Biodegradation of lignin by fungi, bacteria and laccases

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Abstract

Indulin AT biodegradation by basidiomycetous fungi, actinobacteria and commercial laccases was evaluated using a suite of chemical analysis methods. The extent of microbial degradation was confirmed by novel thermal carbon analysis (TCA), as the treatments altered the carbon desorption and pyrolysis temperature profiles in supernatants. Laccase treatments caused only minor changes, though with increases occurring in the 850 °C and char precursor fractions. After fungal treatments, lignin showed a similar change in the TCA profile, along with a gradual decrease of the total carbon, signifying lignin mineralization (combined with polymerization). By contrast, bacteria produced phenolic monomers without their further catabolism. After 54 days of cultivation, a 20 wt.% weight loss was observed only for fungi, *Coriolus versicolor*, corroborating the near-80% carbon mass balance closure obtained by TCA. Compositional changes in lignin as a result of biodegradation were confirmed by Thermal Desorption (TD)-Pyrolysis-GC-MS validating the carbon fractionation obtained by TCA.

Microbial degradation, Laccase, Industrial lignin, Thermal carbon analysis, TD-Pyrolysis-GC-MS

1 Introduction

Lignin, a naturally occurring aromatic heteropolymer, is one of the main building blocks of lignocellulosic biomass providing structural integrity to plant cell walls (Ragauskas et al., 2014). One of the most abundant forms of this biopolymer is Kraft lignin (KL) obtained through an acid precipitation of black liquor, which is generated as a by-product of the alkaline sulfide treatment of lignocellulose characteristic for the paper mill industry (Koljonen et al., 2004). During this process, lignin undergoes structural modifications including the formation of new alkali-stable linkages and various degradation and condensation reactions (Chakar and Ragauskas, 2004). Currently, commercial development of lignin based products, such as adhesives and dispersants, accounts only for 2% of the original lignin, with the rest of it mainly contributing to power generation via combustion (Brebua et al., 2011). Thus, there is a need in increasing the commercial potential of industrial lignin. White and brown rot fungi are well known for their essential role in naturally occurring degradation of lignocellulosic biomass, which is enabled by secretion of extracellular ligninolytic enzymes (Sanchez, 2009).

Table 1 illustrates the use of fungi and bacteria for lignin biodegradation, based on the available literature. For short incubation times, efficient degradation of KL was reported mainly using bacterial strains (Table 1), as the performance of fungi is often unstable when exposed to harsh industrial treatments (Lv et al., 2013). Previous studies focused on the achievement of significant KL biodegradation within a short period of cultivation and thus

did not provide enough detail on the metabolic interactions between the applied microorganisms and lignin, in contrast to microbial degradation of lignocellulosic biomass where such interactions are well documented (Gai et al., 2014). In this study, we selected a longer cultivation time in an effort to gain insights into the degradation pattern of industrial lignin when exposed to various microorganisms. Most of the previous studies used low KL loads thus causing an uncertainty with the scalability of the data obtained. Therefore in this study, a more industrially relevant lignin concentration of 13.3g/L has been used.

Table 1 also shows that gas chromatography with mass spectrometry detection (GC-MS) has been the main method used to characterize lignin degradation products. However, no comprehensive elucidation of degradation processes can be achieved using just the GC-MS results, as only phenolic monomers and dimers are volatile enough to elute from GC columns. Pyrolysis–gas chromatography–mass spectrometry (Pyr-GC-MS) expands the application of GC-MS to phenolic oligomers by converting them to the gas phase through heating in an inert atmosphere. However, previous studies investigating compositional and structural changes in lignin mostly employed a single temperature pyrolytic step (Zhang et al., 2012) (Ohra-Aho et al., 2013). When characterizing lignin degradation products, this method cannot distinguish between the volatile phenolic monomers/dimers formed through microbial treatment from the pyrolysis products obtained as a result of thermal degradation of phenolic oligomers. A recent study by Brzonova et al. successfully characterized lignin degradation products by using fractional thermal desorption TD-Pyr-GC-MS with three temperature steps (350, 450 and 700 °C) to overcome this limitation (Brzonova et al., 2014).

The novelty of this study, besides using higher, industrially relevant lignin concentrations, is in the use of comprehensive analysis of lignin degradation products. First, while still using several traditional methods, e.g., measuring enzyme activities and monitoring lignin degradation gravimetrically, we expanded the application of fractional TD-Pyr-GC-MS to characterization of lignin biodegradation products. TCA and TD-Pyr-GC-MS complemented each other, as the former yielded a complete quantitative carbon evolution profile (including the elemental carbon and its precursors not accounted for by TD-Pyr-GC-MS), thus allowing for mass balance closure on carbon among all fractions, whereas the latter provided a qualitative resolution on the level of chemical species.

The use of comprehensive methods of product characterization allowed us to conduct a direct comparison of fungi, bacteria and purified enzymes (laccases) with respect to lignin degradation. The two fungal strains used, *Coriolus versicolor* (CV) and *Trametes gallica* (TG), are white rot fungi previously applied successfully to black liquor degradation (Hong et al., 2015). Commercial laccases from two similar sources, *Coriolus versicolor* (0.3 U>mg) (Lac I) and *Trametes versicolor* (10 U>mg) (Lac II), were used separately as a control group for a comparative study of microbial lignin degradation. Among the two bacterial strains used, *Streptomyces sp.* (S) was shown to degrade KL (Yang et al., 2012; Giroux et al., 1988) while *Microbacterium sp.* (B) was reported to degrade significantly both lignin and its intermediate biodegradation products, phenolics, during Kenaf biomass treatment (Brzonova et al., 2014; Zhang et al., 2013).

2 Materials and Methods

2.1 Microorganisms and lignin

All microbial strains used in this study were received from the Department of Biology of Lakehead University, Thunder Bay, Ontario, Canada. The cultures were maintained at 4 °C on agar plates mixed with kenaf provided by the Department of Plant Sciences, North Dakota State University (Fargo, ND, USA). Indulin AT, obtained through purification of the sodium salt of Kraft pine lignin (Rojas et al., 2007) was purchased from MeadWestvaco (Richmond, VA, USA). Elemental analysis of indulin AT was performed by Atlantic Microlab Inc. (C%: 64.14, H%: 5.79, N%: 0.46, S%: 1.39). Commercial laccases from *Coriolus versicolor* (0.3 U>mg) and *Trametes versicolor* (10 U>mg) were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

2.2 Inoculum preparation and cultivation

For inoculum preparation, both fungal and bacterial cultures were cultivated on agar plates supplemented with Kenaf at room temperature, a week prior to experiments. For laccase treatment, 0.30 g of laccase powder was dissolved in 5.0 mL of a buffer solution before experiments. The culture medium (pH 6.0) for microbial treatment contained KH₂PO₄ (1.5 g/L), MgSO₄ (0.5 g/L), NaCl (0.5 g/L), CuSO₄ (0.2 g/L), ZnSO₄ (0.2 g/L), sucrose (1.5 g/L) and FeSO₄ (0.1 g/L). The buffer solution (pH 6.0) for laccase treatments contained KH₂PO₄ (1.5 g/L), CuSO₄ (0.2 g/L), ZnSO₄ (0.1 g/L) and FeSO₄ (0.05 g/L).

Indulin AT (1.0 g) was autoclaved at 121 °C for 30 min along with 75 mL of the medium in Erlenmeyer flasks. After cooling down to room temperature, actively growing fungal colonies were selected and four 20 mm × 20 mm fragments from the growing edge of fungal cultures were used for inoculation under sterile conditions at room temperature. Bacterial seed cultures were incubated overnight and then 1mL of such a bacterial

suspension with an OD₆₀₀ of 1.0 was used for inoculation. For laccase treatments, a suspension containing 1.0 g of lignin in 75 mL of the buffer solution was sterilized as described above followed by the addition of 0.50 mL of two commercial laccase solutions.

To ensure the continuous treatment by active laccase through 54 days, 0.50 mL of laccase solutions were added under sterile conditions on the days of sample harvesting. As the laccase treatment served merely as a control for comparison with live cultures, no mediators were added to the solution to increase the enzyme productivity. The other control samples included microbial laccase blanks with just the corresponding media, without any inoculation or enzyme addition. The incubation with lignin continued for 54 days at 31 °C under shaking at 100rpm. Triplicate samples were harvested on days 8, 13, 22, 30, 38, 46, 54 and then the supernatants were separated by centrifugation (1300 rpm, 10 min) and microfiltration for further analysis. For gravimetric analysis and carbon mass balance calculation, flasks of different treatments remained untouched after inoculation until the end of experiment on day 54.

Microbial growth was not monitored because of lignin interference for bacteria and inherent system heterogeneity (thus requiring the digestion of the entire sample) for fungi. The growth was rather small in all cases, as neither the solid nor the liquid fraction eluted increasing amounts of protein markers, nitrogen containing heterocycles, when analyzed by TD-Pyr-GC.

2.3 Determination of the residual lignin content

After 54 days of incubation, the solid lignin portions from triplicate samples were separated from the solutions by vacuum filtration. The residual lignin was dried and weighed gravimetrically, as wt. % with respect to the initial lignin.

2.4 Measurement of ligninolytic enzyme activities

Selected ligninolytic enzyme activities were measured spectrophotometrically using a UV–Vis Evolution™ 600 spectrophotometer (Thermo Fisher Scientific, Madison, WI). The laccase activity was recorded by monitoring absorption changes at 436 nm reflecting the rate of oxidation of 1.0 mM 2,20-azino-bis-[3-ethyl- thiazoline-6-sulfonate] (ABTS) in 0.1M phosphate buffer (pH 6.0). The MnP activity was measured by monitoring the oxidation of DMP (2,6-dimethoxyphenol) at 469 nm. The LiP activity was determined by monitoring the oxidation of Azure B at 651 nm. Reference blanks contained all components except the enzyme. One unit of enzyme activity was defined as the amount of enzyme that transformed 1.0 μmol of substrate per minute.

2.5 Thermal carbon analysis

Samples were analyzed using a Sunset Laboratory Thermal-Optical Carbon aerosol analyzer. Supernatant samples (10 μL) were loaded directly onto a quartz-fiber filter with an area of 1.5 cm^2 and dried at 40 $^{\circ}\text{C}$ for 7 min for moisture removal. Solid samples were dried in an oven at 70 $^{\circ}\text{C}$ overnight and 5 mg of the dried sample were transferred to a separate tube adding 1mL of deionized water. The resulting solution was vortexed for 5 min and then 10 μL of a representative suspension sample were transferred to a quartz filter. Samples were subjected to a stepwise temperature ramp including 200, 300, 400, 500 $^{\circ}\text{C}$ and to a maximum of 850 $^{\circ}\text{C}$ for 6 min at each step under a flow of ultrapure (GC-grade) helium (15 cm^3/min). The carbon evolving from the filter was converted to CO_2 in an oxidizing oven by a MnO_2 catalyst and then reduced to methane to be detected with a flame ionization detector (FID). After the ramp, the temperature was dropped down to 550 $^{\circ}\text{C}$ and oxygen (15 cm^3/min) was flown into the sample with a final temperature being increased to 870 $^{\circ}\text{C}$, so the remaining char precursors (not evolving by evaporation at any reasonable

temperature) were oxidized to CO₂, which then evolved and was detected as methane as described above.

2.6 TD-Pyr-GC-MS

Supernatants from the samples withdrawn from both the experimental runs and controls at the end of incubation, on day 54, were analyzed using a thermal desorption–pyrolysis unit. The CDS Analytical, Inc. (Oxford, PA, USA) 5000 series pyroprobe was run in a trap mode with Tenax-TA^{TM} trap sorbent connecting to an Agilent 7890 GC equipped with an Agilent 5890C mass spectrometer as a detector. A 51-m long HP-5MS column used had a film thickness of 0.25 µm and an inner diameter of 0.25 mm. Prior to analysis by the pyroprobe, quartz tubes filled with quartz wool were cleaned at 1200 °C for 5 s and cooled down before the samples were introduced. After the quartz tube loaded with samples (5 µL) was inserted into the pyroprobe, it was dried at 50 °C for 60 s with a subsequent heating at 10 °C/s to the allotted temperature where it was held isothermally for 30 s. The transfer line and valve oven temperatures were set to 300 and 320 °C, respectively. The trap temperature was 45 °C before heating to 300 °C for 2.5 min, while the pyroprobe was held at 300 °C for 2.5 min. Each sample was analyzed with sequential fractional heating at 150, 220, 320, 400 and 700 °C. The temperature ramp was set up to enable a complete separation of thermal desorption and pyrolytic products. Based on preliminary experiments, the 320 °C step served as a transition between the thermal desorption and pyrolysis, with a consistent minimum appearance of peaks compared to all other temperature steps.

3 Results and Discussion

3.1 Ligninolytic enzyme activities

The profiles of ligninolytic enzyme activity are shown in Fig. 1 for all four strains used. Ligninolytic enzyme activities exhibited a sharp variation over the course of the 54 day long experiment. Expression of lignin modifying enzymes during lignin degradation was reported to depend on both the microbial growth phase and occurrence of secondary metabolism, so subtle changes in the balance between the actively growing and dying biomass could lead to a variation in the extent of extracellular enzyme activities over time (Winqvist et al., 2008). The laccase activities of both fungal strains (CV and TG) turned out to be significantly higher than those of bacteria, reaching the maxima of $(797 \pm 17 \text{ U/L})$ and $(1154 \pm 172 \text{ U/L})$, respectively. However, when paper mill sludge was treated by the same fungal strains in a different study, laccase activity did not exhibit a significant increase ($< 50 \text{ U/L}$) in 25 days (Hong et al., 2015). Predominant fungal laccase activity could be closely associated with lignin polymerization (Nutsuibidze et al., 1998), which was confirmed later in this study through the results obtained from TCA and TD-Pyr-GC-MS.

The *Streptomyces* bacterial strain did not exhibit a noticeable laccase activity in contrast to the earlier study conducted by Yang et al. where other *Streptomyces* strains showed a significant laccase activity ($935.4 \text{ U/g dry cell weight}$) during the degradation of alkali lignin (Yang et al., 2012). Although most of the reported bacterial laccases are expressed intracellularly, some strains of *Streptomyces* sp. were shown to produce extracellular laccases (Sharma, 2007). However, extracellular laccase activity is known to vary significantly depending on solution pH and composition as a result of disruption of electrostatic interactions and hydrogen bonds within the protein tertiary structure (Margot et al., 2013). The lack of significant laccase activity may explain the difference between the outcomes of fungal and bacterial treatments described throughout this study, in terms of the occurrence of cross-linking polymerization.

All four strains investigated showed a pronounced MnP activity throughout all 54 days of cultivation. CV exhibited the highest MnP activity reaching 1455 ± 101 U/L on day 22 while TG had the smallest activity, 937 ± 375 U/L. Compared to fungi, bacteria showed a significant increase of MnP activity toward the second half of the degradation period reaching 304 ± 189 U/L and 470 ± 134 U/L on day 38 and day 46 for B and S, respectively. No LiP activity was detected among all four strains investigated. A similar pattern, with abundant laccase and MnP activities yet low or no LiP activity, was reported among many white rot fungi when treating paper mill sludge and lignocellulosic biomass (Hong et al., 2015).

On the other hand, a significant LiP activity was reported for many bacterial *Streptomyces* strains (Adhi et al., 1989). Camarero et al. claimed that there was no direct correlation between the observed enzyme activity and extent of lignin decomposition (Camarero et al., 1996). The differences observed between this study and previous reports indicate that lignin biodegradation might involve enzymes other than those enzymes we investigated.

3.2 Weight loss of lignin

All microbial treatments after 54 days showed significant differences compared to controls (Table 2, Column a). The fungal treatment, especially with *C. versicolor*, yielded a greater mass decline than bacterial treatments. The greatest lignin weight loss was observed for the *C. versicolor* treatment reaching 45 ± 8 % after 54 days. Taking into account the lignin mass reduction in the control, the lignin removal due to fungal biodegradation was 25 ± 15 %. By contrast, bacterial and laccase treatments led to only a slight decrease in the weight of the treated lignin, if any.

Higher lignin degradation efficiencies by *C. versicolor* (49%) and *Streptomyces* sp. (38%) were previously observed for paper mill effluents and alkali lignin within two weeks of incubation (Wu et al., 2005; Yang et al., 2012). Among the earlier studies on KL microbial treatment, the extent of lignin degradation was most often measured spectrophotometrically. The Kraft lignin concentrations used in previous reports were significantly lower than those applied in this study, thus spectrophotometry was more suitable to assess the lignin loss than the gravimetric method. However, measuring the characteristic lignin band at 280 nm can be skewed due to the interference imposed by other compounds absorbing at a similar wavelength, e.g., degraded carbohydrates and phenolics (Hatfield and Fukushima, 2005; Gould, 1982). Thus, uncertainty remains on whether the earlier published data reflected the true lignin degradation. For these reasons, the gravimetric method was selected for the use in this study, as higher lignin concentrations tend to increase its accuracy. Gravimetric analysis has been used successfully to assess an industrial lignin biodegradation by a fungus, *Pycnoporus cinnabarinus*. After 90 days, 74% degradation was reported using a combination of alkaline lignin (520 mg/30 mL) and wood pulp (2 g) as a substrate (Geng and Li, 2002).

Yet, it should be noted that even correctly conducted gravimetric measurements do not distinguish either between lignin biodegradation and dissolution or between lignin depolymerization to lower molecular fragments and their ultimate catabolism to CO₂ and water. In this study, the mode of lignin biotreatment was investigated using other methods, primarily TCA as shown in the subsequent sections.

3.3 Investigation of carbon distribution and mass balance closure

3.3.1 The solid product portion

Figure 2 shows the thermal carbon elution profile of the solid (water-insoluble) lignin portion after 54 days of treatment. The carbon elution profiles were similar for all solid fractions indicating that all (or most) of the changes resulting from treatments occurred in the liquid phase: Once lignin was modified by the treatment, it (or the products of its degradation) dissolved.

A comparison of the *total* carbon in experimental samples and the corresponding controls showed relatively small but statistically significant changes, particularly, for the CV treatment. Furthermore, a decline in the non-volatilizable char precursors was mainly accounted for by the total carbon decrease as a result of both microbial and laccase treatments. The carbon wt.% representing the solid portion of lignin matches that obtained from the gravimetric analysis, Table 2. The match obtained between the gravimetric measurements and total carbon in the TCA validated both of these methods.

These observations indicated significant modification of lignin samples compared to the control, including the partial dissolution and apparent biodegradation of the most recalcitrant lignin fraction (char precursors). Further insights into the mechanisms of bacterial and fungal action were obtained by analyzing the water soluble, supernatant portion of the reaction products.

3.3.2 The supernatant (water-soluble) portion

3.3.2.1 Laccases

The total amount of carbon present in the supernatants of control samples did not show any significant changes over 54 days. The elution profiles of laccase treated samples were similar to the control, with increases occurring only in the 850 °C and char precursor fractions (Figure 3-a and b). Apparently, laccase produces some water-soluble higher-MW

and mostly cross-linked products thus promoting polymerization rather than depolymerization. The total lignin weight loss observed at the end of experiments may be due to two different factors: 1) Conversion to dissolved or degraded lignin compounds in the supernatant and 2) Conversion to carbon dioxide through microbial metabolism of lignin. In the case of laccase treatments, an increase in the total carbon present in the supernatant was observed along with a corresponding decrease in the solid portion measured by TCA (Table 2, columns b and c), which, in turn, matched the decrease in carbon wt.% loss measured gravimetrically as shown in Table 2, column a. Lignin mineralization is expected to be insignificant for laccase treatments, just as observed. The laccase treatment featured a suitable carbon mass balance closure (Table 2, column d).

3.3.2.2 Fungi

For samples after fungal treatments, carbon wt.% loss in the solid portion measured on day 54 by TCA matched the value obtained from the gravimetric analysis. However, this observed decrease in carbon wt.% was not accompanied by a corresponding increase of carbon wt.% in the supernatant on day 54. Instead, there was a significant drop in carbon wt.% in the supernatants of the samples treated by fungi (Table 2, column c). In contrast to laccase, the thermal carbon elution profiles of the fungally treated lignin changed significantly over 54 days showing a transient decrease of the total carbon present in supernatants (Fig. 3-c and d). The carbon mass balance closed only at $72\pm 8\%$ and $88\pm 14\%$ of the original carbon input (Table 2, column d). This suggests that a portion of lignin is metabolized into volatile products, presumably, CO_2 , at least by CV, as the corresponding gap in the carbon mass balance is statistically significant (Table 2). A similar level of lignin

mineralization to CO₂ was reported when residual lignin in Kraft pulp was treated by *T. versicolor* (Reid, 1998).

A significant amount of carbon released at 200 °C in the microbial control is due to sucrose added to the medium and release of low molecular phenolics during the lignin sterilization. Compared to this control, a significant decrease of the char precursor fraction was observed after the first 8 days of the fungal treatment evidencing lignin breakdown. Surprisingly, the consumption of low molecular weight chemicals occurred rather slowly, until day 22-30, followed by an across-the-board consumption of any nutrients in the supernatant. Finally, toward the end of the 54-day incubation period, an increase occurred in all fractions apparently signifying the dissolution of solid lignin. Delignification by white rot fungi showed similar results in an earlier study, with high molecular weight lignin degrading to yield intermediate molecular weight species while consuming the low molecular weight fraction (Gadd, 2001).

3.3.2.3 Bacteria

Compared to the fungal treatments, the application of ligninolytic bacteria led to a slight yet statistically significant increase of carbon wt.% in the supernatant on day 54 (Table 2, column c). An increase of the total carbon content at the end of experiment indicates that carbon solubilization may not necessarily lead to its mineralization as has been noted previously, especially for actinomycetes (Bandounas et al., 2011). Bacterial solubilization of KL has been previously reported, especially with *Streptomyces* strains (Giroux et al., 1988).

The carbon mass balance closed at 102±9% and 97±11% for B and S, while the carbon wt.% in the solid portion matched that obtained by gravimetric analysis. Unlike the

fungus treatments, only a negligible carbon loss occurred, if any, but 33-36% of the lignin carbon moved into the supernatant. The results indicate that bacteria may employ a different way of metabolizing lignin for growth to produce more water-soluble products of its biodegradation without significant carbon mineralization. This corroborates the previously reported findings where white rot fungi were mostly reported to mineralize lignin whereas bacteria merely caused structural alterations (Thevenot et al., 2010).

The dynamics of changes reflected in the thermal carbon elution profiles as a result of bacterial treatment is shown in Fig. 3-e and f. Unlike the fungal treatment, the total carbon amount remained relatively stable and similar to the control. However, the proportions among the thermally evolving fractions showed significant changes occurring during this process. The observed transient drop in the middle of the incubation period apparently reflects the dissolution of solids followed by the digestion of the resulting dissolved nutrients, low molecular weight fragments of lignin.

Just as for the fungal treatment, the low-MW phenolics evolving at 200 °C and 300 °C exhibited a regular oscillation pattern: They were consumed in the beginning along with the reduction of the total dissolved carbon and then produced again. In the case of bacteria, the changes in 200 °C and 300 °C fractions were less pronounced and accompanied by different changes in the thermal carbon elution profile. First, the low-MW phenolics did not appear at the end of the fungal incubation as they did for bacteria. Also, the char precursor fraction remained to be significant throughout the entire period of bacterial incubation, unlike the fungal treatment.

3.4 TD-pyrolysis-gas chromatography/mass spectrometry analysis

3.4.1 Phenolics elution profile

To obtain a more comprehensive characterization of the lignin treatment products, the percentage of eluting phenolics was estimated in all five temperature fractions based on the normalized integrated area after microbial and laccase treatments, as shown in Fig. 4.

The profiles of phenolics' elution during thermal desorption and pyrolysis after fungal, bacterial and laccase treatments are shown in Fig. 4-a. Given that the 320 °C temperature fraction elutes only traces of chemicals, it marks the boundary between the thermally desorbing and pyrolytic species. To capitalize on this observation, Fig. 4-b depicts the sums of thermally desorbing (150-320 °C) and pyrolytic (400-700 °C) products.

3.4.1.1 Laccases

Compared to the control, the phenolics' elution shifted from thermal desorption to pyrolysis indicating the polymerization imposed by laccase. With decreases in the 150 °C and 220 °C fractions, phenolics eluting at pyrolytic temperatures, especially the 400 °C fraction, showed a significant increase, as can be seen from Figure 4-a. Corroborating this conclusion, a substantial increase in the mean molecular weight was observed when lignin was treated with a purified laccase (Gouveia et al., 2013).

3.4.1.2 Fungi

The phenolic peak distribution profile after the fungal lignin treatments (Fig. 4-a) appeared similar to that after the laccase treatments, featuring the disappearance of monomer phenolics eluting at 150 °C along with an increase of pyrolytic fractions. This shift in phenolics' elution suggests that after 54 days of fungal growth, lignin fragments of high molecular weight were further polymerized whereas the low molecular weight fraction was utilized for fungal growth. The high laccase activity detected during the fungal treatments in this study corroborated this observation. Lara et al. also reported that high

molecular weight lignin was further polymerized with a concomitant release of low molecular weight compounds during the treatment of black liquor by white rot fungi, *Trametes elegans* (Lara et al., 2003). Several other white rot fungi were shown to further polymerize various kinds of industrial lignin (Sain et al., 2015).

Although the char precursors do not evolve any Pyr-GC-MS detectable organic chemicals, the TCA data considered in the previous section showed that fungi can digest even this presumably cross-linked fraction resulting in an across-the-board decrease of the supernatant carbon content (among all TCA fractions). Thus, we may conclude that a balance of two opposite trends may occur throughout fungal treatments, between the loosening of cross-linked structure of lignin and re-polymerization of smaller fragments. This balance may be skewed toward one of these trends within certain treatment phases, depending on the specific environmental conditions, e.g., nutrient source and abundance.

3.4.1.3 Bacteria

Compared to fungal treatments, those conducted with bacteria featured a rather different elution profile, with significantly more phenolics eluting in the low-temperature thermal desorption fractions (Fig. 4-b). Meanwhile, the fraction of phenolics eluting at 700 °C decreased concomitantly (Fig. 4-a), thus indicating once again that bacteria use a different mechanism of metabolizing lignin as a carbon source. Combining this observation with the TCA results shown in Fig. 3 suggests that bacteria not only induced the dissolution and degradation of lignin into smaller-MW fragments but also reduced the char precursor fraction. Thus, some of the observed low-MW phenolics could be produced as a result of loosening the cross-linked lignin matrix.

3.4.2 Compositional changes of treated lignin

All phenolics observed upon thermal desorption at 150 °C and 220 °C using TD-Pyr-GC-MS after different lignin treatments are listed in Table 3. To further elucidate the compositional changes occurring in lignin as a result of microbial and enzymatic treatments, the phenolics' elution across *all* temperature steps (including the pyrolytic fractions) were classified into 6 groups of compounds sharing same base structure as shown in Figure 5. The origin of the lignin feedstock used (pine as softwood) explains the ubiquitous abundance of guaiacol based compounds among all temperature fractions (Figure 5-a). Among its derivatives, guaiacol carbonyls and guaiacol acids were the major products evolving at lower temperature steps, as they are thermally unstable. Conversely, phenol-based compounds, benzenediol-based compounds and aromatic hydrocarbons were pyrolytic products evolving mostly at high temperatures (Brebou and Vasile, 2010; Zhao et al., 2014).

3.4.2.1 Laccases

Laccase treatments led to a decrease in the elution of guaiacols and other lignin base unit compounds at 150 °C along with an increase of their evolution at 400 °C, compared to the control (Figure 5-a). The occurrence of phenolic peaks shifted toward higher temperatures and was more pronounced after the Lac I treatment having a lower enzyme activity compared to Lac II (Table 3). Corroborating these observations, an *in vitro* laccase treatment of Kraft lignin was shown to facilitate mostly the polymerization reactions rather than depolymerization (Gouveia et al., 2013).

Furthermore, guaiacol carbonyls showed up in the high temperature fractions after the laccase treatments (Figure 5-c). Given that these chemicals are thermally unstable, they must be abundant to 'survive' pyrolysis. This observation indicates the formation of

polymers as a result of laccase treatment, which are more oxidized than the original lignin, so these polymers are comprised of guaiacol carbonyl derivatives rather than guaiacol itself. Corroborating this observation, oxidation, demethylation and hydroxylation of Kraft lignin model compounds were shown to occur when treated by laccase from *T. versicolor* (Crestini and Argyropoulos, 1998).

The amounts of pyrolytic products, benzenediol-based compounds (for Lac I), phenol-based compounds and aromatic hydrocarbons for Lac II with a greater enzyme activity also increased in high temperature fractions, which is consistent with the formation of more cross-linked polymers than the original lignin. These features corroborate the conclusion made throughout this paper that the prevalent mode of laccase action is an oxidative polymerization by cross-linking.

3.4.2.2 Fungi

The most significant compositional change in lignin after *fungi* treatments was an increase in phenol-based compounds and aromatic hydrocarbons (Figure 5-d and 5-f, respectively) along with a corresponding decrease in guaiacols and other lignin base unit compounds eluting at 700 °C (Figure 5-a). As we have discussed earlier, pyrolytic phenolics eluting at 400 °C and 700 °C showed a significant increase after the fungal treatments at the expense of monomers eluting at lower temperatures. Therefore, fungi consume low molecular weight phenolics for growth while converting the remaining lignin fragments present in the supernatant into more condensed, recalcitrant structures. This conclusion is also evidenced by the fact that thermally desorbed phenolics originally present in the control (presumably, due to the sample heating during sterilization) disappeared after the treatment and only a few new compounds were identified (Table 3).

The fungal growth thus depends on these intermediates' consumption and mineralization, corroborating the low carbon content of the supernatants on day 54 discussed in the TCA section. A similar observation, i.e., that the majority of aromatic peaks originally present in the medium dissappeared after a fungal treatment of black liquor with *Trametes* sp., was reported earlier (Selvam and Arungandhi, 2013).

3.4.2.3 Bacteria

Unlike lignin after a fungal treatment, a decrease in G elution after the *bacterial* treatments did not lead to an increase in the amount of phenol-based compounds and aromatic hydrocarbons evolving at 700 °C (Figure 5-d and 5-f). Instead, an increase in benzenediol-based compounds eluting at 700 °C was observed (Figure 5-e). However, the increase of the benzenediol-based fraction was less pronounced for bacteria compared to the increase of phenol-based compounds after the fungal treatment. These observations echo a similar difference between a more active Lac II compared to Lac I and corroborate the observed greater production of oxidative enzymes by fungi than by bacteria. A relatively small change in the elution of guaiacols and other lignin base unit compounds compared to the fungal treatments should be considered in combination with the guaiacol acids' elution at low temperatures characteristic only for bacterial samples and observed in stark contrast with the control (Figure 5-a and 5-b). Apparently, bacteria specifically promote guaiacol oxidation.

The other specific feature of bacterial treatments was a significant shift in elution of phenol-based compounds and aromatic hydrocarbons at 700 °C towards lower temperature fractions, which suggests a different structural modification imposed on lignin by bacteria

that involves loosening of condensed structure and depolymerization. Finally, bacterial supernatants continued to elute phenolics at lower temperatures, unlike both fungal and enzyme-treated samples, as shown in Table 3. Results obtained by TCA and TD-Pyr-GC-MS suggested that bacterial treatments led to loosening of the condensed lignin structure and production of low molecular weight phenolic compounds. Corroborating this conclusion, Brown and Chang showed that bacterial treatments yielded small molecular weight aromatic compounds (Brown and Chang, 2014).

However, unlike fungi, bacteria in this study showed no statistically significant lignin mineralization. A similar observation, that bacterial mineralization of lignin was less pronounced compared to that by white rot fungi, was made earlier (Lotfi, 2014). Thus, bacteria appear to employ a different approach in using lignin as a carbon source, with a lesser extent of mineralization compared to white rot fungi (Bandounas et al., 2011) but also with a less pronounced polymerization as shown throughout this study.

Maximum accumulation of low molecular weight lignin products up to 10.5 wt.% (obtained by the TCA analysis) was observed after 54 days of bacterial treatments with only minor, statistically insignificant lignin mineralization. *Streptomyces sp.* thus showed a great potential for the production of lignin-based valuable chemicals from Kraft lignin. By contrast, fungi appear to be more suitable for lignin degradation rather than valorization, although, perhaps, fungal treatment times could be shortened to harvest lignin degradation products before extensive mineralization.

4 Conclusion

Gravimetric measurements may be combined with TCA for an accurate assessment of lignin biodegradation distinguishing it from solubilization and providing both the carbon mass balance and fraction analysis by volatilization. This study showed that fungi

mineralized Kraft lignin to a significant extent whereas bacteria partially degraded and modified lignin. The extent of repolymerization is more pronounced for laccase and fungi as evidenced by a greater char precursor fraction in TCA and pyrolytic fraction in TD-Pyr-GC-MS. Yet, fungi appear to be able to break down even the highly cross-linked lignin fraction, with a delicate balance between the cross-linking polymerization and degradation.

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Figure Caption

Figure 1. Title: Ligninolytic enzyme activity profiles over 54 days

a) Laccase activities of two white rot fungi (CV and TG) and two bacterial strains (B and S) in indulin AT media b) Manganese peroxidase of two white rot fungi (CV and TG) and two bacterial strains (B and S) in indulin AT media. The error bars show the variance of the mean value with one standard deviation obtained from triplicate samples.

Figure 2. Title: Thermal carbon elution profile of the solid (water-insoluble) lignin portion after 54 days of treatment

The error bars show the variance of the mean value with one standard deviation obtained from triplicate samples. See section 2.5 for definitions of temperature fractions.

Figure 3. Title: The thermal carbon elution profiles of the lignin samples in the supernatant with different treatments

a) Laccase (0.3 U>mg), b) Laccase (10 U>mg), c) *Coriolus versicolor*, d) *Trametes gallica*, e) *Microbacterium sp.*, f) *Streptomyces sp.* The error bars show the variance of the mean value with one standard deviation obtained from triplicate samples. See section 2.5 for definitions of temperature fractions.

Figure 4. Title: Phenolics' elution profile of lignin samples in supernatants after 54 days of treatment

a) Distribution of phenolics elution across all temperatures steps in the supernatants.

b) Distribution of phenolics' elution during thermal desorption (150-320 °C fractions) and pyrolysis (400-700 °C) recovered in the supernatants after different lignin treatments and determined using TD-Pyr-GC-MS.

Figure 5. Title: Relative distribution of 6 groups of compounds across five temperature steps after microbial and laccase treatment

Relative distribution of a) Guaiacols and other lignin base unit compounds, b) Guaiacol acids, c) Guaiacol carbonyls, d) Phenol-based compounds, e) Benzenediol-based

compounds, f) Aromatic hydrocarbons. See Supplementary Materials for the full list of compounds.

Tables

Table 1 Summary of recent publications on microbial industrial lignin degradation

Microbial strain	Nutrient	Lignin load	Degradation time	Degradation %	Analysis	Reference
Bacteria						
<i>Pandoraea sp. B-6</i>	+	3g/L	7	46.5%	GC-MS	(Shi et al., 2013)
<i>Novosphingobium sp. B-7</i>	–	3g/L	7	30%	GC-MS	(Chen et al., 2012)
<i>Bacillus sp. Strains CS-1 & CS-2</i>	+	0.05g/L	2	61%	-	(Chang et al., 2014)
<i>Citrobacter freundii (FJ581026)</i>	+	600ppm(w/v)	6	49%	GC-MS HPLC	(Chandra and Bharagava, 2013)
<i>Citrobacter sp. (FJ581023)</i>				54%		
<i>Mixed culture</i>				62%		
<i>Pandoraea sp</i>	+	5g/L	5	39.9%	GPC GC-MS	(Zheng et al., 2013)
<i>Streptomyces spp. strains F-6</i>	+		12	37.6%	GC-MS	(Yang et al., 2012)
<i>Streptomyces spp. strains F-7</i>				38.1%		
<i>Pleurotus ostreatus strain G5</i>				6%		
<i>Bacillus sp.</i>	-	1g/L	6	81.4%	-	(Abd-Elsalam and El-Hanafy, 2009)
<i>Aspergillus sp. strain F-3</i>	+	2g/L	8	65%	GC-MS	(Yang et al., 2011)
Fungi						
<i>P. sulphureus</i>	+	0.5%	10	36%	-	(Arun and Eyini, 2011)
<i>Phellinus sp.</i>				36.52%		
<i>Phellinus sp. – B. pumilus</i>				36.65%		
<i>P. sulphureus – B. pumilus</i>	+	1g/L	30	83.5%	-	(Rodriguez et al., 1994)
<i>P. chrysogenum</i>	+	2g/L	10	35%	-	(Jin, 2012)
<i>Cladosporium Bio-1</i>	+					

<i>Fusarium proliferatum</i>	+	5g/L	30	19.6%	-	(Regalado et al., 1997)

Table 2. Carbon weight % changes in the supernatant after 54 days of treatment.

	Carbon wt.% remaining in solid (GA) ^a	Carbon wt.% remained in the solid (TCA) ^b	Carbon wt.% in the supernatant ^c	Total carbon wt.% ^d
<i>C. versicolor</i>	55±8%	54±11%	18±2%	72±8%
<i>T. gallica</i>	71±4%	72±11%	16±1%	88±14%
<i>Mycobacterium sp.</i>	73±9%	69±7%	33±1%	102±9%
<i>Streptomyces sp.</i>	78±12%	61±12%	36±2%	97±11%
Microbial control	75±17%	71±13%	29±5%	100±15%
Laccase I	77±19%	72±17%	34±1%	106±17%
Laccase II	72±20%	63±12%	35±1%	98±12%
Laccase control	79±12%	73±4%	23±1%	96±17%

a Carbon wt.% in the solid based on gravimetric analysis (GA)

b Carbon wt.% in the solid sample on day 54 measured by TCA

c Carbon wt.% in the supernatant sample on day 54 measured by TCA

d Sum of the values for the solid portion and supernatant, reflecting mass balance closure

Table 3. Products of lignin degradation after 54 days of microbial and laccase treatment evolving at 150 °C and 220 °C. The chemicals highlighted in grey were not present in the control samples and appeared after treatment.

150 °C						220 °C						m/z ^a	Identified compounds
Ret Time	control	CV	TG	S	B	control	CV	TG	S	B			
6.656	+	-	-	-	+	+	+	+	+	+	109, 124, 81	Guaiacol	
7.277	-	-	-	-	-	-	-	-	+	-	138,123,95	Methyl guaiacol	
7.979	-	-	-	+	+	+	-	-	+	+	150,135,107	Vinyl guaiacol	
8.282	-	-	-	+	-	-	-	-	-	-	121,122,93,65	Hydroxy-benzaldehyde	
8.406	-	-	-	-	-	-	-	-	-	+	140, 125, 97	Methoxy-hydroquinone	
8.478	+	-	-	+	+	+	+	-	+	-	151,152,81,109	Vanillin	
8.707	-	-	-	-	-	-	-	-	+	+	164, 149, 77, 91	Isoeugenol	
8.768	-	-	-	-	-	-	-	-	+	+	137, 166, 122	Propyl guaiacol	
8.922	+	-	-	+	+	+	-	-	+	+	151,166,123	Apocynin	
9.132	-	-	-	-	-	-	-	-	-	+	137, 180, 122	Isopropoxy- methoxy-phenylacetone	
9.161	-	-	-	+	+	-	-	-	+	+	152, 137, 123	Homovanillyl alcohol	
9.266	-	-	-	-	+	-	-	-	-	+	137, 138, 123, 182	Vanillyl ethyl ether	
9.286	-	-	-	+	+	-	-	-	+	+	125, 153, 168	Vanillic acid	
9.407	-	-	-	+	-	-	-	-	-	+	151, 182,123	Dihydroxy-methoxy-acetophenone	
9.691	-	-	-	+	+	-	-	-	+	+	137, 182, 138, 122	Homovanillic acid	
9.905	-	-	-	+	-	-	-	-	+	-	137, 210, 164	Hydroxy-methoxy-Phenylacetyl formic acid	
10.083	-	-	-	-	-	-	-	-	+	+	137, 196, 122	Homovanillic methyl ester	
10.111	-	-	-	-	-	-	-	-	+	+	178, 135, 77, 107	Hydroxy-methoxy-cinnamaldehyde	
Ret Time	control	Lac I	Lac II										
5.881	-	-	-	-	+	+	94, 66, 65	Phenol					
6.643	-	-	-	+	+	+	109, 124, 81	Guaiacol					
7.264	-	-	-	+	+	+	123, 138, 95	Methyl guaiacol					
7.761	-	-	-	-	+	-	137, 152, 122	Ethyl guaiacol					
7.968	+	-	-	+	+	+	150, 135, 77, 107	Vinyl guaiacol					
8.202	-	-	-	+	+	-	164, 149, 77, 91	Eugenol					
8.386	-	-	-	-	+	-	140, 125, 97	Methoxy benzenediol					
8.466	+	-	-	+	+	+	151, 152, 81, 109	Vanillin					
8.696	-	-	-	+	+	-	164, 149, 77, 103	Isoeugenol					
8.762	-	-	-	+	+	-	137, 166, 122	Propyl guaiacol					
8.91	+	-	-	-	+	+	151, 166, 123	Apocynin					
9.044	-	-	-	-	-	+	151, 182, 123	Methylvanillate					
9.121	-	-	-	+	+	-	137, 168,122	Homovanillyl alcohol					
9.396	-	-	-	-	+	-	105, 77, 51	Acetophenone					

+Present

- Absent

a Mass to charge ratio

Figure 1-a

Figure 1-b

Figure 2

Figure 3-a

Figure 3-b

Figure 3-c

Figure 3-d

Figure 3-e

Figure 3-f

Figure 4-a

Figure 4-b

Figure 5-a

Figure 5-b

Figure 5-c

Figure 5-d

Figure 5-e

Figure 5-f