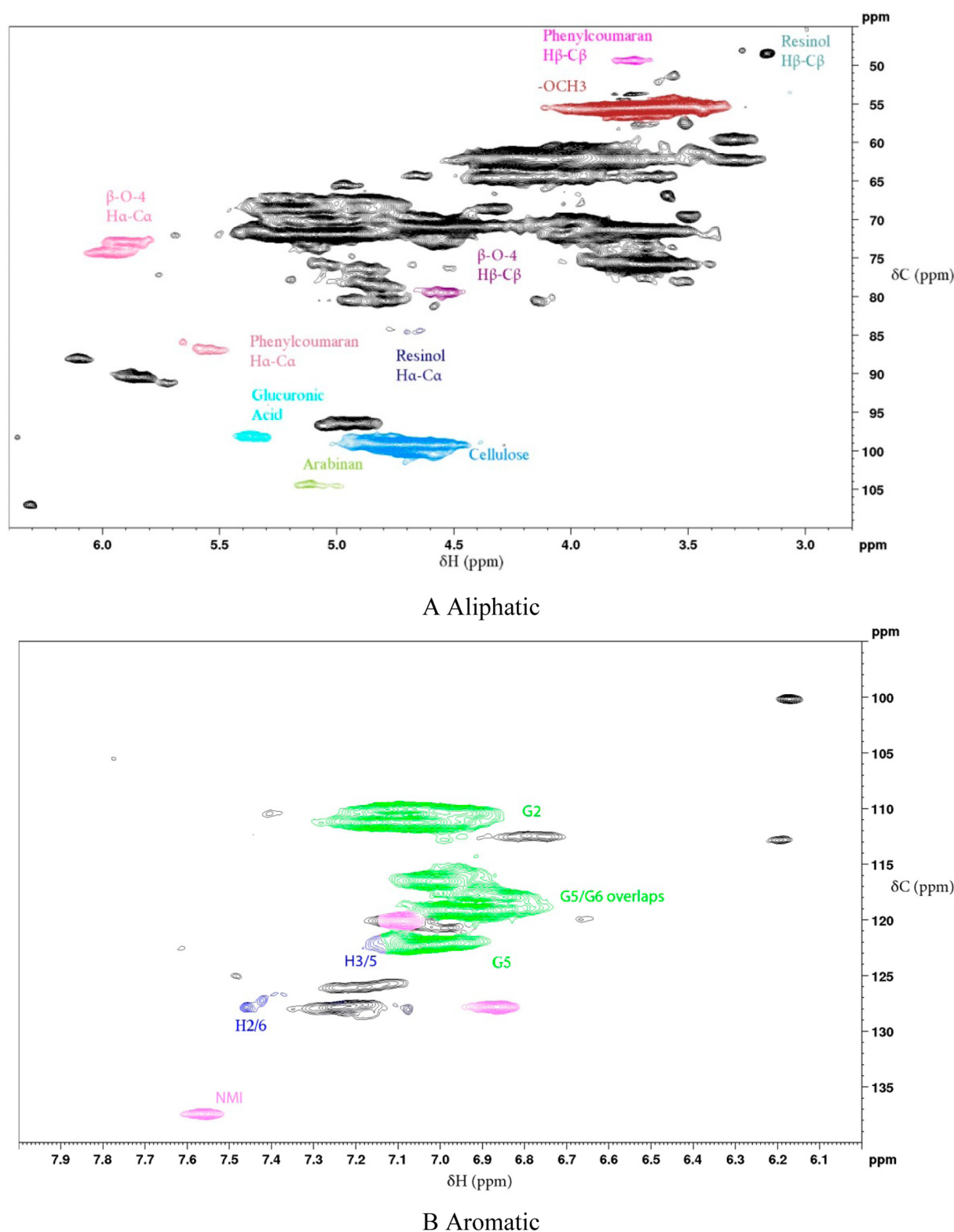


Figure 10. HSQC spectra of ONP: (A) aliphatic region; (B) aromatic region.

contamination due to incomplete washing was detected. This did not affect the NMR quantification as *N*-methylimidazole peaks did not overlap with regions that were used for quantification. The $\text{H}_2\text{-C}_2$ correlation in G units is also well-resolved in the HW aromatic region of the HSQC spectra. The low proportion of H is a characteristic of woody tissues.

As expected, the major lignin inter C_9 unit linkage is $\beta\text{-O-4}$. A slight reduction in the amount of this linkage in HW for mesophilic and thermophilic decomposition respectively was observed (Table 1). Anaerobic decomposition resulted in a decrease in the amount of $\beta\text{-O-4}$ linkage brought about by α -oxidation. For both mesophilic and thermophilic anaerobic

Table 1. Structural Characteristics of Initial and Anaerobically Decomposed Lignocellulose Calculated from Volume Integration of the ^1H – ^{13}C Correlation Signals in the HSQC Spectrum Normalized to Lignin C_9 Unit

linkage/lignin substructures	H–C correlation	linkage amount (mmol/mmol of C_9 unit)								
		HW (<i>Q. rubra</i>)			SW (<i>P. taeda</i>)			ONP		
		I ^a	M ^a	T ^a	I ^a	M ^a	T ^a	I ^a	M ^a	T ^a
β -O-4 structures (A)	$\text{H}_\alpha\text{--C}_\alpha$	0.136	0.124	0.112	0.080	ND	ND	0.085	0.059	0.077
resinol (B)	$\text{H}_\alpha\text{--C}_\alpha$	0.015	0.012	0.012	0.003	ND	ND	0.006	0.005	0.007
phenylcoumaran (C)	$\text{H}_\alpha\text{--C}_\alpha$	0.010	0.004	0.006	0.014	ND	ND	0.018	0.014	0.019
aromatic methoxyl group ^b	H–C	1.744	1.480	1.490	1.010	1.008	0.906	1.097	0.658	0.883
H	$\text{H}_{2/6}\text{--C}_{2/6}$	0.002	0.001	0.001	0.007	ND	ND	0.002	0.002	0.001
G	$\text{H}_2\text{--C}_2$	0.167	0.103	0.099	0.348	ND	ND	0.311	0.195	0.133
S	$\text{H}_{2/6}\text{--C}_{2/6}$	0.249	0.236	0.220	0.007	ND	ND	0.002	0.002	0.001
S/G ratio		1.5	2.3	2.4	0.01	ND	ND	0.04	0.02	0.02

^aI = initial material; M = mesophilic decomposition; T = thermophilic decomposition; ND = no data was acquired for SW degraded samples.

^bDetermined by wet chemistry.

decomposition of HW, α -carbonyl ($\text{S}_{2/6}$ α -ketone) slightly increased (data not shown).

The NMR spectra of decomposed SW were not acquired because there is no evidence of lignin degradation observed using degradative methods as described previously. The spectrum of the initial material is presented here for completeness (Figure 9).

The aliphatic region of the ONP HSQC spectrum (Figure 10A) is quite similar to that of SW with the well-resolved $\text{H}_2\text{--C}_2$ correlation of the G aromatic peak ($\delta_{\text{H}}/\delta_{\text{C}} = 7.01$ ppm/110.8 ppm). The majority of the lignin inter C_9 unit linkages are β -O-4, which was observed to decrease for mesophilic and thermophilic decomposition (Table 1). Decrease in resinol and phenylcoumaran content was observed in mesophilic anaerobic decomposition of ONP.

In the aromatic region (Figure 10B), the absence of the $\text{S}_{2/6}$ peak confirms that the ONP sample was made from SW pulp. The low proportion of the H group is a characteristic of woody tissues as observed in HW and SW. It is interesting to note that, similar to HW, the amount of G decreased when compared with the initial material (Table 1).

The HSQC spectra showed only slight reductions in β -O-4 linkage, suggesting no significant lignin depolymerization for any substrate. The loss of $\text{H}_2\text{--C}_2$ of G units in HW and ONP is surprising as it is well-documented that the S units are more reactive than G units. This could be a subject of further research. Nonetheless, the Klason and acid soluble lignin data as well as CuO lignin data indicate no substantial depolymerization of lignin. It is possible that the loss of the G_2 region in HW and ONP is primarily a result of side chain reactions with no destruction of the aromatic structure. The study suggests that the presence of lignin in its natural form will result in carbon storage from plant biomass in a strictly anaerobic environment such as the deep layer of soil and marine sediments. In addition, the varying reactivity of different plant tissues has implications on the estimates of life cycle emissions of forest products where wood and similar tissues are treated as having the same decomposition rates under current greenhouse gas reporting protocols.⁴⁹

■ ASSOCIATED CONTENT

■ Supporting Information

Details of the CuO oxidation, a summary of the chemical characteristics of the initial materials tested, and cellulose and hemicellulose loss during anaerobic decomposition of different

lignocellulosic materials. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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