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Structural Modification of Lignin and Characterization of Pretreated Wheat Straw by Ozonation

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Supporting Information

ABSTRACT: Ozonolysis is potentially an effective method for pretreating lignocellulosic biomass to improve the production of fermentable sugars via enzymatic hydrolysis. Further understanding of the ozonolysis process and identifying specific lignin structural changes are crucial for improving the pretreatment process. Investigation into pretreatment of wheat straw using ozonolysisis is reported in this paper, with special emphasis on selective modification/degradation of lignin subunits. The ozonolysis was performed for 2 h with less than 60 mesh particles in order to achieve maximum lignin oxidation. The results showed that the lignin structure was significantly modified under these conditions, leading to higher sugar recovery of more than 50% which increased from 13.11% to 63.17% corresponding to the control and ozone treated samples, respectively. Moisture content was found to be an important parameter for improving sugar recovery. Ninety percent (w/w) moisture produced the highest sugar recovery. The concentration of acid soluble lignin in the ozone treated sample increased from 4% to 11% after 2 h treatment. NMR analysis revealed that the S2/6 and G2 lignin units in the wheat straw were most prone to oxidation by ozone as the concentration of aromatic units decreased while the carboxylic acids became more abundant. The experimental data suggest the degradation of β-O-4 moieties and aromatic ring opening in lignin subunits. The pyrolysis-gas chromatography/mass spectrometry results revealed that the rate of lignin unit degradation was in the following order: syringyl > guaiacyl > *p*-hydroxyphenyl. Long ozone exposure resulted in few condensed lignin structure formation. In addition, the formation of condensed units during this process increased the activation energy from ASTM-*E*, 259.74 kJ/mol; Friedman-*E*, 270.08 kJ/mol to ASTM-*E*, 509.29 kJ/mol; Friedman-*E*, 462.17 kJ/mol. The results provide new information in overcoming lignin barrier for lignocellulose utilization.

KEYWORDS: *ozonolysis, pretreatment, lignin modification, wheat straw*

INTRODUCTION

Lignocellulose as the primary component of plant biomass is principally composed of a cellulose and hemicellulose structure that is compactly bound to lignin.[1] Lignocellulosic biomass is an abundant renewable resource that can be used to produce biofuels and bioproducts. Efficient conversion of lignocellulosic biomass into sugars is a prerequisite for improving the economic viability of the biofuels and bioproduct industries. However, lignocelluloses are currently one of the most underutilized types of biomass[2] because of recalcitrance to the cellulolytic enzymes that is primarily due to the presence of lignin. Lignin is a high molecular weight polymer made of three precursors: coniferyl, sinapyl, and coumaryl alcohols. These precursors are called monolignols that contribute to formation of guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units of lignin. There are various interunit linkages (β-O-4, β-5, β-β, 4-O-5, and 5-5) that bind these monolignols to form macromolecular lignin. It has been proved that enzymatic cellulose digestion can be greatly improved through a pretreatment step that removes lignin or hemicellulose.[3] Practically, the S/G ratio is an important factor in determining the requirement of pretreatment chemicals[4] and the efficiency of sugar release.[5]

Ozonation as a pretreatment process has been demonstrated to delignify and brighten wood with minimal effects on hemicellulose[6] and cellulose.[7] Ozonation has been widely used in the pulp and paper industries for bleaching.[8] Additionally, ozone pretreatment has been known to produce low amounts of toxic residues for downstream applications such as saccharification and fermentation.[9] Recent studies have proved the capability of ozone to pretreat lignocellulosic biomass that led to a significant reduction of lignin concentration depending on the substrate, reaction time[10,11] and operating parameters.[12] As well, various mechanisms have been proposed for pure lignin and lignin model compound degradation by ozone including selective reaction with carbon−carbon double bonds,[13,14] high reactivity toward aromatics,[15,16] and glycosidic bond cleavage.[17] Studies investigating ozonolysis of corn stover and softwood found ozone pretreatment over 30 min resulted in condensation of lignin structures.[6] These reports corroborated earlier findings of formation of complex ring structure due to lignin−ozone interaction.[18] However, detailed investigation on lignin structural modification of lignocellulosic biomass during ozone pretreatment is limited.

The purpose of the present study was to identify lignin structural modification during pretreatment of lignocellulose by ozone and to gain insight for improving ozonolysis process. We hypothesized in this study that ozone targets lignin subunits either through structural modification or by opening the aromatic ring to reduce recalcitrance for enzymatic hydrolysis.

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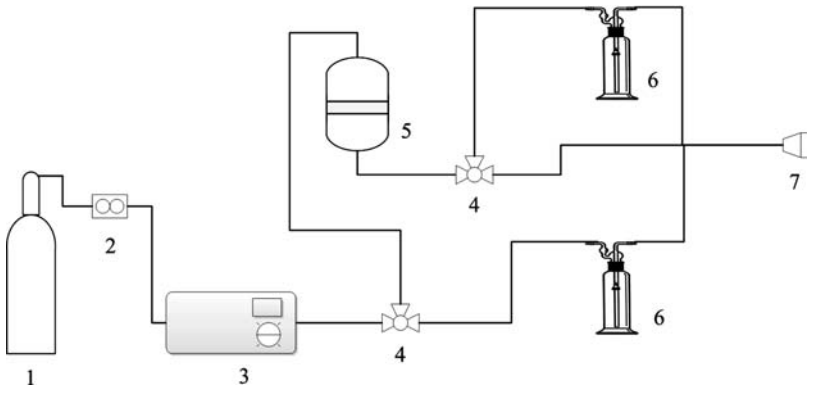

Figure 1. Schematics of ozonation experimental set up: (1) oxygen cylinder, (2) gas flowmeter, (3) ozone generator, (4) three way valve, (5) ozonation reactor, (6) iodide trap to measure ozone concentration, and (7) exhaust.

This hypothesis was built upon existing literature demonstrating the reactivity of ozone with aromatic compounds and the ability to break specific bonds.[13,15−17] We conducted a systematic analysis for testing the hypothesis. Wheat straw lignin modified with ozone pretreatment was analyzed using ^{13}C cross-polarization magic angle spinning with nuclear magnetic resonance (CP/MAS NMR) spectroscopy to understand structural changes. This technique has been known to provide information on chemical changes in the structure of lignocellulosic biomass without degradation or isolation of components.[19,20] Further lignin structural changes were analyzed using solution state ^{1}H, ^{13}C, and two-dimensional (2D) NMR to identify particular molecule degradation or modification by ozone. The data obtained using the NMR study was confirmed by nondegradative attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy analysis. The relative amount of monomeric components generated from lignocellulosic macromolecules after pretreatment was analyzed using three stage pyrolysis-gas chromatography/mass spectrometry (py-GC/MS). Moreover, thermal kinetic studies (thermogravimetry, TG, and differential thermogravimetry, DTG) were performed to determine the effect of particular structure degradation/dissociation of the lignin−hemicellulose−cellulose matrix during ozonolysis, The increased understanding of lignin modification through the study can provide useful information for the development of set ozonolysis conditions as well as lay the groundwork for future studies in development of combined pretreatment processes.

MATERIALS AND METHODS

Wheat Straw Selection and Ozone Pretreatment. Wheat straw was obtained from the Grange Supply Co. in Pullman, Washington, and hammer milled at Washington State University's Wood Materials and Engineering Laboratory. The straw was then sieved through a 60 mesh (0.25 mm) Tyler Standard screen scale. The resulting less than 60 mesh particles were then used for the ozone pretreatment in order to achieve maximum ozonolysis effect on lignin structural modification. Ozone was produced by a L11-L24 ozone generator manufactured by Pacific Ozone, California, and the ozone concentration at outlet was determined according to the procedure adopted by Rakness et al.[21] The wheat straw particles (3 g) were adjusted with moisture (30−90% w/w) and placed into an enclosed stainless steel reactor with 1 cm bed height to ensure equal contact time of the entire particles with ozone and operated in semicontinuous mode (the schematic diagram experimental set up is shown in Figure 1). The ozonation reaction was performed with 5.3% ozone concentration (5.3% w/w) at a flow rate of 2 L/min for 120 min. Final moisture content was not analyzed; rather, samples were dried at 50 °C for 24 h to make sure remaining water was evaporated before characterization experiments. The reaction time was chosen to ensure maximum ozone exposure to the wheat straw. These processed samples were further used for testing. Additional ozonation experiments were also carried out with same condition (90% moisture adjustment, 5.3% (w/v) ozone concentration, and oxygen flow rate of 2 L/min) for varied ozonation times (5, 10, 15, 30, 60, 90, and 120 min) to study the detailed mechanism of ozone reaction with lignocellulosic biomass.

Biomass Compositional Analysis: Acid Soluble and Insoluble Lignin Analysis. Control and ozone pretreated wheat straw samples were dried at 105 °C in hot air oven for 24 h and then used for compositional analyses. Carbohydrate and lignin contents were determined according to the NREL standard laboratory analytical procedure (LAP) for determination of structural carbohydrates in biomass.[22] A 0.3 g amount of sample was weighed and treated in a two-stage acid hydrolysis procedure. After initial hydrolysis at 37 °C with 3 mL of 72% (w/w) sulfuric acid, the samples were diluted with distilled water to a total volume of 84 mL and autoclaved for 1 h in pressure tubes. Sugars in the aqueous phase were determined using ion chromatography (Dionex ICS-3000 with Dionex Pac PA20 column and CarboPac PA20 guard column). The samples were run for 60 min, and the column was flushed between runs with 100% 200 mM NaOH followed by deionized water. Sugar concentration was calculated by comparison to a standard sugar sample, and all measurements were taken in triplicate. Acid soluble and insoluble lignin content was calculated according to NREL's LAP.[22]

Enzymatic Hydrolysis. Enzymatic hydrolysis was performed on control and ozone treated samples to estimate the sugar recovery before and after pretreatment. The hydrolysis was carried out at 1% solid loading in 50 mM sodium acetate buffer (pH 4.8) containing 100 μL of 2% sodium azide, with 30 FPU (per 1 g of biomass) of cellulase (Novozymes NS 50013) and 30 CBU (per 1 g of biomass) of β-glycosidase (Novozymes NS 50010) at 50 °C for 72 h in an orbital incubator shaker (Gyromax 747). The total sugars released after 72 h were used to calculate sugar recovery after enzymatic hydrolysis. The sugar composition of enzymatic hydrolysate was analyzed using DIONEX-IC as detailed in the previous section (Biomass Composition Analysis).

Solid-State ^{13}C CP/MAS NMR Analysis. The ^{13}C CP/MAS solid-state NMR analysis was carried out to study chemical structural changes in the native lignin of both control and ozone pretreated wheat straw biomass without further degradation and/or isolation of its individual components. The finely ball milled wheat straw samples (250 mg) of the control and resultant ozone treated wheat straw biomass were individually packed in a 5 mm pencil-type rotor, and the spectra were recorded under identical acquisition parameters at ambient temperature. The solid-state ^{13}C CP/MAS analysis were carried out at 100 MHz on a Bruker Avance 400 spectrometer (NMR center, Washington State University), equipped with a Chemagnetics double resonance probe. A contact time of 0.5 ms, a proton field of approximately 40 kHz during CP and data acquisition, a relaxation

Table 1. Effect of Moisture Content on Sugar Recovery and Chemical Composition of Wheat Straw by Ozonation

component	control wheat straw (% w/w)	ozone treated wheat straw (% w/w)						
adjusted moisture content (%)	0	30	40	50	60	70	80	90
arabinose	3.10 ± 0.15	3.05 ± 0.22	3.10 ± 0.02	3.21 ± 0.21	2.99 ± 0.13	3.16 ± 0.11	3.00 ± 0.09	2.80 ± 0.21
galactose	0.52 ± 0.03	0.52 ± 0.04	0.50 ± 0.02	0.51 ± 0.03	0.49 ± 0.01	0.47 ± 0.01	0.46 ± 0.02	0.46 ± 0.01
glucose	37.43 ± 1.74	37.21 ± 2.04	37.26 ± 0.08	37.08 ± 0.92	37.33 ± 1.11	36.53 ± 1.01	35.86 ± 0.52	36.40 ± 1.30
xylose	16.84 ± 0.53	17.01 ± 1.02	16.62 ± 0.12	17.24 ± 1.22	16.84 ± 0.53	16.33 ± 0.46	15.51 ± 0.26	13.96 ± 0.82
total sugars	57.89 ± 2.45	57.78 ± 3.31	57.47 ± 0.25	58.03 ± 2.38	57.64 ± 1.78	56.50 ± 0.45	54.83 ± 0.90	53.61 ± 0.28
acid insoluble	17.37 ± 0.48	17.00 ± 0.14	16.83 ± 1.37	13.43 ± 2.97	14.78 ± 0.56	14.22 ± 0.80	13.03 ± 0.99	11.56 ± 0.81
acid soluble	2.16 ± 0.28	2.40 ± 0.63	4.44 ± 0.37	4.58 ± 0.61	4.72 ± 0.44	5.43 ± 0.66	6.38 ± 0.64	9.65 ± 0.18
total lignin	19.53	19.40	21.27	18.01	19.50	19.65	19.41	21.21
sugar recovery (%)[a]	13.11 ± 0.38	32.04 ± 0.09	39.22 ± 1.02	45.72 ± 0.48	46.71 ± 0.51	54.27 ± 0.69	58.65 ± 0.15	63.17 ± 3.93

[a]Sugar recovery and lignin concentration were calculated based on the percentage of available sugars and lignin in control and ozone treated wheat straw samples.

delay of 4 s, and a spinning speed of 5 kHz were applied to obtain the ^{13}C CP-MAS NMR spectra. All the corresponding ^{13}C CP/MAS spectra were derived from 18 500 scans, with the chemical shifts given in δ parts per million.

Lignin Extraction and Solution-State NMR of Lignin Enriched Isolates. To expedite NMR analysis, extractive free cell wall residue was prepared from milled wheat straw (fraction <60 mesh) using ethanol/toluene (50:50) for 24 h, followed by solvation in ethanol (100 mL/g) and water (100 mL/g) for 24 h each. The processed biomass was then freeze dried and ball milled for 24 h in the presence of toluene and a cycle of 2 min run with 1 min pause to avoid any further changes in lignin structure. The ball milled sample was dried and extracted with 94:6 dioxane/water. The resulting preparation underwent purification for wheat straw lignin extraction as per Björkman's method.[23] The purified lignin was used for solution-state NMR analysis.

Solution-state NMR spectra of lignin isolates were recorded on a Varian 400MR NMR spectrometer equipped with a Varian multi-tunable probe operating at 399.77 MHz for proton (^1H) and at 100.53 MHz for carbon (^{13}C), respectively, with the resonance values given in δ parts per million. The lignin isolates (~25 mg) from both control and ozone pretreated wheat straw samples were individually dissolved in DMSO-d_6 (0.5 cm^3), with the ^1H and ^{13}C NMR spectra recorded for each sample. The ^1H NMR spectra of both the lignin isolates were recorded with a spectral width of 6410 Hz, in 16 scans, whereas the ^{13}C NMR spectra were collected with a sweep width of 25 510 Hz, in 25 000 scans. Single bond $^1H-^{13}C$ correlation (HSQC) NMR spectra were acquired using the following parameters: sweep width, 9.0−0.0 ppm in F2 (^1H) using 962 data points (acquisition time, 150 ms) and 160−10 ppm F1 (^{13}C) using 256 increments (F1 "acquisition time" 15.0 ms). The number of scans was 8 with a 1 s interscan delay. Integration calculations of the 2D contours in all spectra was performed using MestRenova software (trial version). All the structural analyses were carried out on comparison of chemical shift values and proton/carbon correlation (in ppm) resonance values for ozone pretreated lignin samples to its control.

ATR-FTIR Analysis of Control and Ozone Treated Wheat Straw. To confirm the changes of specific functional groups during ozone pretreatment after a specific time interval, the FTIR spectra of control and ozone treated (for 5, 10, 15, 30, 60, 90, and 120 min) wheat straw particles (<60 mesh) were recorded on a Shimadzu IR-Prestige FTIR spectrometer. ATR-FTIR spectra were obtained in absorbance mode, and a total of 64 scans were taken from 4000 to 800 cm^{-1} at a resolution of 4 cm^{-1} due to lignin related intensities. All the treated samples were dried at 50 °C for 24h in oven to remove remaining moisture.

Py-GC/MS Analysis. To validate the observation about degradation of specific aromatic lignin moieties (S, G, and H) during ozonation treatment, a continuous three-stage py-GC/MS analysis was carried out. The analysis process was applied to the control and 5, 30, 90, and 120 min ozone pretreated samples for selective separation and identification of components in the biomass. The pyrolysis temperature for the first stage was 260 °C, at which a low-temperature pyrolysis (prepyrolysis) induces release or thermal desorption of absorbed volatile compounds from the substrate, without formation of new compounds. Subsequent 320 °C pyrolysis of biomass residues from the first stage yielded pyrograms that were used to determine hemicellulose, cellulose, and easily pyrolyzed lignin. The remaining residues, which mostly consisted of stable lignin polymer, were subjected to a third pyrolysis stage at 610 °C. Different pyrolytic products at different pyrolysis temperatures revealed the stability of each component, and the change of the pyrolytic products at each pyrolysis stage revealed stability changes in lignin caused by ozone degradation.

The analyses were conducted using a CDS Pyroprobe 5000 connected to an Agilent GC/MS system (7890A GC, 5975C mass selective detector). The control and ozone treated samples were loaded into a quartz tube and gently packed with quartz wool prior to pyrolysis. After a brief incubation at 200 °C for residual oxygen removal, samples were pyrolyzed instantaneously at 260 °C (1st stage), 320 °C (2nd stage), and 610 °C (3rd stage) for 1 min. The inlet temperature was maintained at 250 °C, and resulting pyrolysis vapors were separated by a (5%-phenyl)-methylpolysiloxane hydrophobic column with a 30 m × 25 mm inner diameter. The gas flow rate was 1 mL/min, and the oven was retained at 280 °C for 10 min to prevent any residuals from remaining in the chamber. This gas was then analyzed with a mass spectrometer (Inert XL MSD, Agilent Technologies), and CO_2 was used as the internal standard. The abundance of each derived compound was referenced against the area of the internal standard, and compounds were identified by their electron impact (EI) mass spectra as compared to the National Institute of Standards and Technology (NIST) mass spectral search 2.0 electronic libraries.

TG/DTG Analysis. TG and DTG analyses are valuable tools for analyzing the thermal behavior of biomass during development of thermochemical biomass conversion technologies. Hence, control and ozone pretreated samples were analyzed using TG and DTG to determine the change in activation energy (E) and pre-exponential factor (A). The wheat straw mass at 150 °C was considered to be dewatered, and this was the starting mass point used in all TG calculations. Additionally, calculations for activation energy and pre-exponential factor were performed from 5% to 80%, at intervals of 5%. Values after 80% were not considered due to a rapid increase of activation energy at that point, since the majority of the biomass had turned to char and entered the burnout stage. The analysis was performed using a Mettler Star system (Mettler Toledo TGA/SDTA 851, Switzerland) with 5 heating rates: 10, 15, 20, 30, and 40 °C/min. All the experiments were performed in triplicate under a nitrogen atmosphere with a flow rate of 20 mL/min. On the basis of the

different heating rates, activation energy and pre-exponential factor were calculated using ASTM[24] and the Friedman method.[25]

Both of these analysis methods are based on a differential method requiring the assessment of multiple heating rates. These methods were chosen due to the limitations of obtaining biomass kinetic parameters from a single heating rate.[26] The Friedman method is an isothermal analysis process that can be used to calculate the activation energy (E) and pre-exponential factor of the samples (A) and is expressed as

$$\ln\left(\frac{d\alpha}{dt}\right) = \ln f(\alpha) + \ln(A) - \frac{E}{RT} \quad (1)$$

Where $(d\alpha)/(dt)$ is the instantaneous reaction rate, R is the gas constant, A is the pre-exponential factor, α is the mass fraction remaining at time t, and $f(\alpha)$ is the function regarding the reaction mechanism.

The ASTM procedure is based on an integral method and the assumption that decomposition obeys first-order kinetics to evaluate activation energy and pre-exponential factor:

$$E = -\left(\frac{R}{b}\right)\cdot\Delta(\log\beta)\cdot\Delta\left(\frac{1}{T}\right)^{-1} \quad (2)$$

$$A = \left(-\frac{\beta'}{E_r}\right)\cdot R\cdot\ln(1-\alpha)\cdot 10^{a} \quad (3)$$

where b is the approximate derivative given by the ASTM table of integration constants, β is the heating rate, β' is the heating rate nearest the midpoint of the chosen experimental heating rates, E_r is the refined Arrhenius activation energy, and a is the approximation integral taken from the ASTM table of integration constants.

RESULTS AND DISCUSSION

Effect of Ozone Pretreatment on Wheat Straw Composition and Enzymatic Hydrolysis. Table 1 shows the effect of moisture content on the efficiency of ozone pretreatment in terms of sugar recovery. Increasing the moisture content from 30% to 90% (w/w) improved ozonation efficiency that resulted in enhanced sugar recovery from 13% (with control sample) to 63% (with 90% w/w moisture adjusted wheat straw particles). This was due to the generation of reactive ·OH radicals, which was reported elsewhere[27] or the possible reaction of ·OH in addition to ozone with lignin in the presence of water. These results are in accordance with previous studies that found that moisture content was critical for the reactivity of ozone.[28] Also, Al Jibouri[29] reported that an adequate amount of water was required (more than equilibrium moisture content for improved solubility of ozone in water that is mainly responsible for delignification of embedded lignin molecule) for the ozonolysis process and low moisture content did not favor the delignification process. To study the effect of ozonolysis on the composition of treated wheat straw, sugar, acid soluble, and insoluble lignin content were determined (Table 1), and the result was compared with control wheat straw. Approximately 57.9% of the control wheat straw was composed of cellulose and hemicellulose, and this value decreased slightly to 53.6% after the 2 h ozonolysis pretreatment. This was likely due to oxidation of carbohydrates by ozone. Ben-Ghedalia and Miron[30] reported the loss of cellulose after ozone pretreatment to be less than 5% when working at low pH values. Acid soluble lignin content increased dramatically after ozone pretreatment, from 2.2% to 9.7%. This could be due to the reaction of ozone with lignin polymer and the generation of monomeric lignin fragments during acid hydrolysis (for biomass composition analysis) of pretreated biomass. Accordingly, the acid insoluble lignin content decreased from 17.4% to 11.6%. Ozone is known to be more reactive with lignin than carbohydrates during the pretreatment process and degrades a minimum amount of cellulose.[7]

Solid-State ^{13}C CP/MAS NMR. Figure 2 shows the comparison of ^{13}C CP/MAS NMR spectral patterns of the

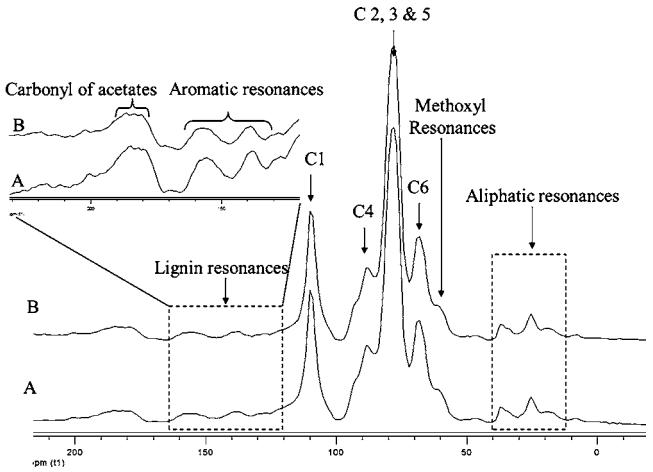

Figure 2. Solid-state NMR of (A) control and (B) ozone treated (conditions 90% moisture content, 5.3% w/v ozone concentration, and oxygen flow rate 2 L/min for 120 min) wheat straw samples.

control and ozone treated wheat straw. Although the spectra shown do not provide quantitative information, both spectra demonstrate characteristic peaks that correspond to components such as cellulose, hemicellulose, and lignin. The peak assignments showing the lignin, anomeric carbon, methoxyl, and aliphatic resonances were based on previous literature report.[19] Additionally, identical acquisition parameters were used to obtain these spectra, and the same amount of sample was used, allowing comparison of the resultant changes in the spectra. As shown in Figure 2, the 40−115 ppm region has peaks typical of polysaccharides, while the side-chain groups (oxygenated Cα, Cβ, and Cγ carbons) of the phenyl-propane lignin structure units also provide a minor contribution. Cellulose resonance signals at different carbon positions were located at 109-C1; 88-C4; 78-C2, C3, and C5; and 68-C6. Signals in the 115−165 ppm region show the difference between aromatic structures of the control and ozone treated wheat straw samples, which is concurrent with a decrease in concentration of the aromatic carbon signal at 153 ppm (due to aryl units) and 137 ppm.[31,32] The modifications were observed at the carbonyl signal (177 ppm) in the ozone treated sample. Change was observed in the intensity of the 60 ppm signal attributed to the methoxy groups in lignin that suggests possible breakdown of ester-linked structures during ozone treatment. These results clearly indicate that lignin was modified during ozone pretreatment.

Solution-State ^{1}H and ^{13}C NMR Spectroscopies of Lignin Isolates. Purified lignin (as detailed in the Lignin Extraction and Solution-State NMR of Lignin Enriched Isolates section) was obtained from control and ozone treated wheat straw particles for ^{1}H and ^{13}C NMR analyses. Figure 3 shows the comparison of ^{1}H NMR spectra obtained from control and ozone treated wheat straw lignin samples, which demonstrates a clear difference in the distribution of the proton aromatic chemical shift values, indicating ozone had a prominent effect

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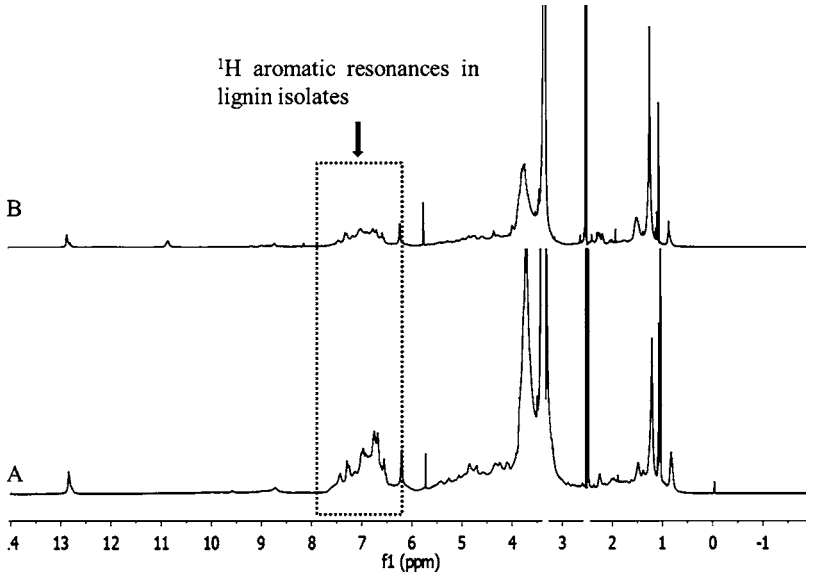

Figure 3. ^{1}H NMR of (A) control and (B) ozone treated (conditions 90% moisture content, 5.3% w/v ozone concentration, and oxygen flow rate 2 L/min for 120 min) wheat straw samples.

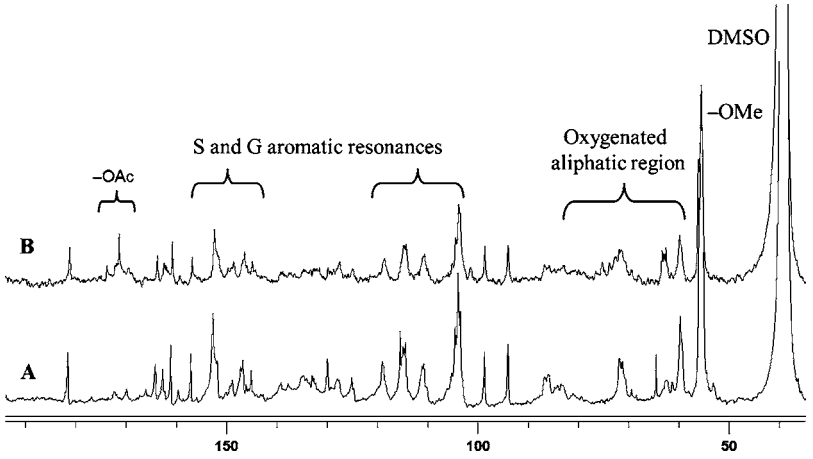

Figure 4. NMR spectra of lignin-derived isolates from wheat straw: (A) 1D ^{13}C NMR spectra of lignin-enriched isolate from control wheat straw; (B) 1D ^{13}C NMR spectra of lignin-enriched isolate from ozone pretreated (conditions 90% moisture content, 5.3% w/v ozone concentration, and oxygen flow rate 2 L/min for 120 min) wheat straw.

on the aromatic component of lignin. These results are in accordance to the Ben'ko et al.[16] and Khudoshin et al.[15] which state that the aromatic component of lignin was very susceptible to ozone reactions. The ^{13}C NMR analysis of ozone pretreated samples showed reduced lignin content as well (Figure 4), which is evident from the decreased intensities of lignin-contributing resonances (both G and S units) within the specific region from 152 to 143 ppm and from 120 to 103 ppm, respectively. The overall pattern of G and S lignin resonances in both the control and pretreated samples showed degradation of both units to the extent. Interestingly, a major difference is observed in the ^{13}C NMR spectra of the ozone treated sample in the region 168−173 ppm when compared to the spectra of the control sample. This region is attributed to the acetate group (−OAc) that is generated from acidic functional groups in lignin during the lignin isolation process (during the dissolution step in acetic acid). Therefore, an increase in the intensities of the acetate region in the spectra of the ozone pretreated sample (Figure 4) shows that there is higher acid functionality due to aromatic ring opening and conversion to carboxylic groups by ozone. These results suggested that ozonation increases the number of carboxyl groups due to ring opening of lignin structures.

The ^{1}H−^{13}C heteronuclear single quantum correlation (HSQC) NMR spectroscopy and the evaluation results of changes in the aromatic resonances of lignin were thoroughly evaluated and marked (Figure 5). The chemical shift of hydrogen atoms was correlated with the carbon to which it is attached and set to contour intensities of the aromatic signals. HSQC has been proved to be a useful tool for analysis of lignin structure.[33] The cross signals in the aromatic region of the 2D HSQC spectra of control and ozone treated wheat straw lignin corresponded to the predominant aromatic rings in syringyl (S) and guaiacyl (G) units. In the control sample, the etherified S2/6-lignin units showed a major signal for $C_{2,6}$−$H_{2,6}$ correlation at δ_C/δ_H 104.64/6.58 and 104.65/6.67 that was decreased significantly in the ozone treated sample where minimal correlation for $C_{2,6}$−$H_{2,6}$ was observed at δ_C/δ_H 104.84/6.60. The $C_{2,6}$−$H_{2,6}$ correlations in C_α-oxidized S units (S′ 2/6) were observed for the control at δ_C/δ_H 105.30/6.97, 105.26/7.04,

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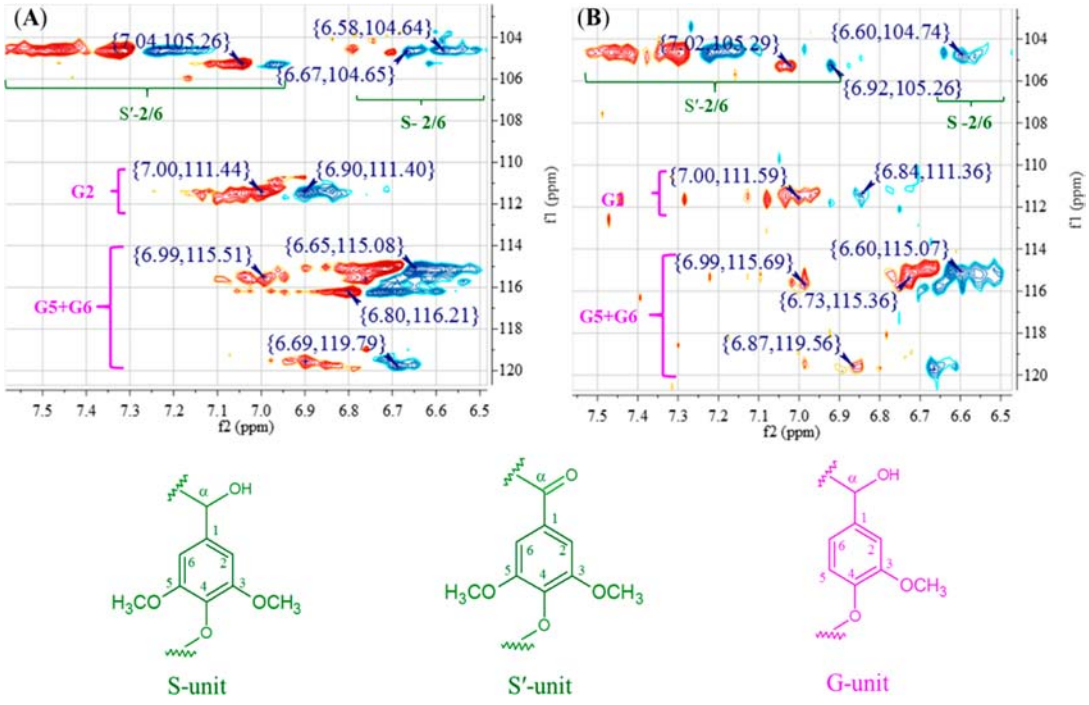

Figure 5. 2D HSQC NMR spectra of lignin-derived isolates from wheat straw: (A) 2D HSQC spectra of selected aromatic region of control wheat straw lignin; (B) 2D HSQC spectra of selected aromatic region of ozone treated (conditions 90% moisture content, 5.3% w/v ozone concentration, and oxygen flow rate 2 L/min for 120 min) wheat straw lignin (Note: all correlations discussed in the Result and Discussion section are not labeled to avoid overcrowding).

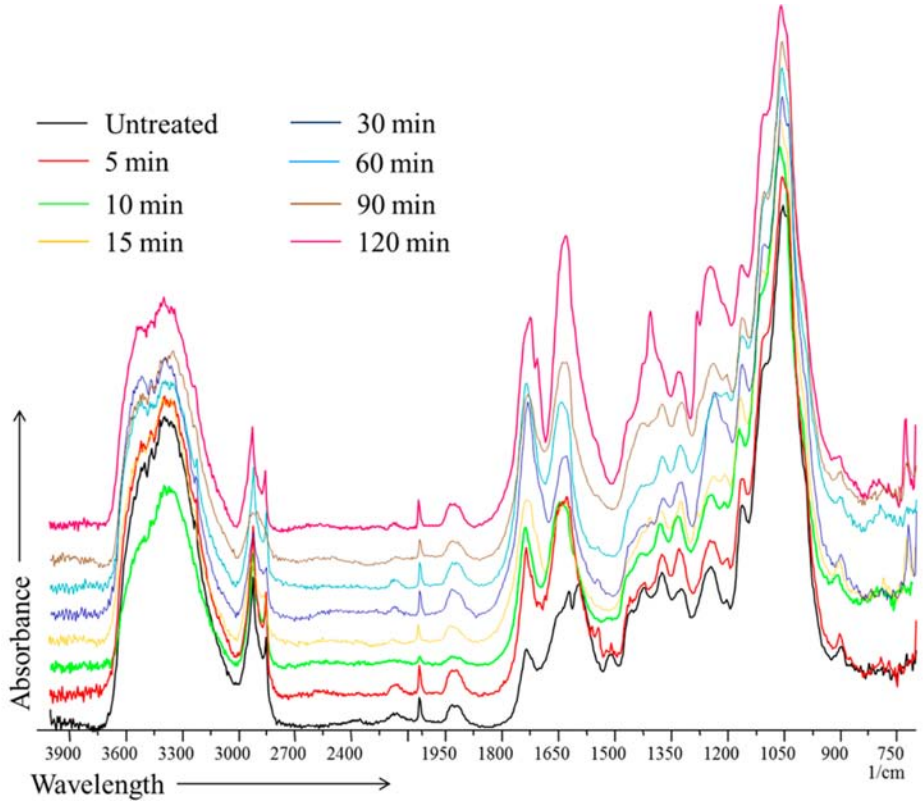

Figure 6. FTIR spectra of control and ozone treated (conditions 90% moisture content, 5.3% w/v ozone concentration, and oxygen flow rate 2 L/min for varying exposure times) wheat straw samples.

104.67/7.20, 104.70/7.32, and 104.59/7.47 out of which signals at δ_C/δ_H 105.26/6.92 and 105.29/7.02 decreased, comparatively. In control wheat straw, the G lignin units produced strong correlations for C_2-H_2 at δ_C/δ_H 111.40/6.90 and 111.44/7.00; for C_5-H_5 at δ_C/δ_H 115.08/6.65, 115.17/6.76, 115.51/6.99, and 116.21/6.80; and for C_6-H_6 at δ_C/δ_H

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Table 2. Assignment of FTIR Absorption Bands of Control and Ozone Treated (120 min) Wheat Straw

		control wheat straw		ozone treated wheat straw (120 min)	
λ (cm^{-1})	attribution and description of FTIR absorption	λ^a (cm^{-1})	T^b (%)	λ^a (cm^{-1})	T^b (%)
3700−3100	O—H stretching vibrations of polymer	3367.71	71.61	3367.71	75.16
3000−2830	C—H stretching vibration (CH_3, CH_2)	2918.30	80.60	2918.30	90.08
		2850.79	87.68	2850.79	94.44
1760−1720	C═O stretching vibrations	1728.22	82.33	1730.15	62.17
1640−1636	C═O stretching vibrations, characteristics of para-substituted aryl skeleton			1641.42	68.54
1660−1590	C═O stretching and aromatic vibrations; S > G; condensed G > etherified G	1595.13	71.21		
1550−1490	aromatic skeletal vibrations; G > S	1512.19	81.86		
1460−1410	aromatic skeletal vibrations	1452.40	72.24		
		1423.47	68.31	1419.61	74.98
1375−1360	C—H deformation of cellulose and hemicellulose	1371.39	64.03	1371.38	67.01
1340−1310	C—H vibration in cellulose and C—O vibration of S ring	1319.31	65.40	1321.24	62.94
1250−1220	methoxyl, C—C and C—O stretching vibrations; C═O stretching vibration	1242.16	54.84	1234.44	52.50
1210−1190	symmetric stretching C—O—C glycoside	1201.65	60.20	1201.65	52.14
1170−1150	C—O—C vibrations in cellulose and hemicellulose	1159.22	42.03	1161.15	41.21
1060−1036	C—O stretch in cellulose	1049.28	10.59	1055.06	11.16
899−887	C—H deformation in cellulose	898.83	66.77	900.76	64.66

[a] λ: wavelength. [b] T: transmittance.

119.79/6.92 and 119.56/6.90. Resonance of the G units in the ozone treated sample was decreased considerably for G2, showing only mere traces for C_2−H_2 at δ_C/δ_H 111.36/6.84. The G5 units did not show C_5−H_5 correlation at δ_C/δ_H 116/20/6.80 and were minimal at δ_C/δ_H 115.07/6.60 and 115.69/6.99 as compared to the control wheat straw response (Figure 5). The response of G6 units was also decreased significantly in the ozone treated sample as compared to the control sample that is evident from the C_6−H_6 correlations at δ_C/δ_H 119.56/6.87 and 119.79/6.69. Overall, it was clear that the nonoxidized S2/6 and G2 correlations were drastically reduced in the 2D HSQC NMR spectra of the ozone pretreated lignin in comparison to the control (Figure 5). Minor structural changes were also observed in the S′2/6 region due to β-O-4 cleavage of the lignin polymer. Thus, nonspecific β-O-4 cleavage of both G and S lignin subunits with generation of acid functional groups during ozone pretreatment is supported by the HSQC data.

FTIR Analysis of Control and Ozone Treated Wheat Straw. The FTIR spectra of control and ozone treated samples (for 5, 10, 15, 30, 60, 90, and 120 min) from 800 to 4000 cm^{-1} is shown in Figure 6. There were several significant changes observed, including a gradual increase of absorbance at 1730 cm^{-1} (due to carbonyl stretch in unconjugated ketones and carboxylic groups), extinction of aromatic vibration at 1594−1609 cm^{-1}, and increase in the C═O stretch of conjugated *p*-substituted aryl ketones. These findings corroborate the ^{13}C NMR (Figure 3) results where the presence of acetyl groups was observed. This could be due to ring opening and formation of an additional carbonyl group[34] after ozonolysis, and these results strongly corroborate the ozone−lignin degradation reaction. The C═O stretching and aromatic vibrations at 1595 cm^{-1} have been shifted toward higher wavelength (1641 cm^{-1} of the C═C weak stretch of a 3-ring compound). This could be due to increased carbonyl groups and condensation of remaining aromatic structures at the surface of ozone treated wheat straw. The most significant difference observed in the FTIR spectra of the ozone treated sample was at 1512 cm^{-1}, where the absorbance peak in the control spectra was almost completely absent in the ozone treated sample. This peak is typically derived from aromatic ring structure, and a decrease in the absorbance of this peak is attributed to the removal of lignin aromatic rings in the biomass. These results were supported by earlier findings in the 2D NMR study where S and G units were seen to be the targets of ozonolysis. Significant decrease in the peaks between 1450 and 1320 cm^{-1} was due to reduced C−C ring skeletal and C−H vibrations, which along with a decrease in C═C absorbance is a result of ozone induced ring opening. All the spectra of (control and ozone treated) wheat straw showed a strong absorbance at 3367 cm^{-1} indicative of O−H stretching, as well as C−H stretch vibrations at 2950 cm^{-1}. The FTIR analysis of different samples pretreated for varying times with ozone concludes increased presence of carboxylic group, diminished aromatic absorbance at a particular wavelength (1512 cm^{-1}), and shift of C═O absorbance attributed due to condensation of a single aromatic ring to double or triple ring structure. The detailed comparison of results for control and 120 min ozone treated wheat straw samples are documented in Table 2, and comparison of the FTIR spectra obtained in transmittance mode was performed according to earlier reports.[35]

Py-GC/MS Analysis. Figure 7a−c depicts three-stage py-GC/MS pyrograms of control, 5, 30, 90, and 120 min ozone treated samples. The corresponding retention time and nomenclature of identified compounds may be found in Table 1 of the Supporting Information. The pyrogram of the control at 260 °C showed lignin-derived compounds representing S, G, and H units. The abundance of S units (peak nos. 5 and 9−12) observed to be somewhat lower than that of G units (peak nos. 1, 3, 4, and 6−8). In contrast, the pyrograms obtained at 260 °C of all the treated samples (5, 30, 90, and 120 min) did not show peaks representing these compounds. This could be due to the destruction of lignin compounds that are readily accessible on the surface of the wheat straw by the ozone treatment. Interestingly, in the 90 and 120 min ozone treated spectra, a significant acetic acid peak was observed at a 3.02 min retention time (data not shown). We postulate that the acetic acid could have been derived from lignin aromatic rings or from dissociated hemicellulose moieties.

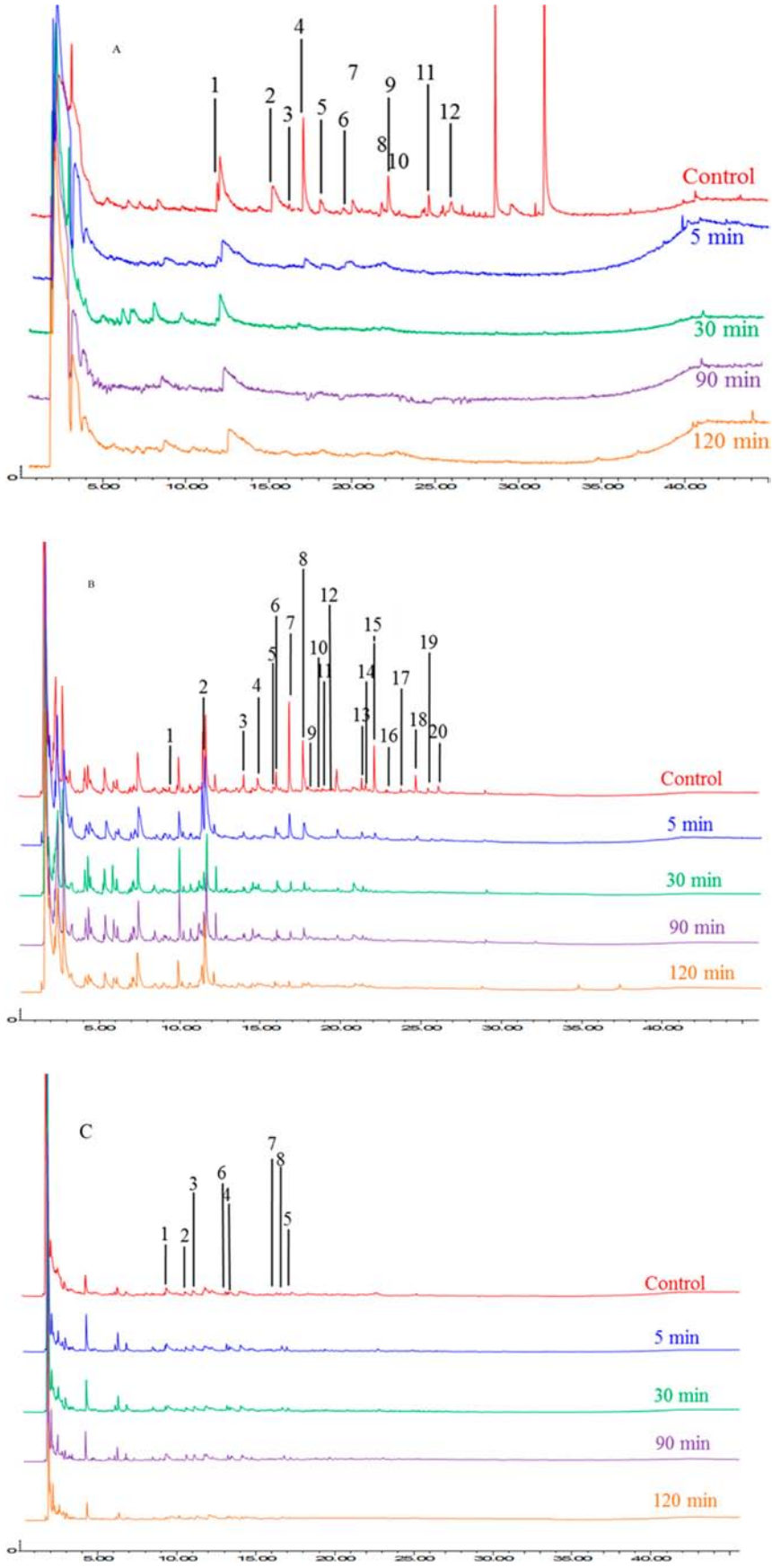

Figure 7. Three-stage py-GC/MS pyrogram of control and ozone treated (conditions 90% moisture content, 5.3% w/v ozone concentration, and oxygen flow rate 2 L/min for varying exposure time) wheat straw: (A) 1st stage at 260 °C; (B) 2nd stage at 320 °C; (C) 3rd stage at 610 °C; the nomenclatures of all identified compounds of the respective stages are listed in Table 1 of the Supporting Information.

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The pyrograms obtained at 320 °C (Figure 7b) showed a selective degradation of S (peak nos. 13 and 15−19) and G units (peak nos. 3, 5, 9, 11, and 14). The abundance of S units observed to getting decreased with increased ozonation time from 5 to 90 min and completely disappeared at 120 ozonation time. In contrast, though G units were also degraded, they were observed to be more resistant than S units as we were able to detect a significant amount even after 120 min of ozonation. Figure 7c shows compounds that were not yet volatilized in earlier 260 and 320 °C pyrolysis experiments. A clear difference was observed between control and treated samples as the later showed few condensed lignin structures (appearance of naphthalene, peak no. 6; naphthalene-1-methy, peak no. 7; napthalen-2-methyl, peak no. 8) which contain two or more aromatic rings. This is consistent with an earlier study[36] which reported that extended ozonation resulted in the formation of condensed lignin oligomers. Earlier peaks in the control wheat straw chromatogram, between 8 and 15 min (with some exception), largely represent phenols derived from H units. The presence of these derived products (peak nos. 1−5) can still be seen in the treated samples, which is consistent with our NMR results and earlier findings that ozone reaction was highly preferential toward S and G lignin subunits.[36] We also observed traces of condensed lignin structures including naphthalene, naphthalene-1, or 2-methyl (Supporting Information, Table 1). These results are in accordance with the FTIR observation where a single aromatic absorbance shift was evidenced. The aromatic compounds observed in control samples pyrolyzed at 260 °C in the pyrogram and the absence of these compounds in samples treated with ozone for 5−120 min support the conclusion that ozonolysis was able to significantly decrease the aromatic ring structure of lignin on the surface.

TG and DTG Analyses. Figure 8A,B presents the TG and DTG curves for the control and samples treated for 2 h, respectively. In both the control and ozone treated wheat straw DTG curves, the highest peak occurs around 330 °C, which corresponds to the temperature at which cellulose was supposed to decompose fastest. However, in the ozone treated DTG, there is a completely new peak centered around 250 °C, and it can also be seen that decomposition begins much earlier compared to the control. Between 150 and 200 °C in the control DTG, it is seen that there is a minimal rate of decomposition for all temperature profiles. However, in the curve for treated sample, decomposition begins earlier and accelerates faster. We hypothesized that this curve splitting was due to separation of the lignin from the hemicellulose in the wheat straw, which allowed the hemicellulose to decompose at a lower temperature. However, the activation energy (E) for the ozone treated sample was significantly higher (ASTM-E, 509.291 kJ/mol; Friedman-E, 462.171 kJ/mol) than that of the control (ASTM-E, 259.738 kJ/mol; Friedman-E, 270.084 kJ/mol). Likewise, Figure 9 shows the activation energy of control and treated samples with respect to decomposition percentage. Considerable increase in activation energy was observed for conversions between 0.2 and 0.5. This increase in activation energy was possibly due to condensation of lignin structures by the ozone treatment. Quesada et al.,[6] have found that treatment of biomass by ozone with times exceeding 30 min have produced condensed lignin structures that are harder to degrade. Further studies need to be performed for identification of different component volatized at various temperatures.

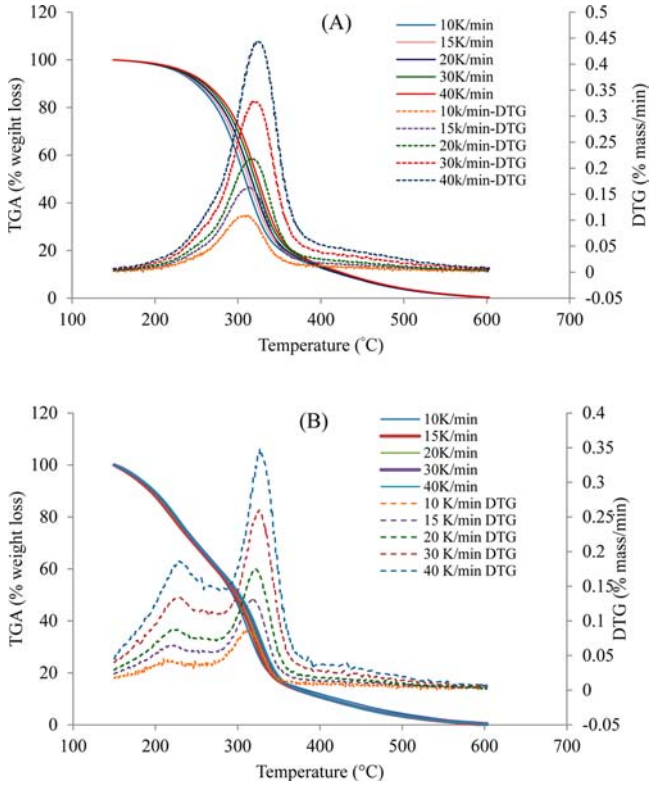

Figure 8. TG and DTG curves for (A) control and (B) ozone treated (conditions 90% moisture content, 5.3% w/v ozone concentration, and oxygen flow rate 2 L/min for 120 min) wheat straw.

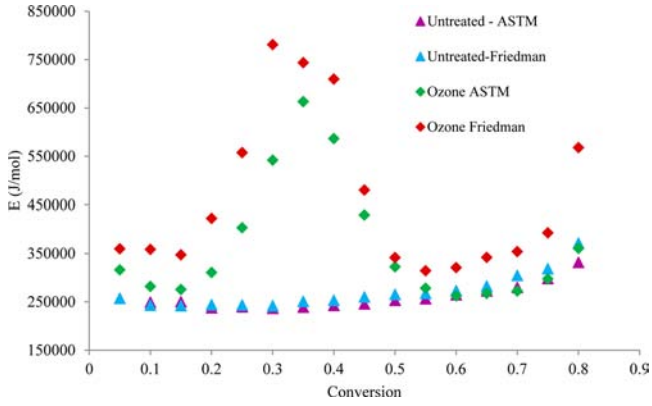

Figure 9. Activation energy of samples compared to conversion amount.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table of nomenclature, retention time, and relative abundance of py-GC/MS products from control and ozone treated wheat straw. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Zaldivar, J.; Nielsen, J.; Olsson, L. Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration. *Appl. Microbiol. Biotechnol.* **2001**, *56* (1), 17−34.

(2) Hendriks, A. T.; Zeeman, G. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* **2009**, *100* (1), 10−8.

(3) Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y. Y.; Holtzapple, M.; Ladisch, M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **2005**, *96* (6), 673−86.

(4) Stewart, J. J.; Kadla, J. F.; Mansfield, S. D. The influence of lignin chemistry and ultrastructure on the pulping efficiency of clonal aspen (*Populus tremuloides Michx.*). *Holzforschung* **2006**, *60* (2), 111−122.

(5) Studer, M. H.; DeMartini, J. D.; Davis, M. F.; Sykes, R. W.; Davison, B.; Keller, M.; Tuskan, G. A.; Wyman, C. E. Lignin content in natural Populus variants affects sugar release. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108* (15), 6300−6305.

(6) Quesada, J.; Rubio, M.; Gómez, D. Ozonation of lignin rich solid fractions from corn stalks. *J. Wood Chem. Technol.* **1999**, *19* (1−2), 115−137.

(7) Sun, Y.; Cheng, J. Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresour. Technol.* **2002**, *83* (1), 1−11.

(8) Roncero, M. B.; Torres, A. L.; Colom, J. F.; Vidal, T. TCF bleaching of wheat straw pulp using ozone and xylanase. Part A: Paper quality assessment. *Bioresour. Technol.* **2003**, *87* (3), 305−314.

(9) Contreras, S. Degradation and biodegradability enhancement of nitrobenzene and 2,4-dichlorophenol by means of advanced oxidation processes based on ozone. Ph.D. Thesis, University of Barcelona, Barcelona, Spain, 2002.

(10) García-Cubero, M. T.; Palacín, L. G.; González-Benito, G.; Bolado, S.; Lucas, S.; Coca, M. An analysis of lignin removal in a fixed bed reactor by reaction of cereal straws with ozone. *Bioresour. Technol.* **2012**, *107* (0), 229−234.

(11) Kojima, Y.; Yoon, S.-L. Improved enzymatic hydrolysis of waste paper by ozone pretreatment. *J. Mater. Cycles Waste Manage.* **2008**, *10* (2), 134−139.

(12) Neely, W. C. Factors affecting the pretreatment of biomass with gaseous ozone. *Biotechnol. Bioeng.* **1984**, *26* (1), 59−65.

(13) Kaneko, H.; Hosoya, S.; Iiyama, K.; Nakano, J. Degradation of lignin with ozone: Reactivity of lignin model compounds toward ozone. *J. Wood Chem. Technol.* **1983**, *3* (4), 399−411.

(14) Kratzl, K.; Claus, P.; Reichel, G. Reactions of lignin and lignin model compounds with ozone. *Tappi J.* **1976**, *59* (11), 86−87.

(15) Khudoshin, A.; Mitrofanova, A.; Lunin, V. Kinetics and mechanism of the reactions of ozone with guaiacol, veratrol, and veratrol derivatives. *Russ. Chem. Bull.* **2008**, *57* (2), 283−288.

(16) Ben'ko, E. M.; Bokova, M. N.; Pryakhin, A. N.; Lunin, V. V. The kinetic patterns of ozonolysis of model lignin compounds in aqueous solutions. *Russ. J. Phys. Chem.* **2003**, *77* (4), 586−589.

(17) Pan, G.; Chen, C.; Gratzl, J.; Chang, H. Model compound studies on the cleavage of glycosidic bonds by ozone in aqueous solution. *Res. Chem. Intermed.* **1995**, *21* (3), 205−222.

(18) Balousek, P. J. The effects of ozone upon a lignin-related model compound containing a β-aryl ether linkage. Ph.D. Thesis, Lawrence University, Appleton, Wisconsin, 1979.

(19) Kolodziejski, W.; Frye, J. S.; Maciel, G. E. Carbon-13 nuclear magnetic resonance spectrometry with cross polarization and magic-angle spinning for analysis of lodgepole pine wood. *Anal. Chem.* **1982**, *54* (8), 1419−1424.

(20) Gao, A. H.; Bule, M. V.; Laskar, D. D.; Chen, S. Structural and thermal characterization of wheat straw pretreated with aqueous ammonia soaking. *J. Agric. Food Chem.* **2012**, in press.

(21) Rakness, K.; Gordon, G.; Langlais, B.; Masschelein, W.; Matsumoto, N.; Richard, Y.; Robson, C. M.; Somiya, I. Guideline for measurement of ozone concentration in the process gas from an ozone generator. *Ozone: Sci. Eng.* **1996**, *18* (3), 209−229.

(22) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. *Determination of structural carbohydrates and lignin in biomass*, Laboratory Analytical Procedure (LAP); National Renewable Energy Laboratory: Golden, CO, 2004.

(23) Björkman, A. Studies on finely divided wood. Part I. Extraction of lignin with neutral solvents. *Sven. Papperstidn.* **1956**, *59* (13), 477−485.

(24) ASTM. *ASTM Standard E1641-07, standard test method for decomposition kinetics by thermogravimetry*; ASTM International: West Conshohocken, PA, 2007.

(25) Friedman, H. L. Kinetics of thermal degradation of char-forming plastics from thermogravimetry. Application to a phenolic plastic. *J. Polym. Sci., Part C: Polym. Symp.* **1964**, *6* (1), 183−195.

(26) Burnham, A. K. Computational aspects of kinetic analysis.: Part D: The ICTAC kinetics project — multi-thermal−history model-fitting methods and their relation to isoconversional methods. *Thermochim. Acta* **2000**, *355* (1−2), 165−170.

(27) Staehelin, J.; Hoigne, J. Decomposition of ozone in water in the presence of organic solutes acting as promoters and inhibitors of radical chain reactions. *Environ. Sci. Technol.* **1985**, *19* (12), 1206−1213.

(28) Secrist, R.; Singh, R. Kraft pulp bleaching. II. Studies on the ozonation of chemical pulps. *Tappi J.* **1971**, *54* (4), 581−584.

(29) Al Jibouri, A. K. H. Effect of intermediate washing on ozonolysis delignification and enzymatic hydrolysis of wheat straw. Masters Thesis, Ryerson University, Toronto, Canada, 2012.

(30) Ben-Ghedalia, D.; Miron, J. The effect of combined chemical and enzyme treatments on the saccharification and in vitro digestion rate of wheat straw. *Biotechnol. Bioeng.* **1981**, *23* (4), 823−831.

(31) Nimz, H. H.; Tschirner, U.; Stähle, M.; Lehmann, R.; Schlosser, M. Carbon-13 NMR spectra of lignins, 10.1 Comparison of structural units in spruce and beech lignin. *J. Wood Chem. Technol.* **1984**, *4* (3), 265−284.

(32) Guadalix, M.; Almendros, G.; Martinez, A.; González-Vila, F.; Lankes, U. A ^{13}C CP/MAS NMR evaluation of the structural changes in wheat straw subjected to different chemical and biological pulping conditions. *Bioresour. Technol.* **1997**, *60* (3), 245−249.

(33) Yelle, D. J.; Ralph, J.; Lu, F.; Hammel, K. E. Evidence for cleavage of lignin by a brown rot basidiomycete. *Environ. Microbiol.* **2008**, *10* (7), 1844−1849.

(34) Areskogh, D.; Li, J.; Gellerstedt, G.; Henriksson, G. Structural modification of commercial lignosulphonates through laccase catalysis and ozonolysis. *Ind. Crops Prod.* **2010**, *32* (3), 458−466.

(35) Pandey, K.; Pitman, A. FTIR studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. *Int. Biodeterior. Biodegrad.* **2003**, *52* (3), 151−160.

(36) Mamleeva, N.; Autlov, S.; Fionov, A.; Bazarnova, N.; Lunin, V. The oxidative destruction of lignin in the ozonation of wood. *Russ. J. Phys. Chem. A* **2009**, *83* (5), 745−751.