Fungi in Bioremediation

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G. M. GADD

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Degradation of plant cell wall polymers

CHRISTINE S. EVANS AND JOHN N. HEDGER

Introduction

Processes of natural bioremediation of lignocellulose involve a range of organisms, but predominantly fungi (Hammel, 1997). Laboratory studies on the degradation of lignocellulose, including wood, straw, and cereal grains, have focused mainly on a few fungal species that grow well in the laboratory and can be readily manipulated in liquid culture to express enzymes of academic interest. Our current understanding of the mechanism of lignocellulose degradation stems from such studies. Although some of these enzymes have economic potential in a range of industries, for example pulp and paper manufacture and the detergent industry, it is frequently expensive and uneconomic to use them for bioremediation of pollutants in soils and water columns. In the successful commercial bioremediation processes developed, whole organisms have been used in preference to their isolated enzymes (Lamar & Dietrich, 1992; Bogan & Lamar, 1999; Jerger & Woodhull, 1999).

Most fungi are robust organisms and are generally more tolerant to high concentrations of polluting chemicals than are bacteria, which explains why fungi have been investigated extensively since the mid-1980s for their bioremediation capacities. However, the species investigated have been primarily those studied extensively under laboratory conditions, which may not necessarily represent the ideal organisms for bioremediation. Fungi in little-explored forests of the world, for example tropical forests, may yet prove to have even better bioremediation capabilities than the temperate organisms currently studied, exhibiting more tolerance to temperature and specialist environments. This chapter discusses the current state of knowledge on the degradation of lignocellulose and how this relates with the ecology of lignocellulolytic fungi. This knowledge is important to modify and enhance the mechanisms of degradation of industrial pollutants such as chlorophenols, nitrophenols and polyaromatic hydrocarbons by these fungi.

Fig. 1.1. Structure of cellulose formed from β-1,4-linked cellobiose units, with hydrogen bonding between parallel chains.

Structure and function of plant cell walls

Plant cell walls offer several benefits to the growing and mature plant. They provide an exo-skeleton giving rigidity and support, enabling the water column to reach the plant apex, and they serve as a protective barrier against predators and pathogens. As the wall is an extracytoplasmic product, it was not considered to be a living part of the cell (Newcomb, 1980), although this view is now challenged and many metabolic processes are now known to occur within the cell wall structure for maintenance and in response to attack by pathogens (Dey, Brownleader & Harborne, 1997a). Every cell of a plant is surrounded by a primary wall that undergoes plastic extension as the cell grows. Some cells such as those of the parenchyma keep a primary wall throughout their lifespan. The primary wall is composed of cellulose fibrils, hemicellulose and protein with large amounts of pectin forming a viscous matrix that cements the wall together. The precise molecular composition varies between cell types, tissues and plant species, although an approximate dry weight ratio would be 30% cellulose, 25% hemicellulose, 35% pectin, and 10% protein (Taiz & Zeiger, 1991).

Cellulose is formed by polymerization of D-glucose molecules linked in the β-1,4 position, resulting in flat, linear chains (Fig. 1.1). Hydrogen bonding between chains leads to the formation of a microfibril up to 3 nm in diameter. These crystalline microfibrils are laid down in different orientations within the primary wall, providing the structural support for the wall. The surrounding wall matrix is composed of hemicellulose, protein and pectin in which the microfibrils are embedded. Hemicelluloses are mixed polymers of different neutral and acidic polysaccharides. They adhere to the surface of the cellulose microfibrils by hydrogen bonding, through OH groups on the sugars, and enhance the strength of the cell wall. Pectic polysaccharides are covalently bound to the hemicelluloses. The protein components of the primary cell wall are hydroxyproline-rich glycoproteins, named extensins, that are involved in cell wall architecture and plant disease resistance (Brownleader *et al.*, 1996; Dey *et al.*, 1997b). As some cell types mature, a secondary cell wall is deposited between the primary wall and the plasmalemma that is more dense than primary walls and binds less water. Wood contains cell walls of the greatest maturity of all plant cell types, with several layers (S1, S2, S3) in the secondary wall (Fig. 1.2). These are composed of cellulose, hemicellulose and lignin but with less pectin than primary cell walls. An approximate ratio of these components would be 35% cellulose, 25% hemicellulose including pectin,
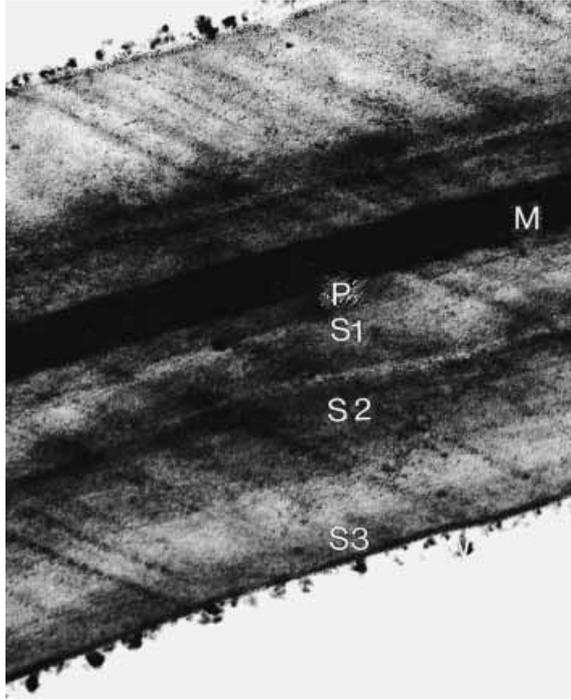
Fig. 1.2. Transmission electron micrograph of an ultra-thin section of beech wood showing the wood cell wall structure. M, middle lamella; P, primary wall layer; S1, S2 and S3, secondary wall layers. Magnification × 28 000.

and up to 35% lignin, depending on the plant species. The cellulose microfibrils are orientated at different angles in each layer of the three secondary wall layers to provide increased strength. Lignin gives rigidity to the wall (Cowling & Kirk, 1976; Montgomery, 1982). Strength is related to these structural components, particularly the orientation and crystallinity of the cellulose microfibrils (Preston, 1974), whereas toughness is a reflection of the elastic component of the cell wall, giving an indication of the potential for plasticity (Lucas *et al.*, 1995).

Lignin is a three-dimensional aromatic polymer that surrounds the microfibrils, with some covalent attachment to the hemicellulose. It is composed of up to three monomeric units of cinnamyl alcohols: coumaryl alcohol, mainly confined to grasses; coniferyl alcohol, the major monomeric unit in gymnosperm wood; and sinapyl alcohol, predominant in angiosperm wood (Fig. 1.3). Polymerization of these monomers is by a free radical reaction catalysed by peroxidase. This results in a variety of bonds
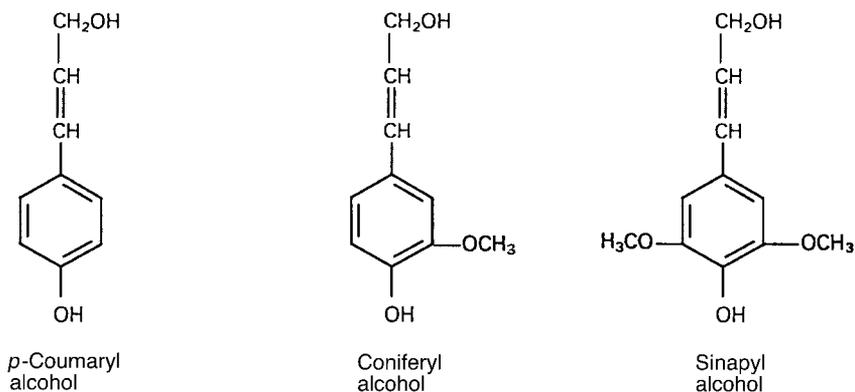
Fig. 1.3. Monomeric phenylpropanoid units that polymerize to form lignin.

in the polymer, with β-O-4 bonds between the C_4 carbon of the aromatic ring and the β-carbon of the side chain being the predominant bond linkage. Other common bonds are α-O-4, C_3 or C_5 aryl-O-4 linkages with some biphenyl linkages. Substitution on the aromatic rings of the monomers determines the type of lignin as syringyl (made from coniferyl and sinapyl alcohols) or guaiacyl lignin (made from only coniferyl alcohol) (Adler, 1977). Side chains of lignin are composed of cinnamyl alcohols, aldehydes and hydroxylated substitutions on the α- and β-carbons. It is the high proportion of ether bonds in the polymer, from the methoxy groups and polymerizing bonds, that gives lignin its unique structure and properties as a strong resistant polymer. It has been found that the polysaccharides in the cell wall can influence the structure of lignin during synthesis. The motion of coniferyl alcohol (one of the lignin monomers) and its oligomers near a cellulose surface can change the course of dehydrogenation polymerization into lignin, as electrostatic forces restrict the motion of the monomer and oligomers (Houtman & Atalla, 1995). This is consistent with experimental observations of the lignin–polysaccharide alignment in cell walls as observed by Raman microprobe studies (Atalla & Agarwal, 1985).

Hemicelluloses in the secondary wood wall vary between plant species, with xylan the predominant polymer. The simple β-1,4-linked-D-xylopyranosyl main chain carries a variable number of neutral or uronic acid monosaccharide substituents, or short oligosaccharide side chains (Fig. 1.4). Hardwood (angiosperms) xylans are primarily of the glucuroxylan type, while softwood (gymnosperms) xylans are glucuronoarabinoxylans (Joseleau, Comtat & Ruel, 1992). The structure
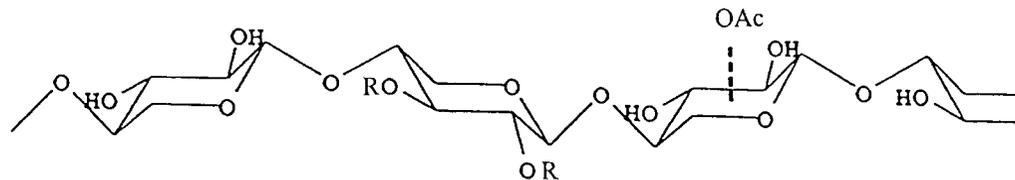
R can be any of the following substituents:

4-OMe-α-D-GlcA (1,2)Xyl   Feruloyl
α-D-GlcA (1,2)Xyl   *p*-Coumaryl
α-L-Ara (1,3)Xyl   Lignin
β-D-Xyl (1,2) α-L-Ara (1,3)Xyl
β-D-Gal (1,5) α-L-Ara (1,3)Xyl

Fig. 1.4. Basic structure of xylan. Me, methyl; GlcA, gluconic acids; Xyl, xylose; Ara, arabinose; Gal, galactose.

of xylans in the wood cell wall is difficult to characterize but it is clear that many covalent linkages occur with other wall polymers, as complete extraction of xylan from wood requires drastic alkaline conditions. Stable lignin–xylan complexes remain in wood pulp after Kraft pulping, and may involve carbon–carbon bonding while other bonds forming acetals or glycosides include oxygen. Other cross-linking polymers in the cell wall are ferulic and *p*-coumaric acids, providing important structural components. Dehydrodiferulate oligosaccharide esters have been extracted from wheat bran but not the free dehydrodiferulate acids, indicating that cross-linkages are formed with hemicellulose components (Kroon *et al.*, 1999).

The secondary wood cell wall structure has been visualized using transmission electron microscopy, revealing distinct layers in the secondary wall (Fig. 1.2). The dense wall structure makes the cells impenetrable to microorganisms without prior degradation of the wall polymers. The size of the pores within the wood cell wall, and hence water-holding capacity of the wall, is low; permeability studies with indicator molecules and dyes indicate that molecules above 2000 Da are unable to penetrate (Cowling, 1975; Srebotnik, Messner & Foisner, 1988; Flournoy, Kirk & Highley, 1991). In wheat cell walls, pores with radii of 1.5–3 nm (measured by gas adsorption) predominate, which are below the size that would allow free penetration by degrading enzymes (Chesson, Gardner & Wood, 1997). Casual predators and pathogens are deterred from establishing an ecological niche in such substrates.

The ecophysiology of lignin degradation

Most reviews of lignocellulose degradation have focused on the mechanisms of the process rather than the ecophysiology of the organisms involved. The Basidiomycota and Ascomycota, mostly in the orders 'Aphyllophorales', Agaricales and Sphaeriales, are considered by Cooke & Rayner (1984) to be responsible for decomposition of a high proportion of the annual terrestrial production of 100 gigatonnes of lignocellulose-rich plant cell wall material, of which lignin alone accounts for 20 gigatonnes (Kirk & Fenn, 1982). The basis of most studies on lignocellulose-degrading fungi has been economic rather than ecological, with focus on the applied aspects of lignin decomposition, including biodeterioration, bioremediation and bioconversion. This has led to an overemphasis on a few fungi as model organisms, particularly in the study of lignin decomposition, without any attempt to decide if they represent the spectrum of lignin-degrading systems in the fungi as a whole. Awareness and understanding of a

wider number of species with good potential for economic use will lead to improvements in bioremediation technology.

Another gap in our knowledge of ligninolytic fungi is that not only have comparatively few taxa been studied but nearly all of them originate from the northern temperate forest and taiga biomes. This is in spite of the fact that the biodiversity of decomposer fungi is much higher in tropical ecosystems, especially tropical forest. In tropical forest, 74% of the primary production is deposited as woody litter and 8% as small litter, 10–35 tonnes litter $ha^{-1} yr^{-1}$, illustrating the enormous quantities of lignocellulose processed by decomposer fungi and termites in tropical forest (Swift, Heal & Anderson, 1976). The tropical forest biome contains 400–450 gigatonnes of plant biomass compared with 120–150 gigatonnes for temperate and boreal forest biomes (Dixon *et al.*, 1994). It is estimated that there are three times more taxa of higher fungi in tropical ecosystems than in other forest ecosystems, of which a much higher proportion are decomposers (Hedger, 1985). In spite of this, the isolation and screening of wood- and litter-decomposing fungi from tropical forests has yet to be systematically commenced (Lodge & Cantrell, 1995).

Lignocellulose degradation by fungi used in bioconversion of lignocellulose wastes

Most world mushroom production is from *Agaricus bisporus*, *Pleurotus ostreatus*, and *Lentinula edodes* and related species (Stamets & Chilton, 1983; Stamets, 1993), all grown on a range of substrates prepared from lignocellulose wastes such as straw and sawdust. These taxa have been widely used in physiological studies of cellulose and lignin decomposition in order to determine the role of lignocellulolytic systems in bioconversion of lignocellulose wastes to fruit bodies. Detailed studies on the ligninases of these taxa provided the early understanding of the ligninase systems (Wood, 1980; Kirk & Farrell, 1987; Hatakka, 1994). However, surprisingly little is known of the ecophysiology of the mycelia of these fungi in their natural environments: soil and litter in the case of *Agaricus* spp. and wood in the case of *Pleurotus* spp. and *L. edodes*. These are very different resources, and the published contrasts in the enzyme systems of these two groups of cultivated fungi may be related to the autecology of their mycelia (Wood, Matcham & Fermor, 1988). However there are no *in vivo* studies of lignocellulose degradation by these fungi. When used for bioremediation, it is the fungal mycelia and not their fruit bodies that transform lignocellulosic wastes and transform aromatic pollutants.

The ecology of wood-rotting fungi

Another source of isolates for the study of lignin decomposition has been the higher fungi, which cause significant economic losses to the timber industry. Pathogens of trees are an obvious example and include fungi such as *Armillaria* spp. and *Heterobasidion annosum*, where pathogenicity includes white rot exploitation of the lignocellulose resource by mycelia of these fungi (Stenlid & Redfern, 1998). Their ligninolytic systems have been studied (Asiegbu *et al.*, 1998; Rehman & Thurston 1992), findings showing that their role in pathogenicity is much less important than in the subsequent phases of saprotrophic exploitation and inoculum production (Rishbeth, 1979).

Decay of timber in-service has also yielded information on the lignocellulose-degrading enzymes produced by fungi like *Serpula lacrymans, Lenzites trabea*, and *Fibroporia vaillentii*. These basidiomycetes are all brown rot fungi, a physiological group that probably coevolved with the Coniferales in the northern taiga and temperate forests (Watling, 1982) and which are important because most in-service timber in Europe and the USA is softwood. Few studies have been made of these economically important fungi in the natural environment. It is salutary to realise that *S. lacrymans*, the dry rot fungus, although the subject of many papers on the nature of its mode of decomposition of cellulose (Montgomery, 1982; Kleman-Leyer *et al.*, 1992), has never been found outside the built environment, although recent studies indicate that it may have its origins in North Indian forests (White *et al.*, 1997). Another well-studied wood-degrading taxon little known outside the laboratory is the white rot thermophilic basidiomycete *Phanerochaete chrysosporium*, which was first considered as a problem in the 1970s in self-heating wood chip piles in its anamorphic state, *Sporotrichum pulverulentum* (Burdsall, 1981). Although this fungus has been the subject of many investigations of cellulases and ligninases because of their potential in bioremediation (Johnsrud, 1988), its natural 'niche' remains unknown.

The ecology of **Trametes versicolor** and the dynamics of wood decay

Up to the early 1980s, most detailed studies on lignin-degrading enzymes were on the 'economically important' fungi discussed above. However, since then the search for ligninolytic systems has been extended to include species of little economic importance but of applied potential because of

their rate of growth and high enzymic activity. The most obvious example is *Trametes (Coriolus) versicolor*, the ligninases of which were first studied by Dodson *et al.* (1987), which causes white rot decay of broad-leaved tree species in temperate forest ecosystems. This fungus has been widely used in bioremediation programmes and characterization of its ligninases is now well understood, providing information that can be related to its ecophysiology in the natural environment. A good example is the regulatory effect of nitrogen on ligninolytic enzyme expression, a reflection of the inductive effect of low nitrogen levels (C : N 200 : 1 to 1000 : 1) found in wood (Swift 1982; Leatham & Kirk, 1983).

Unfortunately, laboratory results of this type have led to the simplistic view that 'success' of fungi in the natural environment can be simply related to the physiology of their mycelia in culture. It might be assumed that active ligninases and cellulases and fast mycelial growth in culture can explain the ubiquity of *T. versicolor* in broad-leaved forest. Fortunately, studies on the ligninases of this fungus coincided with studies on the population dynamics of communities of wood decay fungi, including *T. versicolor* (Rayner & Todd, 1979). The fungus causes a rapid white rot invasion of moribund or fallen trees of species such as birch, beech and oak. Rayner & Webber (1985) have shown that the outcome of primary resource capture by fungi like *T. versicolor* is a result of mechanisms that operate in the early stages of colonization. Early phases of expansion are by a rapidly extending mycelium, which utilizes free sugars in the wood of the tree. Entry into broken or cut ends of the wood from the spore rain means that an individual mycelium is usually restricted to an elongated form because of the faster rates of expansion of mycelia along vessels and tracheids. Following this resource capture, contact between mycelia of genetically distinct individuals of *T. versicolor*, and with mycelia of other species of wood-rotting fungi, results in combative behaviour. *T. versicolor* is typical of early colonizers of wood, an assemblage of fungi characterized by Cooke & Rayner (1984) as disturbance tolerant, with a combative mycelial strategy and active lignocellulose exploitation. The wood volume retained by the mycelium is covered by a melanized pseudosclerotial plate, resulting in a mosaic of individuals – the 'spalted' wood of the turners. White rot exploitation of the wood within these volumes by lignocellulolytic enzymes produced by the mycelia may then take place for a number of years. However, the initial phase of occupation and retention of the resource has little to do with the lignocellulolytic potential of the fungus. Comparison of lignocellulose decomposition by common white rot competitors of *T. versicolor*, for example *Stereum hirsutum* and *Hy-*

poxylon multiforme, show them to produce less ligninases and cellulases; however, they are equally successful colonizers since the initial outcome of competition is solely determined by occupation and retention of substrate (Rayner & Todd, 1979). *T. versicolor* and other 'primary resource captures' may, in fact, be eventually replaced by mycelia of other more combative wood decomposers: 'secondary resource captures', for example *Lenzites betulina* (Rayner, Boddy & Dowson, 1987; Holmer & Stenlid, 1997).

The strategy of many wood-rotting fungi is to exploit the retained wood relatively slowly, their mycelia being characterized as slow growing, stress tolerant, combative and defensive (Rayner & Boddy, 1988; Holmer & Stenlid, 1997). These fungi may persist in wood much longer than *T. versicolor*, although they appear to be less active degraders of lignocellulose under laboratory conditions. Their success is related to slow growth combined with retention of the wood, and tolerance to the developing nutrient stress in the wood as it decays and to extractives in heartwood. Many of these fungi are members of the 'Aphyllophorales', good examples being the genera *Ganoderma*, *Fomes* and *Inonotus*, which may persist for decades on fallen trees. Lignocellulose degradation by such fungi has been little studied, mostly because of their slow growth, difficulties in culturing and little apparent biotechnological potential. However, the later stages in decomposition of wood offer different physiological challenges to mycelia, for example the presence of complex recalcitrant aromatic compounds; consequently their degradative systems may well be of interest.

Another life strategy group of wood decomposer fungi is contained within the ascomycete order Sphaeriales. Genera in this order (e.g. *Xylaria, Daldinea* and *Hypoxylon*) have been studied by Rayner & Boddy (1988), who showed that they occupy and retain volumes of wood in the way described above for combative white rotters like *T. versicolor* but are characterized by a relatively slow white rot and a reduction of the water content of the wood. Physiological studies showed that the mycelia of species in these genera were tolerant of water stress and able to grow at potentials as low as 10–12 Mpa, explaining their successful retention of dry lignocellulose resources (Boddy, Gibbon & Grundy, 1985; Bravo-Velasquez & Hedger, 1988). The operation of ligninases under such low water potentials is of applied interest and the few studies carried out on these fungi have revealed unexpectedly low laccase and manganese peroxidase (MnP) activities, in spite of their *in vivo* abilities to cause extensive white rot (Ullah, 2000).

Litter-decomposing fungi

Studies on lignocellulolytic systems have mostly been limited to wood-rotting fungi, while litter-decomposing fungi that colonize small debris such as leaves and twigs have received little attention. Except for the mushrooms *A. bisporus* and *Volvariella volvacea*, which are both grown commercially on composted lignocellulose, the ligninolytic abilities of other litter-decomposing higher fungi are poorly understood, yet studies on their ecology have shown that they are major processors of lignocellulose in forest ecosystems (Hedger & Basuki, 1982). Frankland (1984), in a study of litter decomposition in Meathop Wood, UK, showed that the agaric *Mycena galopus* was responsible for a large proportion of the breakdown of the leaf litter of oak and other trees.

It is to be expected that the ecophysiologies of other litter-decomposing fungi may be different from those of wood decomposers, given the much lower lignin content of small litter, which consists of leaves, small twigs, seeds and fruits (Swift *et al.*, 1976). What effect this might have on the ligninolytic systems discussed in this chapter is difficult to predict, but needs study. An exception is a study of isolates of litter- and wood-decomposing fungi from a forest in Ecuador in which laccase and MnP activities of 27 different taxa were compared (Ullah, 2000). The 19 wood-rotting fungi had significantly greater laccase activity than 11 isolates from leaf litter, two being close to those of *T. versicolor* control cultures. However, the litter decomposer fungi had significantly higher titres of MnP activity than did the wood decomposers. Such results underline the need for an ecological perspective in the selection of fungal isolates for studies of cellulases and ligninolytic systems, in order to interpret the value of the different components of the system to the ecology of the organisms and perhaps to find novel ligninolytic systems.

Mechanisms of degradation

White rot basidiomycetes that degrade all cell wall polymers are generally considered to be the most effective lignocellulose degraders (Crawford, 1981; Hammel, 1997). However, from the perspective of an individual fungus, this may not be the case. The only reason for fungi to attack lignocellulose is to obtain sufficient carbon and nitrogen for survival. Unless energy used to obtain glucose is less than that resulting from its uptake and metabolism, there is no advantage to the organism in colonizing lignocellulose substrates. In fact, brown rot rather than white rot

basidiomycetes may have the most efficient mechanisms for obtaining glucose from lignocellulose as they are able to extract glucose from the cellulose without expending energy on lignin degradation (Highley, 1977; Micales, 1995). They modify the lignin by methylation but no depolymerization occurs.

The soft rot fungi are a specialized group of organisms that grow in a localized niche within the secondary wood cell wall and degrade the cell wall polymers slowly (Hale & Eaton, 1985; Daniel & Nilsson, 1989). Characteristic patterns of decay in wood are channels within the secondary wall wherein the hyphae lie, degrading polymers immediately around the hyphal surface.

So what are the mechanisms employed by microorganisms to break down lignocellulose? Our understanding of lignocellulose degradation is based on laboratory studies with white rot basidiomycetes, using a selected number of organisms such as *P. chrysosporium* and *T. versicolor*, and, as mentioned above, it is probable that more effective organisms operate in the ecosystem that have not been characterized in the laboratory. All of these organisms have potential for use in bioremediation processes, although to date *P. chrysosporium, T. versicolor* and *P. ostreatus* have been the primary species used in pilot and field bioremediation trials.

Lignocellulolytic enzymes

The usual biological answer to breaking down a polymer is to use highly specific enzymes. This is normally extremely effective as a minimum amount of protein (enzyme) can be synthesized by the organism to cleave a regular repeating bond between units of the polymer. Examples of some of these enzymes are those that catalyse hydrolytic reactions to attack carbohydrates such as cellulose and hemicellulose (Walker & Wilson, 1991; Goyal, Ghosh & Eveleigh, 1991). They tend to be specific to a particular bond chemistry, for instance, β-1,4 specificity is required to hydrolyse cellulose, and α-1,4 specificity for starch hydrolysis.

Cellulases

Our knowledge of the biochemistry of enzymes that depolymerize cellulose is based mainly on studies of *Trichoderma* spp.: the most prolific sources of cellulases known (Mandels & Steinberg, 1976). When enzymes from wood-rotting basidiomycetes have been screened for cellulases, their composition has closely resembled those of cellulases from *Trichoderma reesei*, with five endoglucanases, one exoglucanase and two β-1,4-glucosidases

identified (Eriksson & Pettersson, 1975). Cellulase is a complex of enzyme activities that includes exo- (cellobiohydrolases) and endocellulases, with β-glucosidases; these act in synergy to depolymerize cellulose fibrils, releasing glucose and cellobiose (a glucose dimer). Glucose is readily taken up by the fungus, providing carbon for energy and growth. Cellobiose is converted to glucose by the action of β-1,4-glucosidase (Evans, 1985; Gallagher & Evans, 1990). There are several controlling feedback mechanisms on production of the specific components of the cellulase complex by fungi, for instance glucose represses and cellobiose or cellulose stimulates the production of exo- and endocellulases. Cellobiohydrolases are composed of a cellulose-binding domain linked to a catalytic domain through a proline and a OH-amino acid linker region (Ong *et al.*, 1989). Genes for exo- and endocellulases have been isolated and the gene products characterized, providing an improved understanding of the biochemical mechanisms involved (Beguin, 1990; Covert, Wymelenberg & Cullen, 1992). Feedback control of exo- and endocellulase production is exerted by glucose and sucrose with catabolite repression at concentrations of $1\,\mathrm{g}\,\mathrm{l}^{-1}$, but induction of cellulase occurs with $1\,\mathrm{mg}\,\mathrm{l}^{-1}$ cellobiose or cellulose (Eveleigh, 1987). Figure 1.5 shows a scheme for cellulolysis that represents the biological activities of the cellulase complex.

Although cellulases have been isolated from cultures of brown rot and white rot fungi, there is a fundamental difference in the mechanism of cellulose hydrolysis in the two fungal rots. Brown rots produce complete breakage of amorphous cellulose fibrils while white rots cause a progressive decay from the fibril surfaces (Klemen-Layer *et al.*, 1992; Gilardi, Abis & Cass, 1995). The mechanism that brown rots use to access the cellulose in the wood cell wall is thought to be by generation of hydroxyl radicals from the reaction of H_2O_2 with Fe^{2+} in the Fenton reaction (Koenigs, 1974; Hyde & Wood, 1997). There has been generally less interest in the mechanisms of cellulose degradation by brown rots compared with white rots, as industrial usage of residual lignin is limited. In contrast, removal of lignin but leaving the cellulose intact has great potential in industries such as pulp and paper production.

Enzymes for hydrolysis of hemicellulose have also been identified in many wood-rotting fungi. Hard woods contain xylan, and fungal species colonizing them produce xylanases. Other hemicelluloses are hydrolysed by mannanases, galactosidases and glucosidases. These enzymes have very similar characteristics to the cellulase complex in that different enzymes attack exo- and endohemicellulose (Visser *et al.*, 1992).
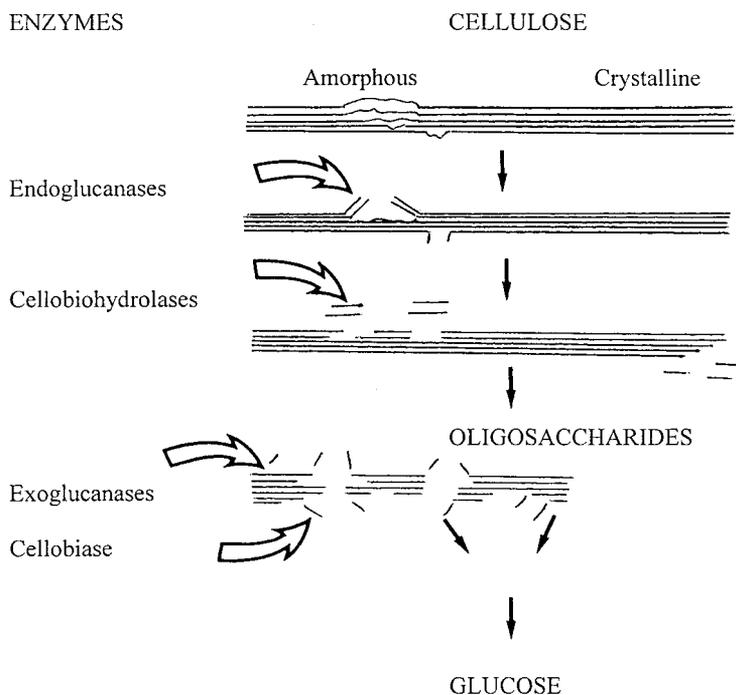
ENZYMES   CELLULOSE

Amorphous   Crystalline

Endoglucanases

Cellobiohydrolases

OLIGOSACCHARIDES

Exoglucanases

Cellobiase

GLUCOSE

Fig. 1.5. Diagrammatic representation of the enzymic digestion of cellu-lose.

Ligninases

As previously described, lignin is not a symmetrical well-ordered polymer with a single repeating bond that links the monomeric units. It is difficult, therefore, to envisage a single enzyme that has the capability to depolymer-ize such an irregular structure. Although many different enzymes could be produced by a degrading organism, this would prove energetically waste-ful, as more energy would be expended in synthesis and secretion of proteins than would be gained from metabolism of the end products. This is particularly so for lignin as there is very little calorific value in the polymer for the fungus, which is unable to survive on lignin as sole carbon source (Kirk, Connors & Zeikus, 1976). It is assumed that white rot species only degrade lignin as a means to access the cellulose in the wood cell wall. The fungal enzymes that are used to degrade lignin are therefore non-specific with respect to substrate. They function mainly by the production of free radicals that are able to attack a wide range of organic molecules.

Peroxidases using H_2O_2, and laccases (polyphenol oxidases) using molecular oxygen are the enzymes responsible for attack on lignin (Field *et al.*, 1993; Evans *et al.*, 1994).

Peroxidases

The first enzyme shown to attack lignin-type compounds (model dimers, trimers and later polymers) was lignin peroxidase or LiP, isolated from *P. chrysosporium* (Tien & Kirk, 1984). LiP is a haem-containing peroxidase ($\sim 42\,000\ M_r$) with an unusually high redox potential. It is highly glycosylated, as are most enzymes secreted for extracellular action. Most but not all white rot species produce it (Hatakka, 1994). It is distinctive in its ability to oxidize methoxyl substituents on non-phenolic aromatic rings by the generation of cation radicals that undergo further reactions. The pH optimum of LiP is below 3.0 but the enzyme shows signs of instability if kept in such acidic environments (Tien & Kirk, 1988). Natural environments in wood cells are acidic, approximately pH 4.0, but localized pockets may occur with lower pH because of secretion of oxalic acid by the fungi (Dutton *et al.*, 1993; Dutton & Evans, 1996). *In vitro* peroxidases can be stimulated by low nitrogen stress on the fungi. Veratryl alcohol is a substrate for LiP and is used in a spectrophotometric assay to monitor its activity by measuring veratraldehyde production in the presence of H_2O_2 (Tien & Kirk, 1984). Veratryl alcohol is produced extracellularly as a secondary metabolite by many white rot fungi and enhances LiP activity (Collins & Dobson, 1995) probably by protecting LiP from inactivation by excess H_2O_2 (Chung & Aust, 1995) (Fig. 1.6).

MnP is also a haem-containing enzyme and is generally considered to be essential for lignin degradation *in vivo*. The catalytic cycle of MnP is similar to that of LiP, in addition to Mn^{2+} being converted to Mn^{3+} during the reaction. In reaction with some substrates such as dimethoxyphenol, MnP activity is independent of manganese (Archibald, 1992). Increased bio-bleaching of Kraft pulps has correlated with purified MnP activity from *T. versicolor* (Paice *et al.*, 1993; Reid & Paice, 1998). The white rot species that have been examined have all shown MnP activity, whereas not all have LiP activity. LiP and MnP both require H_2O_2, which must be generated by the fungus. Fungal enzymes producing H_2O_2 include glucose oxidase (Eriksson *et al.*, 1986), glyoxal oxidase (Kersten & Kirk, 1987) and aryl alcohol oxidase (Guillen, Martinez & Martinez, 1990; Guillen & Evans, 1994). In addition, MnP can generate H_2O_2 through oxidation of organic acids (Urzua *et al.*, 1995), while MnP-chelates can oxidize a range of phenolic compounds, including a variety of synthetic lignins (Masaphy,
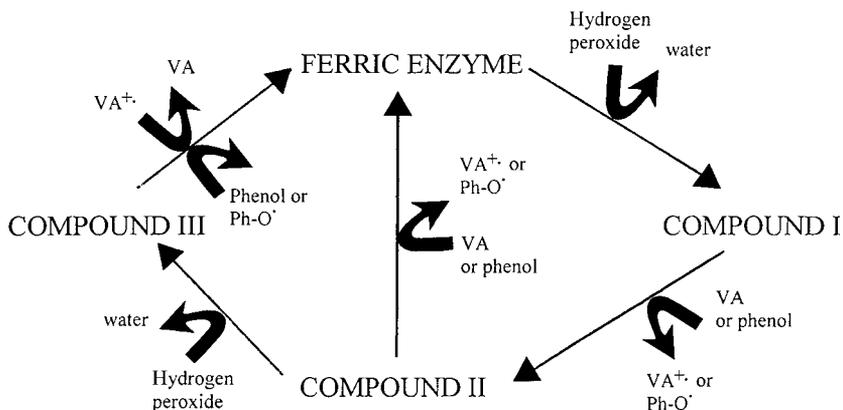
Fig. 1.6. Catalytic cycle of lignin peroxidase (LiP). VA, veratryl alcohol; Ph, phenol.

Henis & Levanon, 1996; Hofrichter *et al*, 1998). Manganese(III)-chelates, such as with oxalate, are small molecules that are able to diffuse into the pores of the wood cell walls that are inaccessible to enzymes (Archibald & Roy, 1992; Evans *et al.*, 1994). The rate of lignin degradation *in vivo* is controlled by the slowest reaction step, which in the case of MnP activity may be availability of H_2O_2 or Mn^{2+} rather than the catalytic rate of MnP. As excess H_2O_2 can destroy the catalytic site of MnP, the rate of production of H_2O_2 may have important effects on the rate of lignin degradation (Palma, Moreira & Feijoo Lema, 1997).

Laccases

The ability to oxidize phenolic compounds extracellularly is used to differentiate white rot fungi from brown rot species. A rapid screening test for white rots based on polyphenol oxidase activity – the Bavendamm test – monitors the development of a brown colouration on agar plates containing guaiacol or gallic acid. Polyphenol oxidase activity is shown by several enzymes including tyrosinase (oxidation of monophenols) and laccase, which oxidizes mono- and diphenols. The majority of white rot fungi produce laccase, frequently as the dominant extracellular enzyme in liquid cultures in the laboratory (e.g. for *T. versicolor* and *P. ostreatus*). Specific isomers of laccase can be induced *in vitro* by addition of compounds such as 2,5-dimethylaniline – a lignin mimic compound. *P. chrysosporium* generally does not produce laccase under artificial growth conditions though it has been reported during growth on a defined medium containing cellulose (Srinivasan *et al.*, 1995).

In the presence of oxygen, laccase converts mono- and diphenolic groups to quinone radicals then quinones in a multistep oxidation process (Thurston, 1994). Fungal laccase is a copper-containing enzyme found in several isoforms. Two are blue isomers with different isoelectric points and different abilities in binding to ion-exchange gels. Yellow laccases, also containing copper centres, have been isolated from cultures of *T. versicolor* and *Panus tigrinus* grown on solid substrates (Leontievsky *et al.*, 1997). Their colour is thought to result from binding of soil phenolics by the enzyme. Fungal laccases were first thought to be enzymes involved in the oxidation of phenolics to effect their removal from the fungal environment, though it was observed that mutant strains of *S. pulverulentum* (*P. chrysosporium*) without laccase were unable to degrade lignin (Ander & Eriksson, 1976). There is now general acceptance that laccases as well as LiP and MnP are involved in lignin degradation through attack on free phenolic groups in lignin and the generation of free radicals. Laccase and MnP both react with free phenolic groups, although in the presence of some low-molecular-mass mediators, laccase can also react with non-phenolic substituents on the aromatic rings. This permits laccase to operate at a higher redox level than normally (Leontievsky *et al.*, 1997).

Different white rot species produce various combinations of LiP, MnP and laccase depending on growth substrates. For example, *P. chrysosporium* secretes mainly LiP and MnP (Glenn & Gold, 1985); *Phlebia radiata* secretes laccase and MnP (Hatakka, 1994); *T. versicolor* synthesizes all three ligninolytic enzymes (Kadhim *et al.*, 1999). Enzymes produced in liquid cultures in laboratories are considered atypical of enzymes produced *in vivo*.

How these enzymes operate *in vivo* has been partially demonstrated using electron microscopy of sections of substrates colonized with fungal hyphae (reviewed by Evans *et al.*, 1991; Daniel, 1994). The technique used was immunogold-cytochemical labelling, which visualized specific enzyme molecules under the electron beam of the transmission electron microscope (Fig. 1.7). These studies have shown that enzymes such as LiP, MnP, laccases and cellulases are too large to penetrate into intact, secondary wood cell walls and remain close to the surface of the fungal hyphae or adhere to the inner surface of the wood cell wall (Evans *et al.*, 1991). Degradation of lignocellulose occurs by surface interaction between cell wall and enzymes, but initiation of decay can occur at a distance from the fungal hyphae, probably involving diffusible small molecular mass molecules such as oxalate, H_2O_2, $Fe^{2+/3+}$ and $Mn^{2+/3+}$ (Evans *et al.*, 1994). A predominant theory on the mechanism of cellulose degradation by brown
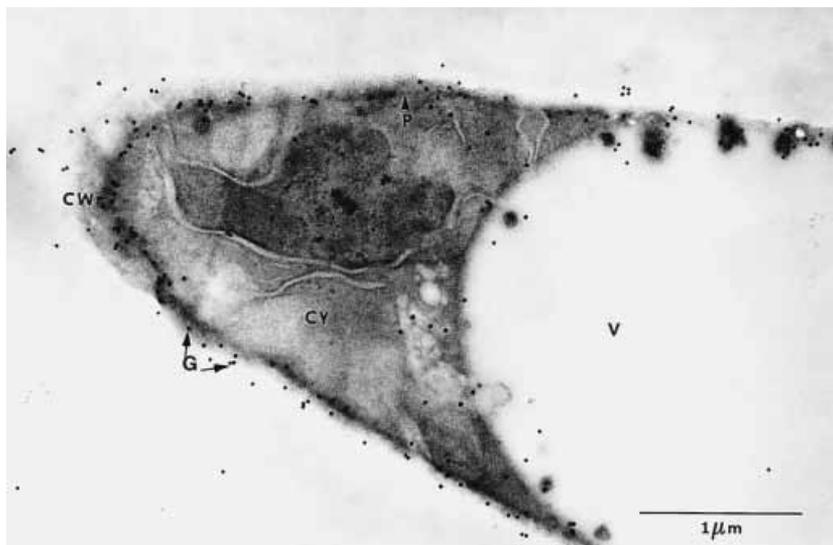
Fig. 1.7. Transmission electron micrograph showing localization of laccase on a hypha of *Trametes versicolor* by immunogold-cytochemical labelling. CW, cell wall; V, vacuole; CY, cytoplasm; G, gold-labelled laccase; P, plasmalemma.

rot fungi (which do not degrade lignin) is that Fe^{2+} reacts with H_2O_2 producing destructive hydroxyl radicals (Koenigs, 1974). Similar radicals can also be released from reactions between oxalate and H_2O_2 (Wood, 1994).

Conclusions

The ligninolytic capacity of white rot fungi makes them the most interesting taxa of fungi for use in bioremediation. Without an understanding of the mechanisms of lignin degradation, it would not be possible to address degradation of pollutants such as chlorophenols, nitrophenols and polyaromatic hydrocarbons in a practical way. These compounds can all be transformed using ligninolytic enzymes because of the free radical reactions, and development of industrial treatments using these fungi are proving successful (Bogan & Lamar, 1999; Jerger & Woodhull, 1999). Much lignocellulose material remains as waste products from industries such as forestry, agriculture and paper manufacture and use, which can be disposed of by accelerated natural biodegradation. The only products of value to accrue from such waste treatment are mushroom production and

composts (Wood, 1989). New opportunities for commercial products and processes in fungal treatment of lignocellulosic materials are likely to arise in the future, and screening for novel organisms should contribute to the development of new technologies.

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