Effects of Extracellular Proteome on Wheat Straw Pretreatment during Solid-State Fermentation of *Phlebia radiata* ATCC 64658

Mahesh Vitthal Bule[†], Innu Chaudhary[†], Allan Haiming Gao, Shulin Chen^{*}

Department of Biological Systems Engineering, Washington State University, Pullman, WA 99164-6120, USA

[†] These authors contributed equally in performing experiments and preparation of this manuscript.

^{}Corresponding author Tel.: +1-509-335-3743; Fax: +1-509-335-2722.*

E-mail addresses:

mahesh.uict@gmail.com (Mahesh Bule)

innu.chaudhary@wsu.edu (Innu Chaudhary)

allan.gao@wsu.edu (Allan Gao)

chens@wsu.edu (Shulin Chen, corresponding author)

Abstract

Biological pretreatment of lignocellulosic biomass potentially offers a less energy intensive alternative to chemical pretreatment for the reduction of recalcitrance towards cellulolytic enzymes, but possesses some disadvantages, including a slow rate and loss of polysaccharides. This study characterizes the biodegradation of wheat straw by *Phlebia radiata*, a white rot fungus capable of secreting three major classes of ligninolytic enzymes: lignin peroxidase, manganese peroxidase, and laccase under natural conditions. We investigated the correlation between synergistic action of fungal enzymes and characteristics of the pretreated biomass. The results showed a sequential expression of enzymes over the course of a three-week pretreatment, with members of the peroxidase family being expressed in week one, followed by laccase expression starting in week two which continued until the course of pretreatment. This highlights the synergy of ligninolytic enzymes in the selective degradation pattern for wheat straw. ^1H - ^{13}C HSQC NMR spectroscopy results demonstrated reduced amounts of syringyl (S) and hydroxyphenyl (H) lignin after pretreatment. Moreover, the reduction in H lignin was also seen in Pyrolysis – GC/MS and FT-IR results. This strongly suggests that this unique lignin modification pattern is associated with *Phlebia radiata* extracellular proteome, as expressed during the solid-state fermentation (pretreatment) of wheat straw.

Keywords

Biological pretreatment; Wheat straw; Lignin; *Phlebia radiata*; fungal secretome

1. Introduction

Biorefineries that produce renewable fuels or chemicals currently face considerable technical challenges. The most pressing one is lowering the cost in conversion of carbohydrates embedded in lignocellulosic matrix into fermentable sugars. One logical strategy is to find the best way to fractionate and separate major components of plant cell walls, such as cellulose, hemicellulose and lignin. The major barrier in plant cell wall deconstruction is lignin that bridges the cellulose and hemicellulose, strengthening and increasing structural integrity of the plant cell wall. Lignin also makes the cellulose framework recalcitrant to enzymes, preventing carbohydrate polymers from being hydrolyzed into sugars (Zheng et al., 1998, Mosier et al., 2005, Chen et al., 2010, Wan and Li, 2012). Therefore, development of pretreatment methods to selectively modify lignin molecules and their association with hemicellulose and cellulose is necessary (Wan and Li, 2012).

Various physicochemical pretreatment methods have been developed to facilitate the degradation of hemicellulose and cellulose to sugars. However, these methods of lignin removal or modification are often energy-intensive, and lack selectivity to purposely deconstruct specific cell wall components and thus reduce recalcitrance (Bak et al., 2009, Haghighi Mood et al., 2013). Although these pretreatment methods are effective, they tend to destroy the basic units of the lignin polymer framework and form undesirable new compounds as by-products. Nature, on the other hand, has created biological degraders with complex enzymatic systems that can selectively modify functional groups on the lignin polymer units by targeting specific chemical bonds of the molecules (Hatakka, 1983, Müller and Trösch, 1986, Zhang et al., 2007, Hendriks and Zeeman, 2009, Chen et al., 2010, Zeng et al., 2011, Galbe and Zacchi, 2012), which loosens the cell wall structure. For example, by producing these enzyme complexes, white rot fungi are considered to be one of the most efficient degraders of lignocellulose. This type of biological pretreatment of lignocellulosic biomass is selective, less energy-intensive, and effective under mild environmental conditions (Forney et al., 1982, ten Have and Teunissen, 2001, Zhang et al., 2007). Many studies have examined delignification of lignocellulosic biomass by white rot fungi, especially by *Phanerochaete chrysosporium*, to improve the efficiency of cellulose hydrolysis and yield monomers. This fungus can produce key enzymes, such as lignin peroxidases (LiPs), manganese peroxidases (MnPs) and esterases (ESTRs) (Van Aken et al., 1999, Kersten and Cullen, 2007, Hammel and Cullen, 2008, Singh and Chen, 2008, Bak et al., 2009, Zeng et al., 2011). Studies on the other hand show that laccase, an important enzyme in the lignin depolymerization process, is not secreted by *Phanerochaete chrysosporium* under normal conditions (Niemenmaa et al., 2006, Wan and Li, 2012). However, another white rot fungus, *Phlebia radiata*, can produce all major ligninolytic enzymes, such as LiPs, MnPs and laccase (Niku-Paavola et al., 1988, Rogalski et al., 1991, Saloheimo et al., 1991, Hatakka, 1994, Vares et al., 1995, Hofrichter et al., 2001, Leonowicz et al., 2001, Mäkelä et al., 2006, Niemenmaa et al., 2006).

Like solid-state fermentation, bio-pretreatment is characterized by fungal growth in a low-moisture environment on a damp, insoluble material. This provides both the source of nutrients and a physical support for the fungus (Osma et al., 2011). *Phlebia radiata* has been shown to utilize and deconstruct lignin more effectively than *P. chrysosporium* under specific experimental conditions, based on differences between their secreted enzymes (Arora et al., 2002). According to Arora et al., (2002), *P. radiata* and *P. floridensis* are the best producers of

MnP and laccases (Arora et al., 2002). The presence of laccases may play an important role in lignin degradation.

Lignin is a polymer of three different p-hydroxycinnamyl alcohol, or monolignol precursors: p-coumaryl, coniferyl, and sinapyl alcohols (Fengel and Wegener, 1983, Boerjan et al., 2003). Each monolignol gives rise to a different type of lignin unit: the H (p-hydroxyphenyl), G (guaiacyl), and S (syringyl) units. These phenolic monolignols can be oxidized into phenoxy radicals by laccases and peroxidases. The laccases catalyze one electron oxidation of the phenolic derivatives, and can also catalyze non-phenolic substrates if the appropriate redox mediators are present. The reaction can further proceed to radical coupling between specific positions of two phenoxy radicals, creating carbon-carbon and ether linkages. The lignin is covalently bound to hemicellulose, which in turn is bound to cellulose through extensive hydrogen bonding (Eriksson et al., 1980). The most frequently suggested lignin and ester linkages occur with acetyl units and hydroxy-cinnamic acids. Although many studies have investigated the mechanism of lignocellulose degradation by *P. chrysosporium* cultures (Singh and Chen, 2008) containing lignin-degrading enzymes, no studies detail the mechanistic understanding of *P. radiata*. Since the ligninolytic enzymes secreted by both of these species are significantly different, it is essential to understand how *P. radiata* breaks lignocellulosic biomass structure efficiently. Moreover, understanding proteome secretion patterns and lignin modification and/or removal during *P. radiata* biopretreatment is an essential step in developing an efficient pretreatment technology that reduces the impact of lignin recalcitrance on cellulose and hemicellulose hydrolysis.

The purpose of this study was to examine lignocellulosic biomass degradation in wheat straw biomass pretreated with the white rot fungus *P. radiata*. Specifically, the connection between degradation reactions by the fungal enzymes and characteristics of the resultant biomass was investigated with the advanced analytical techniques. The extracellular proteome of *Phlebia radiata*, as expressed during the biological pretreatment process using LC/MS-based techniques was analyzed in this study and the effect of the pretreatment on wheat straw degradation was also evaluated by characterizing changes in structure and functional group modification with Py-GC/MS, FT-IR and solution-state ^1H - ^{13}C NMR. The findings provide new insights into enzyme systems for lignin degradation under natural conditions and also provide evidence for sequential secretion of enzymes during the course of three-week solid-state fermentation, to effectively degrade wheat straw biomass by adopting the strategy of synergistic action during the degradation process.

2. Material and methods

2.1 *Phlebia radiata* culture preparation and solid-state fermentation on wheat straw

The *Phlebia radiata* ATCC 64658 strain used in this study was purchased from ATCC. The fungus was cultivated on potato dextrose agar (PDA) plates for 7 days to form vegetative mycelia at $28 \pm 2^\circ\text{C}$, and maintained at 4°C for a month. Wheat straw was obtained from the Grange Supply Co. in Pullman, WA, and used as a substrate for growth of *P. radiata*. To perform pretreatment, 20 g. of chopped wheat straw (~2 to 4 cm) was suspended in 100 ml of distilled water and autoclaved for 20 minutes at 121°C . The sterile wheat straw (substrate) was cooled, and the water was decanted and then inoculated with 5 ml of mycelial suspension (prepared from 2 discs of ~1 cm diameter with a hand homogenizer in saline solution) in a 500 ml Erlenmeyer flask. Samples were collected after 7, 14, and 21 days of bio-pretreatment. Wheat straw treated in the same conditions without fungal mycelia inoculation was used as the control. Experiments were performed in duplicate, and mean values were used for reporting results. Humidity was maintained by incubating flasks in an incubator with humidity control.

2.2 Preparation and analysis of *Phlebia radiata* extracellular proteome samples

The procedure for analysis of *Phlebia radiata* extracellular proteome was as follows (total proteome analysis). After bio-pretreatment, cultures were extracted using acetate buffer (50 mM saline pH = 5 (ABS) containing a 1 mM protease inhibitor cocktail (Sigma). The solution was centrifuged at $10,000 \times g$ for 20 min to get soluble proteins in the supernatant. The protein-buffer solution was concentrated 10 fold, using a 3KDa molecular weight cut-off Amicon Ultra filter. This was desalted by dilution with an acetate buffer (pH = 5) containing a 1 mM protease inhibitor cocktail and then concentrated again. The procedure was repeated in triplicates. Proteins in the concentrate were precipitated overnight at 4°C using 100% ice-cold trichloroacetic acid (TCA), and centrifuged at $10,000 \times g$ for 20 min. Precipitate was washed thoroughly three times with a 5% v/v Tris-buffer (50 mM, pH 7.5) in acetone to remove any traces of TCA. Finally, the precipitate was washed with ice-cold acetone three times, air-dried and stored at -20°C for further analysis.

Extracellular proteins were analyzed with direct liquid chromatography/mass spectrometry (LC/MS) on a Waters Q-ToF Premier quadrupole-time of flight mass spectrometer interfaced with a nanoACQUITY ultra-performance liquid chromatography (UPLC) system (Waters Corp., Milford, MA). The dried, precipitated/extracted proteins were re-suspended in 100 μl of 100 mM NH_4HCO_3 solution. The samples were digested with trypsin (Ham et al., 2005). Insoluble material was removed by centrifugation at 21,952 rcf for 1 min. Eleven μl of 100 mM dithiothreitol (DTT) (Sigma) was added to the supernatant and incubated at 50°C for 15 min. After cooling to room temperature, 27.8 μl of 100 mM iodoacetamide (Sigma) was added to the protein solution, and the mixture was incubated at room temperature for 20 min. Four microliters of trypsin (Promega) was added to the mixture, which was then incubated at 37°C for 16 h. After digestion, 1 μl of formic acid was added, samples were evaporated to dryness with an Eppendorf speedvac for 10 min. One hundred μl of solution containing 5% acetonitrile, 0.1% formic acid, and 94.9% water was added to re-dissolve the peptides, and the samples were subjected to LC/MS analysis (Ham et al., 2005). The mass spectrometer was programmed with a data dependent analysis method where multiple charged analytes likely to be peptides were detected in an MS survey acquisition, which then initiated MS/MS spectrum acquisition from the

selected precursor ion. Raw data were, processed and a database search was conducted with Protein Lynx Global Server version 2.3. Peak list files were analyzed with MASCOT software (Matrix Science, 2008 version).

2.3 Biomass compositional analysis; acid-soluble and insoluble lignin analysis

The oven-dried (50°C) autoclaved wheat straw (control) and the *P. radiata* three-week pretreated wheat straw samples were ground and sieved through a 60-mesh size screen. Ten grams of biomass was extracted with toluene: ethanol (2:1 v/v; 8 h in Soxhlet), as described by ASTM method 1107. The liquor was evaporated under a vacuum, and the extractive content was determined gravimetrically. Control and pretreated wheat straw samples were used for compositional analyses. Carbohydrate and lignin content were determined according to the NREL standard laboratory analytical procedure (LAP) for structural carbohydrates in biomass (Sluiter et al., 2004). Next, 0.3 g of the sample was weighed and used in a two-stage acid hydrolysis method. After initial hydrolysis at 37°C with 3 mL of 72% (w/w) sulfuric acid, 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). Samples were run for 60 min, and the column was flushed between runs with 200 mM NaOH followed by de-ionized water. The sugar concentration was compared to a standard sugar sample, and all measurements were taken in triplicate. The acid-soluble and insoluble lignin content was calculated according to NREL's Laboratory Analytical Procedure (Sluiter et al., 2004).

2.4 Lignin extraction and solution state NMR of lignin enriched isolates

To perform NMR analysis, an extractive free cell-wall residue was prepared from milled wheat straw (fraction <60 mesh) using ethanol: toluene (50:50) for 24 hours in a Soxhlet extractor, followed by solvation in ethanol (100 ml g⁻¹) and water (100 ml g⁻¹) for 24 h each. The processed biomass was then freeze-dried and ball-milled for 24 h in the presence of toluene. A cycle of a two-minute run with a one-minute pause prevented further changes to the lignin structure. The ball-milled sample was dried and extracted with 94:6 dioxane/water. The resulting preparation was purified for wheat straw lignin extraction according to Björkman's method (Björkman, 1956). Purified lignin was used for a 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 (¹³C), respectively, with resonance values given in δ ppm. The lignin isolates (~25 mg) from both the control and three-week fungus pretreated wheat straw samples were individually dissolved in DMSO-*d*₆ (0.5 cm³), with ¹H and ¹³C NMR spectra recorded for each sample. The ¹H NMR spectra of both lignin isolates were recorded with a spectral width of 6410 Hz, in 16 scans. The ¹³C NMR spectra were collected with a sweep width of 25,510 Hz in 25,000 scans. Single bond ¹H-¹³C 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 (¹³C) using 256 increments (F1 'acquisition time' 15.0 ms). Eight scans were conducted with a 1s interscan delay. Integration calculations of the two dimensional contours in all spectra was performed with MestReNova software. All structural analyses were carried out to compare chemical shift values and proton/carbon correlation (in ppm) resonance values for fungus pretreated lignin samples to the control.

2.5 FT-IR analysis

FT-IR spectra of three-week pretreated wheat straw particles (<60 mesh) vs. control samples were recorded on a Shimadzu IR-Prestige FT-IR Spectrometer to detect functional group changes. FT-IR spectra were obtained in transmittance 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.

2.6 Pyrolysis gas chromatography-mass spectrometric analysis (Py-GC/MS)

Py-GC/MS was carried out to verify lignin modification and identify specificity of lignin-S or G degradation during fungal pretreatment. A CDS Pyroprobe 5000 was connected to an Agilent GC/MS system (7890A GC, 5975C Mass Selective Detector). Wheat straw 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 610°C for 1 min. The inlet temperature was maintained at 250°C and the resulting pyrolysis vapors were separated by a (5%-phenyl)-methylpolysiloxane hydrophobic column with a 30 m x 25mm inner diameter. The gas flow rate was 1 ml min^{-1} , and the oven was maintained at 280°C for 10 minutes 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 comparing their mass spectroscopy spectra with the database (Boateng et al., 2006).

3. Results and Discussion

3.1 Extracellular proteome analysis

Extracellular proteomes provide data on probable enzyme complexes secreted by *Phlebia radiata* during growth on wheat straw. The majority of proteins expressed during wheat straw biodegradation are ligninolytic and cellulolytic. Supplementary file 1 summarizes the proteins secreted by fungi obtained through Protein Lynx software. The results indicated that lignin peroxidases (LiP) and manganese peroxidases (MnP), the major ligninolytic enzymes, were expressed with other cellulolytic enzymes during the first week of pretreatment of wheat straw with *P. radiata* cultures. They were also detected during the second and third weeks of incubation.

The phenol oxidase enzyme laccase was detected during the second week of incubation, and continued to express in the third week. *Phlebia radiata* differs significantly from *Phanerochaete chrysosporium*, which is the most extensively studied lignocellulose degrader. *Phanerochaete chrysosporium* only expresses LiP and MnP (Arora et al., 2002); while *Phlebia radiata* harbors all three major ligninolytic enzymes LiP, MnP and laccase as an enzyme cocktail.

Laccase enzyme is involved in the H_2O_2 generation reaction via a series of redox reactions. Monomeric, aromatic alcohols stimulate its production in *Phlebia radiata* non-specifically, while substituted aromatic alcohols stimulate LiP and MnP production (Leonowicz et al., 2001). Laccase acts as an important and strong oxidant for aromatic rings, substituted with groups such as free phenolic hydroxyls in lignin or other related compounds. It acts synergistically with MnP for extensive and efficient degradation of lignocellulose (Leonowicz et al., 2001, Arantes and Milagres, 2007).

However, the results showed that laccase did not appear during the first week of fermentation. This can be explained by previous studies showing that peroxidases generate several reactive phenoxy radicals. These radicals can then subsequently undergo demethoxylation, which is the primary reaction catalyzed by laccase to cleave the ether bonds linking most aromatic subunits. MnP oxidizes the phenolic compounds by catalyzing alkyl-aryl cleavages and α -carbon bonds (Leonowicz et al., 2001).

Other than these specific ligninolytic enzymes, results also show the presence of glucosidase, xylanase and other hydrolases such as Cellobiohydrolase in the secretome, which are related to the degradation of cellulosic chains. In addition, various glycoside hydrolase families of proteins were also identified in the *P. radiata* spent wheat straw extracellular medium (See supplementary file 1).

To understand the probable reason behind secretion of some ligninolytic enzymes earlier in pretreatment relative to others, a schematic has been hypothesized which describes the above mentioned results and elucidate that how synergy between ligninolytic enzymes is strategically used by the fungus to degrade the biomass (Scheme 1).

In scheme 1, MnP takes up a representative lignin model compound and via phenoxy radical formation, generates S and G lignin molecules, which are then catalyzed by laccase to simpler lignin (H) units. According to scheme 1, it is suggested that presence of laccase in the ligninolytic system generates more H-units (preferred demethoxylation reaction by laccase), which are relatively easier to be broken down in the further degradation process, on the contrary, *P.chrysosporium* does not secrete laccase naturally, and hence accounts for relatively less H-unit generation during pretreatment, which results in preferential degradation of G and S subunits.

3.2 Wheat straw chemical composition analysis

The effect of biological pretreatment was analyzed by comparing untreated and three-week pretreated biomass. The experiment was done in triplicates and the results show that a three-week incubation significantly altered wheat straw composition. Table 1 summarizes the chemical composition changes in the biomass during pretreatment (Data not shown for week-1 and week-2, since the changes in biomass composition were not significant).

The data indicate that the insoluble lignin content in the wheat straw decreased due to fungal pretreatment of the biomass. This made the lignin more soluble and ultimately less recalcitrant. Therefore, degradation and modification occurred without much loss in overall lignin content. Total sugars (pentoses and hexoses) in pretreated wheat straw also decreased by approximately 7%, possibly due to utilization of sugar by fungi for their own growth.

3.3 NMR spectroscopic analysis of lignin isolates from control and pretreated wheat straw.

3.3.1 ^1H and ^{13}C NMR Analysis

Purified lignin obtained from control wheat straw and *P. radiata* 3-week treated wheat straw was used for ^1H NMR and ^{13}C NMR analysis, as detailed in the Methods section. Figure 1 compares the ^1H NMR spectra, indicating two primary differences. The two peaks in the oxygenated aliphatic region between 3-4 ppm were significantly reduced. This may be due to modification of lignin side-chain structures, as well as breakdown of hemicellulose-lignin linkages. The presence of hydrolases in this study, as expressed in the secretome of fungi, supports this observation. Past

research indicated that *P. radiata*, as well as other white rot fungi, can produce enzymes such as endo-1, 4- β -xylanase and ferulic acid esterase (Rogalski et al., 1993, Pérez et al., 2002), enabling them to efficiently dissolve hemicellulose linkages as well as break the bond between ferulic acid in the wheat straw and the lignin structure (Jung and Himmelsbach, 1989).

Figure 2 shows that in the ^{13}C NMR spectra, there is a significant amount of degradation across the whole spectrum. With the same sample size of 25 mg used for solid-state ^{13}C NMR and identical parameters used for acquisition for the two samples, the two spectra can be semi-quantitatively compared. Figure 2 clearly shows that *P. radiata* significantly degraded the lignin skeletal structure. This is evidenced by the decrease in peak intensity between 90 ppm and 130 ppm. The decrease of the peak at 58 ppm corresponds to the aliphatic -OH group present on the lignin chain structure. This suggests that the *P. radiata* pretreatment played a significant role in breaking the lignin subunit linkages. In addition, the region at 160 ppm indicates that the methoxyl group (-OCH₃) substitution on the lignin phenolic ring substantially decreased as well.

In combination, the ^1H and ^{13}C NMR spectra indicate a significant degradation of the lignin carbon structure. However, there was little difference between the ^1H NMR of the *P. radiata* sample and the control, suggesting that many of the individual functional groups on the lignin were still present. However, the fungi preferentially targeted the carbon structure.

3.3.2 Two-dimension HSQC NMR Analysis

The heteronuclear single quantum correlation (HSQC) was evaluated between ^1H -spectra and ^{13}C -spectra to understand changes in functional group content on the lignin structure. HSQC has been shown to be a useful analytical technique to determine changes in lignocellulosic structure (Sun et al., 2005, Del Río et al., 2012). Figure 3 shows that lignin resonance ranges between the ^1H spectra of 4.0 to 10.0 ppm and the ^{13}C spectra of 90 to 150 ppm for the autoclaved control and three-week *P. radiata* treated samples. A broad overview of these two spectra indicates that a significant amount of lignin degradation occurred over the three-week fungal pretreatment. Specifically, the major degradation occurred in the regions of (6.65, 105) and (6.65, 115). The HSQC region located at (6.65, 105) is related to position 2 and position 6 carbons of the syringyl unit, while the (6.65, 115) region is related to the para-coumarate linkage between hemicellulose and lignin (Del Río et al., 2012). The diminishment of these two linkages after pretreatment by *P. radiata* indicates that this fungus can dissociate the hemicellulose from lignin, long regarded as a vital step in pretreatment (Iiyama et al., 1994). It can also degrade syringyl lignin, which is one of the recalcitrant lignin subunits.

In the area of (7.05, 110), a significant change was also observed. This area is believed to be associated with the C₂-H₂ bond found in guaiacyl lignin. Notably, the approximate area loss in this region was about the same compared to that of the syringyl lignin. This could be due to comparative loss of lignin in the wheat straw. Our results show that the hydrogen spectra between around 6.5 to 6.6 ppm associated with the C3-H3 and C5-H5 protons in H-unit lignin (Del Río et al., 2012) decreased significantly in the *P. radiata* treated sample. Due to lack of quantification in this NMR analysis, it was inferred that the H-unit lignin was more severely degraded than the G or S units. A quantitative comparison of particular lignin subunit degradation using Py-GC/MS was conducted. Results are discussed in the next section. However, it is apparent that *P. radiata* pretreatment had a significant overall effect on the content of lignin in wheat straw biomass.

3.4 FT-IR Analysis

Figure 4 shows the FT-IR spectra for the autoclaved control and the three-week *P. radiata* treated sample. FT-IR spectra for first and second-week pretreated samples are included in supplementary file 2. Table 2 shows peak assignments. Peak assignments and a detailed comparison of control and treated samples were performed according to earlier reports on FT-IR analysis of lignocellulose (Lawther et al., 1996, Pandey and Pitman, 2003). Several significant changes can be considered, most notably the decrease in absorptions at the peaks of 1604 cm^{-1} , 1371 cm^{-1} , and 1246 cm^{-1} . The decrease at 1246 cm^{-1} , which is attributed to the methoxyl vibrations on the lignin aromatic ring, indicates a decrease of lignin units with $-\text{OCH}_3$ functional groups. These results accord with those of earlier observations of the ^{13}C NMR study in the current research, in which $-\text{OCH}_3$ resonance decreased in the region of 160 ppm. This supports the conclusion that *P. radiata* breaks down G and S lignin subunits.

The peak at 1371 cm^{-1} , attributed to C-H deformation in hemicellulose, showed a significant decrease compared to the control sample. This indicates that hemicellulose-lignin linkages in the wheat straw were degraded, weakening the overall structure of the lignocellulose and breaking the bond between lignin and cellulose in wheat straw (Jung and Himmelsbach, 1989). This corroborates the finding in ^1H NMR of a significant decrease in the aliphatic oxygenate absorbance and consumption of hemicellulose by the fungi.

A peak at 1604 cm^{-1} , which belongs to C=O stretching vibrations and the lignin skeletal aromatic structure of guaiacyl and syringyl units, also decreased in pretreated wheat straw. The destruction of these units, found predominantly in the middle lamella of biomass cells (Saka and Goring, 1988), demonstrates that the ligninolytic enzymes produced by *P. radiata* are effective to degrade areas of biomass with a high lignin content.

Additional decreases in peak absorbance at 1730 and 1548 cm^{-1} , which both indicate aromatic skeletal vibrations, point to an overall decrease in lignin content after pretreatment by *P. radiata*.

3.5 Pyrolysis-GC/MS Analysis

Figure 5 shows Py-GC/MS pyrograms of the control wheat straw and three-week *P. radiata* pretreated wheat straw, respectively. Supplementary file 3 lists the corresponding retention time of lignin-derived compounds. This indicates a clear difference between pyrograms. There was an overall decrease in lignin-derived compounds, represented by peaks from a retention time of 8 min. to around 18 min. H units, represented by peaks from 1 to 10 with the exception of 4, showed a relative decrease in intensity from the control compared with the pretreated sample, with some peaks vanishing entirely (peaks 5-10). On the other hand, peaks derived from guaiacyl and syringyl lignin subunits increased. This indicates that *P. radiata* may have a preferential degradation of H lignin subunits, which are the least recalcitrant of all subunits. This result is also supported by FT-IR and 2D NMR data, where the decrease in $-\text{OH}$ vibrations and H-units were noticeable in pretreated samples. However, the 2D NMR results indicate that degradation of S and G units was nearly the same.

This is supported by Py GC/MS results, which show a relative increase of G and S units and a corresponding decrease of H units. This could be due to the fact that *P. radiata* ligninolytic

373 enzymes targeted least complex H units, rather than G and S due to availability of more H-units,
374 which is accounted for the presence of laccase in the expression system.

375 Since many enzymes from white rot fungi work oxidatively (ten Have and Teunissen, 2001) and
376 *P. radiata* contain additional laccase in the ligninolytic enzyme cocktail, the mechanism of lignin
377 degradation could differ from that of other white rot species such as *Phanerochaete*
378 *chrysosporium*. Oxidative degradation reactions with enzymes MnP and LiP typically occur
379 faster with methoxyl substituted S and G lignin units rather than H lignin. Previous studies on *P.*
380 *chrysosporium*, known to have an oxidative lignin degradation mechanism, also confirm
381 preferential breakdown of guaiacyl lignin over p-hydroxyphenol lignin (Singh et al., 2011).

382 These findings suggest that *P. radiata* may have a different mechanism than other white rot
383 fungi. Since H lignin requires comparatively less energy to degrade, degradation of H-units may
384 be more efficient than the G and S units. If selective degradation of H-units, which comprise
385 only 8-12% of lignin in wheat straw, can effectively pretreat the biomass, the current and further
386 research on *P. radiata* enzymes could suggest the possibilities for developing a new process that
387 targets specific lignin structure for lignocellulosic pretreatment.

388 This study fills an important gap in the literature on the action and expression of ligninolytic
389 enzymes to degrade lignin. The results on the expression levels of all three ligninolytic enzymes
390 over three weeks of fermentation show that H units were selectively cleaved over the substituted
391 G and S units. The most commonly studied white rot fungus, *P.chrysosporium*, selectively
392 degrades G and S units and produces MnP enzymes in relatively higher concentrations than other
393 lignin degrading enzymes, with little or no expression of laccase. The findings elucidate the
394 mechanism in which ligninolytic enzymes target specific units of lignin molecules, as well as the
395 importance of laccase in the degradation pattern (demethoxylation to generate more H-units)
396 These results add new information on the specificity of each ligninolytic enzymes for wheat
397 straw lignin units.

4. Conclusions

In this study, lignin modifications were characterized during biological degradation of wheat straw by white rot fungus *Phlebia radiata*. The pretreatment process is due to the action of ligninolytic enzymes, including manganese peroxidases, lignin peroxidases and laccase. Wheat straw degradation by white rot fungus *Phlebia radiata* enzymes occurs by targeting lignin-hemicellulose linkages, which then proceeds to selective lignin modifications. The major ligninolytic enzymes acts in synergy to modify lignin structure, and laccase presence in the enzymatic cocktail leads to preferential degradation of the H-unit (hydroxyphenyl) of lignin after the pretreatment, which is the least recalcitrant of all lignin subunits (G and S).

Acknowledgements

The authors would like to acknowledge Dr. William Hiscox from the NMR Centre, Washington State University, for his assistance in running the samples for NMR analysis in this study. The authors also wish to acknowledge the University of Idaho Mass Spectrometry Core facility (supported by the UI Office of Research and Economic Development) for proteomic analysis contributions to this work.

This work is supported by Washington State University and partially supported by the National Science Foundation under Grant No. 1231085.

References

- Arantes, V. & Milagres, A. M. F. 2007. The synergistic action of ligninolytic enzymes (MnP and laccase) and Fe^{3+} -reducing activity from white-rot fungi for degradation of Azure B. *Enzyme and microbial technology*, 42, 17-22.
- Arora, D. S., Chander, M. & Gill, P. K. 2002. Involvement of lignin peroxidase, manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. *International biodeterioration & biodegradation*, 50, 115-120.
- Bak, J. S., Ko, J. K., Choi, I. G., Park, Y. C., Seo, J. H. & Kim, K. H. 2009. Fungal pretreatment of lignocellulose by *Phanerochaete chrysosporium* to produce ethanol from rice straw. *Biotechnology and bioengineering*, 104, 471-482.
- Björkman, A. 1956. Studies on finely divided wood. Part i. Extraction of lignin with neutral solvents. *Sven. Papperstidn*, 59, 477-485.
- Boateng, A., Jung, H. & Adler, P. 2006. Pyrolysis of energy crops including alfalfa stems, reed canarygrass, and eastern gamagrass. *Fuel*, 85, 2450-2457.
- Boerjan, W., Ralph, J. & Baucher, M. 2003. Lignin biosynthesis. *Annual review of plant biology*, 54, 519-546.
- Chen, S., Zhang, X., Singh, D., Yu, H. & Yang, X. 2010. Biological pretreatment of lignocellulosics: potential, progress and challenges. *Biofuels*, 1, 177-199.
- Del Río, J. C., Rencoret, J., Prinsen, P., Martínez, A. N. T., Ralph, J. & Gutiérrez, A. 2012. Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-nmr, and reductive cleavage methods. *Journal of agricultural and food chemistry*, 60, 5922-5935.
- Eriksson, Ö., Goring, D. & Lindgren, B. 1980. Structural studies on the chemical bonds between lignins and carbohydrates in spruce wood. *Wood science and technology*, 14, 267-279.
- Fengel, D. & Wegener, G. 1983. *Wood: chemistry, ultrastructure, reactions*, Walter de Gruyter.
- Forney, L. J., Reddy, C. A., Tien, M. & Aust, S. 1982. The involvement of hydroxyl radical derived from hydrogen peroxide in lignin degradation by the white rot fungus *Phanerochaete chrysosporium*. *Journal of biological chemistry*, 257, 11455-11462.
- Galbe, M. & Zacchi, G. 2012. Pretreatment: the key to efficient utilization of lignocellulosic materials. *Biomass and bioenergy*, 46, 70-78.
- Haghighi Mood, S., Hossein Golfeshan, A., Tabatabaei, M., Salehi Jouzani, G., Najafi, G. H., Gholami, M. & Ardjmand, M. 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renewable and sustainable energy reviews*, 27, 77-93.
- Ham, A., Caprioli, R. & Gross, M. 2005. Proteolytic digestion protocols. *The encyclopedia of mass spectrometry*, 2, 10-17.
- Hammel, K. E. & Cullen, D. 2008. Role of fungal peroxidases in biological ligninolysis. *Current opinion in plant biology*, 11, 349-355.
- Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. *Fems microbiology reviews*, 13, 125-135.
- Hatakka, A. I. 1983. Pretreatment of wheat straw by white-rot fungi for enzymic saccharification of cellulose. *European journal of applied microbiology and biotechnology*, 18, 350-357.
- Hendriks, A. & Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource technology*, 100, 10-18.

- Hofrichter, M., Lundell, T. & Hatakka, A. 2001. Conversion of milled pine wood by manganese peroxidase from *Phlebia radiata*. *Applied and environmental microbiology*, 67, 4588-4593.
- Iiyama, K., Lam, T. B.-T. & Stone, B. A. 1994. Covalent cross-links in the cell wall. *Plant physiology*, 104, 315.
- Jung, G. H.-J. & Himmelsbach, S. D. 1989. Isolation and characterization of wheat straw lignin. *Journal of agricultural and food chemistry*, 37, 81-87.
- Kersten, P. & Cullen, D. 2007. Extracellular oxidative systems of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Fungal genetics and biology*, 44, 77-87.
- Lawther, J. M., Sun, R. C. & Banks, W. B. 1996. Fractional characterization of wheat straw lignin components by alkaline nitrobenzene oxidation and FT-IR spectroscopy. *Journal of agricultural and food chemistry*, 44, 1241-1247.
- Leonowicz, A., Cho, N., Luterek, J., Wilkolazka, A., Wojtas- Wasilewska, M., Matuszewska, A., Hofrichter, M., Wesenberg, D. & Rogalski, J. 2001. Fungal laccase: properties and activity on lignin. *Journal of basic microbiology*, 41, 185-227.
- Mäkelä, M. R., Hildén, K. S., Hakala, T. K., Hatakka, A. & Lundell, T. K. 2006. Expression and molecular properties of a new laccase of the white rot fungus *Phlebia radiata* grown on wood. *Current genetics*, 50, 323-333.
- Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzaple, M. & Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource technology*, 96, 673-686.
- Müller, H. & Trösch, W. 1986. Screening of white-rot fungi for biological pretreatment of wheat straw for biogas production. *Applied microbiology and biotechnology*, 24, 180-185.
- Niemenmaa, O., Uusi-Rauva, A. & Hatakka, A. 2006. Wood stimulates the demethoxylation of [¹⁴C]-labeled lignin model compounds by the white-rot fungi *Phanerochaete chrysosporium* and *Phlebia radiata*. *Archives of microbiology*, 185, 307-315.
- Niku-Paavola, M.-L., Karhunen, E., Salola, P. & Raunio, V. 1988. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochem. J.*, 254, 877-884.
- Osma, J. F., Moilanen, U., Toca- Herrera, J. L. & Rodríguez- Couto, S. 2011. Morphology and laccase production of white- rot fungi grown on wheat bran flakes under semi- solid- state fermentation conditions. *Fems microbiology letters*, 318, 27-34.
- Pandey, K. & Pitman, A. 2003. Ftir studies of the changes in wood chemistry following decay by brown-rot and white-rot fungi. *International biodeterioration & biodegradation*, 52, 151-160.
- Pérez, J., Muñoz-Dorado, J., De La Rubia, T. & Martínez, J. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International microbiology*, 5, 53-63.
- Rogalski, J., Hatakka, A., Longa, B. & Leonowicz, A. 1993. Hemicellulolytic enzymes of the ligninolytic white- rot fungus *Phlebia radiata*. Determination of enzyme activities. *Acta biotechnologica*, 13, 47-51.
- Rogalski, J., Lundell, T. K., Leonowicz, A. & Hatakka, A. I. 1991. Influence of aromatic compounds and lignin on production of ligninolytic enzymes by *Phlebia radiata*. *Phytochemistry*, 30, 2869-2872.
- Saka, S. & Goring, D. A. 1988. The distribution of lignin in white birch wood as determined by bromination with tem-edxa. *Holzforschung-international journal of the biology, chemistry, physics and technology of wood*, 42, 149-153.

- Saloheimo, M., Niku-Paavola, M.-L. & Knowles, J. K. 1991. Isolation and structural analysis of the laccase gene from the ligninegrading fungus *Phlebia radiata*. *Journal of general microbiology*, 137, 1537-1544.
- Singh, D. & Chen, S. 2008. The white-rot fungus *Phanerochaete chrysosporium*: conditions for the production of lignin-degrading enzymes. *Applied microbiology and biotechnology*, 81, 399-417.
- Singh, D., Zeng, J., Laskar, D. D., Deobald, L., Hiscox, W. C. & Chen, S. 2011. Investigation of wheat straw biodegradation by *Phanerochaete chrysosporium*. *Biomass and bioenergy*, 35, 1030-1040.
- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. & Crocker, D. 2004. Determination of structural carbohydrates and lignin in biomass. *Nrel, golden, co*.
- Sun, X.-F., Sun, R., Fowler, P. & Baird, M. S. 2005. Extraction and characterization of original lignin and hemicelluloses from wheat straw. *Journal of agricultural and food chemistry*, 53, 860-870.
- Ten Have, R. & Teunissen, P. J. 2001. Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chemical reviews*, 101, 3397-3414.
- Van Aken, B., Hofrichter, M., Scheibner, K., Hatakka, A. I., Naveau, H. & Agathos, S. N. 1999. Transformation and mineralization of 2, 4, 6-trinitrotoluene (tnt) by manganese peroxidase from the white-rot basidiomycete *Phlebia radiata*. *Biodegradation*, 10, 83-91.
- Vares, T., Kalsi, M. & Hatakka, A. 1995. Lignin peroxidases, manganese peroxidases, and other ligninolytic enzymes produced by *Phlebia radiata* during solid-state fermentation of wheat straw. *Applied and environmental microbiology*, 61, 3515-3520.
- Wan, C. & Li, Y. 2012. Fungal pretreatment of lignocellulosic biomass. *Biotechnology advances*, 30, 1447-1457.
- Zeng, J., Singh, D. & Chen, S. 2011. Biological pretreatment of wheat straw by *Phanerochaete chrysosporium* supplemented with inorganic salts. *Bioresource technology*, 102, 3206-3214.
- Zhang, X., Yu, H., Huang, H. & Liu, Y. 2007. Evaluation of biological pretreatment with white rot fungi for the enzymatic hydrolysis of bamboo culms. *International biodeterioration & biodegradation*, 60, 159-164.
- Zheng, Y., Lin, H. M. & Tsao, G. T. 1998. Pretreatment for cellulose hydrolysis by carbon dioxide explosion. *Biotechnology progress*, 14, 890-896.

546 **List of Abbreviations**

- 547 2 D HSQC NMR- Two dimensional heteronuclear single quantum correlation nuclear magnetic
548 resonance
- 549 ABS- Acetate Buffer saline
- 550 ASTM- American Society for Testing and Materials
- 551 ATCC-American type culture collection
- 552 DMSO- Dimethylsulphoxide
- 553 DTT- Dithiothreitol
- 554 ESTRs-Esterases
- 555 FT-IR –Fourier transform Infrared
- 556 G-unit- guaiacyl unit
- 557 H-unit- p-hydroxyphenyl unit
- 558 KDa- kilodalton
- 559 LAP- Laboratory Analytical Procedure
- 560 LC/MS- Liquid chromatography/mass spectrometry
- 561 LiPs- Lignin peroxidases
- 562 MnPs-Manganese peroxidases
- 563 ms- milliseconds
- 564 NREL –National Renewable Energy Laboratory
- 565 *P.chrysosporium- Phanerochaete chrysosporium*
- 566 *P.radiata- Phlebia radiata*
- 567 PDA- Potato dextrose agar
- 568 Py– GC/MS – Pyrolysis- Gas chromatography/mass spectrometry
- 569 S-unit- syringyl unit
- 570 TCA- Trichloroacetic acid
- 571 UPLC- Ultra-performance liquid chromatography

572 **Figure/Scheme Legend**

573 **Scheme 1. Lignin modification.** A mechanism to explain the sequential secretion of
574 extracellular ligninolytic enzymes and synergy between them (hypothesized).

575 **Figure 1 – 1D ^1H NMR analysis.** 1D ^1H NMR spectrum of lignin-rich isolates from control and
576 3-week *P.radiata* pretreated wheat straw.

577 **Figure 2 – 1D ^{13}C NMR analysis.** 1D ^{13}C NMR spectrum of lignin-rich isolates from control
578 and 3-week *P.radiata* pretreated wheat straw.

579 **Figure 3 – 2D (^1H - ^{13}C) NMR analysis.** ^1H - ^{13}C HSQC-NMR analysis of selected lignin region
580 for control and 3-week *P.radiata* pretreated wheat straw sample. .

581 **Figure 4–FT-IR spectra of wheat straw lignin.** (A) Lignin isolated from wheat straw control.
582 (B) Lignin isolated from 3-week spent wheat straw samples with *P.radiata*.

583 **Figure 5- Pyrolysis GC/MS traces of wheat straw lignin.** Pyrograms of (I) wheat straw control
584 and (II) 3-week *P.radiata* pretreated samples. Nomenclature of the compounds is listed in
585 Additional file 3.

586

Scheme 1

Figure 1

Figure 2

Autoclaved Control

3-week P.radiata treated

Figure 3

Figure 4

I

II

Figure 5

Table 1- Chemical Composition of Control and *P.radiata* treated Wheat Straw (All values are expressed as % w/w).

Samples	Acid-insoluble lignin	Acid-soluble lignin	Glucose	Galactose	Arabinose	Mannose/Xylose
Control (untreated)	16.68±0.16	3.81±0.339	44.64±0.424	0.642±0.006	2.96±0.010	26.01±0.25
Sample (pretreated)	15.02±0.22	4.83±0.366	41.71±1.32	0.638±0.066	2.956±0.299	24.65±1.36

Table 2- Percentage intensity of the assigned peaks in the FT-IR spectra for control and *P.radiata* pretreated wheat straw samples.

Peak λ cm ⁻¹	Attribution and Description	%T Control	% T <i>P. radiata</i> Treated
896.83	Deformation of C-H bond in cellulose	94.83	99.19
1043.49	C-O stretching in cellulose	81.33	95.69
1157.29	Ether C-O-C bond in hemicellulose and cellulose	92.43	98.42
1246.02	Methoxyl, C-C and C-O stretching vibrations; C=O stretching vibration	93.68	98.95
1371.39	C-H deformation in hemicellulose	94.8	99.30
1423.47	Aromatic skeletal vibrations	95.41	99.45
1512.19	Aromatic skeletal vibrations from guaiacyl units	96.77	99.76
1604.77	C=O stretching vibrations and aromatic vibrations in order S > G; condensed G > etherified G	96.35	99.67
1730.15	C=O stretching vibrations	96.76	99.82