# 2 Fungal Degradation of Lignin

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## Importance of Lignin Biodegradation

Of all naturally produced organic chemicals, lignin is probably the most recalcitrant. This is consistent with its biological functions, which are to give vascular plants the rigidity they need to stand upright and to protect their structural polysaccharides (cellulose and hemicelluloses) from attack by other organisms. Lignin is the most abundant aromatic compound on earth, and is second only to cellulose in its contribution to living terrestrial biomass (Crawford, 1981). When vascular plants die or drop litter, lignified organic carbon is incorporated into the top layer of the soil. This recalcitrant material has to be broken down and recycled by microorganisms to maintain the earth's carbon cycle. Were this not so, all carbon would eventually be irreversibly sequestered as lignocellulose.

Lignin biodegradation has diverse effects on soil quality. The microbial degradation of litter results in the formation of humus, and ligninolysis probably facilitates this process by promoting the release of aromatic humus precursors from the litter. These precursors include incompletely degraded lignin, flavanoids, terpenses, lignans, condensed tannins, and uberins (Hudson, 1986). Undegraded lignocellulose, e.g. in the form of straw, has a deleterious effect on soil fertility because decomposing (as opposed to already decomposed) lignocellulose supports high populations of microorganisms that may produce phytotoxic metabolites. High microbial populations in undecomposed litter also compete with crop plants for soil nitrogen and other nutrients (Lynch and Harper, 1985). By breaking down the most refractory component of litter, ligninolysis thus contributes to the removal of conditions that inhibit crop productivity.

Conditions that disfavour the biological breakdown of lignocellulose lead to soils with pronounced accumulations of litter. For example, the soils of coniferous forests in the northwest United States may contain 50 years of accumulated litterfall, because the low pH of the litter and the lack of summer rainfall inhibit microbial activity. In mature forests of this type, woody material such as dead trunks and branches can constitute 50–60% of the litter. By contrast, the soils under broadleaf forests in the eastern United States accumulate only a few years' worth of litter, and soils in some tropical rain forests accumulate virtually none, because conditions are more favourable for decomposition (Spurr and Barnes, 1980). Warm temperature, high moisture content, high oxygen availability, and high palatability of the litter to microorganisms all favour decomposition. The more highly lignified litter is, the less digestible it is, and the more its decomposition depends on the unique organisms that can degrade lignocellulose.

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## Ecology of Fungal Lignocellulose Degradation

The organisms principally responsible for lignocellulose degradation are aerobic filamentous fungi, and the most rapid degraders in this group are Basidiomycetes (Kirk and Farrell, 1987). The ability to degrade lignocellulose efficiently is thought to be associated with a mycelial growth habit which allows the fungus to transport scarce nutrients, e.g. nitrogen and iron, over a distance into the nutrient-poor lignocellulosic substrate that constitutes its carbon source. It is curious in this regard that Actinomycetes (i.e. bacteria with a mycelial growth habit) have not evolved the capacity to degrade lignocellulose efficiently. It is possible that they have the ability to modify lignin somewhat, but no evidence has accumulated to show that they can degrade it (Kirk and Farrell, 1987).

### *Fungal wood decay*

The course of fungal lignocellulose degradation is most readily observable in intact dead wood which, despite its complex ultrastructure, is actually the simplest and best characterized form of litter. In wood, three distinct types of fungal decay can be distinguished: white rot, brown rot, and soft rot (Eriksson *et al.,* 1990).

White rot fungi are the most abundant degraders of wood in nature. Their strategy is to decompose the lignin in wood so that they can gain access to the cellulose and hemicelluloses that are embedded in the lignin matrix. Under optimal conditions, the rates at which white rot fungi mineralize lignin rival their rates of polysaccharide degradation. Basidiomycetes and xylariaceous Ascomycetes that cause white rot are the organisms principally responsible for wood decay in hardwood forests and in tropical forest ecosystems, and also play a prominent role in temperate coniferous forests (Eriksson *et al.,* 1990; Blanchette, 1991; Dix and Webster, 1995). Several hundred species in numerous taxa have been described. Communities of white rot fungi and associated organisms in forest ecosystems have been described in extensive studies by British researchers (Rayner and Boddy, 1988; Boddy, 1992; Dix and Webster, 1995).

The brown rot fungi comprise a relatively small group of Basidiomycetes that decay the cellulose in wood preferentially. They do not degrade the lignin extensively, although they modify it by demethylating it. Brown rot fungi thus stand out as an exception to the usually valid observation that lignocellulose must be delignified first if organisms are to gain access to plant cell wall polysaccharides. The biochemical system that enables brown rot fungi to circumvent the lignin while degrading the cellulose and hemicelluloses in wood has not been characterized. Although these fungi secrete cellulases and hemicellulases, the enzymes are too large to penetrate the cell wall matrix in wood, and it is evident that other degradative systems must participate as well. Brown rot fungi make a large contribution to wood decay, especially in coniferous forests (Dix and Webster, 1995), and the residual modified lignin they leave behind is an important humus precursor (Hudson, 1986). They deserve much more research attention, but have been difficult to study because they do not exhibit full degradative activity on defined media *in vitro* (Eriksson *et al.,* 1990).

The soft rot fungi are Ascomycetes and Deuteromycetes that decay water-saturated (but not totally anaerobic) wood, as well as wood prone to fluctuating moisture regimes. Soft rot fungi are slower and less aggressive decayers than white and *brown* rot fungi, and are probably less important degraders in a quantitative sense. They attack the polysaccharide component of wood preferentially, but appear to have some ability to decompose lignin (Dix and Webster, 1995). Soft rot fungi have received little research attention, and their degradative mechanisms remain unknown.

### *Fungal leaf litter decay*

The processes by which fungi degrade leaf litter, as opposed to woody litter, are poorly understood. In some cases, leaves

are colonized shortly after they fall by Basidiomycetes. For example, *Marasmius androsaceus* is an early colonizer and degrader of pine needles, a relatively recalcitrant and long-lived form of leaf litter (Hudson, 1986; Dix and Webster, 1995). Older analyses indicate that conifer needles contain significant levels of lignin (Theander, 1978), but it remains to be shown whether the Basidiomycetes that are early colonizers of leaf litter are ligninolytic.

In most cases, leaf litter decomposition is more complex, involving a succession of biodegradative activities that precede attack by lignocellulose degraders (Hudson, 1986; Dix and Webster, 1995). The process typically begins with colonization by bacteria, Ascomycetes, and imperfect fungi that consume the least recalcitrant components present, e.g. sugars, starch, and low molecular weight extractives. The cellulose present in non-lignified leaf tissues is then attacked by some of these organisms, but there is no evidence that lignin is degraded during this early stage of decay. Subsequently, the remaining lignified litter is modified by fauna such as earthworms, millipedes, slugs, and termites, which macerate lignocellulose mechanically in a process that releases some digestible cellulose. Bacteria and fungi in the guts of these invertebrates then assist in the breakdown of this cellulose, but they do not degrade the lignin component appreciably. Instead, this mechanically modified lignocellulose is released relatively unchanged and becomes part of the soil organic matter. Fragmentation by animals significantly accelerates the degradation rate of the tougher types of litter such as tree leaves, but probably plays a lesser role in the degradation of soft herbaceous litter (Dix and Webster, 1995). Finally, the modified but still lignified litter is colonized by Basidiomycetes that degrade it further.

It is generally assumed that basidiomycete degraders of non-woody litter are ligninolytic, i.e. that they are more like white rotters than brown rotters, but so far little research has been done to confirm this view. The commercial edible mushroom *Agaricus bisporus* is the one litter decomposer whose degradative mechanisms have received some research attention. It degrades both cellulose and lignin, the former more rapidly (Wood and Leatham, 1983; Durrant *et al.,* 1991), and contains ligninolytic enzymes (Bonnen *et al.,* 1994). If *A. bisporus* is typical of other litter-decomposing Basidiomycetes, it is probably correct to infer that fungal ligninolysis is a significant process in non-woody litter. However, it remains unclear to what extent ligninolysis in litter plays the essential role that it does in wood by exposing trapped cellulose to fungal attack. Leaf litter is already finely milled by the time most Basidiomycetes colonize it, and certainly contains bioavailable cellulose, as shown by the fact that non-ligninolytic fungi can deplete cellulose during the comporting of litter (Dix and Webster, 1995).

## Lignin Structure

It is evident from the preceding discussion that fungal ligninolysis is an important component of the process by which some types of litter are degraded. Other, basically non-ligninolytic mechanisms, e.g. those of the brown rot type, may be equally important, but our current understanding of litter decomposition is still so fragmentary that attack of the white rot type is the only degradative component we can discuss at a mechanistic level. To do this, we first need to understand how the structure of lignin makes it the recalcitrant material that it is.

Lignin is formed in vascular plant cell walls by the oxidative coupling of several related phenylpropanoid precursors: coniferyl alcohol, sinapyl alcohol, and *p*-hydroxycinnamyl alcohol (see also Bavage *et al.,* Chapter 16, this volume). Peroxidases or Iaccases in the plant cell wall oxidize these monomers by one electron, yielding transient resonance-stabilized phenoxy radicals that then polymerize in a variety of configurations. The possible ways that the precursors can couple can be portrayed on paper simply by drawing the conventional resonance forms of the phenoxy

radicals, and then by linking the most important of these in various pairwise combinations. This subject has been extensively reviewed (Adler, 1977; Higuchi, 1990), and it will suffice here simply to say that lignin consists primarily of the intermonomer linkages shown in Fig. 2.1, and that the arylglycerol-β-aryl ether structure circled in the figure is quantitatively the most important of these, constituting over 50% of the polymer. Lignin is covalently associated with hemicelluloses in the cell wall via numerous types of linkage. Among the most important are ether bonds between the benzylic carbon of lignin and the carbohydrate moiety, ester bonds between the benzylic carbon of lignin and uronic acid residues, and lignin-glycosidic bonds. In graminaceous plants, hydroxycinnamic acid residues are frequent in the lignin, and are attached to hemicelluloses via ester linkages. The matrix of lignin and hemicellulose encrusts and protects the cellulose of the plant cell wall (Jeffries, 1990).

Fungi that degrade lignin are faced with several problems. Since the polymer is

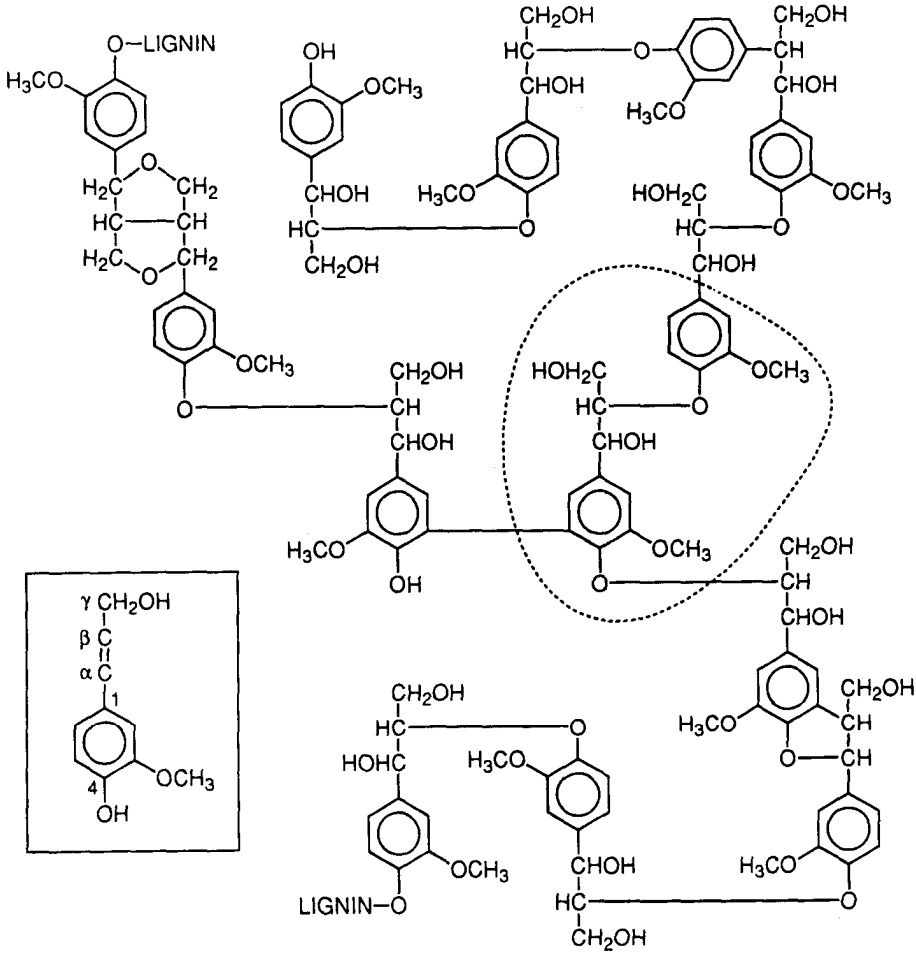


**Fig. 2.1.** Common structures of softwood lignin, with an example of the major arylglycerol-ß-aryl ether structure circled. The inset shows coniferyl alcohol, the phenylpropanoid building block of softwood lignin.

extremely large and highly branched, ligninolytic mechanisms must be extracellular. Since it is interconnected by stable ether and carbon–carbon bonds, these mechanisms must be oxidative rather than hydrolytic. Since lignin consists of a mixture of stereoirregular units, fungal ligninolytic agents have to be much less specific than typical biological catalysts. Finally, the fact that lignin is insoluble in water limits its bioavailability to ligninolytic systems and dictates that ligninolysis is a slow process.

## Measurement of Ligninolysis

The delignification of a solid lignocellulosic substrate is often assessed by the simple procedure of removing its low molecular weight components by extraction, weighing the leftover woody residue, degrading the remaining polysaccharide component with strong acid, and then reweighing the leftover insoluble lignin, which is chemically modified and referred to as Klason lignin. Klason lignin determinations are relatively simple to perform and can be useful if the investigator is confident that interfering substances are not present (Theander and Westerhtnd, 1993). For example, convincing data showing that certain xylariaceous Ascomycetes delignify wood have been obtained with the Klason procedure (Nilsson *et al.*, 1989). However, this method is subject to errors if it is used on plant tissues that contain other high molecular weight components that are not removed in the initial extraction and acid treatment. Interfering substances of this type may include proteins and tannins (Theander and Westerlund, 1993). For example, Klason lignin analyses of dried pine needles indicate that they contain up to 30% lignin by weight (Theander, 1978), but this value exceeds that found even in many woods and is probably too high. On the other hand, the Klason procedure tends to underestimate the amount of lignin in annual plants, because some of the polymer is acid-soluble and consequently lost during the hydrolysis of polysaccharides.

The fungal delignification of woody material can be monitored by electron microscopy (Blanchette, 1991) or light microscopy. with selective staining (Srebotnik and Messner, 1994), although these procedures are relatively complex and only semiquantitative. Microscopy is, of course, useful only when the substrate being investigated still contains lignified cell walls, but these techniques might be used with advantage to assess ligninolysis in relatively intact twigs or conifer needles.

Several chemical procedures have also been introduced for the estimation of lignin content. For example, pulverized wood samples can be treated with acetyl bromide in acetic acid, and the absorbance of the resulting solution at 280 nm can be compared with the absorbance obtained from known lignin standards. Methods of this type are subject to interference from other components, but can be useful for the comparison of closely related lignocellulosic samples (Theander and Westerlund, 1993).

A less direct but very flexible approach to the study of fungal ligninolysis is to assess not whether the growth substrate itself is being delignified, but rather whether the fungus degrades a simpler target molecule whose breakdown indicates that ligninolytic systems must be functioning. Model substrates of this type can be infiltrated into the organism's natural lignocellulosic growth medium, e.g. wood or litter, or they can be used as probes in defined liquid growth media (Kirk *et al.*, 1975, 1978; Srebotnik *et al.*, 1994). The most frequently used probes are $^{14}C$-labelled synthetic lignins, which can be prepared by polymerizing $^{14}C$-labelled *p*-hydroxycinnamyl alcohols (e.g. labelled coniferyl alcohol, see inset to Fig. 2.1) with horseradish peroxidase (Kirk and Brunow, 1988). The advantages of this approach are that synthetic lignins contain the same intermonomer structures that natural ones do (although they differ considerably in the relative frequency of each substructure type), and that they provide a simple and foolproof assay for ligninolysis: $^{14}CO_2$ produced during degradation of the radiolabelled polymer can simply be trapped in alkali and determined by scintillation

counting. The principal disadvantage of the method is that it is expensive and requires facilities for radiochemical organic synthesis: the necessary $^{14}$C-labelled *p*-hydroxycinnamyl alcohols have to be prepared in the laboratory from simpler commercially available labelled precursors. It is also necessary to ensure that the synthetic lignins used are too large to be taken up intracellularly by the organism under investigation - this is generally done by subjecting the synthetic polymer to gel permeation chromatography before it is used and retaining only material with a molecular weight greater than about 1500. Because of their utility, radiolabelled synthetic lignins and newer polymeric lignin model compounds that represent the major substructure in lignin (Kawai *et al.*, 1995) are finding increasing use in studies of fungal ligninolysis.

## Fungal Ligninoiytic Mechanisms

Ligninolytic fungi are not able to use lignin as their sole source of energy and carbon. Instead, they depend on the more digestible polysaccharides in lignocellulosic substrates, and the primary function of ligninolysis is to expose these polysaccharides so that they can be cleaved by fungal cellulases and hemicellulases. In most fungi that have been examined, ligninolysis occurs during secondary metabolism, i.e. under nutrient limitation. With this approach, the fungus avoids synthesizing and secreting metabolically expensive ligninolytic agents when substrates more accessible than lignocellulose are present. The limiting nutrient for fungal growth in most woods and soils is probably nitrogen, and most laboratory studies of ligninolytic fungi have been done in nitrogen-limited culture media (Kirk and Farrell, 1987). However, a few ligninolytic fungi, e.g. some species of *Bjerkandera,* are ligninolytic even when sufficient nitrogen is present (Kaal *et al.,* 1993).

Given the chemical recalcitrance of lignin (Adler, 1977), it is evident that white rot fungi must employ unusual mechanisms to degrade it. Research has characterized several of these mechanisms in some detail, and has shown that they all display one fundamental similarity: they depend on the generation of lignin free radicals which, because of their chemical instability, subsequently undergo a variety of spontaneous cleavage reactions.

### *Lignin peroxidases*

Lignin peroxidases (LiPs) were the first ligninolytic enzymes to be discovered (Glenn *et al.,* 1983; Tien and Kirk, 1983). They occur in some frequently studied white rot fungi, e.g. *Phanerochaete chrysosoporium, Trametes versicolor* and *Bjerkandera* sp. (Kirk and Farrell, 1987; Kaal *et al.,* 1993; Orth *et al.,* 1993) but are evidently absent in others, e.g. *Dichomitus squalens, Ceriporiopsis subvermispora* and *Pleurotus ostreatus* (Perie and Gold, 1991; Kerem *et al.,* 1992; Orth *et al.,* 1993; Rüttimann-Johnson *et al.,* 1993). LiPs resemble other peroxidases such as the classical, extensively studied enzyme from horseradish, in that they contain ferric heme and operate via a typical peroxidase catalytic cycle (Kirk and Farrell, 1987; Gold *et al.,* 1989). That is, LiP is oxidized by $H_2O_2$ to a two-electron deficient intermediate, which returns to its resting state by performing two one-electron oxidations of donor substrates. However, LiPs are more powerful oxidants than typical peroxidases are, and consequently oxidize not only the usual peroxidase substrates such as phenols and anilines, but also a variety of non-phenolic lignin structures and other aromatic ethers that resemble the basic structural unit of lignin (Kersten *et al.,* 1990). The simplest aromatic substrates for LiP are methoxylated benzenes and benzyl alcohols, which have been used extensively by enzymologists to study LiP reaction mechanisms. The $H_2O_2$-dependent oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) to veratraldehyde is the basis for the standard assay used to detect LiP in fungal cultures (Kirk *et al.,* 1990).

The LiP-catalysed oxidation of a lignin substructure begins with the abstraction of one electron from the donor substrate's aromatic ring, and the resulting

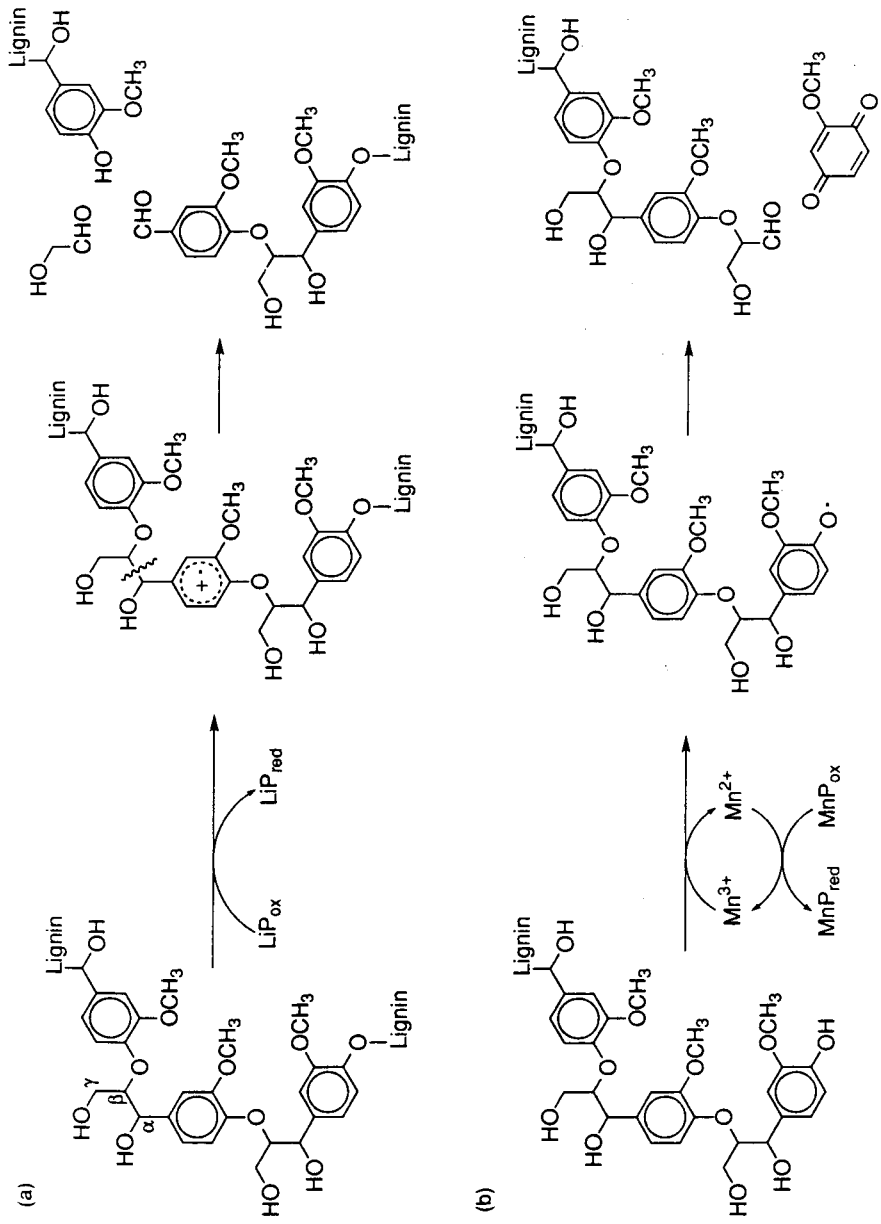


**Fig. 2.2.** (a) Cleavage of a recalcitrant internal non-phenolic arylglycerol-ß-aryl ether lignin structure by oxidized lignin peroxidase. (b) Cleavage of a reactive terminal phenolic arylglycerol-ß-aryl ether structure by oxidized manganese peroxidase.

species, an aryl cation radical, then undergoes a variety of postenzymatic reactions. (Kersten *et al.,* 1985; Shoemaker *et al.,* 1985; Hammel *et al.,* 1986; Kirk and Farrell, 1987). For example, dimeric model compounds that represent the major arylglycerol-β-aryl ether lignin structure undergo $C_\alpha$-$C_\beta$ cleavage upon oxidation by LiP (Kirk *et al,* 1986) (Fig. 2.2). Synthetic polymeric lignins are also cleaved at this position by the enzyme *in vitro,* in a reaction that gives net depolymerization (Hammel *et al.,* 1993). These results strongly support a ligninolytic role for LiP, because $C_\alpha$ -$C_\beta$ cleavage is a major route for ligninolysis in many white rot fungi (Kirk and Farrell, 1987). Other LiP-catalysed reactions that accord with fungal ligninolysis *in vivo* include aromatic ether cleavage at $C_\beta$ and $C_\alpha$-oxidation without cleavage. It has been pointed out that ionization of the aromatic ring to give a cation radical is also what occurs when lignin model substrates are analysed in a mass spectrometer, and indeed the fragmentation pattern obtained by this procedure is similar to that obtained when LiP acts on lignin structures (Dolphin *et al.,* 1987).

There remains an unresolved problem with the proposal that LiP catalyses fungal ligninolysis: LiP, like other enzymes, is too large to enter the pores in sound wood (Srebotnik *et al.,* 1988). If it initiates ligninolysis directly, LiP must therefore act at the surface of the secondary cell wall. Fungal attack of this type is indeed found, but electron microscopic observations also indicate that white rot fungi can remove lignin from the interior of the cell wall before they have degraded it enough for enzymes to penetrate. It has been proposed that LiP might circumvent the permeability problem by acting indirectly to oxidize low molecular weight substrates that could penetrate the lignocellulosic matrix and act themselves as oxidants at a distance from the enzyme (Harvey *et al.,* 1986), but no convincing candidate for a diffusible LiP-dependent oxidant of this type has emerged so far. Notwithstanding these difficulties, LiP remains the only fungal oxidant known that can efficiently mimic, *in vitro, the* $C_\alpha$-$C_\beta$ cleavage reaction that is characteristic of ligninolysis by white rot *fungi* such as *Phanerochaete chrysosporium.* LiP must therefore be considered an important ligninolytic agent, but it may act in concert with other, smaller oxidants that can penetrate and open up the wood cell wall.

### *Manganese peroxidases*

Manganese peroxidases (MnPs) may be the catalysts that provide these low molecular weight oxidants (Glenn *et al.,* 1986; Paszczynski *et al.,* 1986). MnPs occur in most white rot fungi, and are similar to conventional peroxidases, except that Mn(II) is the obligatory electron donor for reduction of the one-electron deficient enzyme to its resting state, and Mn(III) is produced as a result Wariishi *et al.,* 1992). This reaction requires the presence of bidentate organic acid chelators such as glycolate or oxalate, which stabilize Mn(III) and promote its release from the enzyme. The resulting Mn(III) chelates are small, diffusible oxidants that can act at a distance from the MnP active site. They are not strongly oxidizing and are consequently unable to attack the recalcitrant non-phenolic structures that predominate in lignin. However, Mn(III) chelates do oxidize the more reactive phenolic structures that make up approximately 10% of lignin. These reactions result in a limited degree of ligninolysis via $C_\alpha$-aryl cleavage and other degradative reactions (Fig. 2.2) (Wariishi *et al,* 1991; Tuor *et al.,* 1992). It is an interesting possibility that MnP-generated Mn(III) might cleave phenolic lignin structures in this fashion to facilitate later attack by the bulkier but more powerful oxidant LiP.

### *Co-oxidation of lignin via production of oxyradicals*

The LiP- and MnP-catalysed reactions just described cannot provide the only means by which fungi cleave polymeric lignin. LiP, despite its unique properties, is not essential because it is not produced by all

white rot fungi during ligninolysis. MnP-generated Mn(III) cannot be wholly responsible because white rot fungi that lack LiP are nevertheless able to degrade the non-phenolic lignin structures that resist attack by chelated Mn(III) (Srebotnik *et al.*, 1994). Other ligninolytic mechanisms must therefore exist.

Recent work indicates that the production of diffusible oxyradicals by MnP may supply one such mechanism. In the presence of Mn(II), MnP promotes the peroxidation of unsaturated lipids, generating transient lipoxyradical intermediates that are known to act as potent oxidants of other molecules (Moen and Hammel, 1994). The MnP/lipid peroxidation system, unlike MnP alone, oxidizes and cleaves non-phenolic lignin model compounds. It also depolymerizes both non-phenolic and phenolic synthetic lignins, which strongly supports a ligninolytic role for this system *in vivo* (Bao *et al.*, 1994). Although lipid peroxidation has previously been implicated in a variety of biological processes, e.g. aging and carcinogenesis (Halliwell and Gutteridge, 1989), we believe this is the first evidence that microorganisms may use it as a biodegradative tool.

### *Laccases*

Laccases are blue copper oxidases that catalyse the one-electron oxidation of phenolics and other electron-rich substrates. Most ligninolytic fungi produce laccases, *P. chysosporium* being a notable exception. Laccases contain multiple copper atoms which are reduced as the substrates are oxidized. After four electrons have been received by a laccase molecule, the laccase reduces molecular oxygen to water, returning to the native state. The action of laccase on lignin resembles that of Mn(III) chelates, in that phenolic units are oxidized to phenoxy radicals, which can lead to degradation of some structures (Kawai *et al.*, 1988). In the presence of certain artificial auxiliary substrates, the effect of laccase can be enhanced so that it oxidizes non-phenolic compounds that otherwise would not be attacked, but it is not yet known whether natural versions of such auxiliary substrates function *in vivo* in lignin biodegradation (Bourbonnais and Paice, 1992), and indeed, the actual role of laccase has yet to be fully clarified.

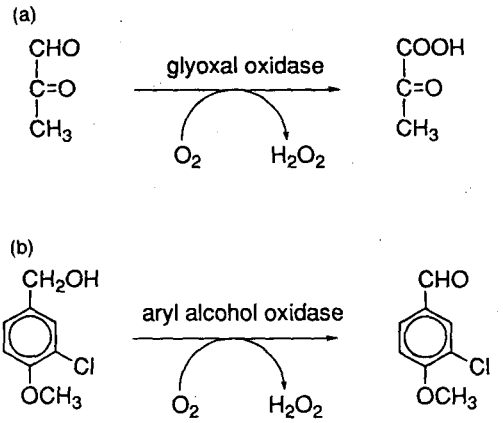


**Fig. 2.3.** Production of extracellular $H_2O_2$ by (a) the glyoxal oxidase of *Phanerochaete chrysosporium* and (b) the aryl alcohol oxidase of *Bjerkandera* sp.

### *Peroxide-producing enzymes*

To support the oxidative turnover of the LiPs and MnPs responsible for ligninolysis, white rot fungi require sources of extracellular $H_2O_2$, This need is met by extracellular oxidases that reduce molecular oxygen to $H_2O_2$ with the concomitant oxidation of a cosubstrate (Fig. 2.3). One such enzyme, found in *P. chrysosporium* and many other white rot fungi, is glyoxal oxidase (GLOX) (Kersten, 1990). GLOX accepts a variety of 1–3 carbon aldehydes as electron donors. Some GLOX substrates, e.g. glyoxal and methylglyoxal, are natural extracellular metabolizes of *P. chysosporium* (Kersten, 1990). Another substrate for the enzyme, glycolaldehyde, is released as a cleavage product when the major arylglycerol-β-aryl ether structure of lignin is oxidized by LiP (Hammel *et al.*, 1994).

Aryl alcohol oxidases (AAOs) provide another route for $H_2O_2$ production in some white rot fungi. In certain LiP-producing species of *Bjerkandera*, chlorinated anisyl

alcohols are secreted as extracellular metabolizes and then reduced by a specific AAO to produce $H_2O_2$ (de Jong *et al.*, 1994). It is noteworthy that, although many alkoxybenzyl alcohols are LiP substrates, chloroanisyl alcohols are not. The use of a chlorinated benzyl alcohol as an AAO substrate thus provides a strategy by which the fungus separates its ligninolytic and $H_2O_2$-generating pathways. A different approach is employed by some LiP-negative species of *Pleurotus,* which produce and oxidize a mixture of benzyl alcohols, including anisyl alcohol, to maintain a supply of $H_2O_2$ (Guillén *et al.*, 1992). In yet other fungi, intracellular sugar oxidases might be involved in $H_2O_2$ generation (Kirk and Farrell, 1987).

### *Detection of ligninolytic enzymes in complex substrates*

Once a fungus has been shown to degrade lignin in experiments with radiolabelled synthetic lignins, the question arises as to which ligninolytic enzymes the organism is expressing. If the degradation experiments have been done in defined liquid media, standard assays for LiP, MnP, laccase, and the various $H_2O_2$-producing oxidases can be done with little difficulty. However defined growth media that elicit the full expression of ligninolysis have not been developed for many fungi. Therefore, in experiments with previously uninvestigated fungi that grow on litter, it is more pertinent to ask what ligninolytic enzymes are expressed in the natural growth substrate. This remains a difficult question because many peroxidases are easily inactivated by phenols or other inhibitors that occur in lignocellulosic substrates, and it is consequently difficult to assay these important ligninolytic enzymes reliably in solid state cultures (Datta *et al.*, 1991). Investigators must therefore turn to indirect methods for the detection of ligninolytic enzymes. One of these is to infiltrate a high molecular weight lignin model compound into the lignocellulosic substrate, and then to determine by subsequent product analysis whether the fungus cleaves it in the same way that purified LiP does (Kawai *et al.*, 1995). Another approach currently under development, and useful when the gene for the enzyme of interest has been sequenced, is to isolate fungal RNA from the substrate and use reverse transcription/polymerase chain reaction techniques to determine whether the gene for the enzyme is being expressed (Gold and Alic, 1993; Lamar *et al.*, 1995). These new research tools should help to alleviate our severe lack of knowledge about degradative mechanisms in litter-decomposing fungi.

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