Significant levels of extracellular reactive oxygen species produced by brown rot basidiomycetes on cellulose

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Abstract **It is often proposed that brown rot basidiomycetes use extracellular reactive oxygen species (ROS) to accomplish the initial depolymerization of cellulose in wood, but little evidence has been presented to show that the fungi produce these oxidants in physiologically relevant quantities. We used [^{14}C]phenethyl polyacrylate as a radical trap to estimate extracellular ROS production by two brown rot fungi, *Gloeophyllum trabeum* and *Postia placenta*, that were degrading cellulose. Both fungi oxidized aromatic rings on the trap to give monohydroxylated and more polar products in significant yields. All of the cultures contained 2,5-dimethoxyhydroquinone, a fungal metabolite that has been shown to drive Fenton chemistry in vitro. These results show that extracellular ROS occur at significant levels in cellulose colonized by brown rot fungi, and suggest that hydroquinone-driven ROS production may contribute to decay by diverse brown rot species.**

Key words: Wood decay; Hydroxyl radical; Aromatic hydroxylation; 2,5-Dimethoxyhydroquinone; *Gloeophyllum trabeum*; *Postia placenta*

1. Introduction

Brown rot basidiomycetes are highly destructive wood decay fungi that have the unusual ability to depolymerize wood cellulose without removing the lignin that encases it. Although cellulases may contribute to this process, they are unlikely to catalyze decay by themselves because enzymes are too large to penetrate the lignin barrier in sound wood [1–3]. A limited role for cellulases is also indicated by the finding that most brown rot fungi evidently lack the complete set of enzymes needed for efficient cellulolysis – when grown on cellulose they produce endoglucanases but not cellobiohydrolases [4]. Moreover, cellulose that has been degraded by brown rot fungi contains carboxylate groups, which suggests that an oxidative agent rather than a hydrolytic one has attacked the polymer [5]. These observations have led to the hypothesis that brown rot fungi employ small extracellular oxidants such as reactive oxygen species (ROS) to depolymerize cellulose during incipient decay [6–8].

The Fenton reaction ($H_2O_2+Fe^{2+}+H^+ \rightarrow H_2O+Fe^{3+}+{}^{\bullet}OH$) could provide a route for extracellular ROS production if brown rot basidiomycetes have extracellular mechanisms to reduce Fe^{3+} and O_2 [5,9]. Evidence in favor of this idea was obtained by Goodell et al., who observed that one of these fungi, *Gloeophyllum trabeum*, produces a mixture of extracellular phenolic compounds that can reduce Fe^{3+} [6]. Recently, two such metabolites, 2,5-dimethoxyhydroquinone and 4,5-dimethoxycatechol, were identified as major extracellular metabolites in glucose-grown cultures of *G. trabeum* [10–12]. Both hydroquinones were shown to reduce Fe^{3+} and O_2 rapidly under physiological conditions, thus generating both Fe^{2+} and H_2O_2 [10,11]. Moreover, the fungus was shown to reduce the resulting dimethoxyquinones back to hydroquinones [10–12], possibly by the action of an intracellular quinone reductase [13]. In this way, glucose-grown *G. trabeum* drives continuous ${}^{\bullet}OH$ production via quinone redox cycling.

Despite these advances, it remains unclear whether brown rot fungi produce significant quantities of extracellular ROS when they degrade cellulose. Various assays, including dimethyl sulfoxide oxidation [14], aromatic hydroxylation [15–17], and polyethylene glycol depolymerization [18], have been used to detect ROS qualitatively in cultures. However, the radical traps used in these assays suffer a variety of drawbacks. Most of them are small enough to penetrate fungal hyphae, where they could be oxidized by monooxygenases [19] or intracellular ROS [18]. High molecular weight polyethylene glycols, although too large to penetrate the fungal cell wall [20], are likely to undergo radical chain propagation reactions that make them unreliable for quantitative estimates of ROS production [18].

To address these problems, we designed a new ROS assay that depends on the oxidation of a polymeric trap, phenethyl polyacrylate, that is too large and insoluble to traverse the fungal cell wall. We included the polymer in cultures of *G. trabeum* and *Postia placenta* that were competent to degrade cellulose, and we obtained an estimate of ROS production from the frequency of aromatic hydroxylation [21]. In addition, we assayed the colonized cellulose for metabolites that are able to drive Fenton chemistry. The results show that these two brown rot fungi produce complete Fenton systems and significant levels of extracellular ROS on cellulose.

2. Materials and methods

2.1. Reagents

β-[^{14}C]Phenylacetic acid was purchased from Sigma/Aldrich and polyacryloyl chloride (approx. 10 kDa) from Polysciences. Standards

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of phenethyl alcohol, 2-, 3-, and 4-hydroxyphenethyl alcohol, 2,- 3,-, and 4-nitrophenethyl alcohol, and 1-phenyl-1,2-ethanediol were obtained from Sigma/Aldrich, as were all other reagents unless otherwise specified.

To prepare radiolabeled phenethyl polyacrylate, β-[^{14}C]phenylacetic acid (0.055 mmol, 9.1 mCi mmol^{-1}) was dissolved in ethereal diazomethane, and unlabeled phenylacetic acid methyl ester (0.445 mmol) was added. The resulting β-[^{14}C]phenylacetic acid methyl ester (0.50 mmol, 1.0 mCi mmol^{-1}) was concentrated almost to dryness by rotary vacuum evaporation, and 2 ml of dry toluene was added. The solution was placed in a flask under dry N_2, the flask was placed in an ice bath, and 3 mmol of diisobutylaluminum hydride in toluene was added slowly to reduce the ester. The mixture was stirred for several hours at ambient temperature, then acidified with HCl and extracted four times with 1 volume of dichloromethane. The pooled extracts were dried over sodium sulfate, concentrated by rotary evaporation, and applied to a silica thin layer chromatography plate (20×20 cm dimensions, 1 mm thickness), which was developed twice in hexane/ethyl acetate, 3:1. The β-[^{14}C]phenethyl alcohol band was scraped from the plate, eluted with ethyl acetate, concentrated to dryness, and redissolved in several milliliters of dry dioxane. The yield of product was 0.40 mmol and its radiochemical purity was 96% as determined by high performance liquid chromatography (HPLC).

The dioxane solution of β-[^{14}C]phenethyl alcohol was transferred to a 20 ml glass vial. With rapid stirring, 0.44 ml of polyacryloyl chloride solution (25% w/v in dioxane, containing approx. 1.2 mmol of acid chloride groups) was slowly added. The solution was stirred at ambient temperature overnight, then transferred into 10 volumes of 0.5 N aqueous K_2HPO_4 to hydrolyze the remaining acid chloride groups. The polymer precipitated as a gum and slowly redissolved with a concomitant drop in pH. Small quantities of 1 N NaOH were added periodically during this process to maintain the pH near 7. The resulting clear solution was dialyzed repeatedly against 10 mM sodium potassium phosphate buffer (pH 7.0), in a bag with a 2 kDa cutoff, until the ^{14}C in the dialysate declined to the background level. The final yield of polymer-linked β-[^{14}C]phenethyl moieties (Fig. 1) was 0.09 mmol.

Hydrolysis of the polymer and HPLC of the ^{14}C-labeled products (see below) showed that phenethyl alcohol was the only compound released. The structure was also confirmed by ^{13}C NMR spectrometry in potassium phosphate/D_2O at pH 7.0. δ in ppm: 38 (α-CH_2 in phenethyl moiety), 39–40 (CH_2 in polyacrylate), 46–49 (CH in polyacrylate), 70 (β-CH_2 in phenethyl moiety), 131 (aromatic C4), 133–134 (aromatic C2, C3, C5, C6), 142 (aromatic C1), 182 (ester carbonyl in phenethyl polyacrylate), 187 (carboxylate anion in polyacrylate). By integrating the methylene signals from the phenethyl alcohol moieties and the polyacrylate backbone, we calculated that approximately 15% of the carboxylate groups in the polyacrylate were phenethylated. The ^{14}C-labeled phenethyl polyacrylate was freely water-soluble at pH 7. It formed an opalescent solution at pH 5 and precipitated at pH 4. It was stored at −20°C as a solution in 10 mM sodium potassium phosphate buffer, pH 7.0.

2.2. Cultures

G. trabeum (ATCC 11539) and *P. placenta* (ATCC 44394) were maintained on potato dextrose agar (PDA) plates, which were stored at 4°C until use. For biodegradation experiments, the fungi were grown on blocks of cellulose that were prepared from sheets normally used as a feedstock for viscose manufacture (Lenzing AG) [22]. The sheets were cut to dimensions of 2.5×2.5 cm and the pieces were shaken gently in water to loosen the fiber structure. For each culture, five wet cellulose sheets were stacked and dried overnight under vacuum at 55°C to give a block with a dry weight of approximately 3 g. The dry weights were recorded, after which the cellulose blocks were autoclaved and dried again under vacuum in a sterile desiccator jar. Each block was infused with 1.5 ml of autoclaved 20% w/v potato dextrose broth containing 4.6×10^5 dpm of filter-sterilized ^{14}C-labeled phenethyl polyacrylate (2.1×10^{-4} mmol of phenethyl moieties). Each block was then placed on a piece of polypropylene mesh over 25 ml of PDA in a 500 ml Erlenmeyer flask that had been inoculated several days beforehand with an agar plug containing one of the fungi. An additional agar plug inoculum was placed on top of each block. The cultures were fitted with gassing manifolds, incubated at 32°C, and flushed daily with moist, sterile air to vent $^{14}CO_2$, which was trapped in an ethanolamine-containing cocktail for quantification by scintillation counting [23]. Fungal hyphae were visible on the cellulose within several days.

2.3. Determination of metabolites and iron in cultures

For each analysis, a block was harvested, weighed to determine its water content, and cut into four pieces. These were moistened with 2 ml of distilled water, placed in the barrel of a plastic 50 ml syringe, and rapidly squeezed to extract as much liquid as possible. A 250 μl sample was analyzed immediately by reversed phase HPLC for hydroquinones and quinones as described previously [10]. These metabolites were identified from their retention times relative to authentic standards and from their UV/visible absorption spectra [10,11], which were obtained with an in-line diode array detector. A portion of the extract was then analyzed for oxalate with a coupled oxalate oxidase-peroxidase assay kit (Sigma/Aldrich) according to the manufacturer's instructions. Total dissolved iron in the extract was determined with a Jobin Yvon-Ultima inductively coupled plasma atomic emission spectrometer. The metabolite and iron concentrations shown below were corrected for the additional water that was added to extract the cellulose blocks.

2.4. Analysis of phenethyl polyacrylate oxidation products in cultures

Because of the labor-intensive nature of the workup, we did not attempt statistical analyses of replicate cultures in these experiments. Instead, four replicate cellulose blocks from each treatment were combined, placed in a Waring blender, and homogenized for 20 s in 200 ml of warm (55°C) 10 mM sodium phosphate buffer at pH 7.0. Viscosimetric analyses (see below) showed that this procedure did not affect the molecular weight of the cellulose. The mixture was shaken at room temperature for 20 min and centrifuged. The supernatant fraction was retained and the pelleted cellulose was subjected to two more cycles of extraction with warm buffer, shaking, and centrifugation. The extracted cellulose was retained for molecular weight analysis (see below). The supernatant fractions were combined, filtered through glass wool, and lyophilized to dryness. The sample was redissolved in 40 ml of distilled water, dialyzed twice against distilled water in a bag with a 2 kDa cutoff, and evaporated to dryness under reduced pressure. The sample was then redissolved in 1.0 ml of 1 N NaOH, stirred overnight to hydrolyze the ester linkages in phenethyl polyacrylate, and adjusted to pH 7 with formic acid. Typical mass balances for ^{14}C during this workup were as follows: 1–4% was mineralized by the fungi and 70–80% was extractable from the cellulose. Of the extracted ^{14}C, 90–95% was recovered as high molecular weight material after lyophilization and dialysis. When this high molecular weight material was hydrolyzed, more than 95% of the ^{14}C was released from the polymer.

To identify the ^{14}C-labeled hydrolysis products, filtered samples (250 μl) were analyzed by reversed phase HPLC on a Synergi Polar-RP column (150×4.6 mm dimensions, 4 μm particle size, Phenomenex). The column was run at 1.5 ml/min and ambient temperature. The eluant consisted of 100% water for 5 min, followed by a 20 min linear gradient to water:acetonitrile, 97:3, then a 25 min linear gradient to water:acetonitrile, 80:20, and finally a 1 min linear gradient to 100% acetonitrile. Fractions (0.5 ml) were collected and assayed for ^{14}C by scintillation counting. Preliminary identifications were made by comparing product elution times with those of the three hydroxyphenethyl alcohol standards. To confirm the identifications, the separated ^{14}C-labeled hydrolysis products and the hydroxyphenethyl alcohol standards were methylated with diazomethane and re-chromatographed on the same system.

2.5. Chemical oxidations of phenethyl polyacrylate

For Fenton oxidation of ^{14}C-labeled phenethyl polyacrylate, the reactions (1.0 ml) contained 0.5 mM each of H_2O_2, $FeCl_3$, ascorbate, and polymer-linked phenethyl moieties at ambient temperature. The reactions were buffered with 100 mM sodium phosphate (pH 7.0) or 20 mM sodium acetate (pH 5.0), and stirred overnight. For singlet oxygen ($^1O_2$) oxidation of the polymer, the reaction (1.0 ml) was done in a stoppered serum vial that initially contained 0.5 mM polymer-linked phenethyl moieties and 150 mM H_2O_2 in distilled water at ambient temperature. Sodium hypochlorite (50 mM final concentration) was added over 30 min with a syringe to the rapidly stirring solution, which was then stirred overnight [21]. For peroxyl radical oxidation of the polymer, the reaction (1.0 ml) contained 0.5 mM polymer-linked phenethyl moieties and 12 mM 4,4′-azobis(4-cyanova-

leric acid) (ACVA) in 50 mM sodium phosphate buffer, pH 7.0 [21]. The reaction was shaken at 60°C overnight. All three chemical oxidation reactions were dialyzed, lyophilized, hydrolyzed, and subjected to HPLC as described above for the fungal experiments.

2.6. Molecular weight analysis of cellulose from cultures

The weight-average degree of polymerization (DP_w) and number-average degree of polymerization (DP_n) of the cellulose samples were determined by high performance gel permeation chromatography (GPC) after tricarbanilation of the cellulose [24]. Cellulose samples were dehydrated by repeated solvent exchange with pyridine, after which 2 mg quantities were dispersed in 3 ml of anhydrous pyridine containing 300 μl of phenyl isocyanate (Fluka). The reactions were stirred for 48 h at 80°C and then stopped with 300 μl of methanol. From the resulting nearly clear solutions, 0.5 ml samples were removed and evaporated to dryness under a stream of argon. The derivatized cellulose samples were dissolved in 2 ml of tetrahydrofuran and filtered portions (60 μl) were analyzed on the following GPC columns (300×8 mm dimensions) with the indicated molecular weight ranges: Shodex KF807 (1000–200 000 000), Shodex KF805 (100–4 000 000), Shodex KF803 (50–70 000), Phenomenex Phenogel 100 Å (500–6000), Polymer Laboratories PLgel 50 Å (100–3000). The columns, linked in series, were run in tetrahydrofuran at 1.0 ml/min and ambient temperature.

To obtain cellulose tricarbanilate molecular weights from the GPC data, polystyrene molecular weight standards were used to construct a universal calibration curve of hydrodynamic volume versus retention volume [25]. Hydrodynamic volume was calculated as the product of molecular weight and intrinsic viscosity by using the Mark-Houwink parameters $k = 0.0112$ and $a = 0.72$ for polystyrene in tetrahydrofuran [24]. The universal curve was fit to a bilinear model by a least squares procedure that used the known weight average molecular weights (M_w), number average molecular weights (M_n), and polydispersities of the standards. Subsequently, the universal calibration curve was used to determine the cellulose tricarbanilate molecular weight distribution by using the Mark-Houwink parameters $k = 0.0053$ and $a = 0.84$ for cellulose tricarbanilate in tetrahydrofuran [24]. DP values were calculated by dividing these molecular weights by 519, the molecular weight of the cellulose tricarbanilate monomer. To calculate the average frequency of glycosidic bond cleavage in cellulose, the following formula was applied:

$$\%\ \text{of glycosidic bonds cleaved} = \frac{\left(\frac{DP_n\ \text{of uninoculated cellulose}}{DP_n\ \text{of sample cellulose}} - 1\right)}{DP_n\ \text{of uninoculated cellulose} - 1} \times 100$$

The viscosimetric degree of polymerization (DP_v) of the cellulose samples was determined as described [26] by dissolving the cellulose in 0.5% cupriethylenediamine solution (GFS Chemicals) and determining its viscosity in Cannon-Fenske capillary viscometer tubes (size 100, Cannon Instruments Co.). DP_v values were calculated based on the calibration determined by Sihtola et al. [27].

3. Results

3.1. Cellulose cleavage

It is generally difficult to elicit cellulolytic activity in brown rot fungi when they are grown in the absence of wood [4]. However, it appeared best to avoid wood-based cultures, because the presence of lignin under oxidative conditions can lead to cross-coupling reactions with phenols. If these reactions occurred, aromatic hydroxylation metabolites derived from our ROS probe might become covalently linked to the growth substrate. Therefore, we attempted a simpler culture system, in which the fungi were grown on blocks of cellulose. Analysis of the recovered cellulose by viscosimetry and by GPC after derivatization showed that both *G. trabeum* and *P. placenta* were clearly cellulolytic in these cultures (Table 1). The depolymerization rates were somewhat slower than the rate reported earlier for *P. placenta* grown on cellulose in the presence of wood [28], but they were sufficient to undertake a search for ROS that could contribute to cellulose degradation.

Table 1
Cellulose cleavage by the fungi[a]

Fungus	Cellulose DP			Scissions per 100 anhydroglucose units in cellulose
	DP_v	DP_w	DP_n	
Uninoculated				
26 days	630	1637	100	0
G. trabeum				
8 days	251	861	85	0.18
17 days	127	528	67	0.50
P. placenta				
10 days	348	1259	86	0.16
27 days	156	694	69	0.45

[a]Values are averages for four pooled replicate cultures.

3.2. Extracellular metabolites

G. trabeum and *P. placenta* both produced 2,5-dimethoxyhydroquinone and 2,5-dimethoxy-1,4-benzoquinone on cellulose (Table 2). The hydroquinone autooxidized to the quinone rapidly after it was extracted from the colonized cellulose, which indicates that the fungi had some mechanism to maintain this metabolite in its reduced form. As expected, the cultures contained significant concentrations of extractable iron, and they also produced oxalate, a strong Fe^{3+} chelator [29]. Hydroquinone autooxidations are generally dependent on metal ions, and previous work has shown that 2,5-dimethoxyhydroquinone reduces Fe^{3+}–oxalate complexes rapidly to generate a Fenton system [10,11]. These results established that our cultures were likely to produce •OH.

3.3. Extracellular aromatic hydroxylation

We incubated [^{14}C]phenethyl polyacrylate in cellulose-grown fungal cultures, hydrolyzed the remaining high molecular weight polyester (Fig. 1), and analyzed the resulting phenethyl alcohol-derived products by HPLC with scintillation counting. The chromatographic data indicated that the fungi produced three monohydroxylated metabolites: 4-, 3-, and

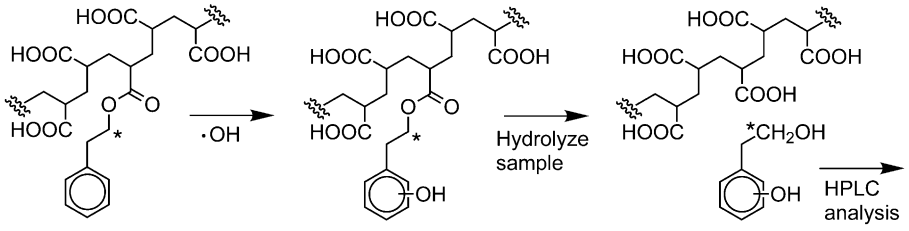

Fig. 1. Structure of [^{14}C]phenethyl polyacrylate and strategy for identification of hydroxylation products. The asterisk shows the position of ^{14}C.

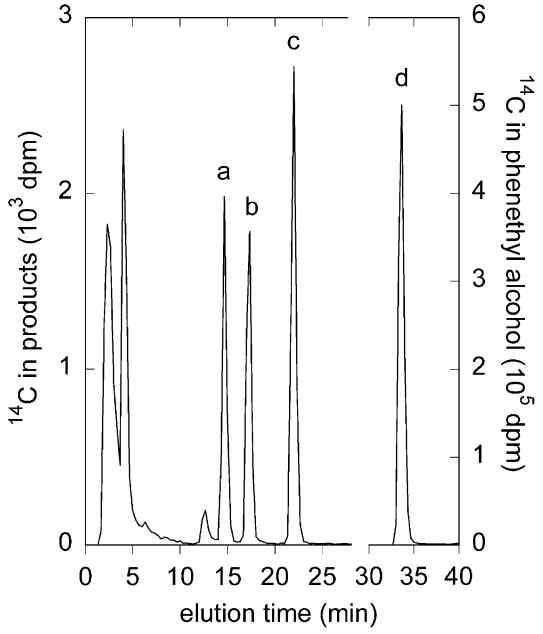

Fig. 2. HPLC analysis of hydrolysis products from [^{14}C]phenethyl polyacrylate that was incubated in *G. trabeum* cultures for 17 days. The peaks indicated are (a) 4-hydroxyphenethyl alcohol, (b) 3-hydroxyphenethyl alcohol, (c) 2-hydroxyphenethyl alcohol, and (d) phenethyl alcohol. The small peak eluting at 12–13 min was tentatively identified from its retention time as 1-phenyl-1,2-ethanediol, but the quantities recovered were too small to attempt confirmation.

2-hydroxyphenethyl alcohol (Fig. 2, products a, b, and c, respectively). Methylation of these products with diazomethane followed by HPLC against standards of the three isomeric methoxyphenethyl alcohols confirmed the identifications (data not shown). The fungi also gave more polar products that we were unable to identify (Fig. 2), but they did not produce nitrophenethyl alcohols (data not shown). The product distributions found in cultures resembled those obtained in vitro with Fenton reagent or singlet oxygen, but not those obtained with peroxyl radicals (Table 3). The relative abundances of the three hydroxyphenethyl alcohol isomers obtained from fungal cultures were similar to those obtained from a Fenton reaction done at pH 5, the closest we could come to the acidic conditions in cultures without precipitating the polymer.

4. Discussion

4.1. Identity of the ROS in cultures

Cellulose-grown cultures of *G. trabeum* and *P. placenta* oxidized the pendant aromatic rings in phenethyl polyacrylate. The reactions involved must have been extracellular, because the polymer we prepared is not only too large to penetrate the

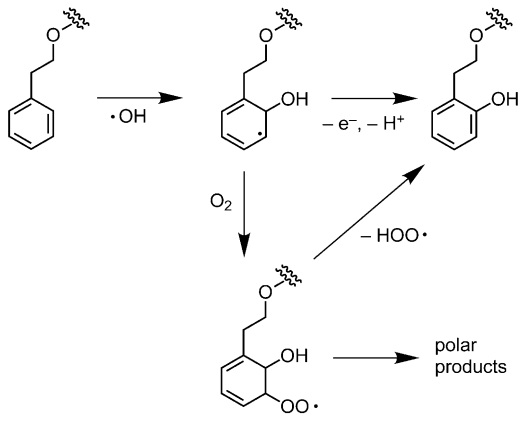

Fig. 3. Examples of typical reactions after addition of •OH to the aromatic ring of phenethyl polyacrylate.

fungal cell wall [20], but also water-insoluble at the low extracellular pH (< 4) generated by the cultures. The three phenolic products that we identified indicate the action of an oxidant with low regioselectivity, and the product distributions agree well with those generated in vitro by •OH at low pH. Moreover, all of the cultures produced 2,5-dimethoxyhydroquinone, which drives Fenton chemistry rapidly at the pH values, iron concentrations, and oxalate concentrations we observed in cultures [10]. These results lead us to conclude that the fungi produced •OH on cellulose.

The initial attack of •OH on the phenethyl alcohol moiety is expected to generate a hydroxycyclohexadienyl radical, which can either reduce some acceptor to become a phenol, or add O_2 to become a hydroxyperoxyl radical. The hydroxyperoxyl radical can lose a perhydroxyl radical to become a phenol, or it can undergo additional oxidoreductions to give products that are more oxidized (Fig. 3) [30]. Most of the polar products that we were unable to identify in the HPLC analyses probably belong in this last category.

Other ROS besides •OH may have contributed to the products we observed in cultures. Since ACVA thermolysis converted some of the polymer's phenethyl moieties to polar products in vitro (Table 3), peroxyl radicals could have been responsible for some of the polar products formed in vivo. Likewise, the ability of H_2O_2/NaOCl to hydroxylate phenethyl polyacrylate means that we cannot rule out singlet oxygen as an oxidant in the cultures. Therefore, although these fungi produced the ingredients needed to drive Fenton chemistry, we refer to the oxidants in cultures generically as ROS.

Other oxidants are unlikely to have contributed significantly to phenethyl polyacrylate oxidation. Although reactive nitrogen species (RNS) such as peroxynitrite can hydroxylate aromatic rings [31], we can probably rule them out because the fungi did not nitrate phenethyl polyacrylate. Moreover,

Table 2
Concentrations of metabolites and iron in the colonized cellulose

Fungus	Concentration (μM) ± S.D.			
	2,5-dimethoxyhydroquinone	2,5-dimethoxy-1,4-benzoquinone	oxalate	iron
G. trabeum (11 days)	18.0 ± 5.1 (n = 3)	27.9 ± 3.7 (n = 3)	205 ± 43 (n = 3)[a]	46 ± 1 (n = 2)
P. placenta (12 days)	6.9 ± 1.4 (n = 4)	13.2 ± 1.2 (n = 4)	977 ± 256 (n = 4)[b]	87 ± 3 (n = 2)

[a]pH of the extracts was 3.5–3.8.
[b]pH of the extracts was 3.3–3.5.

Table 3
Products of phenethyl polyacrylate oxidation

Agent	Product yields (% of ^{14}C in chromatogram)			
	2-hydroxyphenethyl alcohol	3-hydroxyphenethyl alcohol	4-hydroxyphenethyl alcohol	more polar products
Uninoculated[a]				
26 days	0.02	0.02	0.02	0.28
G. trabeum[a]				
8 days	1.69	1.19	1.23	4.89
17 days	3.20	2.30	2.10	8.09
P. placenta[a]				
10 days	0.77	0.55	0.51	1.46
27 days	1.29	0.96	0.89	2.26
H_2O_2/Fe^{2+}				
pH 5.0	1.97	1.59	1.31	6.22
pH 7.0	1.59	1.58	1.67	5.82
H_2O_2/NaOCl	0.14	0.13	0.14	1.71
ACVA thermolysis	0.00	0.00	0.00	8.48

[a]Yields are averages for four pooled replicate cultures.

the extremely low nitrogen content of wood [4] makes it unlikely that fungi would employ RNS as biodegradative agents. It is improbable that an enzyme was directly responsible for polymer hydroxylation in the cultures, because, to our knowledge, no extracellular monooxygenase capable of these reactions has been described from any source.

4.2. Extent of ROS production

The frequency of ROS attack on phenethyl polyacrylate subunits in fungal cultures can be estimated from the ratio of ^{14}C in products to total ^{14}C. If the uncharacterized polar products are included, this calculation yields a frequency of about 16% for 15-day *G. trabeum* cultures and 5% for 27-day *P. placenta* cultures (Table 3). These numbers show that there was a significant accumulation of reactions with ROS on the surface of the cellulose fibers, where the phenethyl polyacrylate had been precipitated to serve as a radical trap. Given the very low selectivity of ROS (and $^{\bullet}OH$ in particular) as oxidants, it follows that the surface of the cellulose must also have been oxidized. Molecular weight data on the cellulose recovered from cultures are consistent with this conclusion (Table 1), but we must ask why the frequency of cellulose cleavage was considerably lower than the frequency of phenethyl polyacrylate oxidation. Three factors probably contributed.

First, dried and reswollen bleached pulps such as the one we used are not sufficiently porous for the Fenton reagents to penetrate the cellulose fibers completely. Data published by Stone and Scallan [32] indicate that Fe^{3+}–oxalate complexes, with molecular diameters around 10–20 Å, are probably excluded from about half of the cellulose. By contrast, 10 kDa phenethyl polyacrylate is too large to penetrate the cellulose fibers significantly, and consequently all of it should be accessible to the Fenton system.

Second, although $^{\bullet}OH$ is likely to cleave a glycosidic bond in cellulose when it abstracts hydrogen from an anhydroglucose unit at C1 or C4, cleavage will not occur when abstraction occurs at C2, C3, C5 or C6 [33]. The relative rates of hydrogen abstraction by $^{\bullet}OH$ at individual carbons in glucosides are not known, but it is evident that measurements of cellulose depolymerization underestimate the extent of cellulose oxidation by $^{\bullet}OH$.

Finally, glucosides react with $^{\bullet}OH$ somewhat more slowly ($2–3\times10^9$ M^{-1} s^{-1}) than does phenethyl alcohol ($6–7\times10^9$ M^{-1} s^{-1}) [34]. Consequently, the proportion of anhydroglucose units oxidized in cellulose is expected to be lower than the proportion of phenethyl units oxidized in phenethyl polyacrylate.

Our data do not rule out the possibility that endoglucanases contributed to cellulose degradation by the fungi. However, it is unlikely that endoglucanases alone were responsible for cellulose cleavage, not only because these enzymes generally fail to cleave cellulose when they act alone [4], but also because ROS production by the cultures was clearly too high to avoid cellulose oxidation at the fiber surface. On the other hand, it is an interesting possibility that endoglucanases and ROS may cleave cellulose more efficiently when they act in concert.

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References

[1] Blanchette, R.A., Krueger, E.W., Haight, J.E., Akhtar, M. and Akin, D.E. (1997) J. Biotechnol. 53, 203–213.
[2] Fluornoy, D.S., Kirk, T.K. and Highley, T.L. (1991) Holzforschung 45, 383–388.
[3] Srebotnik, E., Messner, K. and Foisner, R. (1988) Appl. Environ. Microbiol. 54, 2608–2614.
[4] Eriksson, K.-E.L., Blanchette, R.A. and Ander, P. (1990) in: Microbial and Enzymatic Degradation of Wood and Wood Components (Timell, T.E., Ed.), Springer-Verlag, Berlin.
[5] Kirk, T.K., Ibach, R., Mozuch, M.D., Conner, A.H. and Highley, T.L. (1991) Holzforschung 45, 239–244.
[6] Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., Krishnamurthy, S., Jun, L. and Xu, G. (1997) J. Biotechnol. 53, 133–162.
[7] Hammel, K.E., Kapich, A.N., Jensen Jr., K.A. and Ryan, Z.C. (2002) Enzyme Microb. Technol. 30, 446–453.
[8] Hyde, S.M. and Wood, P. (1997) Microbiology 143, 259–266.
[9] Koenigs, J.W. (1974) Wood Fiber 6, 66–79.
[10] Jensen Jr., K.A., Houtman, C.J., Ryan, Z.C. and Hammel, K.E. (2001) Appl. Environ. Microbiol. 67, 2705–2711.
[11] Kerem, Z., Jensen, K.A. and Hammel, K.E. (1999) FEBS Lett. 446, 49–54.
[12] Paszczynski, A., Crawford, R., Funk, D. and Goodell, B. (1999) Appl. Environ. Microbiol. 65, 674–679.
[13] Jensen Jr., K.A., Ryan, Z.C., Vanden Wymelenberg, A., Cullen, D. and Hammel, K.E. (2002) Appl. Environ. Microbiol. 68, 2699–2703.

[14] Hirano, T., Tanaka, H. and Enoki, A. (1997) Holzforschung 51, 389–395.
[15] Backa, S., Gierer, J., Reitberger, T. and Nilsson, T. (1992) Holzforschung 46, 61–67.
[16] Schlosser, D., Fahr, K., Karl, W. and Wetzstein, H.-G. (2000) Appl. Environ. Microbiol. 66, 2479–2483.
[17] Tornberg, K. and Olsson, S. (2002) FEMS Microbiol. Ecol. 40, 13–20.
[18] Kerem, Z., Bao, W. and Hammel, K.E. (1998) Proc. Natl. Acad. Sci. USA 95, 10373–10377.
[19] Kamada, F., Abe, S., Hiratsuka, N., Wariishi, H. and Tanaka, H. (2002) Microbiology 148, 1939–1946.
[20] Scherrer, R., Louden, L. and Gerhardt, P. (1974) J. Bacteriol. 118, 534–540.
[21] Halliwell, B. and Gutteridge, J.M.C. (1999) Free Radicals in Biology and Medicine, 3rd edn., Oxford University Press, Oxford.
[22] Srebotnik, E., Jensen Jr., K.A., Kawai, S. and Hammel, K.E. (1997) Appl. Environ. Microbiol. 63, 4435–4440.
[23] Kirk, T.K., Connors, W.J., Bleam, R.D., Hackett, W.F. and Zeikus, J.G. (1975) Proc. Natl. Acad. Sci. USA 72, 2515–2519.
[24] Wood, B.F., Conner, A.H. and Hill Jr., C.G. (1986) J. Appl. Polym. Sci. 32, 3703–3712.
[25] Hiemenz, P.C. (1984) Polymer Chemistry: The Basic Concepts, Marcel Dekker, New York.
[26] Technical Association of the Pulp and Paper Industry (1992) Viscosity of pulp (capillary viscometer method), T230 om-89, in TAPPI Test Methods 1992–1993, TAPPI Press, Atlanta.
[27] Sihtola, H., Kyrklund, B., Laamanen, L. and Palenius, I. (1963) Paperi ja Puu 4a, 225–232.
[28] Kleman-Leyer, K., Agosin, E., Connor, A.H. and Kirk, T.K. (1992) Appl. Environ. Microbiol. 58, 1266–1270.
[29] Park, J.S.B., Wood, P.M., Davies, M.J., Gilbert, B.C. and Whitwood, A.C. (1997) Free Radic. Res. 27, 447–458.
[30] Pan, X.-M. and von Sonntag, C. (1990) Z. Naturforsch. B 45, 1337–1340.
[31] Halliwell, B. and Kaur, H. (1997) Free Radic. Res. 27, 239–244.
[32] Stone, J.E. and Scallan, A.M. (1968) Cellul. Chem. Technol. 2, 343–358.
[33] von Sonntag, C. (1980) Adv. Carb. Chem. Biochem. 37, 7–77.
[34] Notre Dame Radiation Laboratory Radiation Chemistry Data Center. URL: http://allen.rad.nd.edu/.