ORIGINAL ARTICLE

Enzymatic demethylation of Kraft lignin for lignin-based phenol-formaldehyde resin applications

Balaji Venkatesagowda [1] · Robert F. H. Dekker [1,2]

Abstract

Wood-rot fungi from the Boreal Forest of Canada were studied for their ability to demethylate Kraft lignin (KL). Demethylation by the action of enzymes (O-demethylases) removed the O-methyl/methoxyl groups of lignin liberating methanol, and produced a demethylated KL enriched in vicinal-hydroxyl groups with potential to serve as lignin-based phenol-formaldehyde polymers. Screening experiments identified the liberation of methanol (measured by selected-ion flow-tube mass spectrometry), lignin-demethylating enzymes, alcohol oxidase, and other ligninolytic enzymes. Highest amounts of headspace methanol (parts-per-billion) were detected in the genus *Aspergillus*, *Ctenomyces*, *Cunninghamella*, *Penicillium*, and *Sporobolomyces*. Methanol generated from lignin demethylation induced alcohol oxidase activity, but which was higher in *Aspergillus*, *Ctenomyces*, *Entoloma*, and non-sporulating fungi. Among the fungi tested, three brown-rot, i.e., *Fomitopsis pinicola* and *Galerina autumnalis* and a mitosporic *Aspergillus* sp.3 BRI 270, were cultured solely on KL, and lignin model compounds (LMCs) to determine lignin demethylation. Various carbohydrate supplements added to nutrient media containing KL significantly influenced demethylating activity. *Aspergillus* sp.3 BRI 270 showed the highest degree of lignin demethylation (30.1%) which no evidence presented for this comment occurred when cultivated on KL media supplemented with birchwood xylan as analyzed by ^{1}H NMR following O-acetylation of modified KLs. All fungi demonstrated considerable demethylating activity by utilizing softwood KL, but OrganoSolv lignins (poplar, willow, wheat straw, and mixed agricultural wastes), because of their harsh chemical treatments of extraction, affected the microbial and enzymatic demethylation. Extracellular demethylating enzymes from *Aspergillus* sp.3 BRI 270 generated high vicinal-diol content (measured by the pyrocatechol titanium(III)-nitrilotriacetate method) in LMCs: 4-hydroxy-3-methoxy cinnamaldehyde (326.00 μmol/mL), syringaldehyde (102.67 μmol/mL), and Kraft lignin (397.46 μmol/mL) as analyzed by ^{1}H NMR analysis.

Keywords Lignin demethylation · O-Demethylase · Methanol · Pyrocatechol · O-Acetylated lignin · Pyrocatechol-titanium(III) nitrilotriacetate complex

1 Introduction

Biological demethylation of lignin occurs enzymatically through reactions catalyzed by enzymes such as the O-demethylases. These enzymes are specifically endowed with the ability to cleave off the O-methyl/methoxyl groups of phenolic and some non-phenolic compounds that constitute the structure of lignin releasing methanol as the end product. Demethylation, albeit chemical or enzymatic, converts lignin into a more widely accessible material enriched in phenolic hydroxyl groups (vicinal diols, pyrocatechols) for applications as biopolymers [1–4].

Lignin is a natural, amorphous heteropolymer found in the plant cell wall, and is composed of aromatic (~ 85%) and phenolic (~ 15%) structures of phenyl-propane repeat sequences (Fig. 1). The degree of methylation of lignin varies with the botanical species but ranges from 14 to 19% in woody plants. Kraft lignin (KL) is extracted by acid precipitation from the black liquor produced through chemical pulping (Kraft process) of wood for the production of cellulose pulp.

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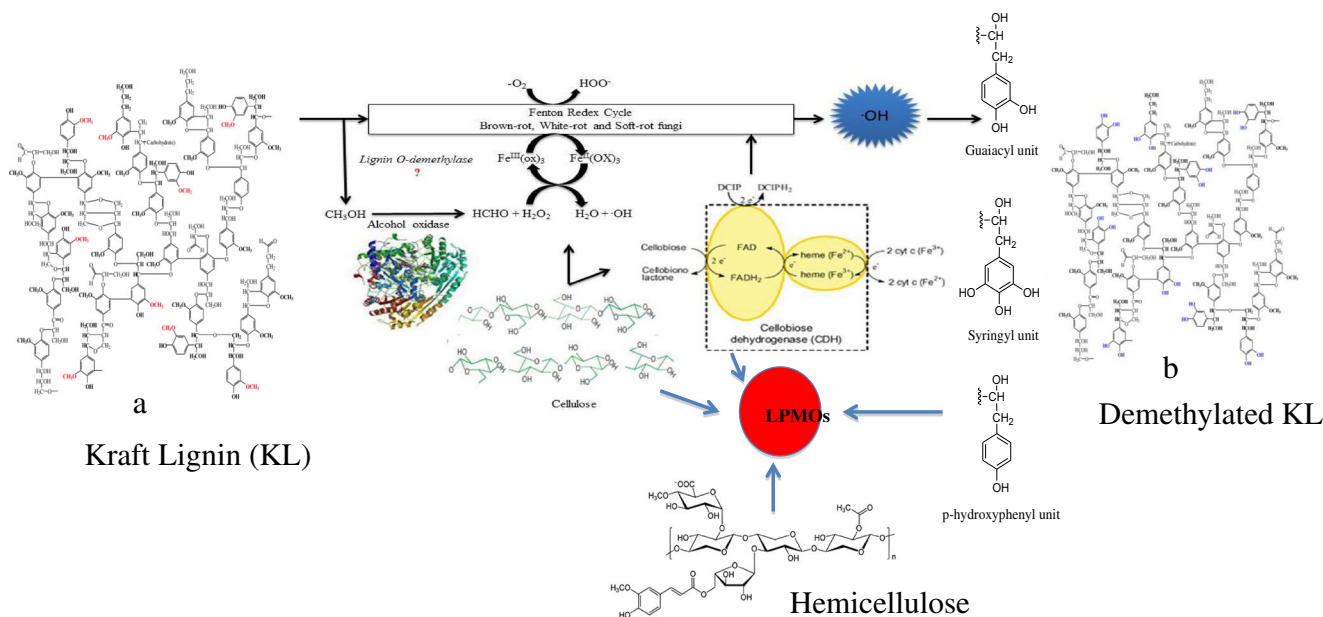
Fig. 1 Enzymatic fungal demethylation of Kraft lignin. (a) Kraft lignin and (b) demethylated Kraft lignin

The extracted lignin contains C–C and ether (C–O–C) bonds that are composed of the following linkages: β-O-4 (phenylpropane β-aryl ether dimer), α-O-4 (phenylpropane α-aryl ether dimer), 4-O-5 (diaryl ether dimer), 5–5 (biphenyl and dibenzodioxocin dimer), β-5 (phenylcoumaran dimer), β-1 (1,2-diaryl propane dimer), and β-β (β-β-linked dimer structures). Functional groups on lignin comprise carbonyl (C=O), alcohols (–CH$_2$OH, –CHOH), free phenolic hydroxyl (–OH), and methoxyl (–OCH$_3$), and are potentially available for chemical, microbial, and enzymatic modification [1–13].

The phenomenon of lignin demethylation has been known as early as 1924 [14]. Furthermore, the enzymatic/chemical demethylation processes of KL and lignin-like model compounds (LMCs) have been well studied [1–3, 5–13]. Certain brown-rot fungi are known to utilize the polysaccharides constituting the plant cell wall as sole carbon source, and modify lignin by a demethylation process. In addition, besides the brown-rot fungi producing polysaccharide-degrading enzymes, they also generate free radicals that can remove *O*-methyl groups from lignin [15]. By contrast, the white-rot fungi simultaneously attack lignin and the carbohydrate biopolymers cellulose and the hemicelluloses, but few can specifically degrade lignin alone [16, 17]. Brown-rot demethylation has been a relatively less-explored process, believed by some to be an enzyme-catalyzed process [5, 18], while others suggest that it is a non-enzymatic event [8].

During the wood degradation reactions, both the polysaccharides and lignin appear to be associated with the action of the lytic polysaccharide mono-oxygenases (LPMO), cellobiose dehydrogenases (CDH), and the *O*-demethylase/ ligninolytic enzymes. Several brown-rot fungi have been reported to be able to demethylate lignin and to induce the overexpression of the enzyme, alcohol oxidase, responsible for the oxidation of the methanol liberated to produce formaldehyde and hydrogen peroxide (H$_2$O$_2$). Besides, H$_2$O$_2$ is derived from the co-metabolism of cellulose and/or hemicellulose [19]. Further, the accessory enzymes, such as oxidases that generate the H$_2$O$_2$ required by the peroxidases lignin (LiP) and manganese (MnP), are known to be involved in the lignin degradation process. Many white-rot and brown-rot fungi utilize the hydrogen peroxide liberated and generate hydroxyl radicals (˙OH) during the course of the Fenton reaction. Ultimately, these free radicals attack the polysaccharides as well as the lignin in the plant cell walls in a non-specific manner, leading to the creation of some cleavage sites, which allow for easier penetration by the enzymes hydrolyzing the components of lignocellulose. It has been suggested that a hydroxyl radical-producing system may be involved in the lignin degradation by means of the LiP or the accessory enzymes [5, 18, 19]. CDH is an enzyme that transfers the electrons from the oligosaccharides produced as a consequence of cellulolytic attack on cellulose to an electron acceptor, such as Fe^{3+} or O$_2$, thereby generating the hydroxyl radicals. It has been demonstrated that these radicals react with the phenolic or non-phenolic LMCs, resulting in the demethylation process [20]. An extracellular redox enzyme, cellobiose dehydrogenase, generates the hydroxyl radicals by reducing Fe^{3+} to Fe^{2+} and O$_2$ to H$_2$O$_2$, and thus plays an important role in the degradation of lignin (and cellulose) by breaking the β-ether linkages, demethoxylating the aromatic/phenolic structures in lignin, and by introducing the hydroxyl groups on the modified lignin yielding a lignin product enriched in phenol hydroxyl groups [21]. A brown-rot fungal degradation pattern of spruce sapwood demonstrated that polysaccharide loss could play a

mechanistic role in the lignin demethylation process that can drive the Fenton reactions. At the same time, catechol/quinone oxidation occurs by a cycling reaction that leads to iron reduction, as observed for *Postia placenta* and *Gloeophyllum trabeum*, where catechol induced the redox reactions with the involvement of the ferric iron generated [8, 22, 23].

In recent years, lignin degradation (and the polysaccharides too) has been gaining more attention, owing to the involvement of some of the accessory enzymes in the generation of H_2O_2, followed by the Fenton reaction. As a consequence, the demethylated lignin produced can act as an electron donor inducing oxidation in the C-1, C-4, and C-6 hydroxyl groups in cellulose and hemicellulose [24, 25].

The brown-rot fungi are believed to possess the ability to secrete a novel enzyme, the lytic polysaccharide mono-oxygenases, which are copper-dependent enzymes requiring an electron donor. These enzymes utilize the insoluble high-molecular-weight lignin and the monomeric forms of catechol, gallic acid, or the oxidized monomers and oligosaccharides of cellulose and hemicellulose generated by cellobiose dehydrogenase, as electron donors. The oxidation by the LPMOs appears to enhance the accessibility of the other hydrolytic enzymes in degrading cellulose and hemicellulose, thereby producing a set of fermentable monomeric sugars [24, 25].

Usually, in situ demethylation of lignin is made possible biologically by the action of certain microorganisms, such as the white-rot and brown-rot fungi. Some bacterial species are also known to secrete enzymes that can act on lignin and LMCs (e.g., vanillate, syringate, and veratrate), and the enzymes involved are known as the vanillate-*O*-demethylases, syringate *O*-demethylases, and veratrate *O*-demethylases [2, 4]. Our interest has centered on searching for *O*-demethylases that are lignin-specific, which essentially strip off the *O*-methyl groups from the phenyl-propanoid units comprising lignin releasing methanol, and modify lignin by increasing the number of phenolic hydroxyl groups. Whereas other ligninolytic enzymes are known to completely alter the basic structure of the lignin skeleton (e.g., laccases, and the non-specific peroxidases, LiP and MnP), *O*-demethylase like enzymes, such as those acting on vanillate, syringate, and veratrate, should also be capable of removing the *O*-methyl groups from lignin without interfering with the complex backbone structure of lignin. The properties of the modified lignin are envisaged to open up new avenues in the applications of lignin as a biopolymer.

The mechanism of action of the *O*-demethylases in lignin degradation still remains obscure and largely unexplored, and their ability to remove the *O*-methyl groups has not been adequately addressed or demonstrated experimentally. Thus, this investigation addresses the demethylation of the KL process by three fungi from our mycological collection, namely, the brown-rot, *Fomitopsis pinicola* and *Galerina autumnalis*, and the mitosporic, *Aspergillus* sp.3 BRI 270, all of which

exhibited high demethylation ability, probably mediated through novel *O*-demethylases. We also investigated the role of the sugars and polysaccharides in the KL demethylation process by the above fungal isolates. The modified demethylated lignins produced were *O*-acetylated to allow analysis of loss of methoxyl groups and determine the extent of demethylation using ^1H NMR. Although the key enzymes responsible for lignin demethylation have not been adequately identified through purification and characterization, we propose that the *O*-demethylases could be a probable candidate for that purpose, in view of their ability to remove the *O*-methyl groups at position C-3 and/or C-4 of the benzyl rings of lignin and the LMCs.

2 Materials and methods

2.1 Chemicals

The chemicals used in this investigation were procured from various sources. Kraft lignin (KL, mixed spruce wood) extracted by acid precipitation from the black liquor was provided by FPI Innovation, Pointe Claire, Quebec (Canada). OrganoSolv lignin preparations were kindly provided and were derived from poplar (Wisconsin Institute for Sustainable Technology—WIST, University of Wisconsin–Stevens Point, WI, USA), wheat straw and willow (Energy Research Center of the Netherlands, ECN), and mixed agricultural waste (American Science and Technology, AST Biorefinery Pilot Plant, Wausau, WI, USA). All the other analytical chemicals were purchased from Sigma-Aldrich, Canada.

2.2 Screening fungal isolates for degradation of Kraft lignin

Around 450 species of the wood-decay fungi from the Mycological Herbarium Culture Collection of the Faculty of Forestry at the *Lakehead University* (LU-CC), and another 300 fungal isolates collected in the Boreal Forest around the city of Thunder Bay, Ontario (Canada) and held by the Biorefining Research Institute Culture Collection (BRI-CC), were raised as axenic cultures and were maintained on the potato dextrose agar (PDA) slants at 4 °C. The fungal isolates were regularly examined for their growth on KL in glass tubes containing a liquid medium comprising Vogel minimal salts medium (VMSM, [26], KL (0.3%, *w/v*), and glucose (0.2%, *w/v*) at pH 5.8. The media were sterilized at 121 °C for 20 min, prior to inoculation with the fungal isolates, and the inoculated cultures were allowed to grow under stationary conditions at 25 °C for a month. The culture tubes were monitored for fungal growth (biomass) and by taking note of the color change of the media. Those isolates that decolorized the KL

strongly were then transferred to the liquid media containing the same amount of KL (0.3%), but with a reduced glucose content (0.05%), and were next left stationary to grow at 25 °C, during which time, the decolorization process was constantly monitored. The best isolates were chosen and were inoculated into fresh liquid media containing only KL (0.3%). In this manner, around a hundred fungal isolates were identified as being capable of growing solely on KL as a carbon source.

2.3 Extracellular ligninolytic enzymes

Out of the abovementioned one hundred fungal cultures, the best isolates (31 in total) were selected and inoculated in the liquid media containing 0.3% of KL as the substrate (pH 5.8), and left to grow at 28 °C for 21 days under stationary conditions. The fungal cultures were then centrifuged ($2200 \times g$/ 20 min), and the supernatants (extracellular fluid, ECF) were recovered and used as the source of enzyme to assay the following ligninolytic enzymes: laccase (LAC), lignin peroxidase (LiP), manganese peroxidase (MnP), methyl alcohol oxidase (MAO), cellobiose dehydrogenase (CDH), versatile peroxidase (VP), and veratryl alcohol oxidase (VAO).

All assays performed for enzyme activity were conducted on 50 μL aliquots of ECF, and were contained in reaction mixtures of a final volume of 1 mL. Appropriate blanks for substrates and ECF were included:

(i) Laccase: the reaction mixture contained ECF and 50 mM ABTS and 0.05 mM citrate/0.1 mM phosphate buffer (pH 3.0), and was incubated at 50 °C for 5 min. The oxidation of ABTS was measured by the increase in absorbance at 420 nm ($\varepsilon = 36{,}000$ M^{-1} cm^{-1}). One unit of laccase activity was expressed as the amount of enzyme oxidizing 1 μmol of ABTS per min [1].

(ii) Lignin peroxidase: the reaction mixture contained ECF and 3 mM veratryl alcohol, 0.33 M sodium tartrate buffer (pH 3.0), and freshly prepared 54 mM H_2O_2, and was incubated at 30 °C for 5 min. One unit of LiP activity was expressed as that equivalent to the number of micromoles of veratryl alcohol oxidized per minute to give rise to veratraldehyde measured at 310 nm ($\varepsilon = 9300$ M^{-1} cm^{-1}) [27].

(iii) Manganese peroxidase: the reaction mixture contained ECF and 0.1 mM $MnSO_4$, 0.1 mM H_2O_2, 0.01% (w/v) phenol red, 25 mM lactate, 0.1% (w/v) bovine serum albumin, and 20 mM sodium succinate buffer (pH 4.5), and was incubated at 30 °C for 5 min. The reaction was terminated by adding 80 mM NaOH. The Mn-dependent peroxidase activity was assessed by measuring the absorbance at 600 nm ($\varepsilon = 4460$ M^{-1} cm^{-1}) [28]. One unit of MnP activity was expressed as that amount of enzyme that catalyzes the oxidation of 1.0 μmol of phenol red per minute.

(iv) Methyl alcohol oxidase: the reaction mixture contained ECF and 100 mM methanol, and 50 mM citrate-phosphate buffer (pH 3.0), and was incubated at 25 °C for 15 min. Thereafter, this was followed by the addition of 0.02 M acetylacetone (2,4-pentanedione in 2 M ammonium acetate and 0.05 M acetic acid), and the contents were incubated at 60 °C for 15 min [29]. Enzymatic oxidation of methanol releases equivalent amounts of formaldehyde (HCHO) and H_2O_2 as the end products. Approximately, neutral solutions of acetylacetone and the ammonium salt were allowed to react with HCHO, which gradually developed a yellow color owing to the synthesis of 3,5-diacetyl-1,4-dihydrolutidine that can be measured spectrophotometrically at 412 nm. AOX activity was estimated from methanol oxidized to release 1 μmol equivalent of formaldehyde estimated from a calibration curve (1–10 μmol/mL).

(v) Cellobiose dehydrogenase: the reaction mixture contained ECF and 100 μM 2,6-dichlorophenolindophenol (DCPIP), 20 mM citrate-phosphate buffer (pH 6.4), 2.5 mM cellobiose, and 20 mM glucono-1,5-lactone (added to inhibit any β-glucosidase activity that would degrade cellobiose [30]), and was incubated at 30 °C. Enzyme activity was measured as the decrease in the absorbance of DCPIP at 600 nm ($\varepsilon = 21$ M^{-1} cm^{-1}) over a 5-min interval. The unit of CDH activity was defined as the amount of enzyme that reduced 1 μmol of DCPIP per min.

(vi) Versatile peroxidase: the reaction mixture contained ECF and 100 mM $MnSO_4$, 100 mM tartrate (pH 5.0), and 100 mM H_2O_2, and was incubated at 30 °C for 10 min. The versatile-peroxidase catalyzed oxidation of Mn^{2+} was assessed by measuring the formation of the Mn^{3+}-tartrate complex ($\varepsilon = 6.5$ mM^{-1} cm^{-1}) by monitoring the production of the Mn^{3+} chelate at 238 nm [31]. One unit of VP activity was defined as the amount of enzyme necessary for the formation of 1 μmol of Mn^{3+} chelate per minute.

(vii) Veratryl alcohol oxidase: the reaction mixture contained ECF and 4 mM veratryl alcohol as the substrate, and 50 mM sodium phosphate buffer (pH 6.0), and was incubated at 30 °C for 5 min. The enzyme activity was assessed by measuring the increase in the absorbance at 310 nm ($\varepsilon = 9300$ M^{-1} cm^{-1}) [32]. One unit of VAO activity was defined as the amount of enzyme necessary to oxidize 1 μmol veratryl alcohol to veratraldehyde per minute.

2.4 SIFT-MS measurement of methanol generated during the lignin demethylation process

The fungal isolates with the ability to decolorize KL were considered ligninolytic with the potential to demethylate KL. Thirty-one ligninolytic fungi were screened for their ability to

release methanol during their growth on KL. The VMSM media were prepared containing 0.3% KL as the substrate (pH 5.8), and the media sterilized at 121 °C for 20 min. Five agar discs cut out from PDA-grown cultures of the fungi were inoculated into the liquid media in 250-mL Erlenmeyer flasks and left stationary at 28 °C for 7 days (Fig. 2). After this time, gaseous methanol released in the headspace of the flasks of the growing fungal cultures was detected by selected-ion

a

b

Catechol-titanium (III) nitrilotriacetate (Ti (III)–NTA) complex

c
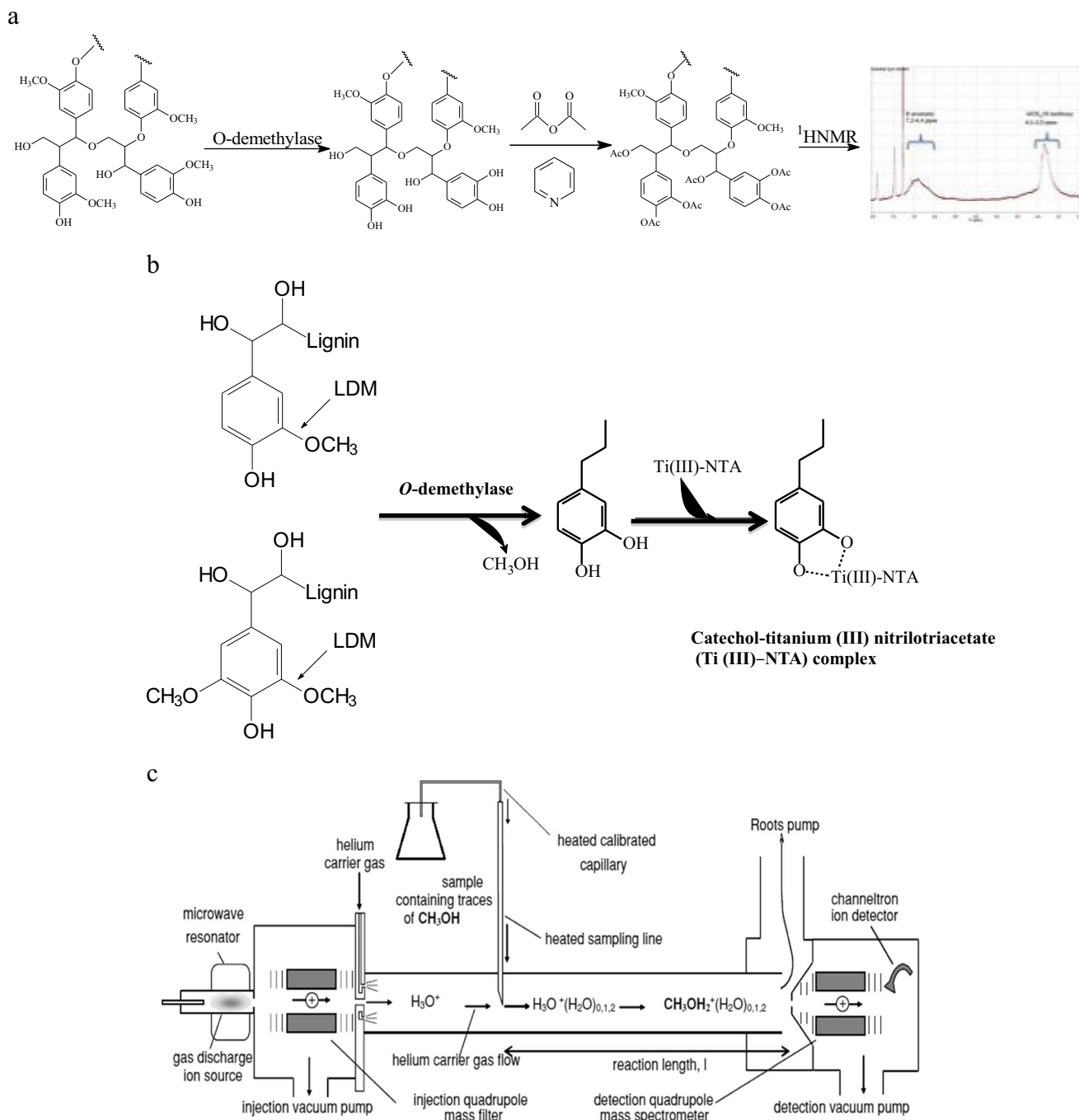
Fig. 2 Kraft lignin demethylation mediated by an *O*-demethylase such as vanillate-*O*-demethylases and syringate-*O*-demethylases. **a** The demethylated lignin was *O*-acetylated and this allowed the detection by 1H-NMR spectral analysis of the difference in the methoxyl groups in the unmodified and modified lignins. **b** Determination of lignin demethylation activity via the Ti(III)-nitrilotriacetic acid assay approach by measuring the pyrocatechol formed following demethylation [9]. **c**

Schematic illustration of the selected-ion flow-tube mass spectrometer (SIFT-MS) and its operation in the determination of methanol released from demethylated lignin model compounds and Kraft lignin. Enzymatically released methanol reacts rapidly with H3O+ precursors, and the reaction CH3OH+ (H3O+ + CH3OH → CH3OH2+ + H2O) is used to quantify methanol by SIFT-MS at the ppb level

flow-tube mass spectrometry (SIFT-MS). The results are expressed as parts per billion (ppb) of methanol released.

2.5 Biological and enzymatic demethylation of Kraft lignin

Three ligninolytic fungi demonstrated to have superior demethylating activity as evidenced by the release of methanol (SIFT-MS results) when grown on KL which were chosen from among the 450-screened fungal isolates, and used to determine their ability to demethylate lignin. They included two brown-rot fungi, i.e., *Fomitopsis pinicola* (Sw.:Fr.) P. Karst and *Galerina autumnalis* (Batsch) Buhner (1935), and the mitosporic fungus, i.e., *Aspergillus* sp.3 BRI 270. The lignin-degrading fungi were inoculated into VMSM media containing 0.3% KL and left to grow at 28 °C under shaking conditions (180 rpm) for 7 days. Thereafter, the spent culture media (ECF) were separated from the fungal pellet (mycelium) by centrifugation at 2000×*g* for 20 min, and used as the source of enzyme in the enzymatic demethylation experiments.

The lignin demethylation products formed by the enzyme-catalyzed reaction (*O*-demethylases) specifically strip off the *O*-methyl/methoxyl groups from the phenyl-propanoid units of KL releasing methanol as the end product, and converts lignin into a pyrocatechol-enriched product (Fig. 2). In order to quantitate lignin demethylation, the following procedures were conducted on the modified KLs recovered: pyrocatechol content was determined by (i) *O*-acetylation of the lignin followed by ^{1}H-NMR analysis, and (ii) vicinal diol detection spectrophotometrically using the Ti(III)–nitrilotriacetic acid (Ti(III)-NTA) method [9].

2.6 Determination of lignin demethylation from the methoxyl content by ^{1}H NMR analysis

Biologically demethylated lignin samples from the culture filtrates containing the dissolved lignin and the undissolved lignin (~ 2 g) were oven-dried at 45 °C for 24 h. The dried demethylated lignin sample (2 g) was dissolved in pyridine (21 mL) and was centrifuged to separate it from the residual fungal mycelium [33]. Next, 2 volumes of pyridine solution were acetylated with 1 volume of acetic anhydride by stirring at room temperature for 24 h. Pyridine was then removed by acidification with 50% HCl (pH 3.0), and this precipitated out the *O*-acetylated lignin at the same time. The precipitates were collected by centrifugation at 4000×*g* for 10 min, and the supernatants discarded. The pellets recovered were washed with distilled water 5 times, followed by re-centrifugation (4000×*g* for 10 min). The pellets were then freeze-dried at − 50 °C for 24 h. The *O*-acetylated lignins prepared were dissolved in a small volume of deuterated chloroform and were analyzed by ^{1}H NMR that allowed the measurement of the extent of demethylation of lignin.

^{1}H NMR spectra were obtained at 298 K on an INOVA-500 Varian-NMR spectrometer operated at a ^{1}H frequency of 500.13 MHz, and were equipped with a 5-mm broadband inverse probe under the following conditions: 2.0-s pulse delay, 30° pulse angle, 15-ppm sweep width, 16 scans, a time domain of 32 K, 16 acquisition transients, and 0.3-Hz line broadening. Tetramethylsilane served as the internal standard. The changes in the amount of demethylated lignin converted into *O*-acetylated lignins were calculated from the ^{1}H NMR spectra [33]. The spectra showed the differences between the syringyl proton signals (6.28 ppm and 6.80 ppm) and the guaiacyl proton signals (6.80 ppm and 8.00 ppm). Also, the theoretical ratios between aromatic and methoxyl protons of guaiacyl and syringyl were 1.00 ppm and 0.33 ppm, respectively. These ratios can actually be measured from the ^{1}H NMR spectra of the *O*-acetylated lignins.

The percentage of lignin demethylation was calculated by the difference between the signals from the aromatic H (6.40–7.10 ppm) and the methoxyl protons (4.1–3.50 ppm) by applying the formula:

$$x = \text{H (aromatic)}/\text{H (methoxyl)}$$

Statistical linear regression analysis produced an angular coefficient $b = 19.750047$ and a linear coefficient $a = 28.28436$.

The following formula was applied to calculate the % lignin demethylation:

$$\%(\text{methoxyl}) = 28.28436 - 19.750047x.$$

The above equation is based on the linear regression of data obtained from a number of different types of lignins [33]. The degree of demethylated lignin (%) obtained following treatment with *Fomitopsis pinicola* (Sw.:Fr.) P. Karst, *Galerina autumnalis* (Batsch) Kühner (1935), and *Aspergillus* sp.3 BRI 270 was quantified from the values obtained from the ^{1}H NMR spectra by applying the above formula. The methoxyl content of the *O*-acetylated starting material (KL) was calculated to be 15.46%.

2.7 Determination of enzymatic demethylation of LMCs and Kraft lignin by the Ti(III)-NTA colorimetric assay

The pyrocatechol (vicinal diols) content of the modified LMCs and KL as a consequence of the enzymatic *O*-demethylation process was determined by the Ti(III)-NTA method [9]. This assay allowed the direct detection of the pyrocatecholic

hydroxyls formed by measuring the formation of a yellow complex between Ti(III)-NTA and the vicinal diols (Fig. 2). The Ti(III)-NTA reagent was prepared as follows: 30 mL of water was sparged for 10 min under nitrogen gas; then, 0.96 g of nitrilotriacetic acid (NTA) was added to the water and the mixture was stirred with a glass rod. The pH of the solution was adjusted to pH 9.0 with 4 M NaOH prior to adding Ti(III)–Cl (the solution does not have to be dissolved before adjusting pH, for it will help dissolve NTA). Then, 1.344 mL of 12% Ti(III)–Cl solution was added dropwise while keeping the pH above pH 2.0 using a saturated solution of $NaCO_3$. The pH was adjusted to 7.0 with $NaCO_3$ after all the Ti(III)–Cl was added.

To initiate the enzymatic O-demethylation reaction, 100 μL of enzyme was added to 50 μL of the substrate (1 mM LMC or 1% (w/v) Kraft lignin) in 50 mM citrate-phosphate buffer (pH 3.0 and 6.0) or 50 mM Tris-HCl buffer (pH 8.0), and left for 30 min at 28 °C. Thereafter, to this was added 100 μL of Ti(III)-NTA, 150 μL of 50 mM Tris-HCl buffer (pH 8.0), and water to a final volume of 1.0 mL. The intensity of the yellow color complex was determined spectrophotometrically at 380 nm [9]. The O-demethylation activity was expressed as 1 μM of the vicinal diols liberated from the substrate per minute. The quantity of the liberated vicinal diols was determined from a standard protocatechuic acid curve.

2.8 Characterization of demethylated Kraft lignin by Fourier-transform infrared spectroscopy

The FT-IR spectra of both the unmodified and demethylated Kraft lignins were recorded on a Bruker Tensor 37 FT-IR spectrometer using potassium bromide pellets. The pellets were prepared from a mixture of 200 mg of potassium bromide, 3 mg of the lignin samples, and 1.5 mg potassium ferricyanide added as the internal standard. The acquisition conditions were spectral width from 4000 to 500 cm^{-1}, 32 accumulations, and 4 cm^{-1} resolution.

2.9 Synthesis of lignin-based formaldehyde resin using enzymatically produced demethylated Kraft lignin

The enzymatic demethylation process was employed to improve the reactivity of Kraft lignin by increasing the pyrocatecholic content. Accordingly, the generation of demethylated lignin with abundant vicinal hydroxyl groups rose considerably when treated with the crude enzyme preparation from *Aspergillus* sp.3 BRI 270. A lignin-based phenol-formaldehyde resin was prepared using the demethylated KL to substitute for phenol. A molar ratio of vicinal diol content in the demethylated lignin of 30.1% (measured by ^{1}H NMR analysis of the O-acetylated lignin samples) to formaldehyde of 1:2 was employed to synthesize the formaldehyde resin.

For that to happen, the enzymatically demethylated lignin (50% by weight) was dissolved in 1 M NaOH (5 mL) and then added to a solution of 37% formaldehyde (10 mL; v/v) in a three-neck glass flask maintained at a temperature of 110 °C under stirring conditions for 3 h. The method to prepare the lignin-based formaldehyde resin was based on that outlined by Ferhan et al. [11] and Song et al. [34].

3 Results and discussion

The primary objective of this study was to demethylate Kraft lignin by means of enzymes, and to explore the appropriate methods to demonstrate whether there were any new enzyme(s) hitherto undescribed in the scientific literature that were specifically associated with the lignin demethylation process (lignin-specific kind of O-demethylase), similar to those described for vanillate-O-demethylases and syringate O-demethylases. Such enzymes cleave off the O-methyl/methoxyl groups from the benzyl rings comprising lignin, releasing methanol and making the modified lignin more phenolic in character (pyrocatecholic), so that it can serve as a phenol substitute for the synthesis of phenol-formaldehyde polymers (Fig. 1).

3.1 Screening wood-rotting fungi for exocellular-produced ligninolytic enzymes

The screening of 450 fungal species and strains from the Mycological Herbarium (LUCC) at Lakehead University was undertaken to assess their ability to grow on lignin as a sole carbon source. In addition, some 300 wood-rot fungal specimens (BRICC) collected from the Boreal Forest ecosystem (mushrooms, bracket fungi, and from decaying wood) were isolated on agar medium (PDA) at 25 °C, and were also used in the screening program. Initially, the 450 LUCC fungal isolates were assessed and identified for their ability to grow on Kraft lignin. These isolates were maintained both on PDA and on malt extract agar media.

The fungal isolates were examined for their growth on liquid media containing VMSM, KL, and glucose under stationary conditions at 25 °C for 1 month. Glucose was added to the nutrient media to stimulate mycelial growth to increase the chances of the fungi producing the right complement of enzymes to modify the KL. The cultures were regularly monitored for growth advancement (biomass), as well as changes in the color of the media. Some fungal specimens actually showed the ability to darken the KL medium to a reddish-brown coloration that was most likely due to the degradation products released into the media during growth. KL itself has a brownish coloration. Those isolates that decolorized KL strongly were then transferred to liquid media containing the same amount of KL, but a reduced glucose content (0.05%),

and left undisturbed at 25 °C. The changes in decolorization were monitored side by side. The best fungal isolates were identified and were chosen for further growth on liquid media (VMSM) containing only the KL. In this manner, some 100 fungal isolates were identified as capable of growing solely on KL as the carbon source. These fungi were then transferred to agar media to measure the radial growth (diameter) of the advancing mycelium. Several of these fungi grew poorly taking longer than 20 days to attain maximum colony diameter, but could decolorize the KL fairly well, while some others grew rapidly but decolorized poorly.

Taking these variables into account, only 31 fungi were selected for further study to determine the secretions of ligninolytic enzyme into liquid media, and their ability of demethylating lignin (Table 1). The 31 best fungal isolates, as judged from the extent of decolorization and growth, under the conditions in which they were cultivated in the liquid media (stationary) on KL, were maintained on KL-VMSM agar slants and stored at 4 °C [1, 4].

3.1.1 Screening for ligninolytic enzymes that catalyze lignin demethylation and modify Kraft lignin

Each of the 31 fungal isolates was cultured on liquid media containing KL and VMSM for 21 days under stationary conditions, and the cell-free ECF was recovered (i) to detect methanol released from lignin demethylation, and (ii) to determine the activities of various ligninolytic enzymes that included laccase, lignin peroxidase, manganese peroxidase, methyl alcohol oxidase, cellobiose dehydrogenase, versatile peroxidase, and veratryl alcohol oxidase.

To confirm that methanol was indeed produced during fungal growth on KL and the lignin-like model compounds tested, methanol released inside the Erlenmeyer flasks was measured by a highly sensitive method at the *parts per billion* level employing SIFT-MS. SIFT-MS analyzes methanol in the head space of the flasks containing the growing fungal cultures. Methanol at levels of 40–180 ppb was observed to have been produced by these fungal isolates, and among these producing the highest parts per billion of methanol were *Sporobolomyces roseus* LU 29, *Ctenomyces serratus* LU 122; *Cunninghamella* sp.2 BRI 139, *Penicillium* sp.1 BRI 259B2, and *Aspergillus* sp.3 BRI 270. Several other fungi among the 31 identified could grow on KL as the sole C source but produced methanol at varying levels of < 50 ppb (Table 1). Sometimes, the levels of methanol in the flasks varied, usually more so after 12-day growth, and it was presumed that this was perhaps due to the methanol liberated during demethylation being consumed by the growing fungi for their metabolic needs. This finding was later corroborated by evidence [29] that some of the fungal isolates evaluated were found to produce the enzyme, alcohol oxidase, with methanol as the substrate.

Wood-rot fungi are known to degrade the wood components, cellulose, hemicellulose, and lignin. Various species are known to be capable of doing so and among them are *Phanerochaete chrysosporium* (white-rot) and *Fomitopsis palustris* (brown-rot). These wood-rot fungi secrete a wide complement of ligninolytic enzymes that can catalyze the generation of the hydroxyl radicals that bring about certain modifications within the lignin subunits, including side-chain oxidation, hydroxylation, demethylation, and depolymerization [1–7]. Four major types of extracellular ligninolytic enzymes are known to be involved in lignin modifications, namely laccase, lignin peroxidase, manganese peroxidase, and versatile peroxidase.

In this work, the fungal isolates cultured on KL were screened for the aforementioned ligninolytic enzymes, besides enzymes capable of demethylating lignin that are induced by Kraft lignin. The enzyme laccase was produced by each of the 31 lignin-modifying isolates. Maximum laccase activity was observed with *Ctenomyces serratus* LU 122, *Aspergillus* sp.1 BRI 122, non-sporulating 3 BRI 215B, *Fusarium* sp. BRI 247A, and *Aspergillus* sp.3 BRI 270 (Table 1). Similarly, *Trametes versicolor*, which is able to grow on the waste by-product of lignin from the pulp and paper industry, has been reported to produce a maximum laccase activity of 1240 U/L [35]. Furthermore, laccase activity was enhanced by the combined action of certain inducers, like xylidine, and the presence of glucose also resulted in high laccase activity (1583 U/mL) [1, 35]. Longer incubation periods appeared to have affected the enzyme activity. Significantly high laccase activities were observed in white-rot fungi such as *Coriolus versicolor* (797 U/L) and *Trametes gallica* (1154 U/L) than in other tested fungal isolates [36, 37]. But in some other studies with the same fungal strains, it was observed that the tested fungi with the paper mill sludge did not induce significant laccase activities (< 50 U/L) even after a 25-day growth period [37]. Laccases from *Trametes cingulata* have been reported to mediate the consecutive polymerization and depolymerization of Kraft lignin [38]. Furthermore, thermal carbon analysis and thermal desorption-pyrolysis-gas chromatography-mass spectrometry analysis confirmed that fungal laccases are involved in the polymerization process [36]. Purified laccases have also been reported capable of demethylating lignin producing methanol [37]. Definitive proof of their involvement in demethylation of lignin was established using Kraft pulp by *Trametes versicolor* laccase in the presence of the mediator ABTS [39, 40].

Manganese peroxidase is known to catalyze the oxidative depolymerization of lignin. This process is believed to occur at the carbon-bridging position in the recalcitrant phenolic aryl-glycerol β-aryl ether linkage and the diarylpropane motifs of lignin, and on lignin model compounds. Attack occurs at the site of the methyl and methylene groups, situated at the *para*-position of the phenolic hydroxyl groups, and is

Table 1 Screening of wood-decay fungi from the Boreal Forest of Canada for ligninolytic enzyme production, and to determine the lignin demethylation abilities

Lignin-demethylating fungi	Ligninolytic enzyme activity (U/L)							
	Methanol content* (ppb)	Laccase	Manganese peroxidase	Versatile peroxidase	Veratryl alcohol oxidase	Lignin peroxidase	Cellobiose dehydrogenase	Alcohol oxidase
Ascodesmis microscopia LU 131	57 ± 9.0	2.7 ± 0.05	ND[#]	0.248 ± 0.02	213 ± 0.06	32 ± 0.71	ND	4.8 ± 0.04
Aspergillus sp.1 BRI 122	30 ± 3.8	330 ± 0.42	ND	0.0309 ± 0.03	289 ± 1.2	575 ± 0.34	ND	9.4 ± 0.06
Aspergillus sp.2 BRI 261B	21 ± 2.7	0.76 ± 0.07	ND	0.225 ± 0.6	132 ± 0.03	330 ± 0.07	ND	2.29 ± 0.01
Aspergillus sp.3 BRI 270	180 ± 3.1	0.1 ± 0.41	0.194 ± 0.02	0.226 ± 0.05	12.5 ± 1.0	26.9 ± 0.09	ND	2.09 ± 0.01
Aspergillus sp.4 BRI 271	19.6 ± 1.3	0.18 ± 0.2	ND	0.339 ± 0.02	35 ± 0.22	68 ± 0.1	60 ± 0.07	1.3 ± 0.02
Aspergillus sp.5 BRI 273	22 ± 13.5	0.3 ± 0.03	ND	0.243 ± 0.06	293 ± 0.42	29 ± 0.21	8.7 ± 0.05	2.07 ± 0.07
Bacillus sp. BRI 141	79 ± 4.3	0.94 ± 0.22	ND	0.331 ± 0.07	46 ± 0.04	769 ± 0.05	ND	1.28 ± 0.07
Ctenomyces serratus LU 122	125 ± 1.8	660 ± 0.45	ND	0.373 ± 0.04	183 ± 0.9	172 ± 0. 31	ND	39 ± 0.06
Cunninghamella sp.1 BRI 138	21 ± 2.6	0.4 ± 0.03	ND	0.235 ± 0.04	4.9 ± 0.08	260 ± 0.86	ND	1.96 ± 0.02
Cunninghamella sp.2 BRI 139	103 ± 2.0	0.75 ± 0.07	ND	0.265 ± 0.03	108 ± 0.5	146 ± 0.03	3.3 ± 0.05	1.56 ± 0.05
Cylindrocladium camelliae LU 120	49 ± 1.7	0.2 ± 0.22	ND	2.62 ± 0.01	48 ± 0.02	76 ± 0.52	ND	1 ± 0.01
Entoloma sp. LU 89	59 ± 2.4	2.7 ± 0.03	0.021 ± 0.02	0.154 ± 0.04	159 ± 0.43	139 ± 0.22	3 ± 0.2	7.91 ± 0.03
Epicoccum purpurascens LU 33	68 ± 1.4	2.6 ± 0.02	0.0395 ± 0.045	0.53 ± 0.03	159 ± 0.22	3.9 ± 0.71	2.2 ± 0.01	1.37 ± 0.08
Fusarium sp. BRI 247A	13.4 ± 0.8	1.4 ± 0.1	ND	0.205 ± 0.03	291 ± 0.4	364 ± 0.45	0.85 ± 0.09	3.67 ± 0.07
Galerina autumnalis LU 86	84 ± 1.7	0.86 ± 0.61	0.372 ± 0.12	0.409 ± 0.06	97 ± 1.7	59 ± 0.45	ND	2.27 ± 0.02
Gliocladium catenulatum LU 111	68 ± 1.4	0.012 ± 0.06	0.162 ± 0.07	0.157 ± 0.08	207 ± 1.5	351 ± 0.7	5.3 ± 0.8	2.76 ± 0.02
Gliocladium roseum LU 08	91 ± 1.8	0.94 ± 0.025	0.00031 ± 0.01	0.356 ± 0.03	145 ± 0.32	58 ± 0.11	123 ± 0.25	2.33 ± 0.03
Gliocladium roseum LU 25	48 ± 8.0	0.49 ± 0.01	ND	0.213 ± 0.021	151 ± 0.74	164 ± 0.32	0.057 ± 0.09	1.12 ± 0.09
Gliocladium viride LU 124	87 ± 1.69	0.76 ± 0.34	ND	0.288 ± 0.06	1.3 ± 0.02	196 ± 0.63	0.8 ± 0.02	2.27 ± 0.01
Mucor sp. BRI 178	60 ± 9.9	0.83 ± 0.04	0.234 ± 0.01	ND	208 ± 0.08	20 ± 0.09	2.4 ± 0.02	1.67 ± 0.02
Non-sporulating 1 BRI 162	74 ± 1.5	0.94 ± 0.51	ND	0.29 ± 0.02	622 ± 0.07	353 ± 0.08	0.7 ± 0.04	1.35 ± 0.3
Non-sporulating 2 BRI 198	62 ± 1.2	0.11 ± 0.06	ND	0.21 ± 0.1	0.015 ± 0.7	144 ± 0.22	ND	0.872 ± 0.9
Non-sporulating 3 BRI 215B	36 ± 4.6	1 ± 0.03	ND	0.506 ± 0.01	42 ± 0.66	347 ± 0.19	ND	1.64 ± 0.01
Non-sporulating 4 BRI 236B	24 ± 3.8	0.68 ± 0.06	ND	0.292 ± 0.06	547 ± 2.1	354 ± 0.90	ND	10.9 ± 0.04
Non-sporulating 5 BRI 249	24 ± 2.8	0.17 ± 0.02	ND	0.237 ± 0.02	161 ± 0.08	79 ± 0.37	4.9 ± 0.3	9.25 ± 0.03
Penicillium sp.1 BRI 259B2	156 ± 3.3	0.35 ± 0.04	0.315 ± 0.03	0.206 ± 0.04	568 ± 1.4	23 ± 0.01	1.8 ± 0.7	2.56 ± 0.09
Penicillium sp.2 BRI 269	112 ± 1.8	0.14 ± 0.03	0.997 ± 0.07	0.289 ± 0.03	118 ± 0.9	418 ± 0.01	0.2 ± 0.01	2.02 ± 0.10
Penicillium thomii LU 32	63 ± 6.4	0.74 ± 0.12	ND	0.291 ± 0.04	159.8 ± 1.03	271 ± 0. 03	4.1 ± 0.05	1.14 ± 0.01
Sporobolomyces roseus LU 29	118 ± 2.3	0.93 ± 0.03	0.00038 ± 0.03	0.22 ± 0.08	40 ± 0.05	71 ± 0.46	2.3 ± 0.03	1.36 ± 0.06

*Measured by SIFT-MS, [#] ND—not detected

accompanied by the reduction of molecular O_2 or H_2O_2 to water [41–43]. Although Mn^{3+} serves as a natural redox mediator in the enzymatic degradation systems, it allows a wide range of substrate specificities for the MnP enzyme. MnP readily acts on the non-phenolic compounds that constitutes from 80 to 90% of lignin at various positions [44]. Among the ligninolytic enzymes, manganese peroxidase plays a key role in the lignin modifications. The KL-induced manganese peroxidase enzyme was investigated with the 31 fungal isolates chosen, where the maximum activity was observed to occur in the case of *Penicillium* sp.2 BRI 269 (0.997 ± 0.07 U/L) and *Penicillium* sp.1 BRI 259B2 **(**0.315 ± 0.03 U/L**)** (Table 1). MnP, like the laccase from *Trametes versicolor*, was also capable of generating methanol during delignification of Kraft pulp [39, 40]. In another study, the MnP activity in four fungal strains tested, among them *C. versicolor*, resulted in maximum MnP activity of 1.455 U/L activity occurring after a 22-day incubation period. *T. gallica* displayed MnP activity of 0.937 U/L.

Versatile peroxidases exhibit bifunctional catalytic properties common to MnP and VP, both of which can oxidize Mn^{2+} to Mn^{3+}, and oxidize aromatic compounds including veratryl alcohol [45–48]. The *P. chrysosporium* versatile peroxidase structure more closely resembles that of the LiP enzyme structure than that of the structure of MnP [47]. In our work, KL-induced versatile peroxidase activity of 2.62 ± 0.01 U/L as observed for *Cylindrocladium camelliae* LU 120 (Table 1). But all the other ligninolytic fungi tested negative by the versatile peroxidase assay. Several wood-degrading fungi, e.g., *Pleurotus* sp., *Bjerkandera* sp., *Pleurotus eryngii*, *P. ostreatus*, *Bjerkandera adusta*, *Bjerkandera fumosa*, and *Pleurotus pulmonarius*, are known as potential producers of versatile peroxidases [46–48].

Maximum veratryl alcohol oxidase activity was observed in the following isolates: non-sporulating 1 BRI 162, non-sporulating 4 BRI 236B, and a *Penicillium* sp.1 BRI 259B2 (Table 1). However, all the other ligninolytic fungi tested negative for the production of veratryl alcohol oxidase.

Lignin peroxidase has a high redox potential, along with a low optimum pH, and is also known to oxidize various phenolic compounds to their respective phenoxy radicals. This enzyme also mediates the oxidation of a wide range of the non-phenolic aromatic substrates, which are not oxidized by the other peroxidases [20, 46], a feature attributable to its high redox potential. During the course of the lignin degradation process, H_2O_2 generated by accessory enzymes (e.g., glyoxal oxidases) acts as a co-substrate for the activity of LiP and oxidizes a variety of lignin compounds [20, 46]. Maximum lignin peroxidase activity was observed in *Gliocladium catenulatum* LU 111, *Gliocladium viride* LU 124, *Aspergillus* sp.1 BRI 122, *Bacillus* sp. BRI 141, and *Penicillium* sp.2 BRI 269 (Table 1). Several wood-rot fungi, such as *P. chrysosporium*, *T. versicolor*, and *Perenniporia medulla-panis*, and some of

the bacteria such as *Acinetobacter calcoaceticus* NCIM 2890 and *Streptomyces viridosporus* T7A, are able to secrete LiP [48]. However, in some recent studies, it was observed that no LiP activity takes place in *C. versicolor*, *T. gallica*, *Mycobacterium* sp., and *Streptomyces* sp., but that high laccase and MnP activities did occur. A similar pattern was observed with the various white-rot fungi acting on paper mill sludge and a lignocellulosic biomass [36, 37].

Cellobiose dehydrogenase oxidizes cellobiose and other disaccharides (e.g., lactose), as well as oligosaccharides possessing β-1,4 linkages [30, 49]. In the wood-rot fungi, the CDH enzyme has been found in association with a cellulose-binding module (CBM). Even in the absence of a CBM, the CDH enzyme still mediates hydrophobic interactions to bind to the cellulose molecules [50]. In another interesting study, some fungi displayed enhanced CDH secretion, and this helped other enzymes such as cellulases and hemicellulases to act under cellulolytic conditions [51, 52]. It is widely accepted that CDH acts by generating hydroxyl radicals by means of the Fenton-type reaction, which mediates the degradation and modification processes of some major components of lignocellulosic residues, like cellulose, hemicelluloses, and lignin [6–8]. Among the various fungal cultures tested, the maximum CDH activity obtained was with *Gliocladium roseum* LU 08 and *Aspergillus* sp.3 BRI 270 (Table 1). All the other ligninolytic fungi tested negative for CDH activity.

In the present study, AOX activity, as induced by KL, has been determined in the 31 potential demethylating fungal isolates that were identified above. The maximum AOX activity was obtained with the following isolates: *Ctenomyces serratus* LU 122, non-sporulating 3 BRI 215B, and the non-sporulating 4 BRI 236B (Table 1). All the other ligninolytic fungi tested failed to secrete the AOX enzyme. There are several reports that the brown-rot fungi operate the Fenton reaction. *G. trabeum* secretes a higher level of alcohol oxidase that is induced by methanol produced during the lignin demethylation process, and the enzyme oxidizes methanol giving rise to formaldehyde and H_2O_2, which together play a vital role in the lignin decay process [8, 29, 53]. Hardwood lignin decayed by brown-rot fungi indicated that some chemical changes do take place, as observed in the NMR spectra, and this led to the conclusion that the demethylation process of syringyl units occurred by the involvement of hydroxyl free radicals generated from H_2O_2, as the production of hydrogen peroxide is of common occurrence in brown-rot fungi. As the brown-rot fungi secrete the enzyme methanol oxidase, this enzyme catalyzes methanol oxidation reactions and produces H_2O_2, besides generating the hydroxyl free radicals via the Fenton reaction as driven by iron (Fe^{2+}) and hydroquinone through a redox cycling process [53–55].

The first ever report on the fungal genome sequence of a brown-rot fungus confirmed that the degradation of cellulose

and the lignin demethylation processes occur as a pivotal step in the degradation of lignocellulose, since methanol was released [6–8, 54–59]. Also, secretome and transcriptomic studies carried out on the brown-rot genome have indicated that the methanol oxidase genes were overexpressed [8, 54–59]. The hydroxyl free radicals are known to mediate oxidative depolymerization of cellulose, and at the same time, these radicals promote a partial demethylation of lignin, a reaction that has been proven by ^{13}C-TMAH thermochemolysis analysis [53, 55]. Interestingly, the overexpression of methanol oxidase is also of common occurrence in the lignin degradation process of the white-rot fungus, *P. chrysosporium*, that could have been mediated by glyoxal oxidase and the methanol oxidases. These accessory enzymes generate H_2O_2 by acting on the lignocellulosic substrates, and at the same time demethylation occurs as a common side reaction after the one-electron oxidation event of the lignin units to their respective cation radicals by the white-rot fungi and their ligninolytic peroxidases [5, 53–60].

A similar process is believed to occur in the brown-rot fungi, since the expression of the aryl-alcohol oxidase has been found to occur in *Phormosoma placenta*, in addition to the main methanol oxidases [54, 58, 59]. This probably suggests that the white-rot and brown-rot fungi mediate the oxidation processes of the substrates, and secondly, that it is the hydroxyl radical generation that mediates the demethylation process of lignin. At the same time, the increased formation of 3-methylcatechol-type compounds detected by analysis of pyrolysis-gas chromatography/mass spectrometry, and the detection of the related diagnostic compounds after pyrolysis/tetrabutylammonium hydroxide, clearly suggests that the demethylation event occurs in the brown-rot lignin degradation process. These findings are consistent with those of [54, 59, 60] and [8]. Further, certain radiolabeling studies revealed that several oxidases are involved in the formation of H_2O_2 by *P. chrysosporium*, as was demonstrated by employing various lignocellulosic substrates [61].

3.2 Kraft lignin demethylation

Lignin demethylation is a rather unusual phenomenon that occurs during the lignin degradation process, and only a few microbes are known to demethylate lignin and produce methanol and pyrocatecholic structures enriched in vicinal hydroxyl groups as the end products (Fig. 1). However, this phenomenon has not been well studied with the fungi as compared to bacteria. More recent literature findings offer some evidence as to the existence of the lignin demethylation phenomenon, and the demethylating enzymes (*O*-demethylases) in both bacteria and fungi [1–8]. The white-rot and the brown-rot fungi have been observed since 1929 to be capable of removing the *O*-methyl/methoxyl groups from lignin (demethylation) [2, 3, 7, 8, 39, 40], thus providing evidence for the existence of specific fungal

enzymes that are involved in catalyzing the cleavage event of the *O*-methyl/methoxyl groups that release methanol and generate the pyrocatecholic structures in the modified lignin (Fig. 2).

Enzymes of this nature (lignin-specific *O*-demethylases) like those reported for LMCs, such as vanillate-*O*-demethylases and syringate *O*-demethylases, are relatively new and have not yet been adequately described in the literature, and may probably have the potential for demethylating lignins in vitro producing modified lignins with applications as biopolymers (Figs. 1 and 2). The wood-decay fungi collected from the Boreal Forests of the northwestern region of Ontario, Canada, were studied for their potential to remove the *O*-methyl groups from softwood KL and OrganoSolv lignins (OL, poplar, willow, wheat straw, and mixed agricultural wastes). Removal of the *O*-methyl groups from these lignin samples liberated methanol, and produced a lignin enriched in the vicinal hydroxyl groups (high phenolic content; pyrocatecholic).

Following an intensive screening program, three fungal isolates, two brown-rot, i.e., *Fomitopsis pinicola* and *Galerina autumnalis*, and the mitosporic *Aspergillus* sp.3 BRI 270, were chosen for studies on the demethylation process of lignin. All 3 isolates were observed to display significant demethylating potential, perhaps by way of utilizing the KL as their sole carbon source.

3.3 ^{1}H-NMR analysis of *O*-acetylated Kraft lignin

In this study, in order to quantify the total phenolic hydroxyl groups for determining the percentage of KL demethylation by *Fomitopsis pinicola*, *Galerina autumnalis*, and *Aspergillus* sp.3 BRI 270, the resulting demethylated KLs produced were *O*-acetylated. This procedure allowed the detection of the two aromatic and methoxyl signals emanating on the ^{1}H NMR spectra, and this was compared with the *O*-acetylated unmodified KL to serve as the control (Table 2, Figs. 3 and 4). The ^{1}H NMR spectral signals emanating from the H aromatic (6.40–7.10 ppm) and the methoxyl protons (4.1–3.50 ppm) allowed the calculation of demethylation as shown in Fig. 3 and Table 2. The percentage lignin demethylation was determined by applying the following formula: $X = (H (Ar)/H (OCH_3))$ [33]. The methoxyl content (–OCH_3) of the *O*-acetylated starting KL material (undemethylated) was calculated from the following formula: % (OCH_3) = 28.28436 – 19.750047x and gave a value of 15.02%.

3.4 Influence of carbohydrate substrates on demethylation of Kraft lignin during fungal growth: ^{1}H-NMR analysis of *O*-acetylated lignin

In some recent studies, it was observed that the degradation of cellulose, hemicellulose, and lignin in lignocellulosic materials by the related enzymes was associated to that of the demethylation of lignin [8, 25, 62, 63]. The influence of the

Table 2 Assignments of signals in the ^{1}H NMR spectrum for *O*-acetylated Kraft lignin and demethylated Kraft lignin

δ (ppm)	Assignment
1.26	Hydrocarbon contaminant
2.01	Aliphatic acetate
2.28	Aromatic acetate
2.62	Benzylic protons in β-β structures
4.1–3.50	Protons in methoxyl groups
4.27	Hγ in several structures
4.39	Hγ primarily, in β-O-4 structures and β-5 structures
4.65	Hβ in β-O-4 structures
~4.80	Inflection possibly due to Hα in pinoresinol units and Hβ in noncyclic benzyl aryl ethers
5.49	Hα in β-5 structures
6.06	Hα in β-O-4 structures (Hα in β-1 structures)
6.93	Aromatic protons (certain vinyl protons)
6.40–7.10	H aromatic
7.29	Chloroform (solvent)
7.41	Aromatic protons in benzaldehyde units and vinyl protons on the carbon atoms adjacent to aromatic rings in cinnamaldehyde units
7.53	Aromatic protons in benzaldehyde units
9.64	Formyl protons in cinnamaldehyde units
9.84	Formyl protons in benzaldehyde units

^{1}H-NMR of *O*-acetylated Kraft lignin samples provides improved spectral resolution of key lignin functionality based on studies of extensive model compounds

sugars comprising lignocellulose and some others on the de-methylation capacity of KL was therefore assessed by the three fungal isolates, i.e., *Fomitopsis pinicola*, *Galerina autumnalis*, and *Aspergillus* sp.3 BRI 270. The fungi were grown on KL nutrient media supplemented with various carbohydrate substrates (mono-, di-, and polysaccharides). The influence of these supplements on the demethylation of KL was estimated by ^{1}H NMR spectral analysis. The results are shown in Figs. 4 and 5, and Table 3.

Fomitopsis pinicola displayed the highest –OCH$_3$ (0.99%) removal ability resulting in a demethylation of 6.4% when cultivated in the presence of galactose, followed next by the filter paper cellulose (0.61% –OCH$_3$ removal and 3.9% demethylation; see Table 3). Similarly, *P. cinnabarinus* was reported to metabolize glucose as the carbon source with ease and shifted the reactions to carry out the reduction of veratric acid, while demethylation was more prominent, but cellulose as the carbon source was more rapidly mineralized [63]. In another study with brown-rot fungi (*Postia placenta* and *Gloeophyllum trabeum*) on lignin demethylation, it was observed that a loss of polysaccharide content occurred and that demethylation of lignin resulted in a higher concentration of the *ortho*-dihydroxy containing lignin fragments that were generated by reactive hydroxyl radicals, which further participated in the condensation process [8]. The brown-rot fungi usually undergo the demethylation process by way of the Fenton reaction, or by the action of some enzymes; polysaccharides are

one of the substrates that generate H$_2$O$_2$ or, alternatively, H$_2$O$_2$ can be generated from some of the demethylated products, e.g., methanol by oxidation with alcohol oxidases.

While examining *Galerina autumnalis* for demethylation of KL in the presence of sorbitol (sugar alcohol), the fungus displayed the highest demethylation percentage (11.7% and a 1.81% of –OCH$_3$ removal) and this occurred as a consequence of the fungal demethylation process, as measured by ^{1}H NMR analysis. Further, cellobiose was observed to have induced a 5.8% extent of demethylation (0.89% removal of the –OCH$_3$) see Fig. 4 and Table 3. Both brown-rot fungi displayed a reasonable level of demethylation of lignin (Table 3). These fungi are known to degrade lignocellulosic materials more efficaciously owing to the presence of their hydrolytic enzymatic machinery.

Fungi are known to possess two types of extracellular enzyme systems—the hydrolases and the oxidative enzymes that mediate polysaccharide degradation events producing residual lignin and further opening up the phenyl-propanoid rings. Therefore, the brown-rot fungi with 3-methoxy and the 4-hydroxyl structures of lignin respond to the ferrous iron and the Fenton products of the formed hydroxyl anions and radicals that mediate the demethylation process, thereby yielding methanol. The modified lignin formed acts as a chelator and an electron source for driving the ferric/ferrous conversion [8].

Some observations with the mitosporic fungus, *Aspergillus* sp.3 BRI 270, on the KL demethylation process were also

Fig. 3 1H NMR spectrum of *O*-acetylated Kraft lignin
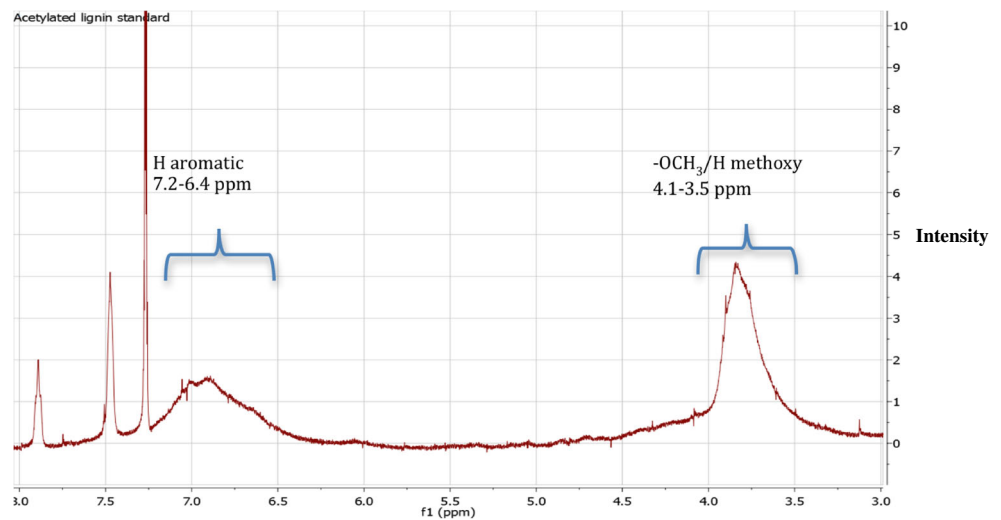
assessed by ^{1}H NMR analysis (Fig. 4). We noted that some fungi grew on the surface of the lignin pellets, among which was *Aspergillus* sp.3 BRI 270 that subsequently exhibited the highest demethylation potential. The influence of carbohydrates on lignin demethylation by this fungal isolate is shown in Table 3. *Aspergillus* sp.3 BRI 270 showed that the highest degree of lignin demethylation (30.1%) occurred when the fungus was grown on KL nutrient media in the presence of a co-substrate, birchwood xylan. Xylan was observed to have influenced the removal (4.66%) of the –OCH$_3$ groups from the KL, as was revealed by ^{1}H NMR analysis (Fig. 4). By contrast, he KL used as control (no carbohydrate supplementation) showed a degree of demethylation of 3.4%. The xylan-mediated demethylation of KL by *Aspergillus* sp.3 BRI 270 displayed a 10.4% content of –OCH$_3$. Next only to maltose, galactose and sorbitol facilitated the demethylation of KL by 15.3, 13.1, and 11.0%, respectively (Fig. 4).

The brown-rot fungi, cultured on KL in the presence of glucose, displayed a lower rate of removal of the *O*-methyl content (0.5%) as observed for *Fomitopsis pinicola*, and 2.8% of the –OCH$_3$ group removal by *Galerina autumnalis.* In another study, in which the influence of glucose was examined using methoxyl-labeled vanillic acid, it was observed that a different pattern of ^{14}CO$_2$ release emerged, giving clear evidence to the operation of glucose-repressed metabolism of the methoxyl group, which gave rise to ^{14}CO$_2$ as a demethylation product. However, methanol and formaldehyde were the other two possible products of the demethoxylation/demethylation process of vanillic acid [64–67]. White-rot and brown-rot fungal decay resulting in syringyl demethylation occurs as a more aggressive process, and demethylation of coniferyl monomers appeared to be more restricted to brown-rot decay [8].

The extent of KL demethylation was observed to be higher when birchwood xylan was used as co-substrate, with the xylan possibly inducing other enzymes that could include the lytic polysaccharide mono-oxygenases (LPMOs). These enzymes might also be involved in the lignin demethylation process, generating the vicinal hydroxyls that can act as the

Fig. 4 1H NMR profiles of demethylated Kraft lignin by *Aspergillus* sp.3 BRI 270 cultivated on Kraft lignin in media containing various carbohydrate supplements ((a) control, (b) cellulose, (c) cellobiose, (d) CMC, (e) fructose, (f) galactose, (g) gluconic acid, (h) glucose, (i) lactose, (j) maltose, (k) sorbitol, (l) sucrose, (m) xylan, (n) xylose)
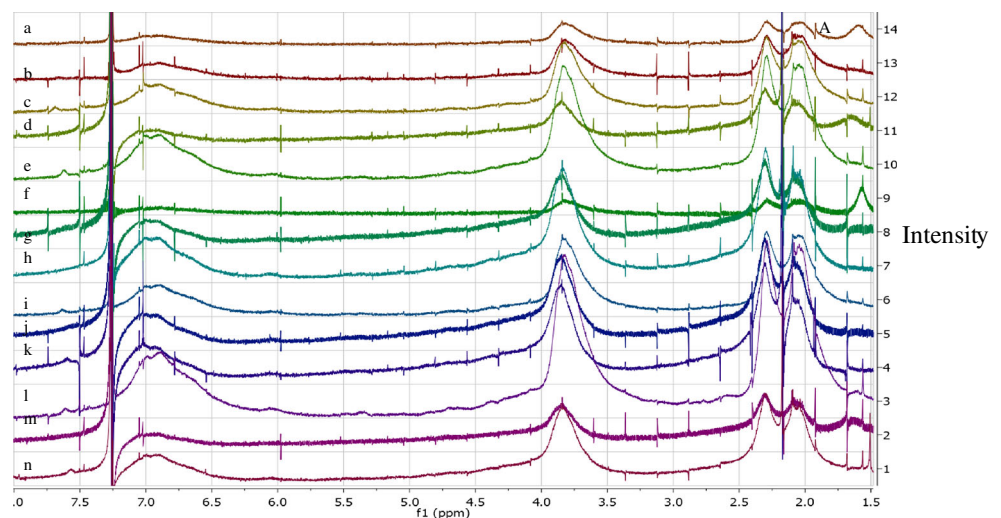
Fig. 5 Different 1H NMR
spectral profiles of lignin
decomposition and demethylation
determined by measuring %
demethylation using the crude
enzyme preparation from
Aspergillus sp.3 BRI 270. **a**
Different lignin controls, and **b**
the corresponding demethylated
lignins therefrom (Poplar
OrganoSolv lignin WIST, ECN
Wheat straw OrganoSolv lignin,
ECN Willow OrganoSolv lignin,
Mixed agricultural waste
OrganoSolv lignin AST)
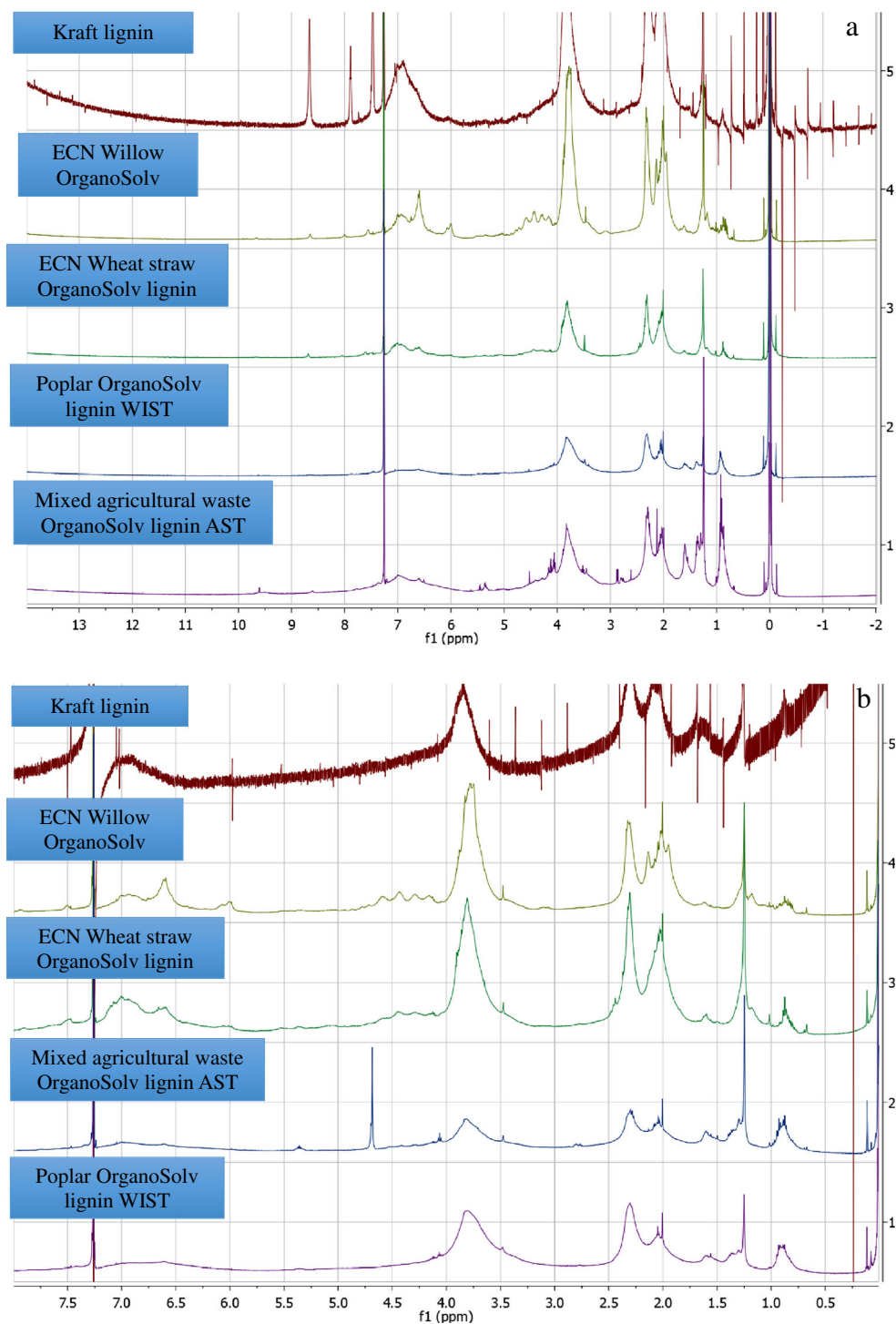
electron donors for xylan oxidation as mediated by the LPMOs [25, 49, 62]. Furthermore, it is possible that once a portion of the lignin is demethylated, the altered lignin backbone itself acts as a chelator and an electron source for the ferric/ferrous conversion. Fungal chelators would only need to be produced initially and in small amounts, and afterwards the catechol/quinone–ferric/ferrous conversion would develop as

a reaction front moving progressively through the lignin backbone [8].

In a recent study, three LPMOs from the fungus, *Myceliophthora thermophila* C1 (Mt LPMO9-A, LPMO9-B, and LPMO9-C), among which LPMO9-A was observed to have oxidized cellulose, xylan, a $(1 \rightarrow 3)(1 \rightarrow 4)$-β-glucan, as well as xyloglucan. Furthermore, Mt LPMO9-C oxidized

Table 3 The effect of different carbohydrate supplements on the fungal decomposition and demethylation of Kraft lignin as determined by ^{1}H NMR following O-acetylation of lignin

Carbohydrate supplements	Fomitopsis pinicola			Galerina autumnalis			Aspergillus sp.3 BRI 270		
	OCH$_3$ content (%)	OCH$_3$ loss (%)	Lignin demethylation (%)	OCH$_3$ content (%)	OCH$_3$ loss (%)	Lignin demethylation (%)	OCH$_3$ content (%)	OCH$_3$ loss (%)	Lignin demethylation (%)
Control (only KL)	15.02	0.44	2.7	15.11	0.35	2.2	14.94	0.52	3.4
Cellulose*	14.85	0.61	3.9	15.38	0.08	0.5	14.47	0.99	6.4
Cellobiose	15.03	0.43	2.8	14.57	0.89	5.8	14.47	0.99	6.4
CMC	14.94	0.52	3.4	15.38	0.08	0.5	15.46	0	0
Fructose	16.02	0.56	3.6	14.93	0.53	3.3	14.47	0.99	6.4
Galactose	14.47	0.99	6.4	15.02	0.44	2.7	13.43	2.03	13.1
Gluconic acid	14.76	0.7	4.5	17.31	1.85	12.0	14.28	1.18	7.6
Glucose	15.54	0.08	0.5	15.03	0.43	2.8	13.87	1.59	10.3
Lactose	15.03	0.43	2.8	14.94	0.52	3.4	14.94	0.52	3.4
Maltose	14.76	0.7	4.5	14.76	0.7	4.5	13.09	2.37	15.3
Sorbitol	14.76	0.7	4.5	13.65	1.81	11.7	13.76	1.7	11.0
Sucrose	15.2	0.26	1.7	14.94	0.52	3.4	15.03	0.43	2.8
Xylan#	15.29	0.17	1.1	15.11	0.35	2.2	10.4	4.66	30.1
Xylose	15.29	0.17	1.1	15.44	0.02	0.1	13.97	1.49	9.6

*Whatman filter paper (no. 1); # birchwood xylan

at the C-4 position of xyloglucan, and a $(1 \rightarrow 3)(1 \rightarrow 4)$-β-glucan (oat spelt) [25, 49, 62]. It was observed that the C-1 and C-4 oxidations enhanced the LPMO activity with different reducing agents, such as the plant-derived flavonoids and the lignin-building blocks, with a 1,2-benzenediol or 1,2,3-benzenetriol moiety giving the highest release of the oxidized and non-oxidized gluco-oligosaccharides from cellulose, as exhibited by all three Mt LPMOs [25]. All three Mt LPMOs were observed to have utilized the electron donors from the 1,2-benzenediol moiety [3-methylcatechol, 3,4-dihydroxyphenylalanine] or from the 1,2,3-benzenetriol moiety [gallic acid], epigallocatechin-gallate appeared to have displayed the highest formation of both the oxidized and non-oxidized gluco-oligosaccharides [25].

In our study with Aspergillus sp.3 BRI 270, the ECF might have oxidized the xylans by secreting LPMOs and simultaneously producing the demethylated lignin by secreting the O-demethylase/ligninolytic enzymes, or through the Fenton reaction mechanism. Up to a 31% of the vicinal hydroxyls generated by the demethylation process can act as the electron

Table 4 The efficacy of Aspergillus sp.3 BRI 270 on the extent of lignin demethylation of different lignin feedstocks as determined by ^{1}H NMR spectral analysis

Lignin feedstock	Methoxyl content (%)[a] Control	Aspergillus sp.3 BRI 270		
		Methoxyl content (%)[a] Modified	Methoxyl loss (%)	Extent of lignin demethylation[a] (%)
Kraft Lignin	15.46	10.4	4.66	30.1
Poplar OrganoSolv lignin WIST	23.23	22.10	1.13	4.86
ECN OrganoSolv lignin wheat straw	18.25	18.05	0.2	1.09
ECN OrganoSolv lignin willow	20.91	20.91	0	0
OrganoSolv lignin mixed Agricultural waste AST	19.18	18.96	0.22	1.14

[a] The percentage of lignin demethylation is calculated by using the difference between the signals from the H aromatic (6.40–7.10 ppm) and the methoxyl protons (4.1–3.50 ppm), and applying the formula $x = (H (Ar)/H (OCH_3))$. The statistical linear regression analysis produced an angular coefficient $b = 19.750047$ and a linear coefficient $a = 28.28436$. The methoxyl content of the acetylated starting material, KL, worked out to be 15.46% (–OCH$_3$). The following formula was applied to calculate the % lignin demethylation: % $OCH_3 = 28.28436 - 19.750047x$

Table 5 Effect of different pH values on the enzymatic demethylation of lignin model compounds and Kraft lignin using the crude enzyme preparation from *Aspergillus* sp.3 BRI 270

| Kraft lignin and lignin model compounds	Lignin demethylation		
	Pyrocatecholic content* (μmol/mL)		
	pH 3.0	pH 6.0	pH 8.0
Kraft lignin	18.67 ± 0.08	200.00 ± 1.07	397.46 ± 0.03
3,4-Dihydroxy-5-methoxy benzaldehyde	263.33 ± 0.03	276.00 ± 2.01	327.00 ± 0.05
Butylated hydroxyanisole	23.33 ± 0.80	26.00 ± 0.89	42.00 ± 0.32
o-Vanillin	39.33 ± 0.2	45.33 ± 0.42	53.20 ± 0.04
Guaiacol	32.00 ± 0.09	26.33 ± 0.54	22.01 ± 0.065
4-Hydroxy-3-methoxy cinnamaldehyde	139.00 ± 1.07	144.00 ± 1.1	326.00 ± 0.22
3-Hydroxyl-4-methoxy cinnamic acid	21.67 ± 0.04	25.00 ± 0.34	34.00 ± 0.07
4-Hydroxy-3-methoxy benzyl alcohol	17.67 ± 0.22	26.00 ± 0.67	37.00 ± 0.76
Syringaldehyde	70.33 ± 0.8	87.67 ± 0.34	102.67 ± 0.99
4-Hydroxy-3-methoxy benzoic acid	21.00 ± 0.03	24.33 ± 0.23	31.22 ± 0.34
3,4-Dimethoxybenzyl alcohol	21.67 ± 0.02	28.00 ± 0.31	36.00 ± 0.62
2,6-Dimethoxyphenol	26.33 ± 0.05	18.67 ± 0.03	19.22 ± 0.33
3-Hydroxy-4-methoxy benzyl alcohol	18.33 ± 0.08	21.00 ± 0.65	33.20 ± 0.72
3,4-Dimethoxycinnamic acid	34.33 ± 0.02	23.00 ± 0.23	44.00 ± 0.61
3,4,5-Trimethoxycinnamic acid	22.33 ± 0.06	27.00 ± 0.12	38.00 ± 0.02
Ferulic acid	21.33 ± 0.18	27.67 ± 0.63	47.22 ± 0.08
3-Methoxy catechol	77.00 ± 0.22	283.33 ± 0.74	298.32 ± 0.04
3-Hydroxy-4-methoxy benzoic acid	24.67 ± 0.01	30.00 ± 0.32	41.34 ± 0.66
Vanillin	31.33 ± 0.07	35.00 ± 0.12	46.50 ± 0.21
Vanillyl alcohol	21.33 ± 0.02	24.00 ± 0.04	13.00 ± 0.72
Sinapic acid	21.33 ± 0.03	22.67 ± 0.32	53.32 ± 0.88
3,4-Dimethoxy benzaldehyde	22.33 ± 0.08	21.67 ± 0.65	38.21 ± 0.39
Syringic acid	20.33 ± 0.32	27.67 ± 0.02	43.22 ± 0.34
3,4-Dimethoxybenzoic acid	15.00 ± 0.02	17.33 ± 0.08	29.00 ± 0.88

*Measured by the Ti(III)-NTA assay procedure [9]

donors for the xylan oxidation process that is mediated by the LPMOs [25, 49, 62]. Other tested lignin feedstocks (OrganoSolv lignins) did not show efficient lignin demethylation, which may have been due to the nature of harsh chemical extraction procedures used that affected the microbial and enzymatic action on these types of lignins (Table 4 and Fig. 5).

Overall, based on the results obtained for the lignin demethylation mechanism initiated by the *O*-demethylases, other ligninolytic enzymes act on the following linkages: C–C, C–O–C, β-O-4, 5-5, β-1, α-O-4, 4-O-5, β-β, and functional groups (C=O, CH_2OH, CHOH, –OH) in softwood Kraft lignin. The *O*-demethylases act mainly on the C-3 and C-5 positions containing –OCH_3 groups and potentially demethylates lignin, which in turn, results in mono-, di-, and triphenolic compounds as well as polymeric lignin. At the same time, non-enzymatic reactions with Fe^{3+} and H_2O_2 (mediated Fenton reaction) can produce oxygen radicals/reactive oxygen species (ROS) and ·OH that oxidize lignocellulosic polysaccharides, and can also demethylate lignin (Fig. 1).

Simultaneously, LPMOs catalyze oxidation within the polysaccharide chain leading to chain cleavage of hemicellulose and cellulose. For example, in cellulose oxidation at C-1 results in the formation of a lactone, which is hydrated to become a reducing-end aldonic acid. But oxidation at C-4 leads to the formation of a keto-aldose at the non-reducing end. Also redox enzyme–based electron systems with small-molecule reductants in the presence of either gallic acid; monophenol; di-, tri-, and polymeric demethylated lignin; and/or cellobiose dehydrogenase are potential electron donors for LPMOs in catalyzing the hydrolysis of hemicellulose and cellulose. In the case of the endo-β-1,4-glucanases (cellulases) and LPMOs that cleave cellulose chains internally releasing chain ends that are targeted by the cellobiohydrolases (exo-β-1,4-glucanases, CBHs), CBHs generate cellobiose or oxidized cellobiose that are subsequently hydrolyzed by β-glucosidase to generate fermentable sugars. In addition, we noticed that when fungal culture filtrates with higher cellobiose dehydrogenase activity, these showed highest *O*-demethylase activity that may indicate

a

b
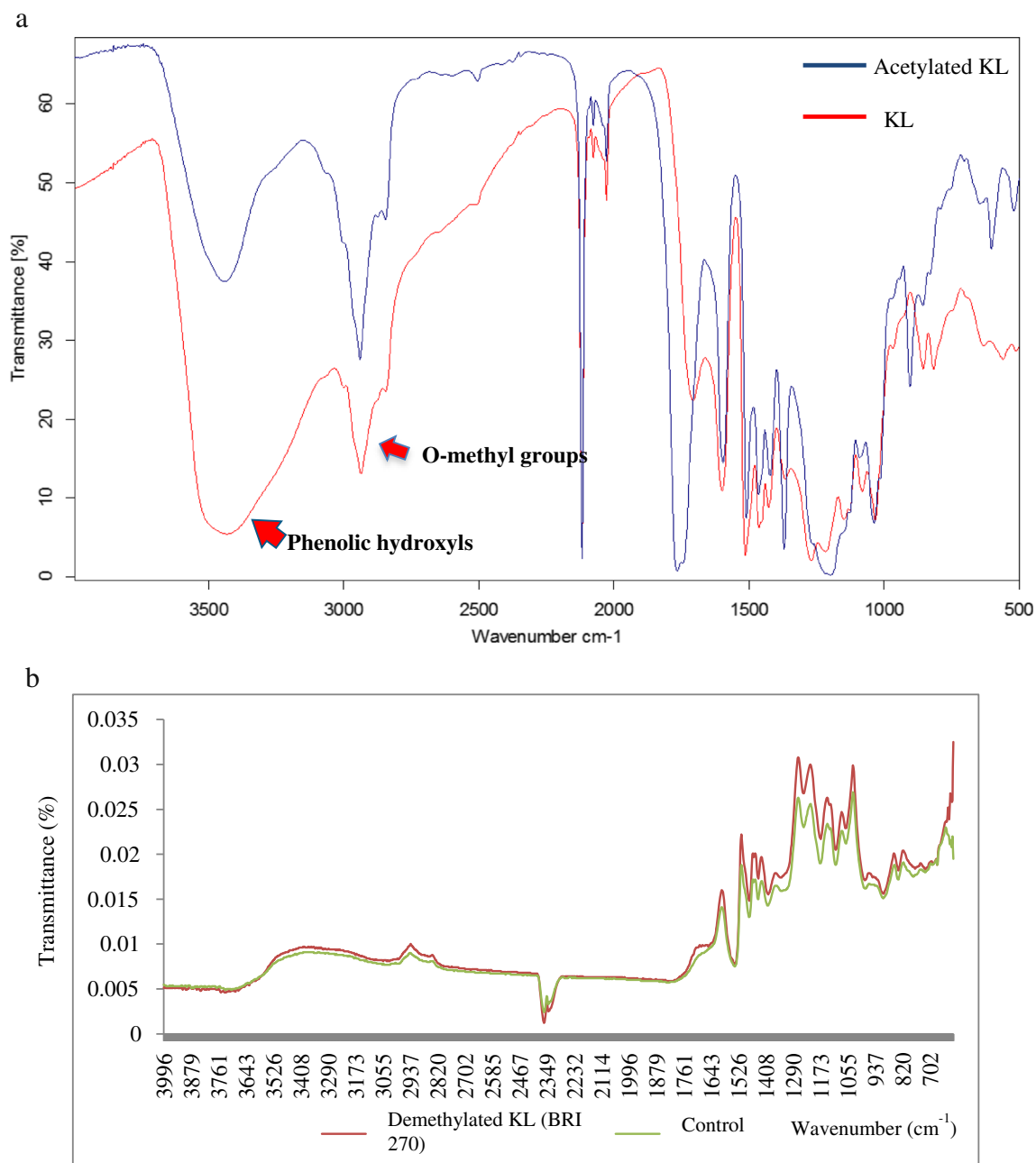
Fig. 6 **a** FT-IR spectral analysis of Kraft lignin and modified-Kraft lignin, and **b** Kraft lignin enzymatically demethylated by *Aspergillus* sp.3 BRI 270

that degradation of lignocellulosic and demethylation takes place by involvement through the cooperation of CDH, LPMOs, and *O*-demethylase activities. Hence, the highest degree of lignin demethylation (30.1%) occurred when the fungus was cultured on KL in the presence of birchwood xylan, which induces the secretion of enzymes such as the lytic polysaccharide mono-oxygenases, which might act on hemicellulose by utilizing the demethylated mono-phenols, and di-, tri-, and polymeric lignin fragments generated through the action of the *O*-demethylases. Furthermore, the LPMOs/xylanase system might cleave the C-4 position of the β-(1 → 4)-xylosyl

bonds in birchwood xylan that potentially leads to the formation of oxidized 4-ketoaldose [25, 49, 62].

3.5 Determination of demethylated KL by the Ti(III) -NTA method

When measuring a large number of samples evaluating fungal enzymes that demethylate KL, it is important to choose a method that is simple, besides being economical. The method should be sensitive and must be rapidly deployed in a biochemical laboratory with relative ease. To achieve this end,

Fig. 7 FT-IR spectra of lignin-formaldehyde resins produced from unmodified Kraft lignin (control), and enzymatically demethylated Kraft lignin
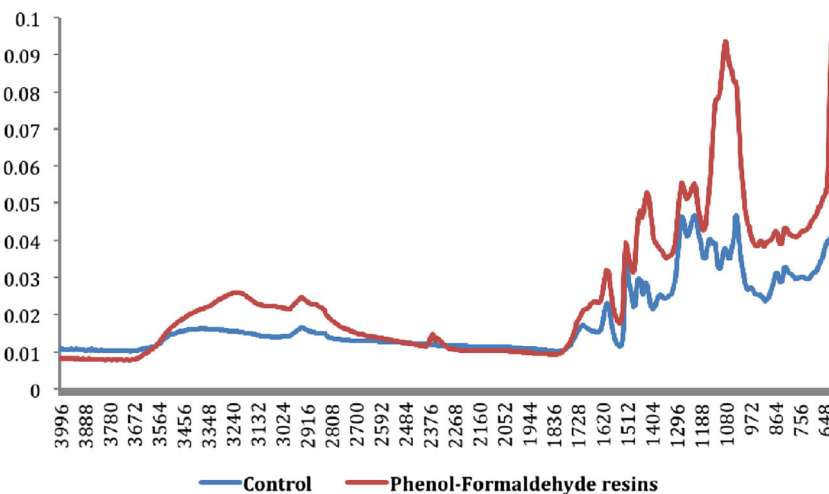
Y axis: Transmittance (%),   X axis: Wavenumber cm⁻¹

certain spectrophotometric methods have evolved to assay for the biomethanol released.

The *O*-demethylase assay was developed using several lignin-like model compounds (guaiacol (2-methoxy phenol), 2,6-dimethoxyphenol; 2-hydroxy-3-methoxybenzaldehyde (*o*-vanillin); 3,4-dimethoxybenzaldehyde; 3,4-dimethoxybenzyl alcohol (veratryl alcohol); 4-hydroxy-3-methoxycinnamic acid (ferulic acid); 4-hydroxy-3-methoxycinnamaldehyde; 4-hydroxy-3-methoxybenzoic acid), as well as KL.

Certain biochemical methods that allow the estimation of enzymatic demethylation (*O*-demethylase activity) have been developed in our laboratory, and include measuring either the amount of methanol released from the lignin-like compounds and KL, or the increase in the vicinal hydroxyl content (pyrocatechols) in the transformed lignin as a consequence of demethylation. The methanol generated by *O*-demethylase enzymes can be oxidized by alcohol oxidase to formaldehyde that in turn can react with either of the two chemical reagents—acetylacetone (3,5-diacetyl-1,4-dihydrolutidine), or acetylacetanilide (3,5-di-N-phenylacetyl-1,4-dihydrolutidine)—to generate complexes that can be measured spectrophotometrically [29]. The Ti(III)-NTA assay procedure allows the measurement in the increase in pyrocatecholic content in the demethylated KL [9].

The procedure for measuring *O*-demethylase activity by the two assays was tested on the crude enzyme filtrate of *Aspergillus* sp.3 BRI 270 with the pH adjusted to pH 3.0, 6.0, and 8.0. The demethylation enzymes by way of the Ti(III)-NTA colorimetric analysis showed highest vicinal diol generation from the model lignin-like compounds (4-hydroxy-3-methoxy cinnamaldehyde (326.00 μmol/mL ± 0.22), and syringaldehyde (102.67 μmol/mL ± 0.99)). That for KL resulted in 397.46 μmol vicinal diols/mL ± 0.03 at pH 8.0 (Table 5). The pH influenced the amount of vicinal

diols produced, being optimal at pH 8.0. Based upon these results, the Ti(III)-NTA assay method was adopted to quantify the extent of microbial demethylation of KL and the lignin-derived compounds.

3.6 Determination of lignin demethylation through FT-IR spectral analysis

Lignin contains various types of functional groups depending upon the wood species and the isolation procedure. Lignins isolated under acid conditions have relatively large amounts of carbonyl groups, but in Kraft lignin, sulfur is introduced into the original lignin structure under the Kraft pulping conditions. However, when compared to the hydroxyl and methoxyl groups, the amount of carbonyl or sulfur groups introduced in the KL was relatively low. Functional groups of KL and that of the modified KL were analyzed in order to study the effect of the fungal enzymatic modification of lignin. The FT-IR spectra of KL, *O*-acetylated KL, and enzyme-modified Kraft lignin, and the peak positions of the major infrared bands and their relative absorbance values are presented in Fig. 6.

Infra-red spectroscopy has proven to be a versatile means of investigating the specific interactions within and between molecules. FT-IR analyses are useful while studying the mechanism of intermolecular interactions brought about by enzymatic demethylation of lignin, both qualitatively and quantitatively. KL was provided as the sole carbon source to rear the lignin-demethylating fungi, which produced a variety of ligninolytic enzymes, including the lignin demethylation enzymes that could alter the lignin functional groups, like carbonyl, hydroxyl, phenolic-hydroxyl, aliphatic-hydroxyl, methyl, and the lignin structural units (guaiacyl and syringyl).

The FT-IR spectrum (Fig. 6) of the lignin samples revealed bands at 1600, 1515, and 1425 cm⁻¹ corresponding

Table 6 Comparison of lignin demethylation mediated by microbes, enzymes, and chemical methods for polymer applications

Demethylation process	Lignin source	Microorganisms/enzymes/catalyst	Conditions	Lignin demethylation	Polymer applications	References
Chemical demethylation	Wheat straw alkali lignin	Lewis acid	Wheat straw alkali lignin with iodocyclohexane in 10:1 molar ratio at 145°C for 3 h with 4 mL DMF under nitrogen atmosphere	$-OCH_3$ decreased from 0.82 to 0.17; phenolic hydroxyl increased from 5.2 to 16.0%	Lignin-based phenol-formaldehyde adhesives	Song et al., [34]
Chemical demethylation	Guaiacyl-type synthetic lignin	1-Dodecanethiol, hydroiodic acid, iodocyclohexane		Higher tannin-like properties	Functional polymer with tannin-like properties and reactive precursors for resins/adhesives	Sawamura et al., [12]
Chemical demethylation	Softwood Kraft lignin	Iodocyclohexane in dimethyl formamide	Softwood kraft lignin (25 mg) dissolved in 2.0 ml of dimethyl formamide and 1.3 ml of iodocyclohexane in reflux condenser for 3h at 145°C	Lignin demethylation resulting yield of 87%		Ferhan et al., [11]
Enzymatic demethylation	Alkali-soluble hardwood lignin	Alkaline lignin oxidase MetZyme® LIGNO™, a genetically engineered laccase of bacterial origin	1-liter twin-pot bioreactor; mediator-syringaldehyde (0.1% w/w); 50 °C; pH 10.5; aeration 0.16 l/min; ~ 5 U enzyme/g lignin for 21 h	30% increase of total number of phenolic hydroxyls and 0.47 mmol of MeOH per gram of lignin were released.	Bio-based materials	Hämäläinen et al., [72]
Enzymatic demethylation	Guaiacol	Cytochrome P_{450} aryl-O-demethylase (aryl-O-demethylation)		Converting guaiacol to catechol	Lignin bioconversion	Mallinson et al., [73]
Bacterial demethylation (*Sphingobium* sp. Strain SYK-6)	Lignin-derived biphenyl (5,5'-dehydrodivanillate, DDVA)	DDVA O-demethylase (*Sphingobium* sp. Strain SYK-6)	200 µl reaction mixture containing FE22 buffer (pH 6.0), LigXa (0.2 M, 10 g/ml of protein), LigXc (6.0 M, 83 g/ml of protein), LigXd (0.4 M, 19 g/ml of protein), 100 M DDVA, and 200 M NADH	LigXa, LigXc, and LigXd converted DDVA into 2,2',3-trihydroxy-3'-methoxy-5,5'-dicarboxybiphenyl		Yoshikata et al., [74]
Biological demethylation	Vanillate, 3-O-methylgallate, and syringate	Tetrahydrofolate-dependent aryl demethylase LigM from *Sphingomonas paucimobilis*	5 mM vanillate, 5 mM H4folate, 76 nM LigM (wild-type or mutant), and 100 mM Tris (pH 8.0) and were run for 2 h at 30°C.	Protocatechuate, or gallate	Fine chemicals, fuels, plastics, biofuel productions	Kohler et al., [3]
Biological demethylation	Vanillate and syringate	Tetrahydrofolate-dependent O-demethylase (LigM)	*E. coli* BL21(DE3) harbouring pELM and pG-KJE7 (300 g of protein) in 100 mM Tris-HCl buffer (pH 8.0) contains 100 M substrate, 1 mM H4folate, under anaerobic conditions at 30°C in an atmosphere of 95% N_2 and 5% H_2 (<100 ppm of O_2).	Protocatechuate and 3-O-methylgallate (3MGA)	High value added products and TCA cycle	Abe, et al., [75]
Biological demethylation	Kraft lignin	Wood-rotting fungi and *B. rhodina* MAMB-05 laccase	Kraft lignin (3 mg/mL) with 0.5 mL (75 U/mL) of laccase (*Lac* 1)	Yield of methanol was 0.382 µg/mL	Phenol-formaldehyde adhesives	Bashtan-Kandybovic et al., [1]
Microbial demethylation	Softwood Kraft lignin	*Aspergillus* sp. and *Galerina autumnalis*	28°C, 180 rpm for 7 days		Phenol-formaldehyde adhesives	Venkatesagowda, [4]
Microbial demethylation	Softwood Kraft lignin/OrganoSolv lignins (poplar, willow, wheat straw, and mixed agricultural wastes)	*Fomitopsis pinicola*, *Galerina autumnalis*, and *Aspergillus* sp.3 BRI 270	28 °C, 180 rpm for 7 days	Lignin demethylation (30.1%)	Phenol-formaldehyde adhesives	Present study

to aromatic ring vibrations of the phenyl-propane skeleton. A strong absorption band present at 3400 cm^{-1} was assigned to the aromatic and aliphatic OH groups, while the bands at 2960, 2925, 2850, and 1460 cm^{-1} were related to C–H vibration of the CH$_2$ and CH$_3$ groups. On comparing the unmodified lignin with the demethylated product (Fig. 6b), it was observed that after the treatment with the fungal ligninolytic enzymes preparation, the methoxyl content declined sharply as expected owing to the occurrence of a series of enzymatic demethylation events. The peak at 3433 cm^{-1} became prominent and wider, suggesting that the hydroxyl group strength increased significantly. This could have been attributable to a ligninolytic enzyme system operating (such as an *O*-demethylase). The peak at 1640 cm^{-1} is due to the conjugated carbonyl group, and its intensity increased considerably, because of the oxidation of the hydroxyl group at C-α. The bands at 1600 cm^{-1}, 1510 cm^{-1}, and 1425 cm^{-1}, however, were not strong enough, perhaps implying that the ligninolytic enzyme system degraded lignin to restructure it with a C=C bond configuration. The spectral differences existing between the KL and the modified KL could be observed in the fingerprint region (1800 and 900 cm^{-1}). In the KL and the modified KL, the bands observed at 1513 cm^{-1} (aromatic skeletal vibration) and 1269 cm^{-1} (guaiacyl ring vibrating with carbonyl stretching) were quite conspicuous (Fig. 6). The intensity of the bands (C–H methyl and methylene deformation) at 1513 cm^{-1} was higher for KL. In addition to the fingerprint region, apparent differences appear to exist between the KL and the modified KL. The band overlap observed in the spectra is associated with the hydroxyl-stretching region (νOH $\approx$ 3700–3000 cm^{-1}). A clear difference in the shape of the band edge can be visualized as in Fig. 6. The modified KL hydroxyl-stretching region was broader, indicating the possible occurrence of a higher degree of demethylation. In both the lignins, however, a broadband center and a shoulder were visible. The broadband center at ~ 3350 cm^{-1} (Kraft lignin) and ~ 3420 cm^{-1} (modified Kraft lignin) and the shoulder at ~ 3500 cm^{-1} have been assigned to the average stretching of the rise in the hydroxyl groups in treated lignin by *Aspergillus* sp.3 BRI 270 (Fig. 6).

3.7 Characterization of the lignin-based formaldehyde resin produced from enzymatically demethylated Kraft lignin

The demethylated lignin prepared from KL displayed enhanced reactivity as a consequence of the increase in the vicinal hydroxyl groups on treating KL with the crude enzyme preparation from *Aspergillus* sp.3 BRI 270. This treatment rendered the modified KL suitable for preparation of a lignin-based phenol-formaldehyde resin (Fig. 7 and Table 6) with application as a wood adhesive [68]. Besides, the vicinal

groups, as introduced by the ligninolytic or lignin-demethylating enzymes, enhanced the number of the cross-linkage sites for a possible interaction with the phenol-formaldehyde resin. Khan et al. [65] reported that up to 50% of phenol could be substituted by bagasse lignin in preparing lignin-based phenol-formaldehyde resins as wood adhesives [69]. The acidification of the pulp from *Eucalyptus* wood produces certain methylated or phenolated compounds that showed reactivity towards a crude "acetosolve lignin" with formaldehyde to prepare certain lignin-phenol-formaldehyde resins [70]. Impurities in the products from biomass pretreatments were analyzed by ^{1}H NMR and ^{31}P NMR spectroscopy [71].

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4 Conclusion

Lignin demethylation can be considered one of the most important processes in the fungal decay of wood. This kind of an unusual phenomenon can occur in a wide range of microorganisms, such as the brown-rot, white-rot, and the soft-rot fungi, besides bacteria. However, the mechanism of lignin demethylation has not yet been elaborated for a large number of lignin-degrading fungi. In the present study, a select number of wood-decay fungi obtained from the Boreal Forest in northern Canada were screened for the lignin demethylation phenomenon, among which, *Fomitopsis pinicola*, *Galerina autumnalis*, and *Aspergillus* sp.3 BRI 270 displayed the highest lignin demethylation activity. We observed that carbohydrate substrates when added to nutrient media containing KL played a key role in the biological demethylation process of lignin. This occurred not only in the brown-rot evaluated, but also with *Aspergillus* sp.3 BRI 270 that employed various lignin feed stocks, as revealed by ^{1}H NMR analysis. Our study clearly revealed the occurrence of a lignin demethylation mechanism, which is capable of removing the *O*-methyl/methoxyl groups from lignin through an enzyme activity typically displayed by *O*-demethylases that have been reported for similar enzyme activities demethylating lignin model compounds (vanillate, syringate, and veratrate), thus providing crucial evidence for the existence of specific fungal enzymes that catalyze this type of reaction. Enzymes of this nature (lignin-specific *O*-demethylases) are relatively new and have not yet been adequately dealt with in the current literature. Enzymes of this kind are considered to offer potential for their application in the production of demethylated lignins enriched in vicinal hydroxyl groups (pyrocatecholic) for possible synthesis of lignin-based formaldehyde polymers.

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