Microbiology (1997), 143, 259–266 Printed in Great Britain

A mechanism for production of hydroxyl radicals by the brown-rot fungus *Coniophora puteana*: Fe(III) reduction by cellobiose dehydrogenase and Fe(II) oxidation at a distance from the hyphae

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In timber infested by brown-rot fungi, a rapid loss of strength is attributed to production of hydroxyl radicals ($HO^{\cdot}$). The hydroxyl radicals are produced by the Fenton reaction [Fe(II)/H_2O_2], but the pathways leading to Fe(II) and H_2O_2 have remained unclear. Cellobiose dehydrogenase, purified from cultures of *Coniophora puteana*, has been shown to couple oxidation of cellodextrins to conversion of Fe(III) to Fe(II). Two characteristics of brown rot are release of oxalic acid and lowering of the local pH, often to about pH 2. Modelling of Fe(II) speciation in the presence of oxalate has revealed that Fe(II)–oxalate complexes are important at pH 4–5, but at pH 2 almost all Fe(II) is in an uncomplexed state which reacts very slowly with dioxygen. Diffusion of Fe(II) away from the hyphae will promote conversion to Fe(II)–oxalate and autoxidation with H_2O_2 as product. Thus the critical Fe(II)/H_2O_2 combination will be generated at a distance, enabling hydroxyl radicals to be formed without damage to the hyphae.

Keywords: cellobiose dehydrogenase, *Coniophora puteana*, free radicals, oxalate, wood decay

INTRODUCTION

Most wood-rotting fungi can be placed in one of three physiological classes: white rot, soft rot and brown rot (Eriksson *et al.*, 1990). The white-rot fungi degrade all components of lignocellulose, while soft rots merely cause a gradual softening at wet surfaces. Decay by brown rot leads to mineralization of polysaccharides, but lignin is not solubilized and remains as an amorphous brown residue. The fungi in this class include *Coniophora puteana* ('cellar rot') and *Serpula lacrymans* (dry rot). Early studies noted a rapid loss of strength prior to loss in weight, leading to the designation 'destruction rot' by Falck (1926). This physical change correlates with cleavage of cellulose into crystallites of about 200 glucose residues (Cowling, 1961). A closer examination revealed that degradation was taking place in an inner layer of the wood cell wall (the S_2 layer) while the layer adjacent to the hyphae (S_3) remained apparently unchanged (Wilcox, 1968; Eriksson *et al.*, 1990). Cowling & Brown (1969) deduced that the causative agent must be smaller than an enzyme, because of the limited size of the wood pores.

The hydroxyl radical is the strongest oxidizing agent in aqueous systems (Wood, 1988). Its standard biological source is the reaction of many forms of Fe(II) with H_2O_2 (the Fenton reaction):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO^{-} \quad (1)$$

Koenigs (1974a, b) presented a detailed comparison of the effects of Fenton's reagent and brown rot on wood. The similarities led him to propose that the early stages of brown rot involve production of hydroxyl radicals. For cellulose, hydroxyl radicals and brown rot fungi both cause selective cleavage within non-crystalline regions and an increased content of carbonyl and carboxyl groups (Highley, 1977; Kirk *et al.*, 1991). As

Abbreviations: CDH, cellobiose dehydrogenase (EC 1.1.99.18); DCPIP, 2,6-dichlorophenol-indophenol.

more direct evidence, the EPR spectrum of an HO$^{\bullet}$ adduct has been observed after incubation of *Poria placenta* with the spin-trap 5,5-dimethylpyrroline-*N*-oxide (DMPO) (Illman *et al.*, 1989). Moreover, growth of *C. puteana* and other brown rots in the presence of phthalic hydrazide led to formation of a 3-hydroxy derivative, as expected for attack by HO$^{\bullet}$ on the aromatic ring (Backa *et al.*, 1992).

A characteristic feature of brown rots is secretion of oxalic acid, leading to a marked lowering of the local pH. The production of oxalic acid was first noted by Shimazono (1955), while data for *C. puteana* are provided by Evans *et al.* (1990). Reported concentrations include 5–7 mM for *Poria placenta* and *Wolfiporia cocos* with pine holocellulose as carbon source (Green *et al.*, 1991; Espejo & Agosin, 1991) and higher values of 22–25 mM in the presence of glucose (Espejo & Agosin, 1991; Dutton *et al.*, 1993). As a working hypothesis, the present study has assumed an oxalate concentration of 10 mM. The pH of wood itself is generally in the range 4–6 (Rayner & Boddy, 1988). However, measurements taken with a pH microprobe for pine-wood infected by *P. placenta* indicated a fall to between pH 1·7 and 2·5 (Green *et al.*, 1991). There are many other reports of pHs close to 2·5 after growth of brown rots (Dutton *et al.*, 1993; Espejo & Agosin, 1991; Micales & Highley, 1989). Such low pHs can be explained by the secretion of oxalic acid, given its exceptional strength as an organic acid ($pK_1 = 1{\cdot}1$). Oxalate is also a moderately strong iron chelator [$K = 10^{20}$ for $Fe^{3+} + 3\,ox^{2-} \leftrightarrow Fe(ox)_3^{3-}$; see below]. In the absence of evidence to the contrary, we have assumed that oxalate is the physiological iron chelator for *C. puteana*.

The flavohaemoprotein cellobiose dehydrogenase (CDH) has been reported from a wide range of wood-rotting fungi (Morpeth, 1991; Bao *et al.*, 1993). It oxidizes the reducing end of cellodextrins, with substrates extending upwards in size from cellobiose to cellulose itself (Kremer & Wood, 1992a):

$$\beta\text{-D-cellobiose} \rightarrow \text{D-cellobionolactone} + 2e^- + 2H^+$$

CDH was first purified from *C. puteana* by Schmidhalter & Canevascini (1993). Postulated roles in other fungi include prevention of snap-back (i.e. making the cellulase reaction irreversible) and reduction of oxidized aromatic species during lignin degradation by white-rot fungi (Ander, 1994). However, CDH can also couple cellodextrin oxidation to reduction of Fe(III) (Kremer & Wood, 1992b). Indeed, the presence of a haem group with axial ligands as for mitochondrial cytochrome *c* (Cox *et al.*, 1992) makes it well fitted for a single-electron reduction.

The aim of this work was to attempt to answer the following three questions, in the context of hydroxyl radical production by brown rots:

- How is Fe(III) reduced to Fe(II)?
- How is O_2 reduced to H_2O_2?
- How can the fungus avoid being killed by the unspecific reactivity of these radicals?

METHODS

Organism, enzyme purification and assay. *C. puteana* (strain PRL-11R, from the Building Research Establishment, Watford, UK) was grown at 26 °C as described by Schmidhalter & Canevascini (1992). The preculture stage was stationary, with 50 ml medium in 250 ml flasks for 8 d. The main culture had 1% (w/v) amorphous cellulose (phosphoric acid treated Avicel) as additional carbon source; four precultures were added to 650 ml medium in a 2 l flask, which was then shaken at 150 r.p.m. for 12 d. CDH was assayed as described for *Phanerochaete chrysosporium*, assuming $\varepsilon_{peak-trough} = 118\ mM^{-1}\ cm^{-1}$ in cellobiose-minus-untreated difference spectra, scanned from 440 to 400 nm (Kremer & Wood, 1992b). Its purification was based on Schmidhalter & Canevascini (1993), including DEAE-Sepharose with a gradient of 0–250 mM NaCl at pH 6·5 (10 mM phosphate buffer), and gel filtration with Sephacryl S-200 (500 ml; pH 6·5).

Materials. Fe(II) was added as Fe(II) sulphate, dissolved in deionized water on the day of use; Fe(III) was added as Fe(III) chloride. Ferricyanide was assayed by measuring A_{420} [$\varepsilon_{420} = 1{\cdot}04\ mM^{-1}\ cm^{-1}$ (Kremer & Wood, 1992b)], and Fe(III) oxalate by measuring A_{340} [$\varepsilon_{340} = 1{\cdot}6\ mM^{-1}\ cm^{-1}$ in 10 mM oxalate (pH 4·0)].

Oxygen electrode, redox potentials. O_2 electrode and redox potential measurements were as described by Kremer & Wood (1992b).

Hydroxyl radical detection. Hydroxyl radicals were detected by salicylic acid hydroxylation as described by Kremer & Wood (1992c). The reaction mixture was quenched with HCl. Aromatics were then extracted in ethyl acetate, evaporated to dryness and redissolved in water. For HPLC, a 50 µl sample was injected into a 25 cm Spherisorb reverse-phase ODS column. The eluant was 97·2% citrate/acetate buffer (pH 4·75) plus 2·8% (v/v) methanol. Detection was by absorbance at 308 nm.

Speciation calculations. Chemical speciation calculations were conducted with the MINTEQA2 computer program (Allison *et al.*, 1990), which includes correction for ionic strength using the Davies equation (Davies, 1962):

$$I = \tfrac{1}{2}\sum(Z_i^2\,C_i)$$
$$\log\gamma_i = 0{\cdot}509\,Z_i^2\{[I^{\frac{1}{2}}/(1+I^{\frac{1}{2}})] - 0{\cdot}24\,I\}$$

where C_i is the concentration and γ_i the activity coefficient of an ion with charge Z_i, and I is ionic strength.

Table 1 shows the relevant stability constants as published and after correction to $I = 0$ for insertion in MINTEQA2. The table uses the standard notation for equilibrium constants: $K_1 = ML/(M.L)$, $\beta_2 = ML_2/(M.L^2)$ and $\beta_3 = ML_3/(M.L^3)$, where M is the cation and L is the ligand (in this case, oxalate^{2-}). The Fe(III) and Fe(II) speciations were evaluated for 10 mM oxalate [i.e. 10 mM total concentration of oxalate in the form of ox^{2-}, Hox^-, H_2ox, and $Fe^{II}(ox)_n$ or $Fe^{III}(ox)_n$ complexes], ionic strength 0·05 M and 25 °C. Total Fe(III) or Fe(II) was set at 10^{-6} M, giving results that are valid for any iron concentration, provided there is a large excess of uncomplexed oxalate (i.e. ox^{2-}, Hox^- and H_2ox) relative to iron. The input included equilibrium constants for $I \rightarrow 0$ from Table 1. Species arising from Fe^{3+} and Fe^{2+} hydrolysis [e.g. $Fe^{II}(OH)^+$ and $Fe^{III}(OH)^{2+}$] were included in the program but were not significant. Redox potentials for $Fe^{III}(ox)_n/Fe^{II}(ox)_n$ couples were calculated from

$$E^0(Fe^{III}/Fe^{II}) = E^0(Fe^{3+}/Fe^{2+}) - (2{\cdot}303RT/F)\,(\log K_{Fe(III)} - \log K_{Fe(II)}),$$

Table 1. Equilibrium constants for computation of speciation

Species	Equilibrium constant (K) in standard notation	log K as measured	Ionic strength (I), temp.	log K corrected to $I \rightarrow 0$	Reference*
Hox^-	K_1		0, 25 °C	4·266	1
H_2ox	β_2		0, 25 °C	5·518	1
$Fe^{II}(ox)^0$	K_1	3·05	1·0, 25 °C	4·21	1, 2
$Fe^{II}(ox)_2^{2-}$	β_2	5·01	1·0, 25 °C	6·07	2
$Fe^{II}(ox)_3^{4-}$	β_3	5·22	0·5, 25 °C	5·22	3
$Fe^{III}(ox)^+$	K_1	7·53	0·5, 25 °C	9·33	4
$Fe^{III}(ox)_2^-$	β_2	13·64	0·5, 25 °C	16·0	4
$Fe^{III}(ox)_3^{3-}$	β_3	18·49	0·5, 25 °C	20·3	4

*References: 1, Martell & Smith (1977); 2, Micskei (1987); 3, Schaap *et al.* (1954); 4, Deneux *et al.* (1968).

assuming $E^0(Fe^{3+}/Fe^{2+}) = +0{\cdot}771$ V for uncomplexed iron (Bard, 1982) and equilibrium constants for $I \rightarrow 0$.

RESULTS

Absence of spontaneous decomposition for Fe(III) oxalate

In a widely cited study, Schmidt *et al.* (1981) claimed that Fe(II) is produced by spontaneous decomposition of Fe(III) oxalate (see Kirk, 1983). We examined this reaction with use of a Pt electrode to monitor the Fe(III)/Fe(II) redox potential. As shown in Fig. 1, a redox potential that was stable in darkness showed a sharp decline on exposure to diffuse daylight, implying photolytic conversion of Fe(III) to Fe(II). Similar results were obtained at pH 2 or pH 4·5. Thus it would seem that Schmidt *et al.* (1981) observed a photochemical decomposition, as discussed below.

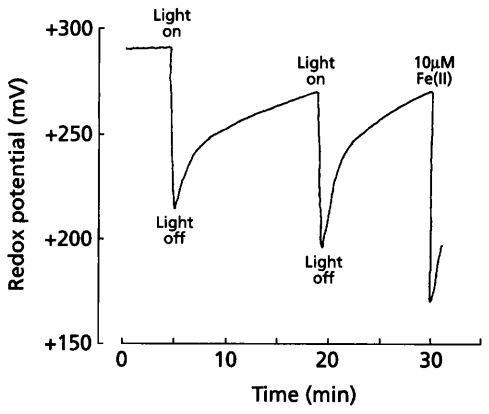

Fig. 1. Light-induced decomposition of Fe(III) oxalate monitored by redox potentiometry. The medium was air-equilibrated 20 mM oxalic acid/potassium oxalate, pH 4·0, plus 30 mM KCl and 1 mM Fe(III), added as $FeCl_3$. The light source was diffuse daylight. The upward rise in potential in darkness is caused by Fe(II) autoxidation by dissolved O_2.

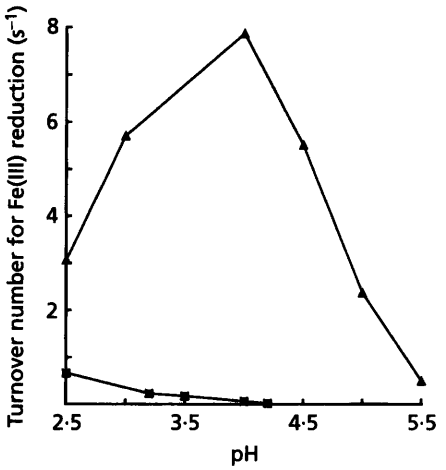

Fig. 2. Ferricyanide and Fe(III) oxalate reduction by CDH as a function of pH. The reduction was monitored spectrophotometrically at 30 °C, using a wavelength of 420 nm for ferricyanide and 340 nm for Fe(III) oxalate. O_2 was previously removed by bubbling with Ar. For ferricyanide reduction, 500 μM ferricyanide and 500 μM cellobiose were added to 50 mM citric acid/sodium citrate buffer at the appropriate pH. Experiments with Fe(III) oxalate used 10 mM oxalic acid/potassium oxalate buffer, with 1 mM cellobiose and 500 μM Fe(III) added as $Fe^{III}Cl_3$. CDH was present at 0·5 μM. Rates obtained from duplicate experiments are expressed as turnover numbers, i.e. [μmol Fe(III) reduced] s^{-1} (μmol CDH)$^{-1}$. ▲, Ferricyanide; ■, Fe(III) oxalate.

Cellobiose dehydrogenase as a source of Fe(II)

Schmidhalter & Canevascini (1993) only reported activity for CDH from *C. puteana* with DCPIP (a quinone analogue) as acceptor. We examined its reduction of Fe(III) (Fig. 2). Initial studies used ferricyanide, as a

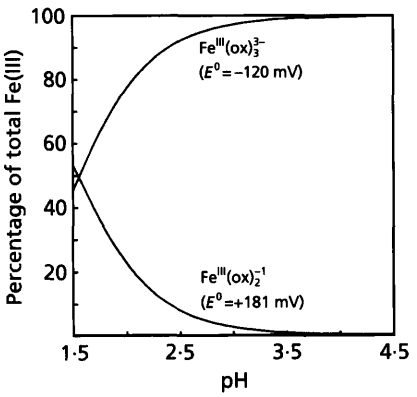

Fig. 3. Fe(III) oxalate speciation as a function of pH. The speciation was calculated for 10 mM total oxalate, $I = 0{\cdot}05$, 25 °C using MINTEQA2. The following species were always less than 1% of the total and are therefore not shown: Fe^{3+}, $Fe^{III}(ox)^+$ [1·1% at pH 1·5].

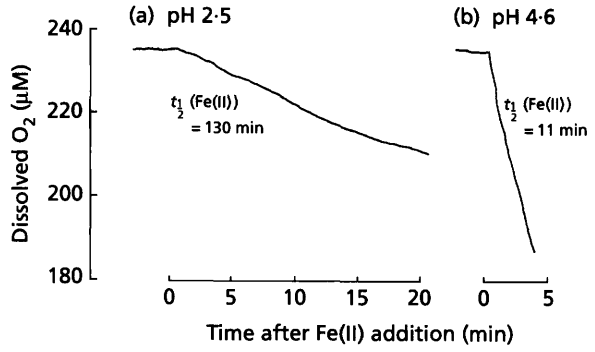

Fig. 4. Fe(II) oxalate autoxidation kinetics studied with an O_2 electrode. The buffer was 10 mM oxalic acid/potassium oxalate at 30 °C; 1 mM Fe(II) was added at zero time. The $t_{1/2}$ for Fe(II) was calculated from the initial O_2 uptake rate, assuming first-order kinetics and 1 $O_2 \equiv 4$ Fe(II).

model Fe(III) complex with a high, pH-independent redox potential. The absolute rates and pH dependence were very similar to earlier studies with CDH from the white-rot fungus *P. chrysosporium* (Kremer & Wood, 1992b, c). Fig. 2 also shows data for Fe(III) oxalate reduction, obtained under anaerobic conditions to prevent reoxidation of Fe(II). Fe(III) oxalate was reduced by CDH, although at a much lower rate than for ferricyanide. The reduction showed a very acid pH optimum.

An explanation for this behaviour with Fe(III) oxalate was revealed by modelling Fe(III) speciation as a function of pH, as shown in Fig. 3. Above pH 3, almost all Fe(III) is complexed by three oxalates to form $Fe^{III}(ox)_3^{3-}$, which has a calculated one-electron redox potential of −120 mV, far below the value of +165 mV measured for CDH from *P. chrysosporium* at pH 4 (Kremer & Wood, 1992b). This complex is therefore almost certainly not reducible by CDH. As the pH is lowered, a complex with two oxalates becomes more important, $Fe^{III}(ox)_2^{1-}$. This has a one-electron potential of +181 mV, allowing reduction by CDH to take place.

Production of Fenton's reagent by Fe(II) autoxidation

Many forms of Fe(II) are autoxidizable, implying reaction with dioxygen by one-electron transfer:

$$Fe^{2+} + O_2 \leftrightarrow Fe^{3+} + O_2^{\cdot-} \quad (2)$$

The superoxide formed by (2) is rapidly removed by reaction with Fe(II) (Brown & Mazzarella, 1987):

$$Fe^{2+} + O_2^{\cdot-} + 2H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (3)$$

Thus Fe(II) autoxidation creates H_2O_2. Moreover, partial autoxidation creates the Fe(II)/H_2O_2 combination required for hydroxyl radical production (Zepp *et al.*, 1992; Wood, 1994). Evidence for HO$^\cdot$ production during autoxidation of Fe(II) oxalate was obtained by the standard test of salicylate conversion to 2,3- and 2,5-dihydroxybenzoate (Grootveld & Halliwell, 1986) (results not shown). Thus the mere presence of Fe(II) oxalate in aerobic solution can lead to hydroxyl radicals, without the need for any separate source of H_2O_2.

Fe(II) lifetime before autoxidation

The rate of autoxidation can be monitored continuously with an oxygen electrode, as shown in Fig. 4. An approximate rate of Fe(II) oxidation can be deduced from such experiments, since Fe(II):O_2 stoichiometries of between 3 and 4 have been obtained with a variety of organic chelators (Burkitt & Gilbert, 1991). The kinetics have been studied for many other Fe(II) complexes, and show a dependence on Fe(II) that lies between first order (rate proportional to [Fe(II)]) and second order (rate proportional to [Fe(II)]^2), depending on the relative rates of reactions (2) and (3) (Brown & Mazzarella, 1987). In the absence of detailed information, a lower limit for the Fe(II) half-life ($t_{\frac{1}{2}}$) can be deduced from the initial rate of O_2 uptake, by ignoring the second-order component and assuming an Fe(II):O_2 stoichiometry of 4. For 30 °C, 10 mM oxalate (as shown), the Fe(II) half-life at pH 2·5 was about 130 min. The rate of oxidation became sharply faster as the pH was increased, with an estimated half-life for Fe(II) of about 11 min at pH 4·6 (Fig. 4b). At lower temperatures, or with less oxalate, the Fe(II) half-life would be longer than these values.

Again, the reason for the sharp pH dependence was revealed by an analysis of speciation (Fig. 5). For 10 mM oxalate, uncomplexed Fe^{2+} predominates at pH 2. As the pH is increased, the dominant species becomes $Fe^{II}(ox)^0$. These species have one-electron Fe^{III}/Fe^{II} redox potentials of +771 and +468 mV respectively, and for thermodynamic reasons cannot be autoxidizable, as discussed by Wood (1994) for ferrocyanide ($E^0 = +430$ mV). However, a pH increase from 2·5 to 4·5 also causes a dramatic increase in the proportion complexed by two oxalates, $Fe^{II}(ox)_2^{2-}$. This has a lower

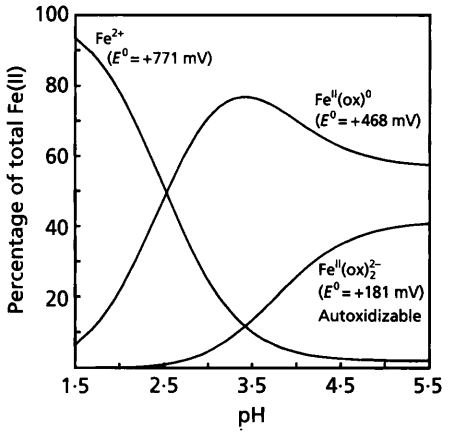

Fig. 5. Fe(II) oxalate speciation as a function of pH. The speciation was calculated for 10 mM total oxalate, $I = 0{\cdot}05$, 25 °C using MINTEQA2. $Fe^{II}(ox)_3^{4-}$ was always less than 1 % of the total and is therefore not shown.

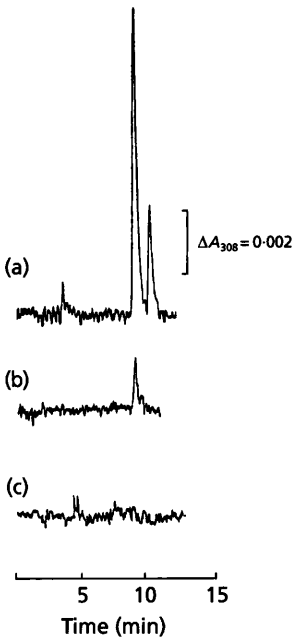

Fig. 6. Reduction of Fe(III) oxalate by CDH as a source of hydroxyl radicals. HPLC profiles obtained after 1 h incubations at 30 °C are shown. (a) Salicylate hydroxylation by Fenton's reagent: 3·3 mM H_2O_2 and 1 mM Fe(II) were added to 10 mM oxalate buffer, pH 3·5, plus 2 mM salicylate. (b) Evidence for HO· production by CDH: 1 μM CDH, 500 μM cellobiose and 50 μM Fe(III) were added to 10 mM oxalate buffer (pH 3·5) plus 2 mM salicylate. (c) Absence of HO· production with catalase present. The conditions were as in (b), apart from addition of 140 units of catalase (assayed as in Kremer & Wood, 1992c). The timescale is with zero at the time of injection. Elution of salicylate took more than 15 min and is not shown.

redox potential of +181 mV, which is consistent with the observed rate of autoxidation. [As a comparison, Fe(II) EDTA, which reacts fast with O_2, has a redox potential of +114 mV (Wood, 1994).]

CDH as a source of hydroxyl radicals

The ability of CDH to reduce Fe(III) leads to the prediction that it should be capable of producing hydroxyl radicals, if present together with a cellodextrin substrate, Fe(III) and dissolved O_2. This was demonstrated by Kremer & Wood (1992c) with CDH from *P. chrysosporium* plus cellobiose, using Fe(III) acetate as an initial model system. For Fe oxalate, the speciation plots in Figs 3 and 5 show that there is no pH at which Fe(III) reduction and Fe(II) autoxidation will both be fast. Nevertheless, it was possible to obtain a measurable yield of hydroxyl radicals; see Fig. 6(b). The reaction was inhibited by catalase (Fig. 6c), which will deactivate H_2O_2 before the Fenton reaction can take place.

DISCUSSION

The complete pathway

The secretion of CDH by the brown-rot fungus *C. puteana* provides a mechanism for Fe(II) production in the presence of oxalate (see Fig. 2). The reaction requires a much lower pH than that of wood itself. Brown rots lower the local pH. At low pH, Fe(II) is resistant to autoxidation and may have a lifetime of an hour or more (see Fig. 4). Throughout its lifetime, Fe(II) will not be static. It will move by diffusion, as for any species of the same size. It also has an exchange reaction with Fe(III). The rate constant is not known for all species of interest, but one example is $k = 4{\cdot}25 \times 10^3\ M^{-1}\ s^{-1}$ for Fe^{2+} and $Fe^{III}(ox)_2^-$ (Horne, 1960):

$$Fe_A^{2+} + Fe_B(ox)_2^- \rightarrow Fe_A(ox)_2^- + Fe_B^{2+}$$

If one partner is present at 50 μM, the pseudo-first-order rate constant for this reaction will be $0{\cdot}2\ s^{-1}$, implying a half-life of about 3·5 s. This is very much faster than the timescale of autoxidation and will greatly accelerate mobility. Diffusion of Fe(II) away from the hyphae brings it to a higher pH and promotes autoxidation. The complete pathway, including reaction of H_2O_2 with unoxidized Fe(II), is shown in Fig. 7. The diffusible

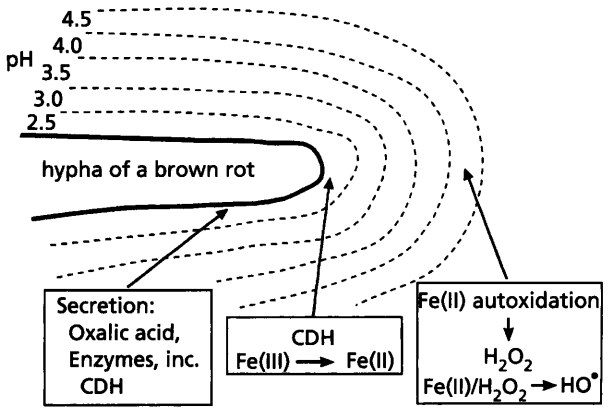

Fig. 7. Model for hydroxyl radical production without damage to the hyphae.

Table 2. Mechanisms proposed for hydroxyl radical production by brown rot fungi

Reference	Species	Source of Fe(II)	Source of H_2O_2
Schmidt *et al.* (1981)	(General model)	Spontaneous decomposition of Fe(III) oxalate	Secreted by the fungus
Enoki *et al.* (1992)	*Gloeophyllum trabeum*	Fe(III) glycopeptide reduction by superoxide (from 1 e^- reduction of O_2)	O_2 reduction by secreted NADH or ascorbate, with Fe-glycopeptide as catalyst
Lu *et al.* (1994)	*G. trabeum*	Fe(III) reduction on binding to a phenolate iron chelator	Not discussed
Hirano *et al.* (1995)	*Tyromyces palustris*	Fe(III) reduction on binding to the chelator; Fe(III) can also be reduced by superoxide	As Enoki *et al.* (1992)
This paper	*Coniophora puteana*	Fe(III) oxalate reduction by CDH	Autoxidation of Fe(II) oxalate, promoted by diffusion of Fe(II) away from the hyphae

species is the extra electron on Fe(II), given the importance of Fe(II)/Fe(III) exchange.

The mechanism of Schmidt *et al.* (1981)

Table 2 presents a summary of the sources of Fe(II) and H_2O_2 in brown rot, as proposed by different authors. In the original mechanism of Schmidt *et al.* (1981), Fe(II) is formed by spontaneous decomposition of Fe(III) oxalate. However, evidence was presented in Fig. 1 for this being a light-dependent reaction. This photosensitivity of Fe(III) oxalate has a long history. Thus Fe(III) oxalate solutions can be used as an actinometer for measuring total exposure to light (Hatchard & Parker, 1956), while the blueprint reprographic process employed paper impregnated with Fe(III) oxalate and potassium ferricyanide and gave a negative image due to light-dependent formation of Prussian blue (Grayson, 1982). The non-enzymic breakdown of Fe(III) oxalate may be of importance for fungal response to light, but cannot provide a general mechanism for timber decay. The mechanism of Schmidt *et al.* (1981) also presumed that H_2O_2 was secreted by the fungus, on the basis of experiments of Koenigs (1972, 1974a). In fact, Koenigs' results can be explained equally well by an indirect mechanism for H_2O_2 production, as proposed in Fig. 7. In this respect, there may be a fundamental distinction between brown-rot and white-rot fungi. In white rots, the H_2O_2 required for lignin and Mn peroxidases is provided by oxidases, with substrates that include glucose and methanol (Eriksson *et al.*, 1990). Such oxidases have not been reported for the standard brown rots. If Fe(II) is important in brown rot as a long-distance mediator, there is a need to avoid production of H_2O_2 close to the hyphae.

Which comes first – oxygen reduction to superoxide, or Fe(III) reduction to Fe(II)?

In the mechanisms of Enoki *et al.* (1992) and Hirano *et al.* (1995), O_2 reduction to superoxide is regarded as the initial step (see Table 2). This pathway for $HO^{\cdot}$ formation has been denoted the 'superoxide-assisted Fenton reaction' (Halliwell & Gutteridge, 1989) and is important inside living organisms, where almost all iron is in complexes that take no part in Fenton chemistry. However, if iron is mainly in low-molecular-mass complexes (as all the studies in Table 2 assume), direct reduction of Fe(III) is likely to be important. Fe(II) offers more scope than superoxide for movement by diffusion, since superoxide will always be subject to decay by dismutation.

The source of reductant

The models of Enoki *et al.* (1992) and Hirano *et al.* (1995) require the presence of NADH or ascorbate as reductant. However, there seems to be no evidence that such species are released from fungal cells. Lu *et al.* (1994) and Hirano *et al.* (1995) also report that Fe(III) is reduced to Fe(II) on binding to a phenolate chelator. This poses the question – is there a mechanism for regenerating the reduced form of the chelator? The source of reductant ceases to be a problem if Fe(III) reduction is catalysed by CDH, since cellodextrin substrates will be provided by cellulases. One must therefore ask, is CDH widely distributed in brown rots? The chief negative evidence has been tests by Ander & Eriksson (1978), who used five species. Since one of these five species was *C. puteana*, a reappraisal is necessary. Our mechanism can also be criticized on the grounds that Fe(III) oxalate reduction by CDH is a relatively slow process, even at pH 2·5 (see Fig. 2). This points to a need to search for alternative mechanisms for Fe(III) reduction. We cannot claim to have definitive answers to the questions posed in the Introduction, but hope the hypothesis in Fig. 7 will be useful for further work.

The iron chelator

This study has assumed that oxalate is the physiological iron chelator, in the absence of evidence to the contrary for *C. puteana*. The large resource of thermodynamic data for oxalate complexes has enabled the paradox of hydroxyl radical production without damage to the hyphae to be explained for the first time. It will be

important to test whether stronger iron-complexing agents are also present, as proposed for *Gloeophyllum trabeum* and *Tyromyces palustris* (see Table 2). Regardless of the iron chelator, our results show the importance of considering Fe speciation and Fe(II) stability as a function of pH.

ACKNOWLEDGEMENTS

We are grateful to L. M. Webb and Professor D. R. Williams (University of Wales, Cardiff) for assistance in speciation computations. This research was supported by the UK Biotechnology and Biological Sciences Research Council.

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Received 21 May 1996; revised 19 August 1996; accepted 27 August 1996.