Direct analysis by Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) reveals action of bacterial laccase-mediator system on both hardwood and softwood samples

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The modification and degradation of lignin play a vital role in carbon cycling as well as production of biofuels and bioproducts. The possibility of using bacterial laccases for the oxidation of lignin offers a route to utilize existing industrial protein expression techniques. However, bacterial laccases are most frequently studied on small model compounds that do not capture the complexity of lignocellulosic materials. This work studied the action of laccases from Bacillus subtilis and Salmonella typhimurium (EC 1.10.3.2) on ground wood samples from yellow birch (Betula alleghaniensis) and red spruce (Picea rubens). The ability of bacterial laccases to modify wood can be facilitated by small molecule mediators. Herein, ABTS, gallic acid and sinapic acid mediators were tested. Direct analysis of the wood samples was achieved by Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS), a surface sensitive mass spectrometry technique that has characteristic peaks for H, G and S lignin. The action of the bacterial laccases on both wood samples was demonstrated and revealed a strong mediator influence. The ABTS mediator led to delignification, evident in an overall increase of polysaccharide peaks in the residual solid, along with equal loss of G and S lignin peaks. The gallic acid-mediator demonstrated minimal laccase activity. Meanwhile, the sinapic acid mediator altered the S/G peak ratio consistent with mediator attaching to the wood solids.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ppl.12688

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The current investigation demonstrates the action of bacterial laccase-mediator systems directly on woody materials, and the potential of using ToF-SIMS to uncover the fundamental and applied role of bacterial enzymes in lignocellulose conversion.

Abbreviations — ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); L, lignin; MCO, multicopper oxidase; PS, polysaccharides; ToF-SIMS, Time-of-Flight Secondary Ion Mass Spectrometry.

Introduction

Lignin is an aromatic heteropolymer that forms through oxidative coupling of three main monolignols, namely para-coumaryl, coniferyl, and sinapyl alcohols (Fig. 1) (Boerjan et al. 2003, Mottiar et al. 2016). Syringyl-type (S) lignin, comprised of coniferyl and sinapyl alcohols, is dominant in deciduous hardwoods (angiosperms), guaiacyl-type (G) lignin comprised mainly of coniferyl alcohols is dominant in coniferous softwoods (gymnosperms), whereas p-coumaryl-type (H) lignin appears most commonly in grasses. Lignin decomposition is key to carbon cycling on earth and is also central to the broader use of renewable biomass (lignocellulose) as feedstocks for production of biofuels, as well as higher value bio-based chemicals and materials (Ragauskas et al. 2014, Li et al. 2016).

The white-rot fungi of the Basidiomycota phylum are especially potent microbial degraders of lignin, which is largely mediated through the action of secreted lignin-active peroxidases (e.g., lignin peroxidases, manganese peroxidases, and versatile peroxidases) characterized by comparatively high redox potential (Hammel and Cullen 2008, Lundell et al. 2010, Pollegioni et al. 2015). Significant achievements relating to the discovery and characterization of these enzymes are advancing their use in industrial bioprocesses (Martinez et al. 2017). However, broad application of fungal-derived lignin-active enzymes has been hampered by challenges to produce such enzymes in the existing industrial host systems (Lambertz et al. 2016).

Bacteria are also thought to contribute to lignin decomposition, mainly through degradation of low molecular weight lignin-derived aromatics by encoded laccases, as well as β-etherases and dye-decolorizing peroxidases (Bugg et al. 2011, Brown and Chang 2014, Bugg and Rahmanpour 2015, Kamimura et al. 2017). Laccases (EC 1.10.3.2; also belonging to the family 1 auxiliary activity (AA1) of the carbohydrate active-enzyme database (Levasseur et al. 2013)) are multicopper oxidases (MCOs) that mediate one-electron oxidation of a broad range of phenolic compounds with concomitant reduction of molecular oxygen to water (Pollegioni et al. 2015). Laccase use of molecular oxygen as a terminal electron acceptor...
is appealing from an application perspective (Cannatelli and Ragauskas 2017); several natural and synthetic mediators of laccase activity have also been identified and reviewed (Cañas and Camarero 2010, Christopher et al. 2014). Whereas most characterized laccases originate from fungi (Baldrian 2006, Lundell et al. 2010, Cannatelli and Ragauskas 2017), genomic and metagenomic analyses have shown that MCOs with laccase activity are also widespread amongst bacteria (Ferrer et al. 2010, Martins et al. 2015). Bacterial laccases, also referred to as laccase-like multicopper oxidases (LMCOs) or small laccases (sLAC), can be readily expressed in recombinant host systems, including *Escherichia coli* (Durão et al. 2008, Ihssen et al. 2015), and have been tested using a broad range of low molecular weight model compounds, and in some cases, isolated lignin fractions (Reiss et al. 2013, Majumdar et al. 2014, Strachan et al. 2014). A recent study also reported bacterial laccase action on lignin in pretreated wood fibre (Sing et al. 2017), confirming the potential of bacterial laccases to catalyze the delignification and lignin depolymerization directly in lignocellulosic biomass (Munk et al. 2015, Singh et al. 2017).

The applied potential of bacterial laccases (and other potential lignin-active enzymes) will benefit from parallelizable assays with ability to (1) test enzyme action directly on diverse lignocellulose preparations, (2) evaluate impact of different enzyme mediators, and (3) distinguish enzyme action leading to delignification versus lignin depolymerization in biomass samples. Such analysis methods would also deepen our understanding of the role such enzymes may play in lignin degradation in nature. Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) is a surface-sensitive mass spectrometry technique that allows for the direct analysis of lignocellulose, permitting advanced enzyme studies beyond the use of model compounds as substrates.

The lignin-characteristic peaks in ToF-SIMS mass spectra have been well established by Saito et al. (2005) for H-lignin (m/z 107 and 121), G-lignin (m/z 137 and 151) and S-lignin (m/z 167 and 181). These lignin-specific peaks have led to several ToF-SIMS chemical imaging studies, wherein differential distributions of H- and G-lignin in spruce compression wood (Tokareva et al. 2007), of S- and G-lignin across cell walls of poplar (Zhou et al. 2011), and of different S/G ratios in early- and late-wood in maple (Saito et al. 2012) were visualized. While ToF-SIMS distinguishes lignin types with unique peaks based on their differing number of methoxy side groups, the assignment of characteristic ToF-SIMS ions to cellulose and hemicellulose has remained more elusive due to the production of many similar fragment peaks by these polysaccharides (Goacher et al. 2011). Furthermore, the peaks that are produced by purified polysaccharides (Tokareva et al. 2011) are not distinctive in whole wood (Goacher et al. 2011).

Although cellulose and hemicellulose lack firm distinction in ToF-SIMS, the action of enzymes on lignocellulose has been characterized by ToF-SIMS through the evaluation of the relative peak ratio of polysaccharide (PS) and lignin (L) peaks, termed the polysaccharide peak fraction, PS/(L+PS) (Goacher et al. 2011).
al. 2012). This ratio is calculated from a list of PS and L peaks that avoids mass interferences from applied protein (enzymes) and the common surface contaminant of poly(dimethyl siloxane) (PDMS) (Goacher et al. 2013). An illustrative ToF-SIMS spectrum with the L and PS peaks marked is shown in Fig. S1. Cellulase treatment has been demonstrated to decrease the PS/(L+PS) peak fraction (Goacher et al. 2012, 2013). If delignification occurs, it is expected that the solubilization of lignin would leave a polysaccharide-rich solid residue, increasing the polysaccharide peak fraction.

The modification of lignin has also previously been detected by ToF-SIMS. In addition to direct fungal treatment of wood samples (Mahajan et al. 2012), ToF-SIMS was previously used to characterize the action of fungal laccase (Goacher et al. 2012) and lignin peroxidase and manganese peroxidase (Macdonald et al. 2016) on ground wood samples. The treatment of aspen and spruce with fungal laccase in the presence of the ABTS (2,2'‐azino‐bis(3‐ethylbenzothiazoline‐6‐sulphonic acid) mediator resulted in a decrease in G- and S-lignin peaks relative to generalized aromatic (Ar) peaks at m/z 77 and 91, causing a decreased lignin modification metric (G+S)/Ar (Goacher et al. 2012). In the study of aspen, birch and maple hardwoods with fungal lignin peroxidase and manganese peroxidase, lignin modification was studied more granularly in terms of the G-lignin modification metric (G/Ar) and S-lignin modification metric (S/Ar) (Macdonald et al. 2016). For these fungal proteins, manganese peroxidase selectively degraded G-lignin more than S-lignin while no lignin preference was detected for lignin peroxidase (Macdonald et al. 2016).

In their work on bacterial laccases, Munk et al. (2015) discussed a need to distinguish between delignification (causing the solubilization and release of lignin) and lignin depolymerization and modification. The data with fungal enzymes indicates that through the lignin-modification metric, ToF-SIMS analysis of lignocellulose can allow for the direct measurement of whether lignin modification has occurred and whether this modification is selective to S- vs G-lignin. Furthermore, it is expected that if delignification occurs to a greater extent than the dissolution of polysaccharides, the residual solid analyzed by ToF-SIMS will be relatively enriched in polysaccharides, causing an increase in the polysaccharide peak fraction (increased PS/(L+PS)).

Therefore, this study aimed to test the action of two bacterial laccases on hardwood (birch) and softwood (spruce) in the presence of the three mediators shown in Fig. 1: ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), gallic acid and sinapic acid. The two bacterial laccases used in the study were CotA from Bacillus subtilis, which has been extensively studied at both biochemical and structural levels (Hullo et al. 2001, Martins et al. 2002, Durão et al. 2006) and YfiH from Salmonella typhimurium strain LT2 (accession number NP_461592), whose crystal structure was deposited to Protein Data Bank in 2003 (PDB code 1rw0). ABTS was included in this study because it is a potent, synthetic redox mediator of laccase action (Bourbonnais et al. 1998, Morozova et al. 2007), while gallic and sinapic...
acids were chosen as possible natural mediators with reported impact on polyaromatic compounds and pretreated pulp samples (Cañas and Camarero 2010, Chandra et al. 2004). These mediators were also chosen because they are colorimetric and we could therefore visualize the activity of the enzymes against them. ToF-SIMS was used to directly analyze the wood powders treated with mediators alone, and with the mediators plus selected bacterial laccases. The dependence of mediator on observed enzyme action was assessed using the above-described peak ratios. The polysaccharide peak fraction was examined to describe overall delignification, while the G-lignin and S-lignin modification metrics were used to describe whether lignin modification occurred. A lignin selectivity metric of S/G lignin was also calculated to determine whether degradation was selective for particular lignin types.

**Materials and Methods**

**Wood Substrates**

Wood samples were heartwood tissue from yellow birch (*Betula alleghaniensis*) and red spruce (*Picea rubens*), which were ground and then sieved through US sieve size 40 mesh to obtain corresponding wood powders. The powders were solvent-extracted using ethanol, toluene and water according to ASTM standard D 1105 (2013). The extraction removes small molecules (extractives), which can overlap with lignin peaks and alter ToF-SIMS peak ratios (Goacher et al. 2013). Since extractives may also play a role in laccase mediation, their removal simplifies the study of different mediators.

**Mediators**

The mediators used were 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS ≥98%, Sigma Life Science, Darmstadt, Germany), gallic acid (97.5-102.5%, Sigma Life Science), and sinapic acid (≥99.0%, Fluka Analytical, Buchs, Switzerland). Stock solutions of the mediators were made in distilled water to a concentration of 10 mM for ABTS and gallic acid, while solubility limited stock sinapic acid to 5 mM.

**Laccase Enzymes**

Two laccase enzymes were used: BSU0630, spore coat protein CotA from *Bacillus subtilis* (accession number BAA22774, PF07731, EC 1.10.3.2, type 2); and STM2661 is a hypothetical protein YfiH from *Salmonella typhimurium* strain LT2 (accession number NP_461592, PF02578, EC 1.10.3.2, laccase). Genes encoding BSU0630 and STM2661 were PCR-amplified from genomic DNA and cloned into a p15Tv-Lic plasmid via a ligation-independent method as previously described (Bonsor et al. 2006). Recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3). The *E. coli* transformants were grown at
37°C with shaking in 1 l of Terrific Broth (100 µg ml⁻¹ ampicillin and 50 µg ml⁻¹ kanamycin) to an optical density (OD 600 nm) of 0.6. Cultures were then supplemented with 1 mM IPTG, 2.5 mM CuCl₂ and grown for 4 h at 20°C with shaking, followed by further overnight incubation at 20°C without shaking.

The *E. coli* cells were harvested by centrifugation, and cell pellets were resuspended in binding buffer (250 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, 5 mM imidazole) followed by sonication at 4°C. Lysates were cleared by centrifugation and the supernatant was incubated with 2 ml of Ni-NTA resin at 4°C for 1 h with gentle shaking. The resin was washed with 50 ml of wash buffer (250 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, 30 mM imidazole) and eluted with elution buffer (250 mM NaCl, 50 mM HEPES, pH 7.5, 5% glycerol, 250 mM imidazole). Purified protein was flash frozen on liquid N₂ and stored at -80°C. Protein purity was evaluated using 10% SDS-PAGE gels.

Prior to reaction with the mediator and wood, enzyme solutions were prepared with either 0.25 µg µl⁻¹ (1x) or 1.25 µg µl⁻¹ (5x) concentrations in 5 mM CuSO₄ in order to ensure sufficient copper in the laccases. Reaction pH was controlled using universal buffers (Britton and Robinson, 1931) that contained 100 mM acetic, boric and phosphoric acids, brought to the desired pH using sodium hydroxide. The pH for each laccase and mediator combination was determined by observing the colorimetric development of the mediator to find the pH where the mediator was most oxidized by the given laccase (Table 1).

**Evaluation of Mediator Interferences**

It is important that the mediators themselves do not interfere with ToF-SIMS measurements of wood, specifically with lignin-related peaks in the mass spectra. Therefore, ToF-SIMS spectra were collected for the solid mediators themselves, and for wood soaked in mediator alone at both the stock concentrations (5-10 mM) and at the 1 mM assay concentration. This allowed the evaluation of whether the mediators may alter the peak ratios of interest without the laccase being present.

For pure mediators, 1-5 mg of solid mediator was applied to double-sided tape on glass microscope slides. Solutions were prepared by soaking 20 mg of extracted birch for 24 h in 750 µl of either the stock mediator (5 mM SA, 10 mM ABTS and GA) or in diluted mediator (1 mM for all). After soaking, vials were uncapped to air dry, without rinsing, for 1 week. This was to simulate a worst-case scenario for residual mediator left on the birch surface.

**Laccase Treatments of Birch and Spruce**

The laccase treatments of ground wood consisted of 20 mg birch or spruce in 1 ml solution. Each solution included 10 µl of 1 M sodium chloride to prevent laccase precipitation. Laccase treatments used 100 µl of either 1x laccase stock (yielding 25 µg laccase) or 5x laccase stock (yielding 125 µg laccase), while controls
replaced this with 100 µl of distilled water. For ABTS and GA, 100 µl of 10 mM mediator stock was used with 790 µl of buffer (see Table 1 for corresponding buffer pH) while for SA, 200 µl of 5 mM mediator stock was used with 690 µl of buffer. This led to all mediators being at 1 mM final concentration in the wood treatments.

Samples were incubated for 18 h at 50°C with shaking at 80 rpm. After incubation, the supernatant was removed and the solids were rinsed with three 20-ml portions of 1 M acetic acid, and one 20-ml portion of distilled water to remove buffer salts (Braham and Goacher 2015). Samples were air dried prior to ToF-SIMS analysis.

**ToF-SIMS Analysis**

ToF-SIMS spectra were obtained using a TOF.SIMS 5 instrument (IONTOF GmbH, Münster, Germany). The instrument was equipped with a bismuth liquid metal ion source incident to the sample at 45°, and with a reflectron-type time-of-flight mass analyzer. All samples were analyzed with 50 keV Bi²⁺ high current bunched primary ions with a pulsed current of ~0.3 pA. A minimum of five positive ion replicate ToF-SIMS spectra were obtained for each sample, with each replicate consisting of 60-120 s of analysis where the primary ions were rastered randomly over 128x128 pixels in a 500x500 µm² area. Charge neutralization was performed using 20 eV electron flooding. The mass spectra were calibrated using SurfaceLab v6.3 (IONTOF GmbH, Muenster Germany) to the CH₃⁺, C₂H₃⁺ and C₃H₅⁺ ions formed during the fragmentation of most organic materials.

Peaks in the mass spectra were exported using nominal mass intervals – roughness of the samples limited mass resolution to m/Δm of 400 to 2,000 at m/z 91. Microsoft Excel was used to calculate peak ratios, and to perform F-tests and unpaired t-tests to determine significant differences in peak ratios. Herein, changes in the ToF-SIMS peak ratios are discussed if they had notable P-values for the t-test comparisons versus the controls. All comparisons that were significant at 95% confidence (P≤0.05) or at 90% confidence (P≤0.1) will be reported, and some P-values between 0.1 and 0.2 will be mentioned if these results support a trend from more significant P-values.

**Results**

**Interference from Mediators**

As mentioned in the introduction, ToF-SIMS spectra can reveal lignin modification through the calculation of specific peak ratios. These include the modification metrics for G-lignin (G/Ar) and S-lignin (S/Ar). The S/G selectivity metric further reflects whether the relative proportions of S- and G-lignin changed. Lastly,
the polysaccharide peak fraction (PS/(L+PS)) indicates whether there was net lignin dissolution and removal from the solid.

The different chemical composition of the mediators themselves compared to the wood samples could cause alterations in these characteristic peak ratios if the mediator were to absorb into or dry onto the wood surface. Accordingly, the pure mediators were analyzed to determine whether the mediators’ chemical compositions would cause increased or decreased peak ratios. Then, to test the extent to which mediator interference with peak ratios could occur, birch wood samples were soaked in the chosen mediators under the reaction condition of 1 mM mediator and under the more concentrated stock mediator solutions (5-10 mM). Unlike laccase treatments, these samples were not rinsed, to allow for a worst-case scenario of mediator interference. The corresponding peak ratios were calculated and compared to birch soaked in distilled water (Fig. S2).

The changes in peak ratios due to mediator presence are discussed more thoroughly in Appendix S1 of the supporting information. To summarize, the ABTS mediator caused the greatest number of peak ratio changes compared to birch soaked in distilled water. The pure ABTS and birch soaked in 10 mM ABTS had decreased PS/(L+PS) (Fig. S2A), decreased S/G (Fig. S2B), and increased G/Ar (Fig. S2C). The decreased S/G ratio also was statistically significant for birch soaked in 1 mM ABTS, although the magnitude of the decrease was less with 1 mM than with 10 mM or pure ABTS (Fig. S2B).

Pure gallic acid had lower PS/(L+PS) (Fig. S2A), lower S/G (Fig. S2B), higher G/Ar (Fig. S2C) and higher S/Ar (Fig. S2D), while birch soaked in 10 mM gallic acid caused only increased G/Ar and S/Ar, and birch soaked in 1 mM gallic acid had no significant differences from birch in water. Lastly, the pure sinapic acid mediator had decreased PS/(L+PS), increased S/G and increased S/Ar, while no changes in peak ratios were evident for birch soaked in either 5 or 1 mM sinapic acid (Fig. S2). Given the impact of higher mediator concentrations on the relevant metrics of wood samples to be analyzed herein, 1 mM mediator concentrations were used in the analysis, and wood treatments lacking enzymes were included as controls.

Wood Treatments with ABTS and Laccases

As described in the methods, CotA and YfiH were incubated with ground birch and spruce in the presence of 1 mM mediator solutions at two enzyme dosage levels: 25 µg (termed “1x”) and 125 µg (termed “5x”). Our preliminary experiments demonstrated that both enzymes were active against ABTS, gallic acid, and sinapic acid used as mediators in this work (data not shown). As is common for laccases, the optimum pH of these enzymes was substrate dependent, where the optimum pH of both enzymes on ABTS was pH 5.0 (Table 1).
The peak ratios for laccase treatments of birch and spruce with the ABTS mediator revealed that while CotA produced significant peak alterations for both birch and spruce, YfiH caused no significant changes to spruce (Fig. 2). Furthermore, CotA demonstrated higher activity than YfiH on birch, with CotA causing higher magnitude peak ratio changes with smaller $P$-values.

Both the 1x and 5x dosages of CotA resulted in an enrichment in polysaccharides for birch and spruce (Fig. 2A). The trend of increased PS/(L+PS) on birch was also observed for the higher 5x dosage of YfiH on birch. The increased PS/(L+PS) ratios are what would be expected for lignin dissolution (delignification), leaving a polysaccharide-rich solid. Accordingly, this analysis confirmed that both enzyme-ABTS systems promote delignification of wood samples, albeit with lower dosage of CotA than YfiH.

Examining the modification of lignin, decreased ratios of G/Ar (Fig. 2C) and S/Ar (Fig. 2D) were observed only for the higher (5x) dosage of both CotA and YfiH on birch, and was also observed with both the 1x and 5x CotA treatments of spruce. The decreased G/Ar and S/Ar ratios indicate lignin modification through the cleavage of the characteristic methoxy and phenol groups from the aromatic ring of lignin, leaving the aromatic core. Although lignin modification was evident, most samples treated with ABTS had unchanged S/G ratios (Fig. 2B), indicating no preferential degradation of G vs S lignin. The one exception was the 5x CotA treatment of spruce, which caused an elevated S/G ratio consistent with more extensive depletion of G-lignin, which is enriched in spruce samples (Fig. 2B).

The raw ToF-SIMS spectra for the birch control with ABTS (pH 5.0) and for the birch treated with the 5x dosage of CotA with ABTS appear in Fig. 3. These spectra illustrate the significant decrease in the intensities of the G-lignin (blue) and S-lignin peaks (green) peaks after treatment with the 5x dosage of CotA in the presence of ABTS.

**Treatments with Gallic Acid**

The results of birch and spruce treatment with CotA at pH 6.0 and YfiH at pH 9.0 in the presence of gallic acid are displayed in Fig. 4. Here it is apparent that neither laccase was active on birch in the presence of gallic acid. This is an interesting demonstration of mediator specificity, as both CotA-ABTS and YfiH-ABTS systems transformed birch samples at the same enzyme dosages (Fig. 2).

The analysis of laccase-gallic acid systems on spruce included two controls because logistics required the ToF-SIMS spectra of the 1x and 5x laccase treatments to be collected on different days (Fig. 4). Thus, each laccase-gallic acid treatment of spruce is compared to the spectra collected for the spruce control samples.
on the same day. The control samples were reproducible between the two days for all but one metric, where
the G/Ar metric of the CotA control on day 2 was elevated (Fig. 4C).

The changes in spruce when treated in the presence of the gallic acid mediator were less significant than
with ABTS. Only treatments using 5x CotA dosage led to a statistically significant increase in the
PS/(L+PS) ratio, consistent with a slight delignification (Fig. 4A). The corresponding S/G ratio also slightly
increased (Fig. 4B) due to a decrease in the G/Ar modification metric (Fig. 4C). Notably, however, when
the 5x CotA treatments are referenced to the 1st control instead of to the 2nd control, the S/G and G/Ar ratios
do not differ. The impact of the YfiH-gallic acid system on spruce was even weaker, where the most
significant change was to the S/G ratio (Fig. 4B), which was mainly explained by decreased G/Ar (Fig.
4C). Overall, the current ToF-SIMS analyses showed that gallic acid appeared to be a less effective
mediator, causing no detectable changes to birch, and causing only slight change to spruce.

Treatments with Sinapic Acid

The results of birch and spruce treatment with CotA at pH 8.0 and YfiH at pH 7.0 in the presence of sinapic
acid indicate a different mode of action than with ABTS (Fig. 5). While CotA with ABTS caused lignin
dissolution (Fig. 2A), both the 1x and 5x CotA treatments with sinapic acid (pH 8.0) decreased the
polysaccharide peak fraction (PS/(L+PS)), with P-values ranging from 0.08 to 0.14 (Fig. 5A). The release
of apparent lignin-carbohydrate complexes from unbleached Eucalyptus kraft pulp through laccase-
mediator treatment was previously reported (Du et al. 2013). Similarly, it is conceivable that action of the
CotA-sinapic acid system on lignin indirectly released polysaccharide components from the wood samples.
Even though released carbohydrates are expected to largely be in oligomeric form, xylose was indeed
detected by liquid chromatography mass spectrometry in reaction supernatants (data not shown).

Considering the lignin compositions in birch and spruce, both laccase-sinapic acid treatments resulted in an
increased ratio of S-lignin to G-lignin (S/G), consistent with preferential transformation of G-lignin
structures (Fig. 5B). This increase in the S/G ratio was more significant for treatments with CotA than YfiH,
although it was observable with both enzymes. Therefore, the CotA enzyme effected greater change than
YfiH for both ABTS and sinapic acid mediators, although the nature of these changes was mediator-
dependent. Lastly, the increase in S/G with sinapic acid was more significant (lower P-values) on spruce
than on birch, consistent with the higher G-lignin content in spruce (Fig. 5B).

The results for G/Ar and S/Ar with the sinapic acid mediator were inspected to provide further insight into
the cause of the increased S/G ratios. Whereas CotA-sinapic acid treatment of birch led to a decrease in the
G/Ar peak ratio consistent with preferential transformation of G-lignin components (Fig. 5C), this was not observed following birch treatment with the YfiH-sinapic acid system. The raw ToF-SIMS spectra in Fig. 6 further illustrate the selective loss of G-lignin for birch treated with the 5x dosage of CotA-sinapic acid. The G/Ar peak ratio of spruce did not change significantly following treatment with either laccase-sinapic acid system. Indeed, the G/Ar peak ratio had considerable spread for many of these treatments. By contrast, both laccase-sinapic acid systems increased the S/Ar ratio of the wood samples; most clearly for spruce samples (Fig. 5D). In light of (1) the similarity of sinapic acid to S-lignin structures (Fig. 1), (2) the early studies to account for interferences from the applied mediators, and (3) the inclusion of mediators in control samples lacking enzyme, the increase in the S/Ar ratio observed for laccase-sinapic acid treatments would seem consistent with laccase-mediated grafting (attaching) of sinapic acid to the wood surfaces.

Discussion

This study demonstrates that bacterial laccases from *B. subtilis* (CotA) and *S. typhimurium* (YfiH) can act directly on lignocellulose components in hardwood and softwood samples. The wood powders in this study received no intensive pretreatment prior to enzyme application, just solvent extraction. This strengthens the literature (Singh et al. 2017) indicating that bacterial laccases can catalyze the modification of lignin in solid wood, going beyond model compounds. Throughout this work, there were greater changes to the wood with CotA versus the YfiH, meriting further study into the abilities of the CotA enzyme.

Furthermore, this work directly studies the impact of different mediators on the ability of bacterial laccases to modify and dissolve lignin. The ToF-SIMS technique allowed for the analysis of the different lignin components and a relative comparison between lignin and polysaccharides at the surface. Comparing the same enzyme dosages, the ABTS mediator facilitated the dissolution of lignin (observed in the increased PS/(L+PS) ratios, Fig. 2A), which was not evident with either the gallic acid or sinapic acid mediators and has not been previously reported. However, the mediator dependence on the PS/(L+PS) ratio was strong. While ABTS facilitated lignin dissolution, the sinapic acid mediator indicated net polysaccharide loss and relative lignin gain (Fig. 5A). The modification of lignin was also different with the three mediators, as ABTS caused decreases in both the G/Ar and S/Ar lignin modification metrics, but little change in the net S/G peak ratio (Fig. 2). Meanwhile, sinapic acid caused increased S/G peak ratios, due to increased S-lignin intensity (increased S/Ar), particularly on spruce, and also due to selective G-lignin loss (decreased G/Ar) for CotA treatment of birch (Fig. 5). These drastically different results for the same laccases and woods emphasizes the role of the mediator in the resulting chemical changes.
In a previous ToF-SIMS study of fungal lignin peroxidase and manganese peroxidase (no mediator),
decreases in G/Ar and S/Ar were detected on hardwoods, with preferential modification of G-lignin for
manganese peroxidase (which would result in higher S/G) and similar modification for G and S lignin for
lignin peroxidase (MacDonald et al. 2016). Herein, the G/Ar and S/Ar peak ratios also decreased similarly
on birch following treatment with both CotA-ABTS and YfiH-ABTS systems (Fig. 2), showing similarity
to the action of lignin peroxidase (MacDonald et al. 2016).

In contrast to MacDonald et al (2016), when Singh et al (2017) studied the action of bacterial laccase (sLac,
small laccase) on steam-pretreated poplar without mediator, their NMR results indicated that S-lignin was
preferentially oxidized. The results of the present work with CotA and YfiH bacterial laccases do not
demonstrate preference for S-lignin oxidation, instead oxidation with ABTS was in equal proportion,
maintaining a constant S/G ratio (Fig. 2), while laccase activity with sinapic acid increased the S/Ar ratio
of the treated wood samples (Fig. 5). This highlights the potential for different lignin modifications as a
function of applied mediators and laccases.

The influence of the reaction conditions (e.g., reaction pH and temperature) on the observed results is
something that deserves further investigation. We have begun to investigate these enzymes and mediators
at other temperatures, starting with 30°C treatments with birch. At this lower temperature, no activity was
detected with the ABTS mediator (data not shown), which contrasts with the significant impacts to wood
compositions following laccase-ABTS treatments at 50°C (Fig. 2) and shows the temperature dependence
of laccase activity with ABTS. By contrast, when birch samples were treated with both CotA-sinapic acid
and YfiH-sinapic acid systems at 30°C (data not shown), subsequent ToF-SIMS analyses revealed increased
S/G ratios (P<0.001), decreased G/Ar peak ratios, and indication of increased S/Ar peak ratios. The
decreased PS/(L+PS) was also reproduced for the CotA-sinapic acid system. The 30°C data are therefore
consistent with the reactions performed at 50°C for sinapic acid (Fig. 5), which are of particular interest
because the increased S/Ar peak ratios appear to be unique among published reports so far.

As illustrated in Fig. 1, sinapic acid has structural similarity to S-lignin, and so the increased S/G and S/Ar
peak ratios with sinapic acid could be explained by bonding or adsorption of sinapic acid onto the birch and
spruce surfaces, appearing to increase S-lignin content. Indeed, the ToF-SIMS analysis of the pure sinapic
acid mediator (Fig. S2) showed decreased PS/(L+PS) (Fig. S2A), a high S/G ratio (Fig. S2B) and a high
S/Ar ratio (Fig. S2D), but as discussed earlier, the peak ratios for birch soaked in 1-5 mM aqueous solutions
of sinapic acid did not differ from birch soaked in water. Moreover, the apparent increase in S-lignin content
was observed for the laccase treatments relative to control reactions that included sinapic acid without
laccase. Accordingly, it is possible that laccase treatments grafted the lignin-like sinapic acid mediator onto
the wood surfaces, leading to an overall increase in lignin peak intensity, also explaining the decrease in PS/(L+PS). This possibility merits further investigation using purified lignin and/or polysaccharides.

Overall, the current ToF-SIMS analysis indicates that the CotA and YfiH bacterial laccases both acted to modify lignin in wood. The different mediators played a strong role in the observed laccase action. Gallic acid was not an effective mediator of laccase action in this work (Fig. 4), ABTS enabled equal attack on G and S lignin moieties and caused lignin dissolution (Fig. 2), while sinapic acid mediation led to more complex laccase action, which appeared to include grafting (Fig. 5). The ToF-SIMS method provided a direct method of analysis, with the ability to distinguish between modification of lignin and delignification – as the lignin modification metrics track the increase and decrease of specific lignin moieties, while the polysaccharide peak fraction indicates net lignin loss or gain at the sample surface. ToF-SIMS therefore has a part to play in understanding laccase action on solid wood substrates, and the development of laccase-mediator systems, as well as reaction conditions, leading to delignification versus fiber modification.

Author contributions
R.G., E.B. and E.M. designed the experiments. A.Y. and R.F. expressed and purified the enzymes and characterized their pH profiles. E.B. executed sample treatments. E.B., R.G. and C.M. analyzed the data. R.G. and E.M. drafted the manuscript, which was approved by all authors.

Acknowledgements – We thank the spring 2017 Instrumental Analysis class and Kylie Kiah at Niagara University for their liquid chromatography mass spectrometry analysis of the supernatants for sinapic acid wood treatments. This work was funded by the Academic Center for Integrated Sciences at Niagara University, the Government of Ontario for the project “Forest FAB: Applied Genomics for Functionalized Fibre and Biochemicals” (ORF-RE-05-005), and a European Research Council (ERC) Consolidator Grant to EM (BHVIE – 648925).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

- **Figure S1.** Representative ToF-SIMS spectrum showing lignin and polysaccharide peaks
- **Figure S2.** ToF-SIMS of pure mediators and of birch soaked in mediators without rinsing
- **Appendix S1.** Additional discussion of ToF-SIMS of pure mediators and birch soaked in mediators
Tables

Table 1. Optimum pH for each the three mediators studied with the CotA and YfiH enzymes.

<table>
<thead>
<tr>
<th></th>
<th>CotA</th>
<th>YfiH</th>
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<tbody>
<tr>
<td>ABTS</td>
<td>pH 5</td>
<td>pH 5</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>pH 6</td>
<td>pH 9</td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>pH 8</td>
<td>pH 7</td>
</tr>
</tbody>
</table>
Figure Legends

**Figure 1.** The three main monolignols of p-coumaryl alcohol (H-lignin), coniferyl alcohol (G-lignin) and sinapyl alcohol (S) that comprise lignin, along with the three mediators used in this study: ABTS, gallic acid and sinapic acid.

**Figure 2.** Peak ratios derived from ToF-SIMS spectra of birch and spruce treated with ABTS and either CotA or YfiH. (A) The polysaccharide peak fraction (PS)/(L+PS). (B) The lignin selectivity ratio (S/G). (C) The G-lignin modification metric (G/Ar), and (D) The S-lignin modification metric (S/Ar). Error bars represent one standard deviation and *P*-values above laccase treatments refer to unpaired t-tests vs their respective controls.

**Figure 3.** ToF-SIMS spectra for (A) the birch ABTS control and (B) the birch 5x CotA-ABTS treatment. The mass range includes characteristic peaks for G-lignin at m/z 137 and 151 (blue) and for S-lignin at m/z 167 and 181 (green).

**Figure 4.** Peak ratios derived from ToF-SIMS spectra of birch and spruce treated with gallic acid and either CotA or YfiH. (A) The polysaccharide peak fraction (PS)/(L+PS). (B) The lignin selectivity ratio (S/G). (C) The G-lignin modification metric (G/Ar), and (D) The S-lignin modification metric (S/Ar). Error bars represent one standard deviation and *P*-values above laccase treatments refer to unpaired t-tests vs. their respective controls. Note that for spruce, the 1x laccase treatments correspond to control 1 while the 5x laccase treatments correspond to control 2.

**Figure 5.** Peak ratios derived from ToF-SIMS spectra of birch and spruce treated with sinapic acid and either CotA or YfiH. (A) The polysaccharide peak fraction (PS)/(L+PS). (B) The lignin selectivity ratio (S/G). (C) The G-lignin modification metric (G/Ar), and (D) The S-lignin modification metric (S/Ar). Error bars represent one standard deviation and *P*-values above laccase treatments refer to unpaired t-tests vs their respective controls.

**Figure 6.** ToF-SIMS spectra for (A) the birch sinapic acid control and (B) the birch 5x CotA-sinapic acid treatment. The mass range includes characteristic peaks for G-lignin at m/z 137 and 151 (blue) and for S-lignin at m/z 167 and 181 (green).
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