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Molecular and structural impacts of fungal depolymerization of corn stover to reduce pretreatment severity

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Abstract

Recalcitrance of lignocellulosic feedstocks to depolymerization is a significant barrier for renewable energy conversion approaches that require monomeric carbohydrates as inputs to conversion. This study assesses feedstock supply chain operations can be transformed into targeted pretreatments. Corn stover was inoculated with lignin degrading filamentous fungi (*Phaenaerochaete chrysosporium*), then stored in aerated bioreactors designed to mimic storage conditions in large-scale storage piles to determine if fungal pretreatment enhanced lignin degradation. Composition changes resulting from *P. chrysosporium* treatment included hemicellulose and lignin degradation. Pyrolysis GCxGC/MS indicate cleavage of glycosidic bonds in hemicellulose resulted in sugar degradation products. Enhanced G and S lignol releases were observed. Dilute acid pretreatment and enzymatic hydrolysis indicated lowering the reaction temperature to reduce pretreatment severity resulted in equivalent xylose release in unstored and fungal treated samples. These results suggest this combined biological, thermochemical pretreatment can augment glycosidic bond cleavage and lignin degradation in lignocellulosic biorefineries.

Introduction

Producing renewable liquid transportation fuels from agricultural residues that would otherwise be discarded is a significant opportunity to reduce the carbon footprint of the transportation sector[1]. Up to one billion tons of sustainably produced biomass and waste materials are potentially available for bioenergy production with >130 million dry tonnes of agricultural residues in the United States presently available[2]. The biorefinery concept takes these carbon rich materials and utilizes integrated biorefineries to create biofuels[3] and bioproducts[4] with a lower carbon intensity compared to petroleum-based counterparts.

The inherent molecular and structural complexity of any lignocellulosic material is a challenge for biochemical conversion systems that are designed for the grain ethanol industry, where starch-rich corn grain is easily broken down enzymatically and fermented to ethanol. The intricately woven matrix of cellulose microfibrils protected by hemicellulose and lignin results in a feedstock that is recalcitrant to physical and biological deconstruction into carbohydrate monomers that can readily be fermented to fuels[5]. Understanding how lignocellulosic material can be deconstructed at a biorefinery using a combination of chemicals, thermal energy, and enzymatic inputs has been a topic of significant interest. Commonly explored pretreatments to reduce recalcitrance prior to fermentation include acid- or alkali-based chemical additions, steam explosion with and without ammonia, mechanical refining, ionic liquid treatments, and enzymes[6],

Long- and short-term storage of lignocellulosic material is required to provide a year round supply of seasonally available biomass sources, and short term storage at a biorefinery gate allows for a readily available supply of feedstock to feed a reactor[7]. Innovative approaches to reduce biomass recalcitrance during storage have the potential to reduce the energy required for bioconversion and improve the sustainability of bioenergy systems. This study aims to investigate biological approaches to reduce recalcitrance. Success of this approach has the potential to transform short term storage in a queuing operation into a preprocessing step that adds value to the material by reducing the energy required to convert lignocellulosic biomass into fuel, in a step that currently is a net cost to the operation.

A key player in biomass degradation during aerobic storage are filamentous fungi. Filamentous fungi have been characterized in their role of decaying woody biomass on forest floors and are

well recognized for their ability to degrade lignin in order to make cellulose more accessible[8]. Filamentous fungi have been studied in terms of bioenergy conversion, biopulping, and biobleaching for their role in degrading lignin and reducing recalcitrance in lignocellulosic feedstocks[9, 10]. Lignin molecules are formed when *p*-coumaryl, coniferyl, and sinapyl alcohols are oxidatively polymerized into p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units. Lignin can consist of hundreds of these units and can be linked to hemicellulose, pectin, and cellulose through direct ester or ether bonds or through acid bridges such as ferulic acid or hydroxycinnamic acid[11, 12]. The aromatic nature of lignin provides potential to create for numerous bio-based products including fuels, chemicals, and as substrate for compounding and building materials. Gillet et al. have provided a comprehensive review of lignin structure and isolation approaches[13].

Long term storage offers itself as a unit operation in which lignin depolymerization can begin[14]. The pulp and paper industry has employed lignin degradation in a short term "seasoning" step prior to processing. This is accomplished primarily through the use of white-rot fungi that produce laccase and peroxidases that cleave lignin bonds and expose hemicellulose and cellulose[15]. Numerous studies have shown that filamentous fungi can initiate lignin degradation during storage, effectively pretreating the biomass, making it more susceptible to conversion to biofuels[9, 16-18]. A comprehensive review of studies assessing fungal-assisted pretreatments is available and highlights the opportunity space for the scientific community[19].

Three primary classifications of filamentous fungi are widely recognized: white-rot, brown-rot, and soft-rot. White-rot fungi are the most commonly studied in terms of biomass pretreatment because their targeted mode of action is lignin degradation facilitated by the secretion of enzymes including lignin peroxidase, manganese peroxidase, and laccase[20]. Brown rot fungi have evolved significantly from the white rot to utilize non-enzymatic approaches to lignin decomposition, and they are hypothesized to occur through Fenton reactions[21]. Fenton reactions are based on the formation of highly reactive hydroxyl radicals (·OH) formed from the oxidation of Fe^{2+} to Fe^{3+} by H_2O_2. The resulting hydroxyl radicals oxidize the bonds in cellulose, hemicellulose, and lignin, resulting in near complete degradation of the biomass. Soft-rot fungi lack lignin degrading enzymes and instead target cellulose as a carbon source through the utilization of cellulases and hemicellulases[10, 22]; hence,

soft-rot fungi have limited applicability in biomass pretreatment but are important enzyme producers in biomanufacturing.

The most prominent lignin-modifying enzymes, lignin peroxidase, manganese peroxidase, and laccase, all result in oxidation of lignin molecules but have unique reaction mechanisms. Lignin peroxidase targets non-phenolic portions of lignin through oxidation of lignin molecules and reduction of H_2O_2 resulting in radical cation production[20]. Manganese peroxide is oxidized from Mn^{2+} to Mn^{3+}, which forms a complex with an organic acid that can then oxidize phenolics in lignin[23]. Laccase utilizes molecular oxygen to oxidize phenolics in lignin[20]. Hatakka summarized the presence of lignin-modifying enzymes and associated isoenzymes in a range of fungal species[20]. Su et al. developed a high throughput method for evaluating fungal strains based on their levels of laccase, lignin peroxidase, and manganese peroxidase expression during biomass degradation[24]. Similarly, Sista Kameshwar and Qin characterized the genomic-level prevalence of these enzymes across white-, brown-, and soft-rot fungi[22]. The prevalence and high activity levels of these lignin degrading enzymes have resulted in broad utilization of filamentous fungi-secreted enzymes to depolymerize a range lignocellulosic-based product streams for biomanufacturing purposes.

Impacts of fungal treatment within the context of a biorefinery are often limited to metrics such as lignin loss and downstream carbohydrates released in conversion. A full understanding of storage performance and associated conversion impacts coupled with molecular characterization is necessary to further understand the mechanisms of this complex system. In the present study, corn stover was assessed for the potential impact of fungi-induced lignin degradation in a simulated outdoor environment conducted using 100L working volume aerated bioreactors. This was coupled with analytical investigations performed to better understand the mechanisms of degradation in the context of bioenergy systems. Compositional changes in macromolecular components measured using traditional methods were characterized with analytical pyrolysis coupled with two-dimensional gas chromatography mass spectrometry (GCxGC/MS) to assess molecular changes. The production of small molecules during analytical pyrolysis was correlated with ^{13}C cross-polarization magic-angle-spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy to understand the structural impacts on the biomass and suggest mechanisms of fungal attack. Finally, a

combination of fungal pretreatment and acid-based pretreatment were assessed to show the impact of fungal treatment on hemicellulose removal and remaining cellulose accessibility to enzymatic depolymerization.

Experimental

Filamentous Fungi Cultivation

Experiments were performed using *Phaenaerochaete chrysosporium* (NRRL 6370), a non-selective lignin degrader using methodology described in Saha et al.[25]. The fungi were first grown on Yeast Mold Agar (BD Difco™, Franklin Lakes, NJ) and then in Yeast Mold Broth (BD Difco™, Franklin Lakes, NJ) with shaking at 50 rpm at 28 °C. *P. chrysosporium* fungus was grown for seven days where it reached a density of 8.4 mg dry weight/mL. The cultures were pelleted by centrifugation at 11,260 x g for 10 minutes, resuspended with 25 mL of a 10 mM phosphate buffer and homogenized in a Waring blender (Waring Commercial, model 51BL31) for 5 seconds on low speed twice, and once on high speed for 2 seconds. The culture was further homogenized using a glass tissue homogenizer. The cultures were pelleted once more by centrifugation then resuspended in 150 mL of sterile tap water. Dry fungal weight was calculated based on OD_{600} measurements and correlated to the fungal slurry weight after drying overnight at 105 °C.

Corn Stover Source and Storage Procedure for Large Scale Experiment

Corn stover was sourced from Hubbard, IA in 2018. Corn stover was size reduced using a Schutte Buffalo hammer mill fitted with a 6 mm screen. Duplicate 100 L storage reactors were used for *P. chrysosporium* and compared to a moisture only control, with methods similar to those described previously[26]. Briefly, the reactor design consisted of stainless-steel chamber insulated with a temperature-controlled water jacket in stainless steel chassis surrounded by fiberglass insulation. Airflow was controlled with mass flow controllers and introduced from the bottom of the reactor and exiting the top of the reactor. Inlet air was kept at or near 100% relative humidity by sparging through a water bath that was maintained at the same temperature as the reactor. Gas exiting the reactors traveled through a vapor condensing column to remove moisture. Gas was then pumped to a MicroGC 3000 gas chromatograph (Agilent, Santa Clara, CA) with a PLOT U column and thermal conductivity detector for carbon dioxide measurement. CO_2 was a product of aerobic microbial respiration that can be used to assess

degradation in real time according to what is described in McGechan (1989)[27], where $(CH_2O)_n$ relates to the carbohydrate loss calculated based on CO_2 mass measured using the molar ratio of 1:1 known for microbial respiration.

$$\% DML = \frac{\Sigma(CH2O)_n}{g\ Biomass_{\ pre\ storage}} x100 \qquad Equation\ 1$$

The reactors were modified with stainless steel screens and spacers such that two zones for biomass were available for sampling at one and two weeks, respectively. Corn stover (10 kg) was rehydrated to 55% moisture overnight at 4°C, and then the following day it was inoculated with 2,500 ml solution containing 3.34 g of *P. chrysosporium* to reach a moisture content of 60% wet basis (w.b.) and mixed thoroughly. Inoculated corn stover was then loaded into the duplicate reactors, and moisture content for each reactor was assessed on three samples taken throughout the loading process to account for associated moisture losses during loading. Uninoculated corn stover was also rehydrated overnight and used as a control, although inconsistent mixing resulted in moisture contents of 59.5% and 70.3% for the one- and two-week zones in Reactor 1, respectively, and 51.5% and 65.7% for the same zones in Reactor 2. Moisture content for the *P. chrysosporium* inoculated stover in Reactors 3 and 4 was between 59.0% and 59.5% for all zones. An airflow of 1 L/min filtered room air, corresponding to complete air exchange every 100 minutes, was applied to the reactors and humidification of the air at the bottom of the chamber occurred, similar to previous studies[26, 28, 29]. Temperature changes were measured at the interior of each zone within the reactor using resistance temperature detectors collecting measurements continuously over the course of the experiment.

Compositional Analysis

Chemical compositional analysis was performed using standard Laboratory Analytical Procedures on duplicate samples[30]. This procedure began with corn stover undergoing 100°C water and subsequent ethanol extraction using an automated solvent extractor ASE 350 (Dionex, Sunnyvale, CA). Extracted biomass is then subject to two-stage acid hydrolysis to solubilize structural carbohydrates. Monomeric carbohydrates in the liquors were quantified using high performance liquid chromatography and a refractive index detector (Agilent, Santa Clara, CA) and Aminex HPX 87P column (Bio-Rad, 300 x 7.8 mm, Hercules, CA). The column temperature was held at 85°C with water as the eluent at a flow rate of 0.6 ml/min. Acid-soluble lignin was calculated with a Varian Cary 50 ultraviolet-visible spectrophotometer

(Agilent, Santa Clara, CA). Gravimetric differences were used to quantify acid insoluble lignin, structural ash, and total ash. Acetate was measured using the HPLC described above but with an HPX-87H ion exclusion column (Bio-Rad, 300 mm × 7.8 mm, Hercules, CA, USA) and UV detector; column temperature was 50°C with 0.01N H_2SO_4 as the eluent at a flow rate of 0.6 ml/min. An additional sample was taken through all steps through the two-step acid hydrolysis to collect a lignin-enriched step used for further molecular analysis.

Molecular Characterization with Two-Dimensional Gas Chromatography and Mass Spectrometry (GCxGC/MS)

Approximately 300 μg of corn stover milled to pass a 0.2 mm screen was weighed and added into 38 mm analytical pyrolysis tubes fitted with a 19 mm quartz spacer (CDS Analytical, Oxford, PA) and a small quartz wool plug. A second quartz wool plug was added to secure the biomass followed by 3 nanomoles of the internal standard 9-(9H)-fluorenone. The fluorenone was injected into the plug as 1 μl of a 3 millimolar solution in acetonitrile. Alternatively, a 1 nanomole aliquot of biphenyl was used as an internal standard. The internal standards were used to provide quantitation for a select number of pyrolysis products, and both fluorenone and biphenyl provided comparable results. Analytical pyrolysis occurred in a CDS Analytical 5250 pyrolyzer equipped with a 36 sample autosampler. Briefly, the pyrolysis experiment occurred when the sample was lowered into the pyrolysis chamber, initially subjected to a 2 second drying time at 100°C, and then held for an additional second at 100°C. Temperature within the chamber increased at 50°C/second to the maximum pyrolysis temperature of 400°C (T_{max}) and then was held at T_{max} for 5.00 seconds followed by ejection of the sample tube. A cleaning cycle followed each run, heating the pyrolysis chamber to 1,200°C for 10 seconds to remove residual solids.

Two-dimensional gas chromatography (GC) was performed as described previously[31] using an Agilent 7890 gas chromatograph that separates compounds first on the basis of boiling point and then by polarity. GCxGC is enabled by a four-jet modulator and a secondary oven within the primary oven. The first chromatographic dimension used a 28 m L x 0.25 mm i.d. column with a 0.5 μm Rxi-5ms (Restec, Bellafonte, PA) stationary phase consisting of 5% diphenyl/95% dimethyl polysiloxane. The second chromatographic dimension used a 1 m L x 0.1 mm i.d. column with a 0.1 μm Rxi-17 (Restec, Bellafonte, PA) stationary phase consisting

of 50% diphenyl/50% dimethyl polysiloxane. The addition of a second gas chromatograph dimension in the pyrolysis allowed for detection of pyrolysis products that would otherwise co-elute, such as guaiacol and anhydro sugars. A transfer capillary between the secondary oven and the mass spectrometer consists of a 21 cm section of the Rxi-17 column. He carrier gas flow was maintained at 1 mL/min occurred throughout the analysis.

The analytical pyrolysis compounds were split in the heated injector (300°C) using a split ratio of 20:1. Upon initiation of the analysis, the primary column was held at 50°C for 0.5 min, then ramped at 7.5°C/min to a target temperature of 260°C and held constant for an additional 3.00 min. The secondary oven and 4-jet modulator were maintained at 5 and 15°C, respectively, above the temperature of the primary oven. After a 3.00 sec modulation period, hot and cool pulse times were varied to efficiently trap and desorb compounds of increasing mass over the course of the experiment, with smaller compounds eluting first. Hot pulse and cool times were 0.50 and 1.00 sec, respectively, before a retention time of 394 sec. This was repeated twice per modulation cycle by the 4-jet modulator. After 394 sec, the hot pulse and cool times were 1.00 and 0.50 sec, respectively. The transfer capillary to the mass spectrometer was maintained at 280°C.

Mass spectrometry was performed using a Leco Pegasus 4D instrument (St. Joseph, MI). An acquisition delay of 220 seconds allowed very light compounds to pass through the time-of-flight mass spectrometer before analysis was initiated. The instrument was scanned from *m/z* 43 to 300 at a rate of 200 spectra/second, a scan rate that enabled deconvolution of closely eluting compounds. The electron impact ion source was operated at 250°C with an ionization energy of 70 volts. Mass spectra for pyrolysis-generated compounds were generated using the Leco ChromaTOF software using automatic smoothing and a baseline off-set value of 2.0, which discriminated against the spectrometer noise level. Compounds were identified by library searching mass spectra against the NIST and Wiley mass spectral libraries, and identification was based on forward and reverse similarity indices, probability[32], and the judgment of the analyst.

Three dimensional chromatograms (3D chromatograms) were generated using both the total ion- and extracted ion-chromatographic data. The resulting plots provide a color-mapped qualitative assessment of the differences between the pyrolysis behavior of the unmodified and

fungal-pretreated samples. The z-axes were scaled to enable pairs of 3D chromatograms to be normalized for differences in the initial sample masses. Pyrolysis efficiency data was also assessed (Supplemental Information).

Solid-State 13C [1H] -CP/MAS NMR Spectroscopy

Solid corn stover samples milled to 0.2 mm minus were loaded into 4 mm ZrO rotors and capped with Kel-F rotor caps. The spectra were measured using a standard Bruker HX magic-angle spinning (MAS) probe as part of a Bruker Avance III spectrometer with a field strength of 9.4 T (^{1}H ν = 400.03 MHz, ^{13}C ν = 100.59 MHz). All samples were spun at ν_R = 10 kHz. The basic Bruker cross-polarization (CP) pulse program was used for all samples.[33] Proton nutation frequency was set at 92.6 kHz with a decoupling field strength of 48.1 kHz (under the SPINAL64 decoupling program).[34] The Hartmann-Hahn condition (contact time) was optimized at 1.5 msec using the unstored corn stover and used for the remaining samples. The first spectra collected were from unstored corn stover and *P. chrysosporium*-inoculated corn stover that had been stored for 1 or 2 weeks in the aerated bioreactors. The relaxation delay for these experiments was set to 4 sec, the sweep width was set to 497 ppm, and the total number of transients per experiment was 16,384 (for a total experimental time of 2.85 days). Spectra were normalized to the peak at 105 ppm which coincides with the C1 bond in cellulose to allow for comparative analysis. The Crystallinity Index (CrI) was assessed by subtracting the peak area of the crystalline portion of the area under C4 bond curve from the total area of the C4 region[35]. The second set of spectra were collected from samples containing acid insoluble lignin residues collected according to the procedure outlined in the "Compositional Analysis" section above. Due to the small amount of material from these samples, the mass of the solid was enhanced by addition of sodium chloride, which provided a ^{13}C NMR invisible matrix while allowing for the rotors to be fully filled. The number of scans used in these experiments was 2048, the number of points used in the acquisition was 4,994, and the relaxation delay was set to 4 sec, which amounted to a total experimental time of 2.3 hours per sample. The spectra were normalized to the peak at 54.8 ppm that represents methoxyl groups in lignin. The total number of points for each analysis was 4,994 points but this was truncated during processing to 900 points to reduce the amount of noise in the spectra, as the free-induction decay had reached the noise level at that point.

Dilute Acid Pretreatment and Enzymatic Hydrolysis

Dilute acid pretreatment was performed in triplicate on the unstored and fungal-treated biomass from Reactor 3 using a Dionex ASE 350 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA) based on a method described in Wolfrum et al.[36]. Briefly, 3 g (dry weight equivalent) of corn stover knife milled to pass a 2 mm screen was loaded manually into a Dionium extraction cell. Sulfuric acid (30 ml of 1% sulfuric acid w/w) was loaded into the cell to achieve 10% (w/w) solids loading, followed by a six-minute ramping to the desired temperature and a seven-minute incubation period. This liquor was expelled from the cell and collected in glass jars followed by neutralization of the solids with 100 ml of nanopure water and collection of this rinse water. All samples were analyzed in quadruplicate to ensure triplicates were available for statistical analysis. Residence temperatures of 110°C, 130°C, and 160°C, corresponded to a combined severity factor of 1.31, 1.73, and 2.61, respectively. This factor calculates severity of the pretreatment based on reaction time, temperature, and pH[37].

$$(CSP) = Log\left(t \times exp\left(\frac{T-100}{14.75}\right)\right) - pH \qquad \textit{Equation 2}$$

Monomeric and polymeric sugars were measured using HPLC, as described above.

Pretreated solids were then subjected to enzymatic hydrolysis based on a method designed previously[38]. Hydrolysis was performed in shake flasks with 10 ml total volume at 10% (w/w) solids loading in 50 mM citrate buffer, pH 4.8 supplemented with 0.02% of sodium azide to prevent microbial contamination. Enzyme complexes Cellic® Ctec2 and Cellic® Htec2 (Novozymes®, Franklinton, NC) were added at a loading rate of 20 mg protein/g glucan and 2 mg protein/g glucan, respectively. Ctec2 and Htec2 had cellulase activities of 99 FPU/ml and 77 FPU/ml, respectively. Hydrolysis occurred at 50°C with mixing at 150 rpm for a 5-day period, after which the liquid was filtered and analyzed for soluble carbohydrates as described above. All samples were run in triplicate, and analyzed in duplicate. Total glucan and xylan were determined using the structural components as well as the soluble fraction of each carbohydrate (from compositional analysis) and were used as the basis to estimate the maximum theoretical carbohydrate yield per the following equations:

$$\%\ Glucose\ Yield = \frac{Glucose_{Enzymatic\ Hydrolysis}}{Glucan_{Total}\, x\, (\frac{1}{0.9})} x\ 100 \qquad \textit{Equation 3}$$

$$\%\ Xylose\ Yield = \frac{Xylose_{Enzymatic\ Hydrolysis}}{Xylan_{Total}\ x\ (\frac{1}{0.88})}\ x\ 100 \qquad Equation\ 4$$

Mass differences of 0.88 and 0.9 in the monomeric versus polymeric forms of glucose and xylose, respectively, were accounted for in all calculations.

Statistical Analysis

Single-factor one-way analysis of variance (ANOVA) was performed in JMP 14.2.0 (SAS, Cary, NC) to identify significant differences, and Tukey's honest significant difference (HSD) test was performed for multiple-level comparison of statistical equivalency if the ANOVA was significant at $p < 0.05$.

Results and Discussion

This study assesses the impact of fungal pretreatment in corn stover in terms of compositional, structural, and convertibility changes on biomass recalcitrance in the context of a biorefinery. Dry matter losses and compositional changes were used as a guide to understand the working envelope of a non-selective (*P. chrysosporium*) lignin degrading fungus. This was followed by an in-depth investigation of *P. chrysosporium* degradation in a simulated outdoor storage pile combined with powerful analytical tools to understand the mechanisms of degradation and the impact on conversion performance and cost.

Storage performance and Compositional Impacts of P. chrysosporium inoculation in Aerated Bioreactors

Filamentous fungi require a sufficient supply of oxygen to maintain optimal growth, and lack of available oxygen may hinder the use of fungi as a primary pretreatment approach[39]. Aerated storage bioreactors were designed previously at Idaho National Laboratory to create a controlled environment to mimic larger storage systems while gaining crucial mass balance information that is nearly impossible to gather in outdoor settings[40]. The reactors enable real-time estimation of dry matter loss through hourly measurement of carbon dioxide emitted by bacteria and fungi as they respire carbohydrates. The reactors' exteriors are jacketed, and a circulating water bath adjusts the jacket temperature to match the temperature at the center of the reactor, where respiratory self-heating is occurring. These reactors have been used to study corn stover degradation as a function of aeration[26, 28], initial moisture content[29], and humidity[40]. Scale up of *P. chrysosporium* as a microbial pretreatment was conducted in these reactors over

a 14-day period with sampling at seven days to provide essential understanding the degradation rate of corn stover as well as any temperature changes associated with microbial decay. The experiments used non-sterile corn stover to replicate the interactions of the native microflora with the fungal amendments, as would occur in a commercial storage environment.

Storage induced losses and temperature changes due to fungal treatment

P. chrysosporium-inoculated corn stover rehydrated to 60% moisture content was stored in duplicate highly controlled and instrumented aerobic bioreactors and compared to control samples with no fungal treatment. Mean interior temperature in each reactor increased due to microbial heating and caused all reactors to heat from 20°C to 40°C within the first hours of storage (Figure 1). A lag then occurred until 3 days of storage after which temperature spiked to 51°C and 57°C in the control reactors by 4.5 days. The variation in maximum temperature was likely attributed to differing initial moisture contents as demonstrated previously in corn stover trials using these reactors[29]. Elevated internal temperature correlated with elevated dry matter loss rates calculated based on CO_2 evolution; dry matter losses for Reactors 1 and 2 after 1 week were 9.2% and 7.6%, respectively. Reactors with *P. chrysosporium*-inoculated corn stover heated at a slower rate, only reaching 46°C, which suggested that fungal growth was preventing other microorganisms from thriving. Sulej et al. (2019) showed an increase in antimicrobial production against gram-positive bacteria by *P. chrysosporium*, which may explain the decreased respiratory self-heating seen in the fungal-amended corn stover[41]. Similarly, dry matter loss in the corn stover were reduced in these reactors, which only experienced 4.4% and 2.8% in Reactors 3 and 4 after 1 week, respectively. All reactors cooled slightly during the 1-week sampling, but temperatures increased again once the reactors were sealed and aerated. However, the control reactors steadily cooled, an indication that the readily accessible carbohydrates had been consumed and the microbial community could no longer sustain the same growth rate. Approximately 5% additional dry matter loss was incurred in the second week of storage. In contrast, the *P. chrysosporium*-inoculated corn stover resumed heating, a sign of increased respiratory activity, likely due to fungal-based depolymerization of the corn stover to release additional carbon sources. Temperature increases of up to 42°C have been measured in fungal inoculated, unventilated wood chip piles[42]. The rate of dry matter loss in the fungal inoculated stover increased dramatically between 1 and 2 weeks of storage; 2-week dry matter loss was 13.9% and 7.2% in Reactors 3 and 4, respectively. These trends

indicate that once *P. chrysosporium* had depolymerized the corn stover to a certain extent, fungal growth rates increased dramatically. These results are consistent with the findings of Adav et al.[43] that suggested the lignin degrading peroxidase activity of *P. chrysosporium* only increased after initial hemicellulase and cellobiose dehydrogenase activity had liberated sufficient hemicellulose and cellulose for consumable carbohydrates to facilitate further growth and lignin breakdown.

Compositional changes due to fungal treatment

Macromolecular changes in composition were assessed on corn stover stored in aerated bioreactors with and without *P. chrysosporium* inoculation (Figure 2, Supplemental Information Tables A1-A4). Xylan was reduced from 20.9% to as low as 16.8% after 2 weeks of storage with *P. chrysosporium* (Reactor 3). Lignin content was reduced slightly from 18.2% to 17.2% in this reactor after 2 weeks. However, lignin content was increased to 19.1% in the duplicate Reactor 4. Reactor 4 temperature increased to >50°C, similarly to the early temperature increases seen in the control reactors. Final structural xylan concentrations in Reactor 4 were similar to those seen in the two control reactors and higher than those in Reactor 3. Additionally, the final acetate concentrations (Supplemental Information Table A2) for Reactor 4 were lower and more similar to the controls than the fungal-amended Reactor 3. Lignin enrichment has been reported as a function of degradation loss in aerobically stored corn stover due to the preferential utilization of cellulose and hemicellulose components[29]. The apparent lignin enrichment in Reactor 4 combined with the compositional changes in the hemicellulose components xylan and acetate suggest the impact of increased bacterial rather than fungal activity in Reactor 4 after the one-week sampling event.

Total extractives in the *P. chrysosporium* inoculated corn stover in the aerated bioreactors stayed relatively constant (13.07% initially and after 2 weeks only 11.68% and 12.70% in Reactors 3 and 4, respectively). It is possible that microorganisms originally present on the corn stover at the time of harvest consumed these soluble products to support respiration. Total extractives decreased from 13.07% to 8.8% in both control reactors in the aerated bioreactors, although soluble arabinan did increase slightly. However, the fungal inoculated aerated reactors contained a higher ratio of non-quantified (unknown) to quantified (e.g., soluble sugars) extractives, which presumably indicates that soluble lignin degradation products were

produced as a function of inoculation and storage. Acid soluble lignin and ethanol extractives decreased to a greater extent than acid insoluble lignin. Based on these cumulative results the *P. chrysosporium*-inoculated corn stover from Reactor 3 was selected for further analysis of changes on molecular compositional and conversion yields given that it experienced the greatest hemicellulose and lignin loss, a significant indicator of the intended mode of degradation.

Structural Impacts of P. chrysosporium inoculation in Corn stover

Fungal Degradation Products Measured by Pyrolysis GCxGC/MS

Traditional chemical compositional analysis methods, such as those used in this work, fractionate biomass into soluble and structural components. However, high solids storage treatments that aim to depolymerize structural lignocellulose components may cleave structural bonds without fully solubilizing a carbohydrate molecule or creating a measurable lignin oxidation product. Pyrolysis coupled with two dimensional GC/MS has been used to measure the abundance of the compositional subunits released from lignocellulose by means of thermal energy[31, 44]. The goal of this analysis was to understand if pyrolysis at 400°C could be used to assess partial cleavage of the backbone of cellulose, hemicellulose and associated side chains or substitutions. A pyrolysis temperature of 400°C is lower than that typically used for biomass characterization; however, in previous studies lower temperatures enhanced differences between biomass samples from different pretreatment environments.[31] Quantitative analysis of pyrolysis production efficiency from S, G, and H lignols released as a function of fungal treatment also provide insight into the mechanisms of degradation given the complexity of native corn stover.

Comparative 3D chromatograms from early eluting compounds (220 to 920 s retention time in the first chromatographic dimension, rt1) indicate that 2-oxopropanal, acetic acid, and acetic anhydrate are present in greatest abundance in the unstored corn stover with several additional oxygenates present in lower concentrations (Figure 3). In comparison, *P. chrysosporium* treated corn stover showed markedly enhanced acetol and anhydro sugar compared to the unstored corn stover, corresponding to the reduction of acetate binding to hemicellulose as well as the increase in soluble hemicellulose components. Anhydro sugar production in pyrolysis of the fungal treatment could result from partially hydrolyzed hemicellulose or

cellulose with enhanced dangling ends that have a greater propensity for producing pyrolysis products at 400°C. Xylan degradation in pyrolysis begins to occur at 200°C whereas cellulose degradation has been reported at 300-375°C in thermogravimetric analysis[45]. Furfural, furyl alcohol, and 2(5H)-furanone were also produced in pyrolysis of corn stover, and these 5-carbon monomers are all xylose or arabinose degradation products. Quantitative analysis of the pyrolysis production efficiencies of select oxygenates indicated slightly enhanced production of 2,3-butanedione, acetoxyacetone, and 2(5H)-furanone in *P. chrysosporium* treated corn stover (Supplemental Information Figure A2). Elevated pyrolysis efficiency of 5-methyl furfural, a degradation product of 5-hydroxymethyl furfural[46] thermally produced from glucose degradation[47], correlates to the enhanced soluble glucose content in the unstored corn stover, whereas consumption of this soluble sugar occurred during fungal attack. Overall, the changes in these early eluting compounds in pyrolysis indicate changes consistent with chemical composition results suggesting glucose consumption and hemicellulose degradation seen in *P. chrysosporium* treated corn stover.

Lignin-degradation products formed during pyrolysis are observed as later-eluting compounds (920 to 1620 s rt1) in comparative 3D chromatograms (Figure 4). 4-vinyl phenol, a decarboxylation product of p-courmaric acid and a common pyrolysis product from herbaceous biomass such as corn stover[48], is the most prominent peak along with the internal pyrolysis standard of 9(9H)-fluorenone. Visualizing the 3D chromatographs in the range of 1051 to 1600 s (Figure 5) shows the differences in the lower concentration lignin pyrolysis products. Unstored corn stover has elevated 4-formyl phenol from H lignols, whereas production of 4-vinyl-guaiacol and 4-formyl-guaiacol are enhanced after *P. chrysosporium* treatment. This suggests that G-residues in the lignin polymers produced an enhanced number of G-lignols, which would be consistent with lignin degradation forming dangling guaiacyl moieties that are precursors for the formation of the G lignols seen in the pyrolysis experiments. Zeng et al. also documented lignin degradation and G lignol changes due to *P. chrysosporium* treatment as indicated pyrolysis 610 °C coupled with GG/MS detection[49].

Pyrolysis efficiencies of lignols confirm enhanced production of guiacol, 4-vinyl guaiacol, 4-formyl guaiacol, and phenol in fungal treated biomass but reduced 4-formyl phenol (Supplemental Information Figure A3). Pyrolysis efficiency measurements also indicate

enhanced syringol after *P. chrysosporium* treatment, the only pyrolysis product observed originated from an S-lignin. Corn stover has been noted to contain less than 10 wt % S lignols and near equal distribution of H and G lignols[50].

In summary, these findings confirm that G and S lignols in corn stover were targeted by *P. chrysosporium* but that H lignols were impacted at a lesser extent. Changes in the relative H, G and S lignol distributions have been shown to be feedstock dependent; herbaceous crops have different lignin and cellulose ratios compared to softwood and hardwoods therefore fungal degradation impacts on lignin are often feedstock specific[39]. Assessing the pyrolysis products that are produced at 400°C allows for enhanced observation of the mechanisms of *P. chrysosporium* attack, which would be more difficult to observe using higher temperature pyrolysis, where fast pyrolysis kinetics would overshadow more subtle differences originating from fungal degradation.

Structural binding profiles observed with Solid-State 13C-CP/MAS NMR Spectroscopy

^{13}C cross-polarization magic-angle-spinning (CP-MAS) NMR is a solid-state technique that uses the connected hydrogens in the structure to increase the signal of the carbon backbone, and this technique is useful for analyzing biomass samples that are rich in cellulose and hemicellulose. Analysis of the unstored, one- and two-week fungal inoculated samples from aerated bioreactors indicate slight changes in the NMR spectra (Figure 6). One notable difference is in the cellulose peaks at ~70-80 ppm, which correspond to the C2, C3 and C5 positions. Differences appear in the signal intensity, yet they are unlikely to represent changes in the cellulose monomer given the strong hydrogen bonding in this polymer. On the other hand, differences are likely due to the reduction of hemicellulose and lignin in the fungal treated samples; these carbon atoms are observed at the same ^{13}C chemical shift in hemicellulose and cellulose. This is supported by the gross chemical composition data for these samples. Similarly, significant differences in the peak intensities are observed at 21.1 and 171.5 ppm, which represent methyl (CH_3) and carboxyl (COOH) groups, respectively. The peak height at both these locations was enhanced in fungal inoculated corn stover after two weeks of storage compared to both the unstored and 1-week stored samples, likely a function of the increased degradation rate between one and two weeks of storage. It is likely that the methyl and carboxyl groups are formed after fungal degradation (including acetate hydrolysis) of the

hemicellulose, which is reflected in gross compositional changes from 3.6% to 3.3% and 2.1% after one and two weeks of storage, respectively. Lastly, no changes in crystallinity were observed as a function of treatment, corresponding to x-ray diffraction spectra reported in the Supplemental Information.

Lignin isolation in the unstored and *P. chrysosporium*-inoculated was accomplished with a two-stage hydrolysis aiming to solubilize all carbohydrates and leave lignin in the solid phase. ^{13}C-CP/MAS NMR spectra from the lignin isolated samples provide dual functions of providing higher resolution spectra of the lignin and insight into the structural changes in corn stover as a function of *P. chrysosporium* inoculation over the two-week storage period (Figure 7). Comparison of the spectra of the unstored sample and fungal treated corn stover reveal additional molecules in the lignin-rich fungal treated sample. Enhanced signals of the C1-C6 carbons in cellulose after fungal treatment suggest irreversible binding between lignin and cellulose occurred as a function of treatment. Lignin coalescence has been demonstrated in during pretreatment previously in low solids pretreatment[51]. It is hypothesized that lignin or other molecules solubilized due to the oxidative conditions in the fungal treatment, as supported by the increase in total extractives noted above, condensed on the cellulose after the acid treatment, creating a lignin-carbohydrate complex.

Carboxyl groups at 171.5 ppm were present in the lignin-rich sample, and like the unstored corn stover the fungal treated sample, this peak was slightly reduced in intensity, suggesting fungal treatment impacted these functional groups in lignin. One other notable change was a peak at 32.2 ppm, which is prominent in the fungal treated sample. It is hypothesized that it is a CH_2 group based on NMR peak libraries[52], potentially an aliphatic hydrocarbon that is a lignin degradation product or a product formerly attached to lignin. Methylene present in dangling phenylpropanoid moieties formed from lignin degradation is another possible explanation. The slightly increased signal in the fungal treated sample at 29.5 ppm, which corresponds to the methyl group, suggests that perhaps lignin degradation product is contributing to this intensity. Overall, the use of ^{13}C CP/MAS NMR analysis demonstrated that the lignin rich fungal sample had enhanced cellulose contamination, a key data point that may be useful to predict potential responses in downstream conversion operations.

Carbohydrate Yield in Hydrolysis as a Function of Thermochemical Severity

The goal of enzymatic hydrolysis in the context of a biorefinery is to provide the maximum theoretical free sugar levels for the next unit operation, in this case microbial fermentation to fuels or fuel precursors. A study was done to further understand if the cellulose was indeed inaccessible to enzymatic attack due to a complex with coalesced lignin. Unstored corn stover, and stover inoculated with *P. chrysosporium* and stored for one and two weeks was size reduced and subject to hydrolysis with glycosidases over 120 hours. Results, as reported in the Supplemental Information, indicate that less glucose is solubilized in the presence of glycosidic enzymes after one and two weeks of fungal degradation. The reduced yields of glucose as a function of fungal treatment and time support the hypothesis stated above that lignin coalesces in a complex with carbohydrates, making it less accessible to enzymatic depolymerization.

The low carbohydrate yields shown in the Supplemental Information suggest a more severe pretreatment method than mechanical processing alone is necessary to achieve a high solubilization rate of the corn stover for downstream fermentation. Corn stover inoculated with *P. chrysosporium* and stored for one and two weeks were also assessed using a dilute acid pretreatment and enzymatic hydrolysis method. Dilute acid is a pretreatment that targets hemicellulose hydrolysis so that cellulose can be exposed to cellulytic attack, and it was used in this study to investigate any synergies in fungal depolymerization that solubilized hemicellulose. Washing is typically performed after dilute acid pretreatments to create a neutral pH for hydrolysis, and it would have the combined effect of removing any competing fungal enzymes. Dilute acid pretreatment combined with enzymatic hydrolysis has been proposed by Wolfrum et al. as a means to screen biomass for reduced recalcitrance[36], and these conditions have been used previously to assess the impact of corn stover storage on carbohydrate release[28, 29]. Three severity levels with varied temperatures were chosen to determine if fungal pretreatments could reduce the energy requirements of the dilute acid pretreatment. *P. chrysosporium* treatment showed a reduction in hemicellulose recalcitrance during dilute acid pretreatment and subsequent enzymatic hydrolysis compared to unstored corn stover. Figure 8 shows that more xylose is released from the fungal-stored corn stover at all pretreatment temperatures tested. Fungal inoculated samples stored for one week displayed ~ 30% more xylose released compared to unstored samples for the experiments conducted at 110°C (severity factor of 1.31), but an additional week of storage had no further impact.

Storage duration resulted in enhanced xylose release for the two experiments conducted at higher temperatures of 130°C and 160°C, (severity factor of 1.73 and 2.61, respectfully). Additionally, this experiment indicated that similar xylose yields (82.9% ± 1.4%) were achieved after two-week fungal treatment with a pretreatment temperature of 130°C, as compared to the xylose yields for untreated corn stover subjected to a pretreatment temperature of 160°C (80.2% ± 0.5%). These results suggest the combined fungal treatment to liberate hemicellulose and oxidize lignin increased the ability of dilute acid pretreatment to depolymerize hemicellulose to a greater extent than in the untreated corn stover.

Glucose yield corresponded to the reaction temperature increases associated with increased combined severity factor. Yields of 37% glucose released were measured in experiments conducted at the lowest temperature (110°C, severity factor 1.31), significantly higher at 40% after 1 week of *P. chrysosporium* treatment, but then decreased to 30% after 2- weeks of treatment. Glucose yields increased to approximately 60% and 95% at the 1.73 and 2.61 severity factors, but no significant difference was observed as a result of treatment. This study did not explore changing enzyme loading or concentrations, but it is hypothesized that increasing the cellulase loading during hydrolysis would increase the glucose release from what was observed in the 1.73 severity treatment.

Others have shown that reduced severity pretreatment was possible, for example Kuhar et al. indicated sulfuric acid loading could be reduced from 3.5% to 2.5% in fungal pretreated wheat straw[53]. Energy cane biomass subjected to *P. chrysosporium* treatment combined with reduced sulfuric acid pretreatment maintained high yields during ethanol fermentation[54]. Combined fungal and phosphoric acid pretreatment has been shown to result in nearly 90% conversion of glucose to ethanol in residues from oil palm[55]. Future opportunities to optimize fungal pretreatment with existing and emerging thermal and chemical treatments have the possibility to dramatically change the operating conditions in lignocellulosic biorefineries by reducing the costs associated with higher severity pretreatment conditions.

The economics associated with fungal-assisted storage of corn stover at a biorefinery gate have been reported by Wendt et al[56]. This study varied the impact of corn stover moisture content, storage residence times, and fungal strain and identified that residence times greater than 2 weeks resulted in increased dry matter loss. The corresponding techno-economic analysis

defined the cost of fungal assisted storage at a biorefinery gate as a function of dry matter loss and reduced biorefinery processing needs, and the primary driver for cost-effectiveness was reducing storage degradation by reducing residence time. Such cost-benefit tradeoffs will continue to be a critical element for designing sustainable approaches for reducing biomass recalcitrance at low severity levels.

Finally, the study suggests that monitoring temperature within storage piles is a straightforward indicator to monitor the type of degradation occurring and could be used to characterize conditions conducive to fungal-assisted delignification. Temperature monitoring has been described previously for corn stover and woody biomass storage[28, 57]. Additional studies to correlate microbial activity and temperature increases, composition changes, and convertibility impacts would be beneficial in developing predictive models to show the impact of storage performance on biorefinery performance.

Conclusion

Filamentous fungi effectively reduce the recalcitrance of lignocellulosics to thermal and caustic pretreatments, resulting in increased hydrolysis to fermentable carbohydrate monomers. Key findings in this study indicate that *P. chrysosporium* degradation on corn stover increased the release of hemicellulose and G and S lignols in pyrolysis, that condensed lignin may be cross-linking the cellulose as indicated by ^{13}C-CP/MAS NMR, and fungal treatment over a two-week period facilitated reduction of pretreatment temperature from 160°C to 130°C while achieving a similar xylose yield. Additional investigation is warranted to further improve performance of fungal pretreatment feedstocks in mechanical, thermal, or enzymatic depolymerization approaches.

Conflicts of interest

There are no conflicts to declare.

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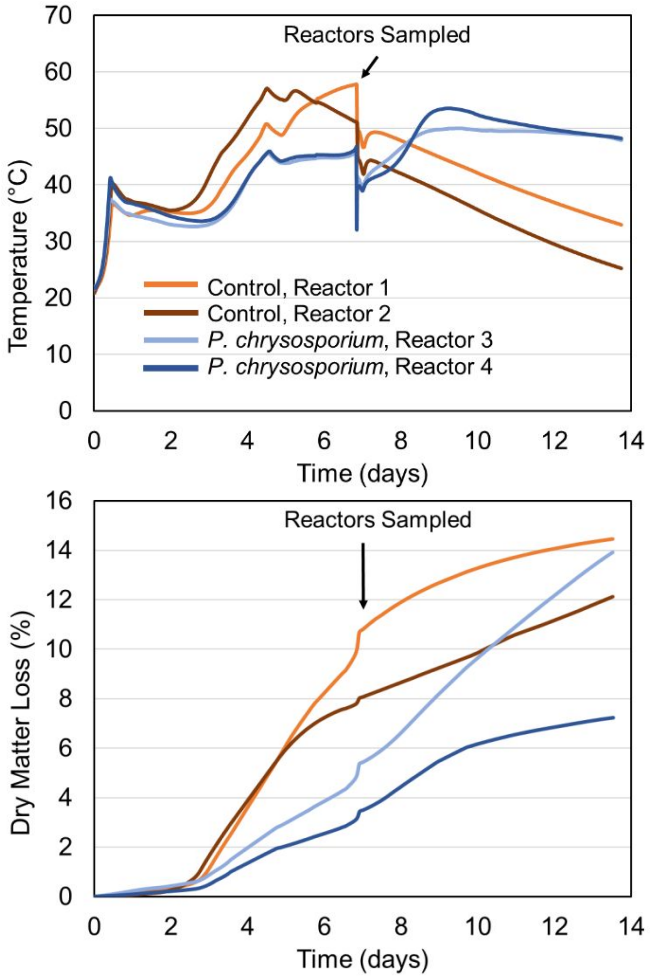

Figure 1. Temperature (top) and dry matter loss (bottom) profiles of corn stover inoculated with P. chrysosporium and stored in aerobic bioreactors at 60% moisture for two weeks

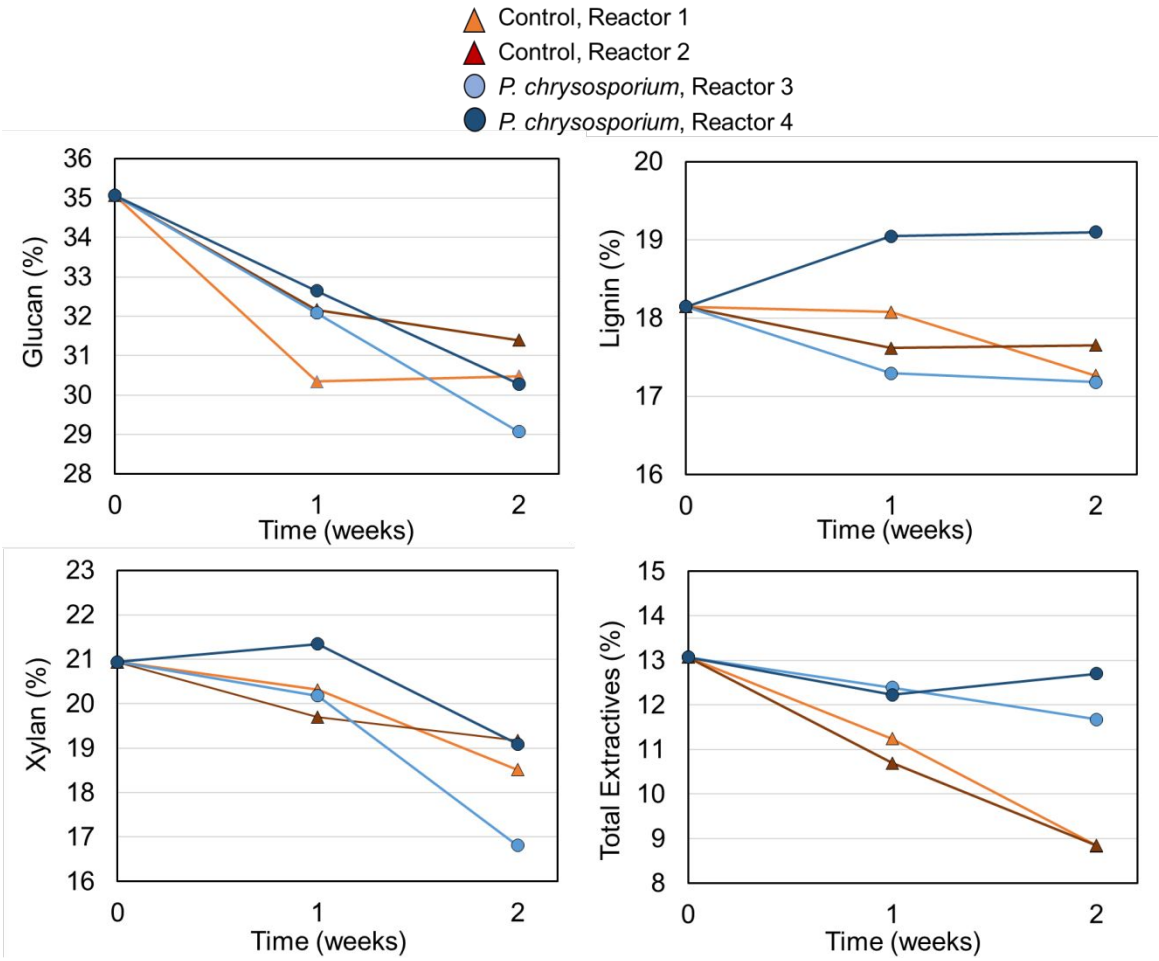

Figure 2. Compositional changes weighted with dry matter loss as a function of time. Corn stover inoculated with P. chrysosporium was compared alongside uninoculated stover. Both fungal-inoculated and control experiments were conducted in aerobic bioreactors at 60% moisture for two weeks.

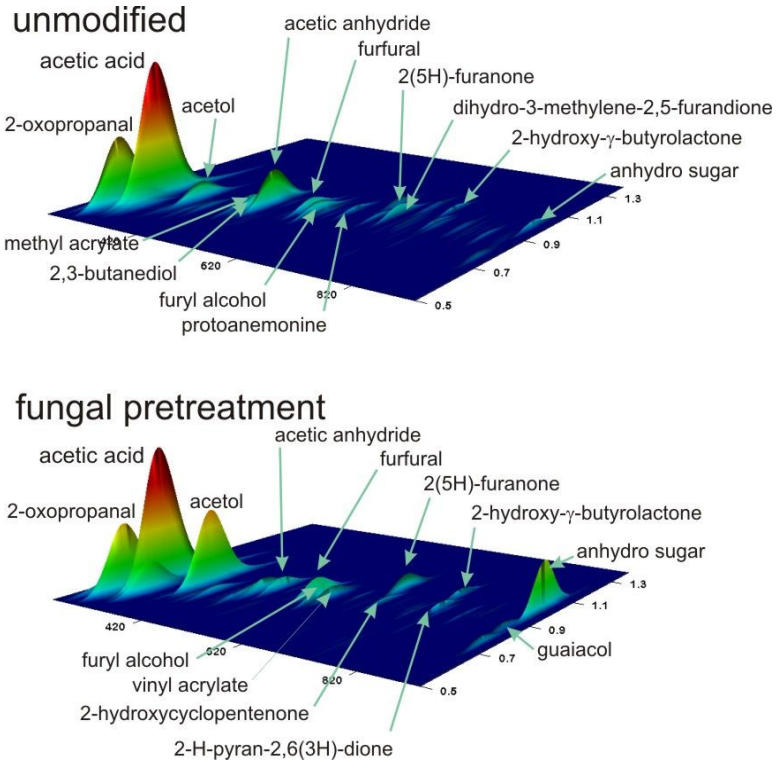

Figure 3. 3D total ion chromatograms generated from pyrolysis/GCxGC/MS of corn stover samples, using maximum pyrolysis temperature of 400°C. Chromatograms display the early eluting peaks (220 – 920 s in the first chromatographic dimension). Top - unmodified; bottom – fungal pretreatment

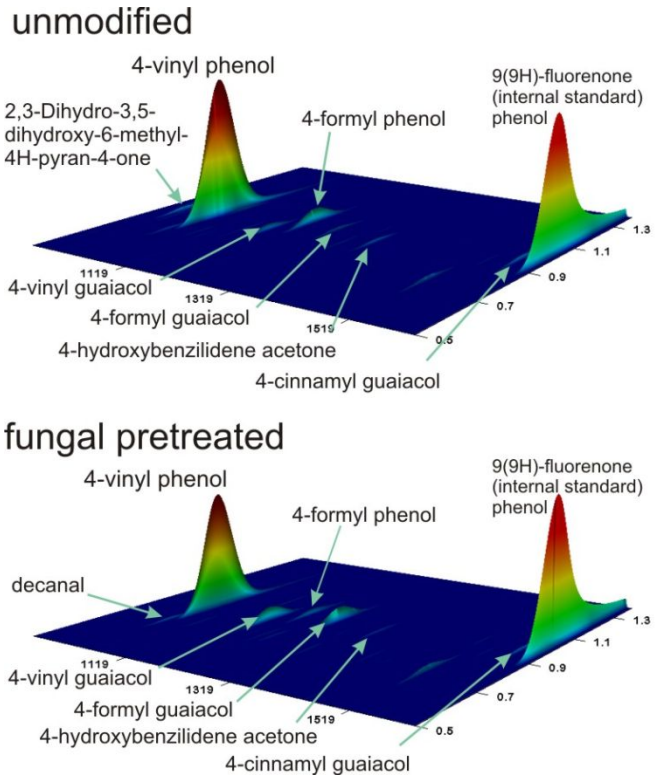

Figure 4. 3D total ion chromatograms generated from pyrolysis/GCxGC/MS of corn stover samples, using maximum pyrolysis temperature of 400°C. Chromatograms display the late eluting peaks (920 – 1620 s in the first chromatographic dimension). Top - unmodified; bottom – fungal pretreatment

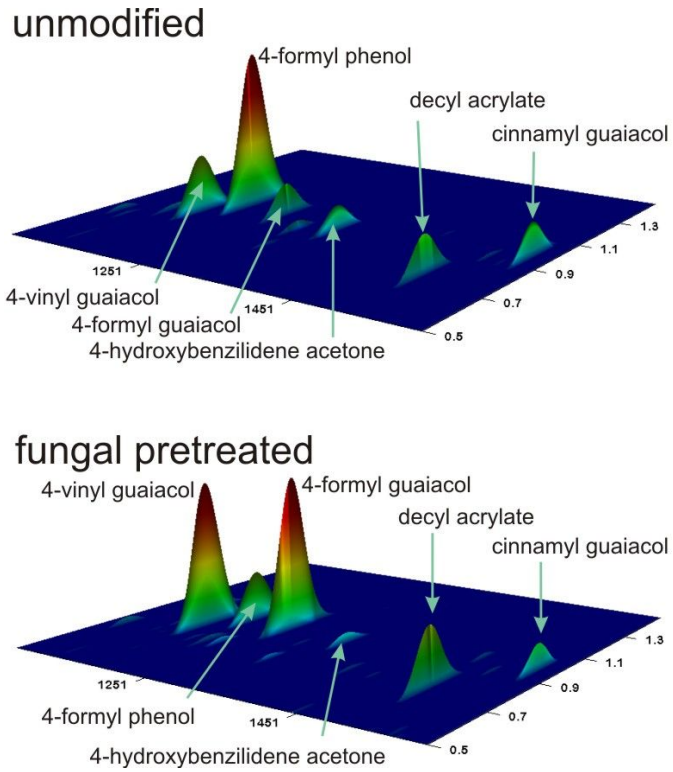

Figure 5. 3D total ion chromatograms generated from pyrolysis/GCxGC/MS of corn stover samples, using maximum pyrolysis temperature of 400°C. Chromatograms display the region form 1150 – 1550 s (first chromatographic dimension) where the majority of the lignols elute. Z axis is expanded compared to Figure 4 and Figure 5. Top - unmodified; bottom – fungal pretreatment.

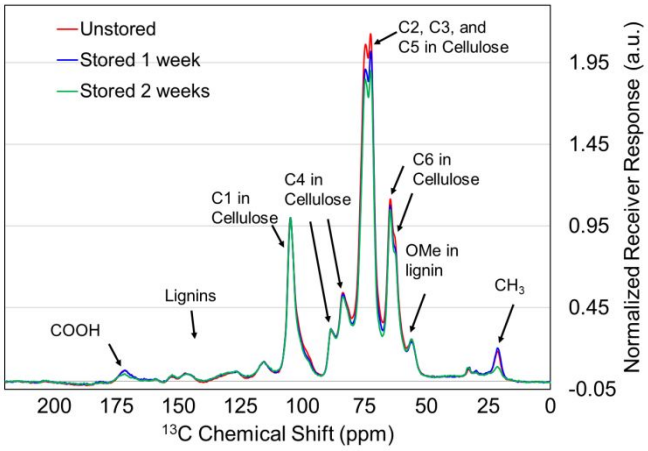

Figure 6. Solid-State ^{13}C-CP/MAS NMR profiles of unstored and P. chrysosporium-inoculated corn stover.

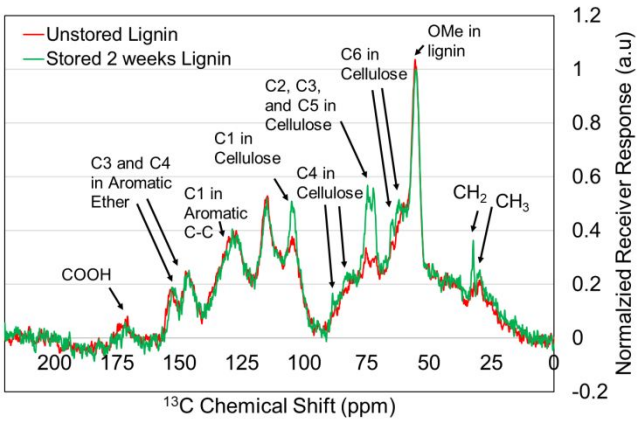

Figure 7. Solid-State ^{13}C-CP/MAS NMR profiles of a lignin rich sample of unstored and P. chrysosporium-inoculated corn stover.

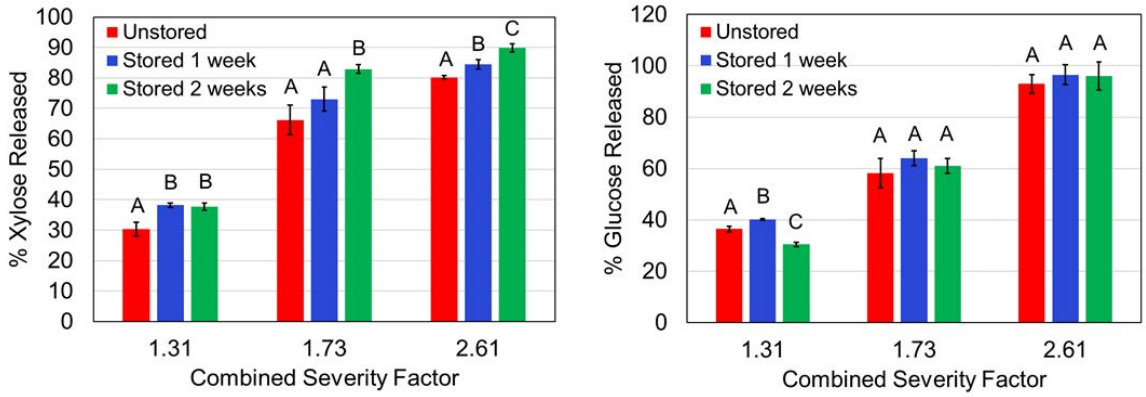

Figure 8. Combined dilute acid and enzymatic hydrolysis yields corn stover subject to P. chrysosporium treatment over a two-week period. Error bars represent standard deviation, n=3 or 4. Letters represent significant differences based on a Tukey Kramer test.