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ORIGINAL PAPER

Lignocellulosic polysaccharides and lignin degradation by wood decay fungi: the relevance of nonenzymatic Fenton-based reactions

Valdeir Arantes · Adriane M. F. Milagres · Timothy R. Filley · Barry Goodell

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Abstract The brown rot fungus *Wolfiporia cocos* and the selective white rot fungus *Perenniporia medulla-panis* produce peptides and phenolate-derivative compounds as low molecular weight Fe^{3+}-reductants. Phenolates were the major compounds with Fe^{3+}-reducing activity in both fungi and displayed Fe^{3+}-reducing activity at pH 2.0 and 4.5 in the absence and presence of oxalic acid. The chemical structures of these compounds were identified. Together with Fe^{3+} and H_2O_2 (mediated Fenton reaction) they produced oxygen radicals that oxidized lignocellulosic polysaccharides and lignin extensively in vitro under conditions similar to those found in vivo. These results indicate that, in addition to the extensively studied *Gloeophyllum trabeum*—a model brown rot fungus—other brown rot fungi as well as selective white rot fungi, possess the means to promote Fenton chemistry to degrade cellulose and hemicellulose, and to modify lignin. Moreover, new information is provided, particularly regarding how lignin is attacked, and either repolymerized or solubilized depending on the type of fungal attack, and suggests a new pathway for selective white rot degradation of wood. The importance of Fenton reactions mediated by phenolates operating separately or synergistically with carbohydrate-degrading enzymes in brown rot fungi, and lignin-modifying enzymes in white rot fungi is discussed. This research improves our understanding of natural processes in carbon cycling in the environment, which may enable the exploration of novel methods for bioconversion of lignocellulose in the production of biofuels or polymers, in addition to the development of new and better ways to protect wood from degradation by microorganisms.

Keywords Lignocellulose · Brown rot fungi · White rot fungi · Biodegradation · Fenton reaction · Cellulose · Lignin · Iron reducing activity

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Introduction

Wood decay fungi and their degradative systems (and biomimetic systems) have been evaluated for bioremediation and bioconversion of recalcitrant wastes [1–6], bioconversion of lignocellulosic biomass into biofuels [7, 8], biopulping of wood chips [9], biobleaching of cellulosic pulps [10–14], biosorption of dyes and heavy metals [5, 15], paper deinking [16, 17], lignin adhesive pre-treatment [18, 19], and many others. The efficiency of such biotechnological applications and the development of new ones depend primarily on understanding the chemical and biochemical mechanisms involved in lignocellulosic polysaccharides and lignin biodegradation, especially by brown and white rot fungi, which are the most effective decomposers of lignocellulosic materials in nature.

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Despite the fact that considerable progress has been made concerning the enzymology of wood biodegrading fungi, uncertainties still remain regarding the chemical and biochemical mechanisms responsible for lignocellulosic polysaccharides and lignin biodegradation as the actions of extracellular fungal enzyme systems alone are unable to initiate or advance significant degradation of the intact wood cell wall, especially when considering the action of brown rot, and selective and lignin-peroxidase-deficient white rot fungi [20–22]. In selective white rots, lignin is removed preferentially, with limited attack on cellulose at early stages of degradation [23].

It has been observed in brown rot and selective white rot fungal attack, that wood cell wall degradation occurs at a distance from fungal hyphae, and that this is initiated in the early stages of wood decay [20]. Many studies have demonstrated (recently reviewed by Arantes and Milagres [24]) that lignocellulolytic enzymes are too large to penetrate into the intact wood cell wall and cause the extracellular degradation of the macromolecular constituents located there, and also that nonenzymatic degradative systems involving low molecular weight compounds play a crucial role in the overall lignocellulose biodegradation process by brown and selective white rot fungi [20, 21]. The role of low molecular weight compounds is also important in simultaneous white rot fungi, with several low molecular weight compounds having been identified as mediators that function with specific lignin-degrading enzymes [1, 25, 26]. However, the mediators or other low molecular weight agents (e.g., metals, H_2O_2) reportedly involved with the enzyme systems of simultaneous white rot fungi do not appear to diffuse as far from the enzyme system to produce lignin depolymerization as in the case of selective white rotters [27].

A variety of nonenzymatic low molecular weight compounds (LMWC) reported to be secreted by brown and selective white rot fungi have been indicated as potential diffusible agents in hypothesized mechanisms supporting either the direct attack of both polysaccharides and lignin or as mediators of ligninolytic enzymes for indirect attack of lignin [24, 28]. Some of the more widely reported low molecular weight degradative systems that have been proposed, and the target macromolecular plant cell wall constituents of these systems are listed in Table 1. As research has advanced over time, some of these hypotheses have been well supported, whereas others remain equivocal.

Among the nonenzymatic oxidative systems listed in Table 1, the mediated Fenton reaction, which involves low molecular weight Fe^{3+}-reductants, has been widely accepted as a mechanism for generation of the OH radical through Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + {}^{-}OH$) [12, 21, 29–31]. In this mechanism, Fe^{3+}, which is present in the wood, is reduced to react with hydrogen peroxide and permit the Fenton reaction. The OH radical is a powerful oxidant that can depolymerize polysaccharides via hydrogen abstraction and can also attack lignin, for instance in demethylation/demethoxylation [29, 32].

In white rot fungi, the involvement of Fenton-based ·OH-producing reactions has received far less attention. Previously, we demonstrated that mediated Fenton reactions might also play an important role in wood biodegradation by

Table 1 Low molecular weight compounds (LMWC) produced by wood decay fungi, the proposed or hypothesized degradative systems they function within, and the target macromolecular wood constituent (adapted from [24])

LMWC	Degradative system	Decay	Wood constituent
3-Hydroxy-anthranilic acid	Laccase/mediator	WR	L
Veratryl alcohol (VA)	AVA/LiP	WR	L
2Cl-1,4DMB[a]	2Cl-1,4DMB/LiP	WR	L
	2Cl-1,4DMB/LiP/oxalate-Mn^{3+}	WR	L
Unsaturated fatty acids[b]	Fatty acid peroxidation	WR	L
Carboxylic acids[c]	Oxalate (direct attack)[d]	BR, WR	C, H
	Oxalate-Mn^{3+}/MnP	WR	L
Fe^{3+}-reducing compounds[e]	Mediated-Fenton reaction	BR, WR	C, H, L
	Mediated-Fenton reaction/Laccase	WR	L
	Mediated-Fenton reaction/MnP	WR	L

BR Brown rot decay, *WR* white rot decay, *C* cellulose, *H* hemicellulose, *L* lignin

[a] 2-chloro-1,4-di-methoxy-benzene

[b] Linoleic acid

[c] Oxalic acid

[d] Supported by only limited research

[e] Phenolate derivative compounds and peptides

selective and lignin-peroxidase-deficient white rot fungi [1]. The oxidation of phenolic and nonphenolic lignin model compounds by Fenton reactions mediated by low molecular weight biomimetic Fe^{3+}-reductants has been demonstrated [33], as has the oxidation of nonphenolic lignin compounds and the recalcitrant dye Azure B by the synergistic action between the partially purified low molecular weight Fe^{3+}-reductants and laccase or manganese-peroxidase from cultures of different white rot fungi [1]. It is thought that the OH radical generated through nonenzymatic Fenton-based system reacts with the nonphenolic substructures of lignin and the oxidized products could then be further oxidized by manganese-peroxidase and/or laccase, a pathway that can, at least in part, explain complete lignin degradation by lignin-peroxidase deficient white rot fungi.

To attack wood constituents via Fenton chemistry directly or indirectly, brown and white rot fungi require the presence of specific mechanisms to solubilize ferric iron from iron oxy(hydr)oxides present in wood. The iron must then also be reduced to its ferrous state, and additional mechanisms are also needed to produce H_2O_2. It has been widely accepted that both oxalate and low molecular weight phenolate-type compounds with the ability to chelate and reduce Fe^{3+} are implicated in this nonenzymatic mechanism [21, 24, 34–36]. Nevertheless, most of the research on the isolation and characterization of these Fe^{3+}-reducing compounds has been conduced with compounds obtained from cultures of the brown rot fungus *Gloeophyllum trabeum* [11, 25, 28, 32, 34, 37–39]—a model brown rot fungus. In addition, there is no conclusive experimental evidence demonstrating the involvement of this nonenzymatic system in white rot decay.

In an effort to strengthen our current knowledge of the nonenzymatic mediated-Fenton reactions in brown and white rot fungi as well as their relevance in overall lignocellulose biodegradation, this work aimed to isolate low molecular weight Fe^{3+}-reducing compounds from liquid cultures of the brown rot fungus *Wolfiporia cocos* and the selective white rot fungus *Perenniporia medulla-panis*. These compounds were then characterized structurally and their function in Fenton-based reactions with cellulose, hemicellulose and lignin was assessed under conditions similar to those found in vivo during wood biodegradation.

The goal of this research was to determine the relevance of nonenzymatic mediated-Fenton reactions in fungi outside of *Gloeophyllum* brown rot species, and determine whether this low molecular oxidative system involving Fe^{3+}-reductants to generate OH radicals via Fenton-based reactions is a common degradative pathway in other brown rot or selective white rot fungi. A related goal was to evaluate the extent of polysaccharide and lignin degradation caused by the action of the nonenzymatic system. In addition, a further goal of this work was to assess the importance of Fenton-based ·OH producing reactions mediated by these phenolates in selective white rot fungi to better understand how, together with enzyme systems, low molecular weight phenolates or other low molecular weight compounds might play a role in the complete degradation of lignocellulose.

This research improves our understanding of natural processes in carbon cycling in the environment, which may enable the exploration of novel methods for bioconversion of lignocellulose in the production of biofuels or polymers, in addition to the development of new and better ways to protect wood from degradation by microorganisms.

Materials and methods

Fungal strains and culture conditions

The brown rot fungus *W. cocos* (ATCC 62778) and the selective white rot fungus *P. medulla-panis* (ATCC 42463) were cultured for 30 days in liquid media previously selected as optimum for production of low molecular weight Fe^{3+}-reducing compounds [39]. It consisted of 1% microcrystalline cellulose (Avicel) supplemented with 0.2% glucose for *W. cocos* and 0.2% L-ornithine hydrochloride for *P. medulla-panis*, and Vogel's mineral solution. All other cultivation conditions were as previously described [33].

Isolation of low molecular weight Fe^{3+}-reducing compounds

After harvesting (30 flasks for each fungus), the supernatants were filtered through Whatman no. 1 filter paper, combined and concentrated ten-fold by lyophilization. The concentrated samples were then partially purified by ultrafiltration using Millipore Ultrafree-15 Membranes (cut-off 5,000 Da), and the low molecular weight fractions (<5 kDa) were collected for isolation of peptides and phenolate derivative compounds as described below.

Isolation of phenolate compounds

Phenolate-derivative compounds were isolated from the partially purified low molecular weight fractions (<5 kDa, pH between 2 and 3) by mixing 100 ml of the ultrafiltrate with 1 g of the nonpolar hydrophobic crosslinked polystyrene copolymer Amberlite XAD-2 resin (Aldrich, St. Louis, MO) and stirring for about 1 h as described elsewhere [40]. Briefly, the resin was pretreated by adding a double volume of methanol and stirring for about 10 min at 540 rpm (this step was repeated twice) and washed with

a double volume of water. A column (1 × 10 cm) packed with the resin-adsorbed phenolates was washed with 5–10 column volumes of deionized water. The adsorbed compounds were eluted with two column volumes of methanol, and the methanol extracts were evaporated under reduced pressure. Unless otherwise noted, the term 'Pmp reductant' and 'Wc reductant' in this paper are used to refer to the mixture of ultrafiltered, XAD-2 extracted, Fe^{3+}-reducing phenolate compounds produced by *P. medulla-panis* and *W. cocos*, respectively.

Isolation of peptides

Low molecular weight peptides were isolated from the ultrafiltrates by precipitation with trichloroacetic acid (TCA) [41]. Samples (3 ml low molecular weight fraction) were extracted with TCA, at a final concentration of 30% (w/v). The mixture was homogenized, kept in an ice bath for 1 h and then centrifuged at 7,000 *g* for 15 min at 4°C. The supernatant was carefully removed and the residue was washed with cold acetone (−20°C). After centrifuging the mixture at 7,000 *g* for 15 min at 4°C, the residue containing the precipitated peptides was redissolved in 0.2 ml 1 M NaOH.

Fe^{3+}-reducing activity

The reduction of Fe^{3+} to Fe^{2+} was determined using ferrozine, a colorimetric reagent that forms a stable magenta complex with Fe^{2+} [42]. Samples containing Fe^{3+}-reducing compounds were incubated with 50 µl $FeCl_3$ 20 mM (freshly prepared) in sodium acetate buffer (50 mM, pH 4.5). After 30 min, 50 µl NaF (10%) and 400 µl ferrozine (1%) was added, mixed, and the solution immediately read at 562 nm [33]. All procedures were conducted in the absence of light. The control contained all reagents listed above with deionized water substituted for the sample. The results are expressed in terms of Fe^{2+} generated in the reaction mixture (ε_{562nm} = 27,900 M^{-1} cm^{-1}) [42].

In another set of experiments, the effect of oxalic acid and pH on Fe^{3+}-reduction by Wc and Pmp reductants was also evaluated according to Arantes et al. [39]. The reaction mixture consisted of 2.4 mg Wc reductant (or 2.9 mg Pmp reductant), 50 µM Fe^{3+} (as $FeCl_3$ freshly prepared) and 0–10 mM oxalic acid. These assays were carried out at pH 2.0 (KCl–HCl Clarke and Lubbs buffer, 100 mM) and pH 4.5 (sodium acetate buffer, 100 mM). Oxalic acid and iron were mixed in the presence of buffer and allowed to react for 18 h. Wc reductant (or Pmp reductant) was added with mixing. After 5 h, ferrozine was added to the solution, mixed and immediately read at 562 nm. All procedures were conducted in the absence of light. The control contained all reagents listed above with deionized water substituted for Wc and Pmp reductants. The results are expressed in terms of Fe^{2+} generated in the reaction mixtures (ε_{562nm} = 27,900 M^{-1} cm^{-1}) [42]. Experimental results of Fe^{3+}-reduction by Wc and Pmp reductants as a function of oxalate concentration were plotted against a chemical speciation model (Visual MINTEQ software—version 2.53) for the interaction of the same components at both pH 2.0 and 4.5.

Detection of phenolate compounds and peptides

Phenolate-derivative compounds were determined according to Arnow [43] using 2,3-dihydroxybenzoic acid as the standard. Peptides were detected using the Lowry assay [44].

Identification of LMWC by ^{13}C-TMAH/GC–MS

Pmp and Wc reductants were identified using a gas chromatograph equipped with a mass spectrometer (GC–MS). Samples were methylated inline using tetramethylammonium hydroxide (TMAH), which, in alkaline conditions, methylates OH groups generating OCH_3 [45]. To distinguish the OCH_3 groups originally present in the compounds from those generated during methylation with TMAH, Wc and Pmp reductant samples were also methylated using ^{13}C-labeled TMAH (^{13}C-TMAH). ^{13}C-labeled TMAH was synthesized as described [45]. Samples (Wc or Pmp reductants) were weighed (typically 100–150 µg) into a 3 × 3 mm platinum bucket containing eicosane as the internal standard. After sample addition, 3 µl of a 25 wt% solution of TMAH (or ^{13}C-TMAH) was added to the bucket and allowed to diffuse into the sample for 5 min. Chromatography and mass spectral analysis of the products was performed on a Shimadzu GC17A gas chromatograph interfaced to a Shimadzu QP5050A quadrupole mass spectrometer collecting in the mass range m/z 40–550. Chromatographic separation was performed on a fused silica column (Restek RTx-5, 30 m, 0.25 mm i.d., film thickness 0.25 µm). The oven was temperature programmed from 60 (1 min hold) to 300°C at 7°C/min and maintained at that temperature for 15 min.

Analyses were conduced in triplicate and compound identification was based upon comparison of the mass spectra of the products to spectra of unlabeled TMAH products in NIST21 and NIST107 spectral libraries as well as detailed analysis of the individual spectra.

Oxidative degradation experiments

To evaluate the oxidative effect of Fenton reactions mediated by Pmp and Wc reductants on cellulose and hemicellulose, various cellulosic (Avicel, carboxymethylcellulose and

p-nitrophenyl-β-D-glucopyranoside—pNPG) and hemicellulosic (xylan and p-nitrophenyl-β-D-xylopyranoside—pNPX) models were used. Milled red spruce wood (*Picea rubens*) was used to assess the effect of the Fenton reaction on lignin.

To prepare samples for the oxidative degradation assays, each substrate was treated using one of the reaction conditions outlined in Table 2. For each condition, the treatment was performed in three replicates, in the absence of light and with constant gentle shaking. Fe^{3+} was added last to initiate the reaction.

The extent of substrate degradation (oxidation) was determined as follows:

Xylan: the reducing power of the solution was measured at different time periods after adding 1.5 ml DNS (3,5-dinitrosalicylic acid) reagent, boiling the mixture for 5 min, cooling, and measuring the absorbance of the final solution at 540 nm [33]. Results are expressed as the increase in absorbance at 540 nm for an average of three replicates.

pNPG and pNPX: release of *p*NP was monitored at different time periods after adding 2.0 ml 10% sodium bicarbonate and measuring the absorbance of the final solution at 410 nm [33]. Results are expressed as the increase in absorbance at 410 nm for an average of three replicates.

Avicel: the reducing power of the solutions was measured at different time periods. After each incubation time, the reaction mixture was centrifuged for 10 min at 3,000 *g*. The supernatant was collected and assayed for reducing power as described for xylan; 1 ml sodium acetate buffer (50 mM, pH 5.0) was added to the residue (previously washed with deionized water) and the reducing power of the final mixture was determined with DNS as described above for xylan. Results are expressed as the increase in absorbance at 540 nm for an average of three replicates.

CMC: the reducing power and viscosity of the solutions was measured as follows. After each incubation time, 1 ml reaction mixture was collected and assayed for reducing power using DNS as described for xylan. The remaining volume was assayed for viscosity by transferring the sample to a 200 Fenske-Oswald viscometer for 5 min to bring the solution to bath temperature (25°C) before beginning the assay. The change in viscosity of CMC solutions was determined by the change in the time required for solution flow-through the viscometer (average of four measurements) [33]. The reciprocal of specific viscosity ($1/n_{sp}$) was calculated by the formula: $1/n_{sp} = t_o/(t - t_o)$, where t and t_o represent the time of outflow of the reaction mixture and buffer, respectively [46].

Table 2 Chemicals[a] and LMWC loadings in samples[b] prepared for oxidative treatment of the xylan, Avicel, p-nitrophenyl-β-D-xylopyranoside (*p*NPX), p-nitrophenyl-β-D-glucopyranoside (*p*NPG), carboxymethyl cellulose (CMC), and milled wood

Substrates: Xylan, Avicel, *p*NPX and *p*NPG					
Reaction times (1, 3, 6, 24 and 48 h), Temperature (50°C)					
Run #	Substrate (µl)	Wc (mg)	Pmp (mg)	Fe^{3+} (µl)	H_2O_2 (µl)
1	800	2.4	–	50	50
2	800	–	2.9	50	50
3	800	2.4	–	–	–
4	800	–	2.9	–	–
5	800	–	–	50	50
6	800	–	–	–	–
Substrate: CMC					
Reaction times (1, 3, 6, 24 and 48 h), Temperature (50°C)					
Run #	Substrate (ml)	Wc (mg)	Pmp (mg)	Fe^{3+} (µl)	H_2O_2 (µl)
1	8.0	24.0	–	500	500
2	8.0	–	29.0	500	500
3	8.0	24.0	–	–	–
4	8.0	–	29.0	–	–
5	8.0	–	–	500	500
6	8.0	–	–	–	–
Substrate: milled wood					
Reaction time (40 h), Temperature (25°C)					
Run #	Substrate (mg)	Wc (mg)	Pmp (mg)	Fe^{3+} (µl)	H_2O_2 (µl)
1	50	2.4	–	50	50
2	50	–	2.9	50	50
3	50	2.4	–	–	–
4	50	–	2.9	–	–
5	50	–	–	50	50
6	50	–	–	–	–

[a] Xylan (Sigma, St. Louis, MO): 1% w/v in 50 mM sodium acetate buffer pH 5.0; CMC (Sigma): 1% w/v, medium viscosity, in 50 mM sodium acetate buffer pH 5.0; Avicel (Fluka, Milwaukee, WI): 1% w/v in 50 mM sodium acetate buffer pH 5.0; pNPX and pNPG (Sigma): 0.1% w/v in 50 mM sodium acetate buffer pH 5.0; milled wood: milled red spruce (*Picea rubens*) wood (40 mesh) in 50 mM sodium acetate buffer pH 5.0; H_2O_2 stock solution 40 mM; $FeCl_3$ stock solution 4 mM (freshly prepared)

[b] Mixture of ultrafiltered, XAD-2 extracted, Fe^{3+}-reducing phenolate compounds produced by *Wolfiporia cocos* (Wc) and the selective white rot fungus *Perenniporia medulla-panis* (Pmp)

Viscosity measurements were plotted against reducing power measurements as described above.

Milled wood (lignin): the extent of demethylation of the lignin in the treated wood residues was monitored using ^{13}C-TMAH thermochemolysis. After the incubation time, the reaction mixtures were centrifuged and the treated and

untreated wood residues were freeze dried in the absence of light. Before ^{13}C-TMAH thermochemolysis, the dried wood residues were ball milled to a powder (~15 s). ^{13}C-labeled TMAH was synthesized as described [45] and analysis was conducted as outlined previously [47], which assessed the LMWC chemistry of this system.

Structural mass spectrometry was used to determine ^{13}C-enrichment (% ^{13}C) of the major ^{13}C-methylated guaiacyl monomers released after ^{13}C-TMAH thermochemolysis of wood residues as described [47]. The eight major monomers investigated are listed elsewhere [47]. Comparison of the ^{13}C-content of each monomer released from treated wood residues with the untreated wood residues indicates the degree of demethylation and specifically the loss of the methoxyl carbon on the 3-position of the aromatic ring [46].

Results and discussion

Isolation and properties of low molecular weight Fe^{3+}-reductants

Low molecular weight fractions (<5 kDa) obtained from concentrated culture broths and ferrozine tests demonstrated the presence of Fe^{3+}-reducing activity in the ultrafiltrates (Table 3). The ultrafiltrate obtained from *W. cocos* culture broth possessed greater iron reducing capacity compared to the *P. medulla-panis* ultrafiltrate, which is consistent with previous studies [11, 39].

Phenolate compounds were produced by both fungi and detected in the low molecular weight fractions (LMWF; Table 3). However, *W. cocos* produced slightly higher amounts. Peptides were also produced by both fungi and detected in the same ultrafiltrates after precipitation with TCA. As indicated by the increase in absorbance in the Lowry test, *W. cocos* produced about a 1.6-fold greater amount of peptides compared to *P. medulla-panis* (Table 3). The greater production of the Fe^{3+}-reducing phenolates, and potentially peptides, by *W. cocos* explains the higher Fe^{3+}-reducing activity of the ultrafiltrates obtained from its culture broth.

Fe^{3+}-reducing activities obtained with the isolated low molecular weight peptides were only about 3% and 10% of the activity detected with the original ultrafiltrates from *P. medulla-panis* and *W. cocos*, respectively. This finding indicates that even though low molecular weight peptides with Fe^{3+}-reducing capabilities were produced by both fungi, at least under the growth conditions used in these experiments, these peptides were not the major compounds responsible for the high Fe^{3+} reduction activity detected in the LMWF of both fungi. Thus, in order to further purify the phenolate-derivative compounds with high Fe^{3+}-reducing activities produced by *P. medulla-panis* and *W. cocos*, a liquid–solid extraction step was carried out by batch loading the phenolate-derivative compounds present in the ultrafiltrates onto an Amberlite XAD-2 resin and eluting them with methanol.

The Amberlite XAD-2 isolated phenolate-derivative compounds (Wc for *W. cocos* and Pmp for *P. medulla-panis*) had high Fe^{3+}-reducing activity, and, again, the compounds produced by *W. cocos* displayed higher reductive activity per milligram of isolated compounds than those produced by *P. medulla-panis* (Table 3).

Effect of oxalic acid and pH on Fe^{3+}-reducing activity

Oxalic acid has been cited as the main organic carboxylic acid produced by wood decay fungi, especially brown rot fungi, and this acid is also responsible for iron solubilization during wood biodegradation [21, 38, 49, 50]. While fungal secretion of oxalic acid may enhance the availability of Fe^{3+} during brown rot degradation by reducing pH and solubilizing bound Fe^{3+}, high oxalic acid concentrations could impede nonenzymatic Fenton-based degradative mechanisms by forming highly stable Fe–oxalate complexes [36, 50, 51]. Thus, degradative fungi appear to have the ability to regulate oxalate production and even

Table 3 Chemical composition and Fe^{3+}-reducing activity of the low molecular weight fractions (LMWF; <5 kDa) from cultures of *P. medulla-panis* and *W. cocos*

Fungus	LMWF		TCA-LMWF[a]	XAD-2-IC[b]	
	Phenolates (μM)	FeRA[c] (μM Fe^{2+})	Peptides (Abs 750 nm)	FeRA[c] (μM Fe^{2+})	FeRA[c] (μM Fe^{2+}/mg)
P. medulla-panis	21.1 ± 0.8	40.5 ± 0.4	0.211 ± 0.015	1.5 ± 0.2	47.9 ± 1.3
W. cocos	24.1 ± 1.2	46.0 ± 0.6	0.333 ± 0.011	4.4 ± 0.7	60.4 ± 0.9

[a] Peptides isolated from LMWF after precipitation with trichloroacetic acid (TCA)

[b] Phenolate-derivative compounds isolated from LMWF with XAD-2 resin

[c] Fe^{3+}-reducing activity

the balance between soluble and insoluble oxalate forms [50–53].

We have previously reported the production of oxalic acid by both *P. medulla-panis* and *W. cocos* [11], and, in this study, to evaluate the effect of pH and oxalic acid on the Fe^{3+}-reducing activity of Pmp and Wc reductants, they were assayed for Fe^{3+} reduction at pH 2.0 and 4.5 in the presence of increasing concentrations of oxalic acid (0–1 mM) and fixed Fe^{3+} concentrations (50 μM), creating an oxalate:Fe^{3+} molar ratio in the range of 0:1–20:1 (Fig. 1). When the experimental results of Fe^{3+} reduction by the Wc and Pmp reductants were plotted against the oxalate:Fe speciation data, Wc and Pmp reductants were shown to reduce Fe^{3+} at pH 2.0 and 4.5 and in the presence of oxalic acid only when the oxalate:Fe^{3+} molar ratio was lower than 18 (oxalate <900 μM) at pH 2.0 and lower than 10 (oxalate 500 μM) at pH 4.5. Under these conditions, most of the iron was in the form of uncomplexed Fe^{3+} [mostly oxy(hydr)oxides at pH 4.5] and/or $[Fe(C_2O_4)^+]$, suggesting that $[Fe(C_2O_4)^+]$ is the only Fe-oxalate complex form that is reduced by the isolated fungal low molecular weight phenolate compounds. Oxalate concentrations greater than 900 μM at pH 2.0 and higher than 500 μM at pH 4.5 completely inhibited Fe^{3+}-reducing activity by Wc and Pmp reductants. Under these conditions, the predominant Fe-oxalate species were $[Fe(C_2O_4)_2^-]$ and $[Fe(C_2O_4)_3^{3-}]$, indicating that when iron is complexed to oxalate, these forms cannot be sequestered and subsequently reduced by the fungal isolated phenolates, and that $[Fe(C_2O_4)^+]$ is the preferred species for reduction. Therefore, it is expected that where high oxalate conditions prevail, such as would occur immediately surrounding the fungal hyphae, iron-reduction would be prevented and this would also provide a mechanism to prevent hydroxyl radical production in close proximity to the fungal hyphae, preventing the destruction of these hyphae.

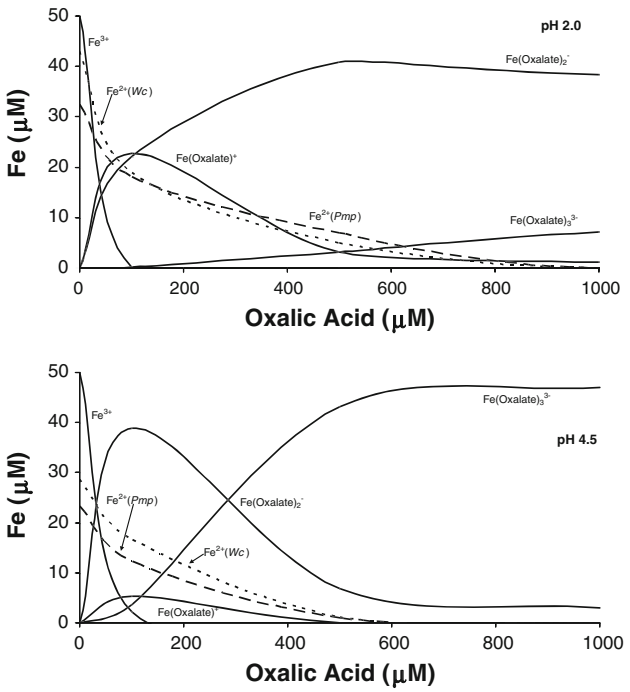


Fig. 1 Fe^{3+} reduction by Wc and Pmp reductants (isolated from *Wolfiporia cocos* and the selective white rot fungus *Perenniporia medulla-panis*, respectively) as Fe^{2+} and Fe-oxalate speciation [as either Fe^{3+}, $Fe(C_2O_4)^+$, $Fe(C_2O_4)_2^-$ or $Fe(C_2O_4)_3^{3-}$] over a range of oxalic acid concentrations at pH 2.0 and 4.5, and 50 μM Fe^{3+}

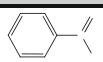
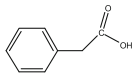
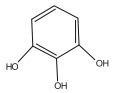
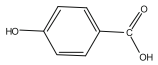
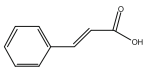
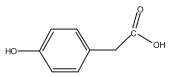
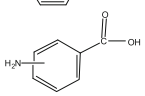
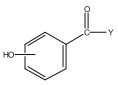
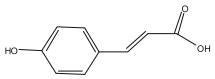
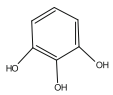
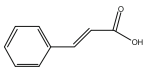
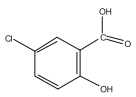
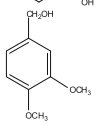
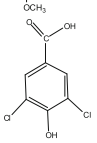
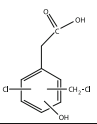
Identification of low molecular weight Fe^{3+}-reductants

Several low molecular weight compounds were produced by both *W. cocos* and *P. medulla-panis*. Proposed structures and relative concentrations of these compounds are shown in Table 4. Among the compounds produced by *W. cocos* were derivatives of hydroxybenzene, benzoic acid, cinnamic acid and phenyl acetic acid, as well as a hydroxyphenone and long chain saturated fatty acids (Table 4). The LMWC produced by *P. medulla-panis* included hydroxybenzenes, cinnamic acid, veratryl alcohol, chlorinated aromatic compounds, and long chain saturated fatty acids (Table 4). 1,2,3-Trihydroxybenzene (pyragallol) and 3-phenyl-2-propenoic (cinnamic acid) were produced by both fungi. There were also some abundant compounds produced by both fungi that have not yet been identified.

The Fe^{3+}-reducing ability of di- and tri-hydroxybenzene derivatives is well known, and that of 4-hydroxy-cinnamic acid was demonstrated recently [54]. The Fe^{3+}-reducing ability of 4-hydroxy-phenylacetic acid was confirmed with Fe^{3+} reduction assays using authentic standards (data not shown). Thus, among the LMWC isolated and identified from cultures of *W. cocos*, those with Fe^{3+}-reducing ability were 2-methyl-catechol, pyrogallol, 4-hydroxy-cinnamic acid and 4-hydroxy-phenylacetic acid; with 4-hydroxy-phenylacetic acid being the most abundant. The benzoic amino acid compound produced by *W. cocos* may also reduce Fe^{3+} depending on the position of the amino group on the aromatic ring, as reported for anthranilic acid (2-amino-benzoic acid) [55]. The Fe^{3+}-reducing compounds produced by *P. medulla-panis* were catechol, pyrogallol and veratryl alcohol.

The structural differences in the Fe^{3+}-reductants produced by both fungi confirm what had previously been proposed [33]. In this work, there was no correlation between the amount of phenolate-derivative compounds detected by the Arnow assay and the levels of Fe^{3+}-reducing activity displayed by *P. medulla-panis* and *W. cocos*, even though the greatest iron-reducing activity for both fungi was detected in culture media containing the largest concentration of phenolates. Compounds

Table 4 GC-MS data and proposed structures for the low molecular weight compounds isolated from cultures of *W. cocos* and *P. medulla-panis*

Fungus[1]	Compound	Structure	Relative Concentration[3]
Wc	Benzoic acid		1.308
Wc	Phenyl acetic acid		0.440
Wc	1,2-dihydroxy-3-methyl benzene[2]		0.126
Wc, Pmp	1,2,3-trihydroxybenzene (pyrogallol)[2]		0.217
Wc	4-hydroxybenzoic acid		1.886
Wc, Pmp	3-phenyl-2-propenoic (cinnamic acid)		0.530
Wc	4-hydroxyphenylacetic acid[2]		1.520
Wc	Amine-benzoic acid		0.186
Wc	Hydroxyphenone derivative		0.292
Wc	4-hydroxy-cinnamic acid[2]		0.173
Pmp	1,2-dihydroxy benzene (catechol)[2]		0.416
Pmp	1,2,3-trihydroxybenzene (pyrogallol)[2]		0.386
Pmp	3-phenyl-2-propenoic (cinnamic acid)		0.883
Pmp	5-chloro-2-hydroxybenzoic acid (5-chlorosalicylic acid)		0.238
Pmp	3,4-dimethoxybenzyl alcohol (veratryl alcohol)[2]		0.336
Pmp	3,4-dichloro-4-hydroxybenzoic acid		0.547
Pmp	Chloro-hydroxy-mehtyl choloro phenilacetic acid		0.319

[1] Wc *Wolfiporia* cocos, Pmp *P. medulla-panis*

[2] Compounds with a known Fe^{3+}-reducing ability

[3] Internal standard was used to normalize TIC-area values

containing only one OH phenolic group react weakly, or do not react at all in the Arnow assay, while those containing two or three OH phenolic groups react strongly. Thus, considering that the Fe^{3+}-reductants produced by *P. medulla-panis* are mainly di- and tri-hydroxy aromatic compounds, whereas for *W. cocos* they are mainly a mixture of mono-hydroxy aromatic compounds and di- and tri-hydroxy aromatic compounds, differential responses would be expected with the Arnow assay for Fe^{3+}-reductants produced by different fungi. This indicates that care should be taken when using the Arnow assay for quantitative comparisons between different samples.

The isolation and identification of LMWC with Fe^{3+}-reducing capabilities from the culture broths of *P. medulla-panis* and *W. cocos* provide experimental evidence that both fungi possess the means to reduce Fe^{3+} during wood biodegradation (considering that these reductants are also produced in wood undergoing fungal decay).

Palmitic and stearic acids have been reported to be produced by some white rot fungi such as *Ceriporiopsis subvermispora* [56]. However, limited information is available on the production of fatty acids by brown rot fungi, and mechanisms for participation of lipids in brown rot mechanisms have not been proposed. Chlorinated organic compounds have been reported to be produced by wood- and forest litter-degrading fungi [26, 57] and found in decayed wood [58]. The physiological role of fungal chlorinated aromatic compounds have been proposed to be low molecular weight mediators for lignin-peroxidase [59] or substrates for aryl alcohol oxidase for generation of the extracellular hydrogen peroxidase required for the action of peroxidases such as lignin-peroxidase and manganese-peroxidase [26]. *P. medulla-panis* did not produce lignin-peroxidase under the growth conditions used in this work data not shown) nor did it in previous work when grown in synthetic culture media [1, 60] or in eucalyptus wood chips [61]. This indicates that this fungus may not produce lignin-peroxidase. Therefore, it seems that the chlorinated aromatic compounds produced by *P. medulla-panis* may be involved in the production of hydrogen peroxide required for the action of manganese-peroxidase, which is produced by *P. medulla-panis* [1, 61] and/or for the Fenton chemistry to occur.

Nonenzymatic-Fenton-based oxidation of polysaccharides and lignin

Cellulose and hemicellulose

The release of pNP, the breakdown product of pNPG, was detected after all treatments, except when Pmp and Wc reductants were used in the absence of Fe^{3+} and H_2O_2 (Fig. 2). Release of pNP was faster and more efficient when pNPG was treated with Wc-Fe^{3+}/H_2O_2 or Pmp-Fe^{3+}/H_2O_2 than with the neat Fenton reaction. The extent of pNPG degradation with Wc-Fe^{3+} and Pmp-Fe^{3+} alone was very low.

Degradation of hemicellulosic (xylan) and hemicellulosic model (pNPX) substrates was associated with an increase in the reducing power and release of pNP, respectively, after oxidative treatments. The degradation pattern of both xylan and pNPX was very similar to that

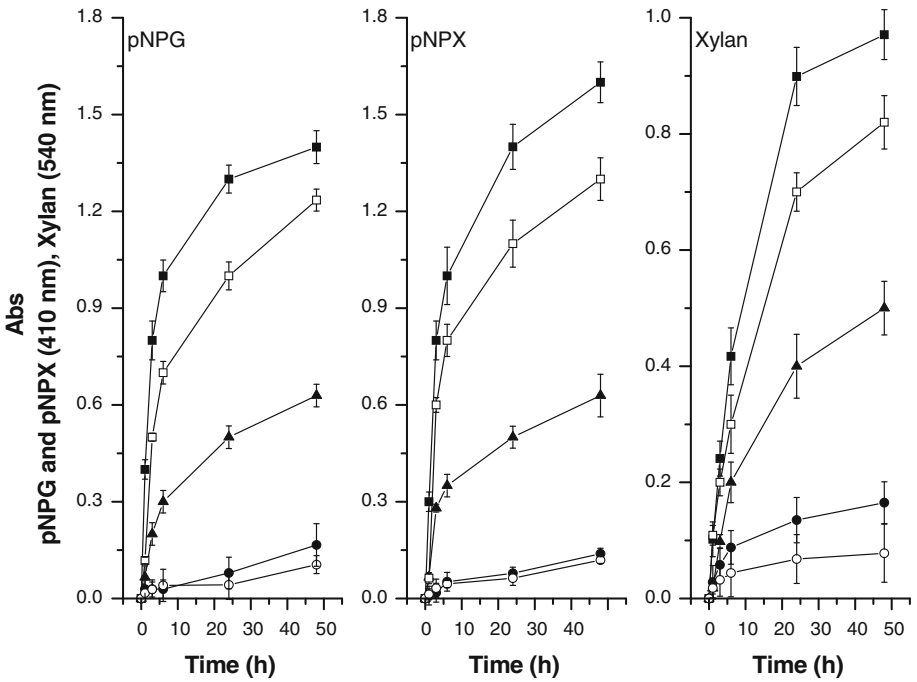


Fig. 2 Time-course of p-nitrophenyl-β-D-glucopyranoside (pNPG), p-nitrophenyl-β-D-xylopyranoside (pNPX) and xylan degradation as indicated by the increase of the absorbance at 410 and 540 nm for pNPG/pNPX and xylan, respectively, due to the release of *p*-nitrophenol from pNPG and pNPX, and reducing sugars from xylan after treatment with Wc-Fe^{3+}/H_2O_2 (*filled squares*), Pmp-Fe^{3+}/H_2O_2 (*open squares*), Fe^{3+}/H_2O_2 (*filled triangles*), Wc-Fe^{3+} (*filled circles*) and Pmp-Fe^{3+} (*open circles*)

observed with pNPG. That is, the mediated Fenton reactions (Wc-Fe^{3+}/H_2O_2 and Pmp-Fe^{3+}/H_2O_2) were much faster and more efficient in degrading both substrates than reactions with only iron and hydrogen peroxide. The extent of pNPX and xylan degradation with Wc-Fe^{3+} and Pmp-Fe^{3+} were very low, whereas the Fe^{3+}/H_2O_2 system promoted about half of the degradation extent obtained with mediated Fenton reactions. Pmp and Wc reductants alone did not cause any oxidation of either pNPX or xylan.

Parallel to the study on the degradation (oxidation) of CMC determined by measurement of the reducing power increase in CMC solutions, the change in CMC chain length (depolymerization) was also determined by calculating the reciprocal of the specific viscosity of the solution ($1/n_{sp}$; Fig. 3). Each method provides different information about depolymerization, as a random cleavage in the cellulose chain at points remote from the end of the chain may cause a large decrease in viscosity (a parameter related to chain length), while the reducing power of the solution would not be significantly affected [33].

Oxidative degradation of CMC with Fenton (Fe^{3+}/H_2O_2) and mediated Fenton reactions (Wc-Fe^{3+}/H_2O_2 and Pmp-Fe^{3+}/H_2O_2) showed two distinct phases (Fig. 3). The first was characterized by extensive cellulose chain depolymerization within the first 3-h reaction period and by low CMC oxidation. Specifically, a 3-h treatment of CMC decreased the viscosity to about 80% of the maximum viscosity reduction that was obtained with a 48 h treatment while, concurrently, the number of reducing ends produced in the CMC solutions (indicating CMC oxidation/degradation) was less than 15% of theoretical reducing ends that could potentially be generated after complete hydrolysis. The second phase (3–48 h treatments) was characterized by extensive CMC degradation but with cellulose chain depolymerization proceeding at an increasingly slower rate.

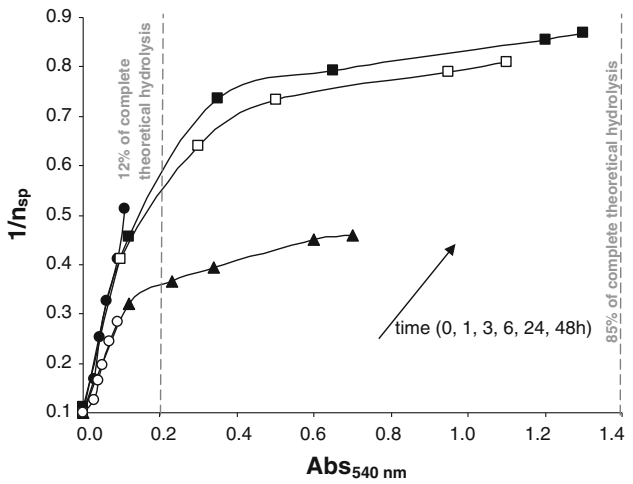


Fig. 3 Time-course of carboxymethyl cellulose (CMC) depolymerization/oxidation as indicated by the increase in reducing power of the CMC solutions (Abs_{540nm}) and change in the reciprocal of the specific viscosity ($1/n_{sp}$) after treatment with Wc-Fe^{3+}/H_2O_2 (*filled squares*), Pmp-Fe^{3+}/H_2O_2 (*open squares*), Fe^{3+}/H_2O_2 (*filled triangles*), Wc-Fe^{3+} (*filled circles*) and Pmp-Fe^{3+} (*open circles*)

CMC was also depolymerized with Wc-Fe^{3+} and Pmp-Fe^{3+} (minus H_2O_2) systems, as indicated by an increase in the $1/n_{sp}$ values. However, the extent of depolymerization promoted by these systems was much lower than that obtained with mediated Fenton reactions (Wc-Fe^{3+}/H_2O_2 and Pmp-Fe^{3+}/H_2O_2; Fig. 3). The Wc-Fe^{3+} and Pmp-Fe^{3+} systems promoted only limited cellulose oxidation as the maximum reducing power of CMC solutions with 48 h treatment were equivalent to only 5% of the theoretical reducing ends that could be obtained with complete hydrolysis of CMC. Wc and Pmp reductants alone caused neither CMC viscosity loss nor an increase in reducing ends in comparison to untreated CMC solution.

No increase in the number of reducing ends in the supernatants obtained from Avicel treatments in comparison to the untreated Avicel was detected. It is likely that the insoluble residue of the microcrystalline Avicel-cellulose remaining after treatment was modified but not extensively enough to release reducing sugars. We confirmed this using the DNS assay on both treated and untreated Avicel residues, which showed a minor increase in reducing ends only in the Avicel residues obtained from the treatment with Fenton reactions in the presence of Wc or Pmp reductants (Wc-Fe^{3+}/H_2O_2 and Pmp-Fe^{3+}/H_2O_2; data not shown). This indicates that only the mediated Fenton reactions were capable of promoting an increase in the reducing ends of microcrystalline cellulose. This increase could have been the result of either generation of short fibers due to oxidative breakdown of cellulose chains, or due to the disruption of hydrogen bonding between cellulose chains within the highly ordered and tightly packed regions of the cellulose microfibrils. The latter, in turn, would be expected to expose new reducing ends of the cellulose molecules buried within previously intact cellulose microfibrils for interactions with reactants. This phenomenon has been referred to as amorphogenesis [62, 63], a proposed initial step during cellulose biodegradation that would render the highly ordered regions more susceptible to the subsequent hydrolytic action of cellulases.

In this context, it appears that mediated Fenton reactions could, particularly in brown rot fungi, be a potential candidate for the C_1 component of the C_1–C_X mechanistic model introduced by Mandel and Reese about 60 years ago to explain microbial degradation of cellulose [64, 65]. Briefly, this original model hypothesized that a component of the cellulase systems (C_1, the so-called 'swelling factor') opened up the cellulose matrix, allowing the now more accessible substrate to be depolymerised by cellulose-degrading enzymes (C_X) [64, 65]. Although many hydrolytic enzymes that could account for the suggested C_X

action have been identified and characterized, so far the identification and characterization of the C_1 factor remains elusive [63]. Although the disruption of the highly ordered and tightly packed regions of Avicel was not monitored directly in this work, it is expected that it would occur, as observed by Wang and Gao [31], who demonstrated this phenomenon by observing an ~50% decrease in the crystallinity of cotton fibers after a mediated Fenton reaction treatment.

Lignin

The syringyl lignin monomers released from the ^{13}C-TMAH thermochemolysis of treated and untreated red spruce milled wood were assessed for the extent of demethylation (or increase in hydroxy content), by calculating the level of ^{13}C-methyl addition at position 3 on the aromatic ring of the eight monomers investigated. These monomers are characteristic for a coniferyl-based gymnosperm lignin [66]. Any increase in the % ^{13}C with respect to the untreated wood residue was presumed to be the result of demethylation.

Lignin in the milled wood treated with Fenton reaction mediated with Wc and Pmp reductants showed significant demethylation as signified by the increase in the hydroxyl content of up to 7% of all monomers, compared to the untreated wood (Fig. 4), indicating that demethylation of the 3-methoxy carbon occurred during treatments with mediated Fenton reactions, generating 3,4-dihydroxy phenyl structures in the lignin. G8 and G6 displayed the greatest increase in hydroxyl content, followed by G18 and G4, with minor increases in G7, CA, G14 and G15. G14 and G15 contain the fully methylated glycerol side chain and are near to the structure of the complete methylated lignin monomer. These compounds are also thought to be indicative of undegraded β-O-4 linkages and monomers with intact glycerol side chains in lignin [45, 67]. Their presence in the TMAH thermochemolysis products of natural lignin-containing samples indicate that some portion of the sample contains lignin fragments that have not undergone complete side chain oxidation. Therefore, an increase in the yield of the highly oxidized monomer G6, relative to the control, has been interpreted as the extent of lignin degradation [68]. Both Wc-Fe^{3+}/H_2O_2 and Pmp-Fe^{3+}/H_2O_2 treatments promoted lignin oxidation as indicated by the statistically significant increase in G6 values compared to the untreated spruce (control; Table 5). Previous studies demonstrated that an increase of the G6 to G4 (acid/aldehyde) ratio indicates a higher degree of lignin side-chain oxidation [66, 68, 69]. Statistically significant lignin side-chain oxidation relative to untreated milled wood was observed only with Wc-Fe^{3+}/H_2O_2 treatment (Table 5).

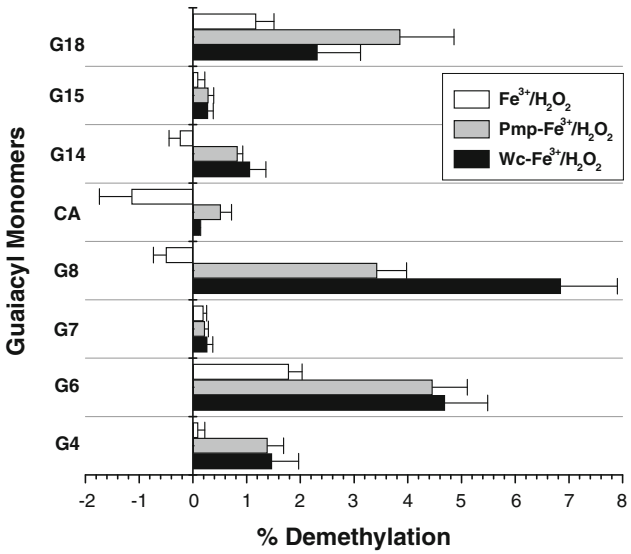


Fig. 4 Degree of demethoxylation of lignin monomers released after ^{13}C-tetramethylammonium hydroxide (TMAH) thermochemolysis of red spruce wood (*Picea rubens*) treated with Wc-Fe^{3+}/H_2O_2, Pmp-Fe^{3+}/H_2O_2 and Fe^{3+}/H_2O_2 relative to the untreated control sample. G4 3,4-dimethyoxybenzaldehyde, G6 3,4-dimethoxybenzoic acid, methyl ester, G7 cis-1-(3,4-dimethyoxyphenyl)-2-methoxyethylene, G8 trans-1-(3,4-dimethyoxyphenyl)-2-methoxyethylene, G14 threo-1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane, G15 erythro-1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane, *CA* coniferyl alcohol, G18 3-(3,4-dihydroxyphenyl)-2-propenoic acid, caffeic acid

Table 5 Concentration (mg/mg wood sample) of G6 and G6/G4 released after ^{13}C-tetramethylammonium hydroxide (TMAH) thermochemolysis

Treatment system	G6	G6/G4
Untreated spruce	2.48 ± 0.15 a	0.28 ± 0.04 a
Pmp-Fe^{3+}/H_2O_2	2.92 ± 0.25 b	0.32 ± 0.04 a
Wc-Fe^{3+}/H_2O_2	4.72 ± 0.81 c	0.38 ± 0.06 b

Values with the same lower case letter do not differ significantly at the 95% confidence levell

Upon Fe^{3+}/H_2O_2 treatment, G6 and G18 were demethylated to about 1.8 and 1.2%, respectively, whereas G8, CA and G14 displayed a decrease in the hydroxyl content relative to the untreated sample. The decrease in hydroxyl content may be an indication of further aggregation or selective polymerization of the ortho-hydroxy functionality. Previous work found that some of the monomers released after ^{13}C-TMAH thermochemolysis of the brown rot-decay wood residue had a lower hydroxyl content than fresh wood [48].

Implications for brown and white rot fungi

The results presented here demonstrate that Fenton-based ·OH-producing reactions mediated by the isolated

phenolate-derivative compounds (Wc-Fe^{3+}/H_2O_2 and Pmp-Fe^{3+}/H_2O_2) displayed high degradation activity towards lignocellulose carbohydrates and lignin. These findings indicate that, besides *G. trabeum*, other wood decay fungi (including selective white rot fungi) possess the means to carry out mediated Fenton chemistry during lignocellulose biodegradation. This pathway, which has generally been reported to be the main non-enzymatic degradative system of *G. trabeum* [12, 21, 24, 29, 31], has not been well explored in other degradative fungi, and our current research supports the hypothesis that this mechanism can be used by other wood decay fungi to carry out Fenton-based, ·OH-producing, degradative reactions.

Since both brown and selective white rot fungi seem to possess the means to carry out Fenton chemistry, the difference in Fe^{3+}-reducing activity and also in the lignocellulolytic enzymatic repertories between brown and white rot would be expected to have a significant impact on overall plant cell wall biodegradation.

For brown rot fungi, high levels of Fe^{3+}-reducing activity (and therefore high levels of hydroxyl radical generation via the Fenton reaction) would lead to depolymerization and also some oxidation of polysaccharides at early stages of plant cell wall biodegradation. At more advanced stages in brown rot, when Fenton-based ·OH-producing reactions would have promoted an increase in plant cell wall accessibility, carbohydrate-degrading enzymes could then diffuse into the cell wall and continue cellulose and hemicellulose biodegradation. Brown rot fungi do not typically produce lignin-degrading enzymes and structural lignin changes that occur in brown rotted lignocellulose are believed to be caused by the action of OH radicals generated via Fenton reaction [21, 29]. In this context, high levels of Fe^{3+}-reducing activity would lead to an increase of O:C ratio in the remaining lignin (common in lignocellulose undergoing brown rot decay) as a consequence of aryl methyl ether cleavage, which in turn results in an increased number of phenolic groups as observed in the current work. The strong acidic conditions typically found in wood undergoing brown rot decay [21] would significantly limit solubility and diffusion of the brown rotted lignin fragments. This would also promote the repolymerization of lignin fragments, increasing their susceptibility to repolymerization within the wood cell wall.

In our previous work [47], we demonstrated the diffusibility of lignin fragments after chlelator-mediated Fenton system (CMFS) treatment but, in brown rot degradation this diffusion is likely to be limited by the pH of the microenvironment, thus resulting in the brown, modified lignin residue commonly observed after brown rot decay of wood.

In selective white rot fungi, it would be expected that the more limited Fe^{3+}-reducing activity observed in the *P. medulla-panis* model would cause only moderate depolymerization and oxidation of the carbohydrate fraction as well as moderate demethylation of lignin compared to that observed in our brown rot. In addition, the limited production of cellulose-degrading enzymes by these fungi [60] can explain the moderate degradation of the carbohydrate fraction in wood that occurs with wood biotreated with selective white rot fungi [23]. Considering that lignin demethylation leads to an increase in the production of phenolic groups, this makes lignin more reactive, as ligninolytic enzymes such as manganese-peroxidase and laccase are unable to oxidize non-phenolic lignin [72]. These enzymes could then further degrade lignin that has been attacked by OH radicals generated through mediated Fenton reactions and explain, at least in part, the extensive degradation of lignin by selective white rot fungi as well as lignin-peroxidase-deficient white rot fungi. The existence of such synergism between fungal Fe^{3+}-reductants and ligninolytic enzymes in white rot fungi was demonstrated recently during oxidation of nonphenolic lignin model compounds with manganese-peroxidase, laccase, and a mixture of low molecular weight Fe^{3+}-reductants, all of them partially purified from various white rot fungi [1]. However, for this to occur, selective white rot fungi require a mechanism that permits the diffusion of lignin fragments generated during Fenton-based ·OH-producing reactions. Diffusion permits these fragments to move beyond the wood cell wall to microvoid spaces large enough that the ligninolytic enzymes can access, since these enzymes cannot penetrate the intact cell wall until significant degradation has occurred.

The less acidic conditions commonly found in wood undergoing white rot decay in comparison to brown rot decay may permit diffusion of the demethylated lignin fragments into the wood cell lumen space or into regions that have been sufficiently degraded to allow penetration by the relatively large ligninolytic enzymes, which will further oxidize the fragments. The higher pH environment of the wood cell wall would also limit the repolymerization of lignin fragments that is typically observed in brown-rotted wood, but not in white-rotted wood. Moreover, it has been speculated that the polysaccharidic fraction in wood, which is only partially removed during the initial stages of selective white rot decay, may also help to promote the swelling or partial dissolution of oxidized lignin fragments in the cell wall [70], based on the finding that lignin is readily soluble in glycols [71]. White rotted lignin fragments may be soluble for some time and it is possible that some may function to bind and reduce iron as has been previously proposed for participation in chelator-mediated Fenton reactions [38, 68].

Conclusion

Both the brown and selective white rot fungi examined in this work produced low molecular weight peptides and phenolate-derivative compounds with Fe^{3+}-reducing capabilities. Phenolate compounds were the major Fe^{3+}-reductants produced under the growth conditions used, and these phenolate compounds were able to reduce Fe^{3+} even in the presence of physiological concentrations of oxalic acid. Fenton-based ·OH-producing reactions mediated by the isolated phenolate-derivative compounds were efficient in oxidizing lignocellulose carbohydrates and lignin, indicating that both brown and white rot fungi possess the means to promote Fenton chemistry. Micro-site environmental factors that are different in white rot environments than brown rot environments may play a significant role in the overall degradation of plant cell wall components, especially in the diffusion of the Fenton-based oxidized lignin fragments, which seems to occur in selective white rot fungi but not in brown rot fungi. In selective white rot this work suggests that, to some extent, oxidatively degraded plant cell wall components may also be degraded by operating separately or synergistically with lignocellulolytic enzymes to achieve effective biodegradation. Moreover, it is believed that the levels of Fe^{3+}-reducing activity as well as lignocellulolytic enzyme levels would play a significant role in overall plant cell wall biodegradation.

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