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Biotechnology in the Study of Brown- and White-Rot Decay

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2.1 Introduction

Many different types of organisms deteriorate wood, but the greatest damage results from fungi. Decay is the most serious form of microbiological deterioration because it can cause structural failure, sometimes very rapidly. Because widespread damage to wood from decay is seldom spectacular, the tremendous economic and resource loss resulting from decay is often overlooked. This oversight is exemplified by a failure to recognize that biodeterioration of wood products is important and that extending the service life of wood is a sure way to prolong the available timber supply. Knowledge about the biochemical systems of decay fungi can serve another purpose as well. Decay fungi can be used for the bioconversion of lignified tissue, such as biopulping or enzymatic treatment of pulps (Kirk and Hammel, 1992). Recent research has advanced our understanding of how wood components are degraded by microorganisms and their enzymes, permitting rapid advances in biotechnology (Wainwright, 1992).

The most important and potent wood-destroying organisms are white- and brown-rot fungi, which attack various components of the wood cell wall. Most white-rot fungi utilize cellulose and hemicelluloses at approximately the same rate relative to the original amounts present, whereas lignin is usually utilized at a somewhat faster relative rate. A few white-rot fungi remove lignin and hemicelluloses preferentially, but ultimately they degrade all wood cell wall components. White-rot fungi cause the wood to become pale, eventually reducing it to a fibrous, whitish mass. Brown-rot fungi utilize cell wall hemicelluloses and cellulose, leaving the lignin essentially undigested. However, brown-rot fungi do modify lignin as indicated by demethylation and accumulation of oxidized polymeric lignin-degradation products. These fungi cause the wood to darken, shrink and break into brick-shaped pieces that crumble easily into a brown powder.

Brown- and white-rot fungi decay wood by distinctly different mechanisms. However, in spite of considerable research the biochemical bases for the different morphological and chemical changes are not clear (Figures 2.1 and 2-2). Many factors have hindered progress - in particular, the complexity of the wood substrate

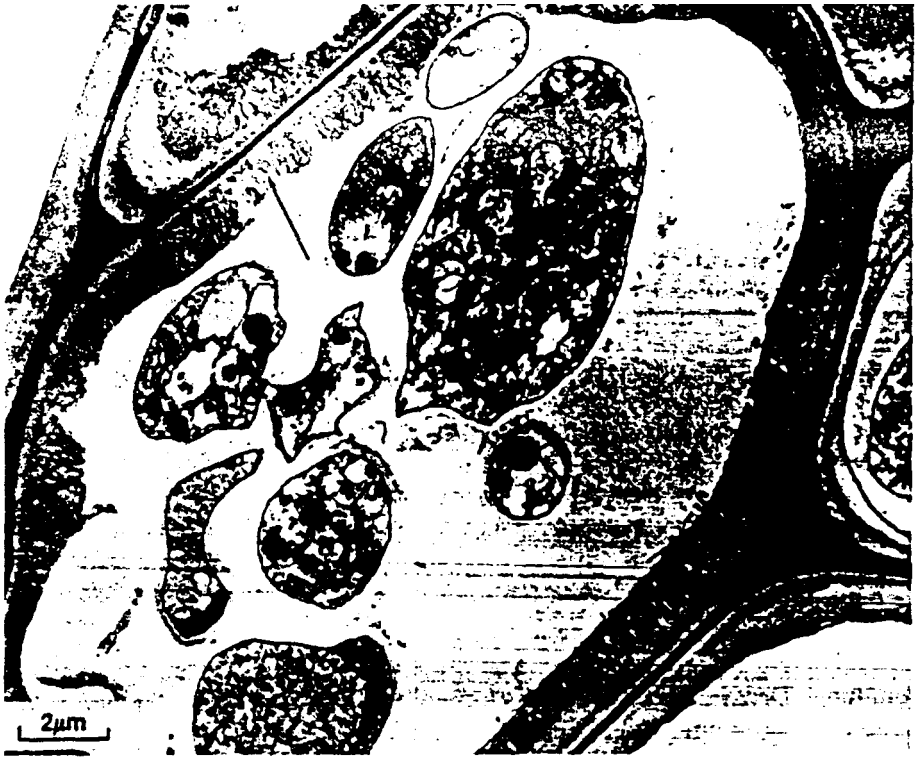

Figure 2.1 Advanced cell wall degradation in sweetgum by hyphae (H) of the brown-rot fungus *Postia placenta* The S_2 layer was apparently attacked first; the S_1 and S_3 layers appear relatively free of attack. Arrow indicates area where decay affected the entire wall. Aldehyde-OsO_4 fixation, 3000 ×

and the multiplicity of enzymes produced. The applications of classical and molecular genetics to wood-degradative systems of decay fungi have progressed rapidly and can be used to elucidate the mechanisms of wood degradation. This chapter reviews the decomposition of wood by white- and brown-rot fungi and discusses how recent advances in biotechnology and their application to the study of wood decay have augmented knowledge about the fungal mechanisms of wood deterioration. White-rot fungi have received by far the most attention, probably because they produce more enzymes that may have biotechnological application.

2.2 Mechanism of White-Rot Decay

2.2.1 Cellulose Degradation

Phanerochaete chrysosporium has served as a model organism for white-rot degradation studies of wood. The enzyme mechanisms involved in cellulose degradation by this white-rot fungus were extensively investigated by Eriksson (1978). It is well established that the degradation of crystalline cellulose by white-rot fungi, similar to that of other fungal cellulases, is carried out by a multicomponent enzyme cotmplex

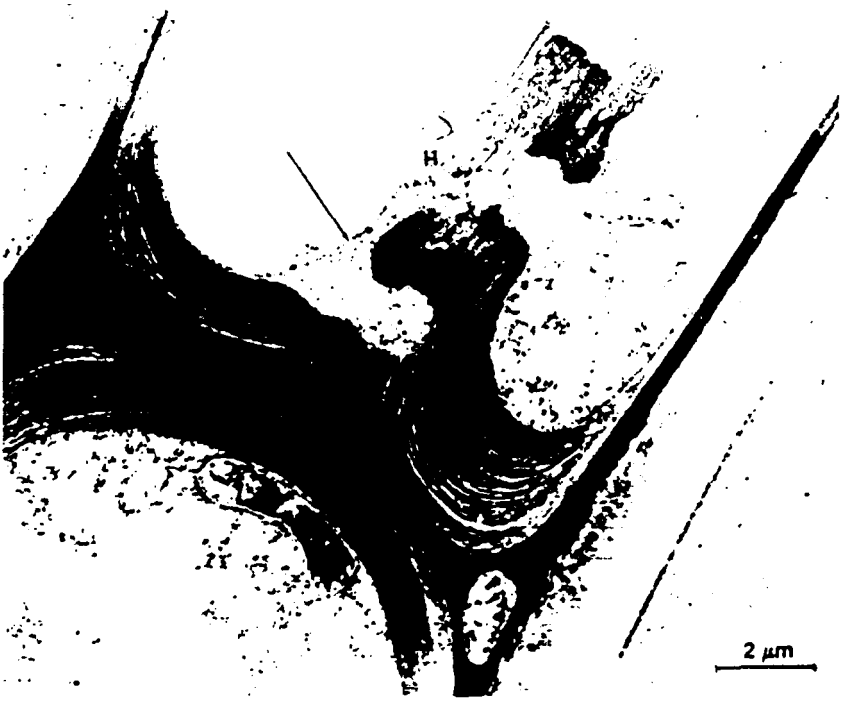

Figure 2.2 Hypha (H) of *Trametes versicolor* attached to sweetgum cell wall by sheath (arrow). Cell wall is degraded beneath the sheath and away from hypha towards the middle lamella. Note localized degradation at cell corners and erosion troughs. $KMnO_4$ fixation, 4100 ×

in which the individual components interact synergistically to degrade cellulose to glucose. Endogiucanses (EGs) act randomly over the exposed surfaces of cellulose microfibrils, exposing non-reducing termini that are hydrolyzed by cellobiohydrolases (CBHs), producing cellobiose. Cellobiose may be cleaved by β-glucosidase, yielding glucose. Eriksson and Pettersson identified five EGs and one CBH Eriksson and Pettersson. 1975a. b). as well as two glucosidases from *P. chrysosporium.* Multiple CBH-like enzymes were subsequently characterized (Uzcategui *et al.,* 1991).

Relatively little work has been done with regard to cellulase regulation in fungi. The precise mechanism or mechanisms of cellulase induction are unknown. With white-rot fungi, cellulase synthesis is induced by cellulose and repressed by glucose. The most generally accepted view of the induction process is that the organisms produce a basic level or a constitutive amount of cellulase that produces soluble hydrolysis products of cellulose that function as inducers. Cellobiose, a product of cellulase action, both induces and inhibits cellulase of *P. chrysosporium* (Eriksson and Hamp. 1978). This fungus can control cellobiose concentration in at least four ways (Eriksson, 1978). The first way is via the hydrolytic enzyme β-glucosidase. which hydrolyzes cellobiose and oligosaccharides to glucose. Second, cellobiose can also be eliminated by transglucosylation reactions. The other two ways involve at least two oxidative enzymes that have been implicated in cellulose degradation by

P. chrysosporiurn and seem to be important in preventing enzyme inhibition by cellobiose. The first of these, cellobiose oxidase, catalyzes the oxidation of higher cellodextrins to their corresponding gluconic acids (Ayers *et al.,* 1978). The second oxidative enzyme is cellobiose:quinone oxidureductase (CBQase) (Westermark and Eriksson, 1974). This enzyme is important for both lignin and cellulose degradation. It is relatively specific for cellobiose but much less so for its quinone substrates. However, recent work showed that CBQase does not affect phenoxyl radicals produced by lignin peroxidase (LiP) or phenol polymerization catalyzed by LiP *in vitro* (Odier *et al.,* 1988). The cellobionic acid formed on oxidation of cellobiose by the two oxidative enzymes of *P. chrysosporium* can be hydrolyzed by β-glucosidase (Eriksson, 1978). However, the product of this degradation, gluconolactone, strongly inhibits β -glucosidase (Deshpande *et al.,* 1978). Lactonase decreases the inhibitory effects of both glucono- and cellobionolactones on β-glucosidase and particularly the effects of cellobionolactone on the complete cellulase system. Lactonase is therefore a component of the synergistic attack of this system.

Genetic analysis of cellulolytic fungi has been extremely limited. Analyses of single basidiospore cultures were used to determine allelic relationships and to generate a genetic map using restriction fragment length polymorphisms (RFLP) (Kockert 1991; Cullen and Kersten, 1992). Linkage between a *cbhl* gene (Sims *et al.,* 1988) and an Lip gene was established using the restriction fragment length polymorphism (RFLP) map (Raeder *et al.,* 1989). Development of transformation systems for *P. chrysosporium* paved the way for more detailed genetic analyses (Alic *et al.,* 1989).

Studies of bacterial and fungal cellulases have revealed a common structural design composed of discrete functional domains: a catalytic core, a conserved cellulose-binding terminus, and an intervening highly glycosylated hinge region (Covert *et al.,* 1992a). Cellulases of *P. chrysosporium* also appear to be organized in accordance with this model. For example, as with other cellulases, papain cleavage of *P. chrysosporium* CBH separates the catalytic domain from the hinge and binding domains (Uzcategui *et al.,* 1991). Also, a CBH gene cloned from *P. chrysosporium* ME446 (Sims *et al.,* 1988) is similar in sequence to other fungal CBH genes (Shoemaker *et al.,* 1983; Azevedo *et al.,* 1990).

Restriction mapping and sequence analysis of cosmid clones revealed a cluster of three structurally related CBH genes in *P. chrysosporium,* one of which lacked the cellulose-binding domain common to other microbial cellulases (Covert *et al.,* 1992a,b). *P. chrysosporium cbhl-1* and *cbhl-2* are separated by only 750 bp and are located approximately 14 kb upstream from a cellulase gene previously cloned from *P. chrysosporium* (Sims *et al.,* 1988). Within a well-conserved region, the deduced amino acid sequences of *P. chrysosporium cbhl-1* and *cbh-2* are, respectively, 80 and 69 per cent homologous to that of the *Trichoderma reesei* CBH I gene. The conserved cellulose-binding domain typical of microbial cellulases is absent from *cbhl-1* gene product. Transcript levels of the three *P. chrysosporium* genes varied substantially, depending on culture conditions. The *cbhl-1* and *cbhl-2* genes were not induced in the presence of cellulose, nor did they appear to be subject to glucose repression. Therefore, Covert *et al.,* (1992a,b) concluded that aspects of the chromosomal organization, structure and transcription of these genes are unlike those of previously described cellulase genes.

Eriksson (1981) suggested an additional mechanism for cellulose degradation in white-rot funqi, wherein hydrolysis by cellulases is combined with an oxidative step.

Oxidative enzymes may produce reactants such as superoxide anion ($O^{\cdot}_2$), singlet oxygen ($^1O_2$, or hydroxyl radical ($OH^{\cdot}$) that are involved in the primary attack of crystalline cellulose. $^1O_2$ and $OH^{\cdot}$ are particularly reactive radicals (Pryor, 1976). These reacants are small enough to penetrate the cellulose microstructure and predispose it to attack by endo- and exoglucanases of white-rot fungi. While-rot fungi have been reported to produce radicals that could react with cellulose. Nakatsubo *et al.* (1982) observed $^1O_2$ formation by *P. chrysosporium* and Eriksson (1981) found that $O^{\cdot}_2$ is produced extracellularly by this fungus as well as other wood-destroying fungi. Einoki *et al.* (1991) isolated an extracellular substance from *Irpex lacteus* that produced and reduced H_2O_2 to $OH^{\cdot}$. He concluded that this substance is involved in the degradation of wood cellulose and lignin. The origin of radical-generating oxidases and their involvement in eelluloase degradation by white-rot fungi warrant further study.

Immunocytochemical techniques (Polack and Priestly, 1992) have been beneficial in localizing cellulases in white-rotted wood. Ruel *et al.* (1989) localized cellulases in poplar wood decayed by *P. chrysosporium* using a post-embedding immunoelectron microscopic technique. Polyclonal antibodies were formed with a mixture of EG and CBH injected into rabbits. The purified immunoglobulins (IgG) were tagged and used for various Vocalizations of the cellulases in relation to the physiological state of the fungus. These immunoglobulins can be found in intracellular vesicles, concentrated along the plasma membrane, and penetrating a very short distance (0.2 to 0.5 μm) inside the wood cell wall. The presence of gold labelling in wood was only observed in areas of low electron density, which might have been previously degraded by a mechanism that was not necessarily of enzymatic origin. Non-enzymatic degradation of the wood cell wall could effectively be obtained by activated oxygen species generated from ferrous and manganese salts in the presence of hydrogen peroxide.

Transmission electron microscopy (TEM) of immunogold-labelled ultra-thin sections of *Coriolus versicolor* hyphae grown on malt agar and on solidified carboxy - methylcellulose (CMC) medium was used by Gallagher and Evans (1990) to localize β-glucosidase of the cellulase complex. β-Glucosidase was mainly detected in the hyphal sheath where dense labelling was observed. Label density was greater in hyphae grown on CMC-containing medium than on malt agar. Labelling, though virtually absent within the cytoplasm, also occurred in the hyphal cell wall and on the plasmalemma. This pattern of labelling revealed that more enzyme was secreted under conditions of growth on cellulose than on malt agar. This substantiates the reported increase in activity of β-glucosidase in the culture medium when cellulose is the carbon source. Sythesis of β-glucosidase may occur at the plasmalemma-cell wall interface since the enzyme is not found intracellularly.

2.2.2 Hemicellulose Degradation

Hemicellulose is structurally more complex than cellulose, which contains only 1.4-β-glycosidic linkages. Hemicelluloses area group of homo- and heteropolymers consisting largely of anhydro- β -(1 → 4)-D-xylopyranose, mannopyranose, glucopyranose and galactopyranose main-chains with a number of substituents. The enzymes that degrade hemicellulose are similarly complex. Compared with cellulases, little is known about the molecular genetics of hemicelluloses. There have been no reports

of xylanase clones from fungi (Cullen and Kersten, 1992). However, because hemicellulases have shown promise as bleaching agents in pulp and paper production, research has intensified in recent years.

Degradation of hemmicellusoes by white-rot fungi proceeds in a manner roughly analogous to that of cellulose, but the mechanism of attack has been studied in much less detail. The hemicellulose chains are attacked first by endo-enzymes (mannanases, xylanases) that produce progressively shorter chains, which are hydrolyzed to simple sugars by glycosidases (mannosidases, xylosidases, glucosidases). It is not known whether exe-enzymes are involved. The enzymes involved in the removal of side-chain substituents (arabinose, uronic acids, acetyls) have received little attention (Kirk and Cowling, 1984).

As with cellulases, simple sugars repress the production of most hemicellulose-degrading enzymes by white-rot fungi. Cellulose apparently is the only carbon source necessary to induce the formation of hemicellullose-degrading enzymes by these fungi. Eriksson and Goodell (1974) proposed that a single regulatory protein governs the induction of cellulase, mannanase and xylanase in *Bjerkandera adusta (= Polyporus adustus). They* further indicated that this single regulatory protein could be used to adjust simply and effectively the rate of wood decay by controlling enzyme induction.

Blanchette *et al.* (1989) used polyclonal and monoclinal antisera to xylanase in conjunction with immunocytochemistry to determine the ultrastructural localization of xylanase within cell walls of wood decayed by white-rot fungi. Labelling with gold-tagged antibodies to xylanase occurred primarily in the inner regions of the S_2 and S_1 layers and middle lamellae. Intercellular regions within the cell comers of the middle lamella were less dense but labelled positive with anti-xylanase gold. The remaining secondary wall exhibited little labelling with the xylanase-gold complex. Erosion troughs that reached the S_1 layer or middle lamella had less labelling in the remaining cell wall.

2.2.3 ***Lignin Degradation***

Unlike cellulose and hemicellulose, lignin is not principally linear but is a complex, heterogeneous, non-stereoregular aromatic polymer composed of phenylpropanoid units. White-rot fungi are the only known organisms that are capable of completely degrading lignin to carbon dioxide and water. Rapid progress has been made in recent years regarding the biochemistry and molecular genetics of lignin biodegradation, primarily through studies of the white-rot fungus *P. chrysosporium,* and this work has been extensively reviewed (Buswell and Odier, 1987; Alic and Gold, 1991; Kuan *et al.,* 1991; Cullen and Kersten, 1992, 1996). This section briefly summarizes some of these findings.

The ligninolytic system of P. *chrysosporium* is not induced by lignin but appears constitutively as cultures enter secondary metabolism: that is. when primary growth ceases because of depletion of some nutrient (Kirk *et al.,* 1978). Secondary metabolism is triggered by nitrogen, carbon or sulphur limitation but not phosphorus limitation (Jeffries *et al.,* 1981). Apparently, the regulation of secondary metabolism, including lignin degradation, is connected to glutamate metabolism (Kirk, 1981). Cultures can obviously cope well with nitrogen limitation, so that sustained lignin degradation occurs. In this connection, Kirk and Fenn (1982) speculated that lignin

degradation is a secondary metabolic event because of the very low nitrogen content of wood (Merrill and Cowling, 1966). Soon after a white-rot fungus invades wood, nitrogen becomes limiting and secondary metabolism, including lignin degradation, begins.

Although nitrogen repression of lignin degradation in white-rot fungi is common, it may not always be the rule (Leatham and Kirk, 1983). Thus, addition of nitrogen to certain white-rot fungi in different biotechnical applications utilizing lignin or lignin-related compounds may increase the efficiency of these fungi.

Enzymes thought to play a leading role in lignin depolymerization include two extracellular haem peroxidases, manganese peroxidase (MnP) and LiP, and an H_2O_2-generating system. However, white-rot fungi secrete unique combinations of peroxidases and oxidases (Perie and Gold, 1991). *Trametes* (= *Coriolus) versicolor* and *Phlebia radiata* each produce one or more laccases in addition to LiP and MnP (Fahraeus and Reinhammar, 1967; Niku-Paavola *et al.,* 1988). *Pleurotus sajor-caju* secretes an aryl alcohol oxidase (Bourbonnais and Paice, 1988), a laccase and several peroxidases (Fukuzumi, 1987). *Bjerkenderu adusta* secretes an aryl alcohol oxidase (Muheim *et al.,* 1990), and *Rigidoporus lignosus* and *Dichomitus squalens* secrete a laccase and a MnP (Galliano *et al.,* 1988). Thus, a variety of oxidative enzymes may be utilized by white-rot fungi for lignin degradation. An essential component of all white-rot ligninolytic systems, however, is a source of H_2O_2. Two enzymes have been proposed to fulfil this task: MnP itself, which can form H_2O_2 from O_2 when NADH, NADPH or glutathione are present; and glyoxal oxidase (GLOX), a novel enzyme produced during secondary metabolism in *P. chrysosporium* and activated by LiP plus veratryl alcohol (Kersten, 1990).

Considerable progress has been made in recent years concerning the molecular genetics of lignin biodegradation by white-rot fungi, primarily with *P. chrysosporium.* Standard methods have been established for auxotroph production, recombination analysis and rapid DNA and RNA purification (Cullen and Kersten, 1992). Since Tien and Tu (1987) first reported cloning of the cDNA-encoding lignin peroxidase H8. much has been learned about the number, structure and organization of the *P. chrysosporium* peroxidase genes. However, there is still considerable uncertainty about the exact number and structure of LiP genes. The number of LiP genes in *P. chrysosporium* has been variously reported - from live to 15 (Gaskell *et al.,* 1994). An RFLP-based genetic map localized LiP genes of *P. chrysosporium* isolate ME446 to two linkage groups (Raeder *et al.,* 1989). Following chromosome separation by clamped homogeneous electrical field (CHEF) electrophoresis, five LiP genes were assigned to a single chromosome (Gaskell *et al.,* 1991). In agreement with the RFLP map, another Lip clone (GLG4) was assigned to the same chromosome as a *cbhl* cluster (Covert *et al.,* 1992a,b; Gaskell *et al.,* 1994). The number of MnP genes and their chromosomal organization have not been reported, although preliminary unpublished) results show that at least one MnP gene resides on the same chromosome as do five LiP genes (Cullen and Kersten, 1992).

A cDNA-encoding MnP (Pribnow *et al.,* 1989) and its corresponding genomic clone (Godfrey *et al.,* 1990) were isolated from *P. chrysosporium* and sequenced (Pease *et al.,* 1989). The amino sequence homology to LiP H8 is 50 to 60 per cent. and the residues essential for peroxidase activity are conserved.

Research on the transcriptional regulation of peroxidases has been hampered by difficulties in distinguishing closely related genes (Cullen and Kersten, 1992). It is clear, however, that LiP genes are transcriptionally regulated and that expression of

MnP genes is Mn^{2+} dependent (Brown *et al.*, 1991); the specificity of the transcripts observed on Northern blots is questionable (Cullen and Kersten, 1992).

To quantify closely related LiP transcripts, Stewart *et al.* (1992) devised a quantitative polymerase chain reaction (PCR) approach that offers high levels of sensitivity and specificity. The technique is particularly suited to LiP gene families. The authors found that transcriptional regulation of LiP genes GLG4, GLG5 and V4 is dramatically affected by nutrient limitation. Their transcripts are almost mutually exclusive; GLG5 and V4 are present in nitrogen-limited cultures but undetectable or present in very low levels in carbon-limited cultures, whereas the GLG4 level is one-thousand-fold higher in carbon-limited cultures than in nitrogen-limited cultures. Analysis of concentrated culture filtrates with isoelectric focusing (IEF) gels showed a major pI 4.6 band in carbon-limited cultures. The absence of GLG5 from carbon-limited cultures is consistent with the isozyme pattern observed by Glumoff *et al.* (1990) in such cultures. The significance of these patterns of regulation in wood remains to be established, although it is clear that wood is nitrogen limited. In any case, the PCR system of Stewart *et al.* (1992) may be adapted to identify specific transcripts in such complex substrates.

Stewart *et al.* (1992) used Southern blot analysis of CHEF gels to map the GLG4 gene to a dimorphic chromosome separate from the other LiP gene. This chromosome was previously reported by Covert *et al.* (1992a,b) to contain a cellulase gene cluster. Subsequent work by the same researcher indicated that the cellulase genes in this cluster are expressed during carbon limitation (unpublished data). Thus, there may be a link between the organization and regulation of these genes involved in lignocellulose degradation.

Glyoxal oxidase (GLOX) was previously mentioned as a source of the extracellular H_2O_2 that is required by ligninolytic peroxidases. As a first step towards elucidating the molecular genetics of GLOX, Kersten and Cullen (1993) cloned and sequenced a cDNA-clone-encoding GLOX. They demonstrated that GLOX expression is transcriptionally regulated and co-ordinated with LiP and, MnP genes. Subsequently, Kersten *et al.* (1995) showed that GLOX is encoded by a single gene with two alleles, that the gene is located on a dimorphic chromosome unlinked to known LiP, MnP and CBH genes, and that GLOX is efficiently expressed in *Aspergillus nidulans.* The results of Kurek and Kersten (1995) suggest that ligninolysis by peroxidase could be regulated by GLOX and influenced by the presence of veratryl alcohol, lignin and lignin degradation products. They further note that such coordinated metabolism would influence the kinetics of free radical generation by LiP and, therefore, the overall efficiency of lignin. Ultimately, the catalytic mechanism and interactions of GLOX with peroxidases will be elucidated through crystal structures, site-specific mutagenesis of active sites, and gene disruptions (Kersten and Cullen. 1993). The cloning and sequencing of GLOX is an important step towards this objective.

The molecular genetics of other lignin-degrading fungi has received little attention (Cullen and Kersten, 1992). Saloheimo *et al.* (1989) cloned and sequenced an LiP gene from *Phlebia radiata.* The deduced amino acid sequence of this clone is 62 per cent, identical to that of the *P. chrysosporium* isozyme H8 (ML1). From Southern blot hybridization to the H8 gene, multiple LiPs apparently are present in *Bjerkandera adjusta. Coriolus verslcoior* and *Fomes* lianosus (Huoponen *et al.*, 1990).

Electron microscopic analyses of *P. chrysosporium* inoculated on wood have suggested various locations for Lip or MnP: in the hyphal wall or slime layer (Daniel

et al., 1989, 1990; Ruel and Joseleau, 1991); in the periplasmic space (Forney. *et al.*, 1982; Srebotnik *et al.*, 1988; Daniel *et al.*, 1989, 1990); or in association with the plasma membrane and with membranes or luminae of cytoplasmic vesicles (Garcia *et al.*, 1987; Srebotnik *et al.*, 1988; Daniel *et al.*, 1989, 1990). However, these studies which used wood as a substrate, do not permit a satisfactory spatial and temporal overview of peroxidase secretion with respect to the whole mycelium (Moukha *et al.*, 1993). Also, primary and secondary (idiophasic) growth cannot be distinguished as in artificial media.

Moukha *et al.* (1993) attempted to localize the secretion of LiP and MnP with hyphal growth in *P. chrysosporium* by using cultures sandwiched between perforated polycarbonate membranes. Comparison of sites where newly synthesized protiens and immuno-detected peroxidase were released into the medium suggests that enzyme diffusion from the walls is a limiting step in the release of peroxides. Microautoradiography of colonies revealed apical growth of thin hyphae and branches in the central secreting area. These secondary hyphae possessed peroxidase activity and reacted with LiP antibodies. The results suggest that LiP and MnP are initally secreted at the apex of secondary growing hyphae and later slowly released into the surrounding medium.

Since the discovery of lignin-degrading enzymes, there has been considerable progress in characterizing their properties and understanding their catalytic mechanisms. However, most of the detailed mechanistic studies have been conducted using simple model compounds. Questions remain as to how lignnin-degrading enzymes attack native lignin. In fact, nither the purified lignin-degrading enzymes nor the crude filtrates duplicate the extensive extracellular degradation of lignin observed in intact cultures of ligninolytic fungi. Biotechnological techniques should help identify the apparently missing components of the isolated lignin-degrading machinery.

2.3 Mechanism of Brown-Rot Decay

2.3.1 Cellulose *Degradation*

Brown-rot fungi are unique among cellulose destroyers because they are the only known microbes that can degrade wood cellulose without first removing the lignin. These fungi leave a brown residue (hence the name) of partially demethylated lignin. Furthermore, brown-rot fungi degrade cellulose in an unusual manner that differs from that of other cellulolytic organisms. Shortly after colonizing wood, the fungi cause a rapid and extensive depolymerization of cellulose to the ‘limit’ (length of cellulose crystallite) or crystalline degree of (polymerization (DP) at low weight loss. Acid hydrolysis has a similar effect on cellulose, as do various strong oxidants. The cellulose that remains has an average DP of 150-200 and is more crystalline than non-depolymerized material because the cleavages occur in the amorphous non-crystalline regions. The biochemical agent - or the system that produces it - that causes this initial depolymerization is Clearly a small diffusible agent because enzymes are too large to penetrate wood to reach cellulose (Cowling, 1961; Cowling and Brown. 1969). Furthermore, brown- rot fungi appear to degrade Cellulose by a mechanism different from that of the synergistically acting systems involvingexo-glucanases present in white-rot fungi and *Trichoderma* (Highley, 1973). Uemura *et al.* (1993) found that the cellulase system from brown-rot fungi gave a negative

response towards antibodies to *Trichoderma* CBH, suggesting the absence of homologous sequences and structures with the *Trichoderma* CBH.

Attempts to identify the cellulose depolymerizing agent produced by brown-rot fungi have frustrated researchers for years. More than 25 years ago, Cowling and Brown (1969) recognized that even the smallest cellulases are too large to penetrate the pores of wood. Also, cellulases do not mimic the action of brown-rot fungi in generating cellulose crystallite (Chang *et al.*, 1981; Phillip *et al.*, 1981). Cowling and Brown thus proposed that a non-enzymatic oxidative agent might be involved in depolymerization of cellulose by brown-rot fungi. They noted that Halliwell (1965) had described the degradation of cotton cellulose by Fenton's reagent (H_2O_2/Fe^{2+}), which generates a hydroxyl radical or a similar oxidant (Halliwell and Gutteridge, 1988). Based on these observations, Halliwell was the first to propose the possible existence of a non-enzymatic celluloytic system involving peroxide and iron. Subsequently, Koenigs (1972a,b, 1974a,b, 1975) demonstrated that cellulose in wood can be depolymerized by Fenton's reagent, that brown-rot fungi produce extracellular hydrogen peroxide, and that wood contains enough iron to make Halliwell's hypothesis reasonable.

Enoki *et al.* (1989) reported the ability of brown-rot fungi to oxidize 2-keto-4-thiomethylbutyric acid (KTBA) to ethylene; KTBA is converted to ethylene by one-electron oxidants such as the hydroxyl radical. Ethylene production was correlated with weight loss but not cellulose depolymerization. More recently, Enoki *et al.* (1990) reported the isolation of an extracellular protein from cultures of *G. trabeum* which requires H_2O_2 and is capable of KTBA oxidation. These authors partially purified the protein and reported it to be an iron-containing glycoprotein of molecular weight 1600-2000. Based on their work, Enoki and co-workers suggested the existence of 'a unique wood-component degrading system that participates directly or indirectly in the fragmentation of cellulose as well as of lignin in wood and oxidizes KTBA to give ethylene'. However, it is yet to be established whether the H_2O_2-dependent KTBA-oxidizing ability of this protein is related to cellulose depolymerization. Similar glycoproteins were isolated from both white-rot and soft-rot fungi (Enoki *et al.*, 1991), raising questions about their role in wood decay.

Hydrogen peroxide is an important component of Fenton chemistry. Probably the strongest argument against the occurrence of the Fenton system in brown-rot fungi is that more than two decades of research on H_2O_2 production by brown-rot fungi have not definitely established that these fungi synthesize extrucellular H_2O_2 (Highley and Flournoy, 1994). Presumably, the conflicting reports of whether wood decay fungi produce extracellular H_2O_2 can be explained by the transient uppearance of H_2O_2 in culture and the lack of a selective assay for the reagent.

The presumptive role of H_2O_2 is the generation of the hydroxyl radical in a reaction with either a metal or a metal chelate. Hydroxyl radical has been detected in liquid media, agar media, or wood by various methods, including *p*-nitrosodium methylaniline (Highley, 1982), desilvering (Veness and Evans, 1989), electron spin resonance (Illman *et al.*, 1989b) and chemiluminescence (Backa *et al.*, 1992). The hydroxyl radical would need to be formed at its site of action because it is very reactive with a very short fife time and, therefore, would not diffuse into wood. Lu *et* al., (1994) reported that *G. trabeum* produces low moleular weight phenolate chelators, which the authors propose diffuse into wood and, in the presence of iron and H_2O_2, produce radical species within the wood ceil wall. Earlier TEM immunolabelling studies using an antibody to a low molecular weight fraction from *G.*

trabeum (Jellison *et al.,* 1991) showed that the chelator was present within the decayed and undecayed regions of the wood cell wall. Hyde and Wood (1995) recently proposed a model for attack at a distance from the hyphae of the brown-rot fungus *Coniophora puteana,* based on the formation of H_2O_2 by autoxidation. This fungus produces cellobiose dehydrogenase, which reduces Fe^{3+} to Fe^{2+}. Diffusion of Fe^{2+} from the hyphae in a low pH environment promotes conversion to Fe^{2+}-oxalate and subsequent autoxidation with H_2O_2 as the product. The critical Fe^{2+}/H_2O_2 combination is therefore formed at a distance from the hyphae. Flournoy (1994) noted that because most reports concerning free radicals are phenomenological in nature, it is difficult to assign any meaning to them and little can be concluded regarding their significance in brown-rot decay.

Oxalic acid, an important physiological metabolize of brown-rot fungi, may be produced at concentrations inhibitory to the Fenton reaction. Oxalic acid was proposed by Schmidt *et al.* (1981) to play a role in reduction of Fe^{3+} to Fe^{2+}, which increased cellulose decomposition by the Fenton reaction. However, Schmidt *et al.* (1981) and Tanaka *et al.* (1994) reported that at higher concentrations of oxalic acid, cellulose degradation by the Fenton system is inhibited. *Postia placenta* and *Serpula incrassata* were shown to accumulate oxalic acid in wood (Green *et al.,* 1992b).

Brown-rotted cellulose depolymerization products have been chemically characterized and compared with other depolymerized cellulose samples (Kirk *et al.,* 1989, 1991). The following depolymerized cellulose samples were prepared from pure cotton cellulose: acid-hydrolyzed (HCl) to the limit DP; $H_2O_2/FeSO_4$-oxidized (Fenton-oxidized); HIO_4/Br_2-oxidized; and brown-rotted *(P. placenta).* These samples were characterized as to molecilar size distribution, yield of glucose on complete acid hydrolysis and carboxyl, uronic acid and carbonyl contents, as well as sugar acids released on acid hydrolysis. Consistent with earlier results, the Fenton system, but not the other oxidation system, mimicked the brown-rot system in nearly all measured characteristics. The acid-hydrolyzed sample also possessed similar characteristics. Glyceric, erythronic, arabonic and gluconic acids were identified by GC/MS in the hydrolysates of the brown-rotted and Fenton-oxidized samples. These results are consistent with the depolymerizing agent being related to the Fenton system, but Flournoy (1994) noted that the authors did not establish that the fungi employed such a system. Shimada (1993) stated that:

> the identification of these aliphatic acids does not always provide proof of' the involvement of Fenton's system in physiological wood decay processes, since enzymatic evidence for the production of these acids has not yet been offered. Furthermore, Fenton's system yields such destructive OH radical to living matter as to degrade their own cell polymers.

The Fenton system is powerful enough to decompose cellulose to carbon dioxide under appropriate Conditions us long as hydrogen peroxide is continuously supplied (Schmidt *et al.,* 1981).

Flournoy (1994) found several pitfalls in the studies of Highley (1977) and Kirk *et al.* (1991), The authors did not establish a correlation between the oxidation of cellulose and depolymerization. It is unknown, for instance. whether oxidation of the cellulose precedes depolymerization, whether oxidation is a result of post-depolymerization modification, or whether oxidation and depolymerization are coupled. The cellulose used in these studies was highly degraded. Samples from early decay were not examined, which would be the samples of most interest in

understanding the depolymerization mechanism. In summary, although the literature indicates that chemical alterations in brown-rotted lignin are oxidative in nature, oxidative changes in cellulose as a result of brown-rot decay have not been unequivocally demonstrated.

Assays for metals in brown-rot wood decay have been performed in an attempt to clarify their role in cellulose breakdown by metal-catalyzed oxidation. Electron spin resonance (ESR) spectrometry was used to detect and follow changes in the oxidative states of paramagnetic metals during brown-rot decay (Illman *et al.*, 1989a). Alterations in low-spin iron could not be detected at room temperature, and changes in high-spin iron were not tested at low temperature (Illman *et al.*, 1989a). A comprehensive study of iron oxidation states using ESR has yet to be made.

Changes in manganese were observed with ESR after inoculation of susceptible species of wood with the brown-rot fungus *P. placenta* (Illman *et al.*, 1989a). These alterations were manifested as increases in the size of the sextet spectra specific for manganese (Mn^{2+}). The ESR spectra for Mn^{2+} were taken over a 4-week period from fungal-inoculated white fir, Douglas-fir, sweetgum and redwood. The increases in Mn^{2+} signals correlated with wood susceptibility to brown-rot decay. Little or no increase was found in wood species resistant to brown-rot decay (Illman *et al.*, 1989a). The chemical basis of the Mn^{2+} change was not determined.

In treating white fir slivers with oxalic acid, Illman and Englebert (unpublished data) found that the ESR signal for Mn^{2+} increased with increasing oxalic acid concentration, although the Mn^{2+} signal did not increase as much as that in *P. placenta* decayed wood. The enhanced Mn^{2+} signal may be a result of lowered pH with acid solubilization of the metal. Oxalic acid from several species of wood-decay fungi can mobilize calcium from glass and concrete. Alternatively, the increase in the Mn^{2+} signal may be due to chelation of the metal. Oxalate is a known chelator of several elements, including manganese, calcium and potassium. The chemical basis of possible oxalic acid effects on wood needs further investigation.

2.3.2 Hemicellulose Degradation

Brown-rot fungi secrete a number of hemicellulose-degrading enzymes, and the mechanism of hemicellulose breakdown appears similar to that for white-rot fungi (King, 1966: Keilich *et al.*, 1970; Highley, 1976). However, little definitive work has been done on the hemicellulases of brown-rot fungi with respect to specificity, molecular size and other properties such as active sites. The few hemicellulose-degrading enzymes that have been isolated and 'purified' from brown-rot fungi are not substrate specific. A purified β-glucosidase from the brown-rot fungus *G. trabeum* also hydrolyzed β-xyloside (Herr *et al.*, 1978); the purified xylanase from the brown-rot fungus *Tyromyces palustris* also had endoglucanase but no glycosidase activities (Ishihara *et al.*, 1978). The purified endoglucanase from the brown-rot fungus *Polyporus schwenitzii* was accompanied by mannanase and xylanase activities (Keilich *et al.*, 1970). A multiple glycan and glycoside hydrolase were isolated from the brown-rot fungus *Poria placenta* (Highley *et al.*, 1981). However, glycanase activities were later separated from glycosidase activities (Green *et al.*, 1989).

Brown- and white-rot fungi differ in their regulation of hemicellulase synthesis. Many brown-rot fungi exhibit good hemicellulase as well as cellulase activities during growth on simple sugars. whereas white-rot fungi do not (Eriksson and

Goodell, 1974; Highley, 1976). Regulation in wood-decay fungi, however, has not been examined in great detail and warrants additional study.

Green *et al.* (1991b, 1992a) utilized gold-labelled monoclinal antibodies to xylanase to localize xylanase of *Postia placenta* grown on agar medium. Xylanase was associated with the hyphal surface, within the hyphal sheath matrix, and on fibrillar elements of the sheath structure.

Oxalic acid may also be involved in the solubilization and hydrolysis of hemicellulose, thus making the cellulose fibres more accessible to cellulases (Beth-Anderson, 1987). Green *et al.* (199la, 1992b) expanded on this hypothesis, suggesting that the acidic conditions in wood, caused by oxalic acid production, are responsible for early acid hydrolysis and depolymenzation of hemicellulose and amorphous cellulose, thereby increasing wood porosity. Enzymes and other degrading agents in the hyphal sheath would then have access to the remaining cellulose and cause its final removal.

2.3.3 Lignin Degradation

Chemical analysis of brown-rotted wood indicates that brown-rot fungi do not utilize lignin to an appreciable extent (Cowling 1961; Kirk and Highley, 1973). The main effect of the fungi on lignin is demethylation of aryl methoxyl groups (Kirk and Adler, 1970), although oxidative changes occur, including some cleavage of aromatic rings (Kirk, 1975). Cowling (1961) also showed that lignin decayed by *P. placenta* had appreciably greater volubility in water and 1 per cent NaOH than did lignin in sound wood. Haider and Trojanowski (1980) demonstrated that brown-rot fungi can be induced to metabolize lignin to some extent. They found that isolated lignins were degraded to carbon dioxide to a limited extent by brown-rot fungi in liquid culture. Microscopic studies (Highley *et al.*, 1985) suggest that brown-rot fungi cause degradation of the cell wall, including the lignin-rich middle lamella and cell corners. Thus, brown-rot fungi may have a greater ligninolytic capacity than previously thought. The ligninolytic agent produced by brown-rot fungi is possibly the same or similar to the degrading agent that initiates the rapid depolymerization of cellulose.

Harvey *et al.* (1986) discussed the possibility that brown-rot fungi degrade lignin by a single-electron oxidation similar to that of white-rot fungi. Both types of fungi demethylate methoxyl groups in phenolic- and non-phenolic-containing lignin structures (Kirk and Adler, 1970: Kirk and Chang, 1974) as well as hydroxylate aromatic rings (Kirk and Adler, 1970). Both types of decay fungi promote C*x* side-chain oxidations and aromatic ring cleavage reactions (Kirk and Adler, 1970: Kirk, 1975: Kirk and Chang, 1975). Lignin peroxidase production has been reported by the browt-rot fungus *Polyporous ostruformis,* but at a low level compared with that by white-rot fungi (Dey *et al.*, 1991) The oxidative processes caused by brown-rot fungi, in contrast to white-rot fungi, result in the formation of polymeric lignin fractions (Kirk, 1975). Harvey *et al.* 1986) proposed that brown-rot fungi repolymerize lignin because highly unstable radical cation intermediates formed from phenolic-containing aromatic compounds are oxidized by a single electron. These phenoxy radicals undergo further reactions, including oxidative carbon-to-carbon and carbon-to-oxygen coupling reactions, to produce higher molecular weight products that the brown-rot fungi evidently cannot metabolize.

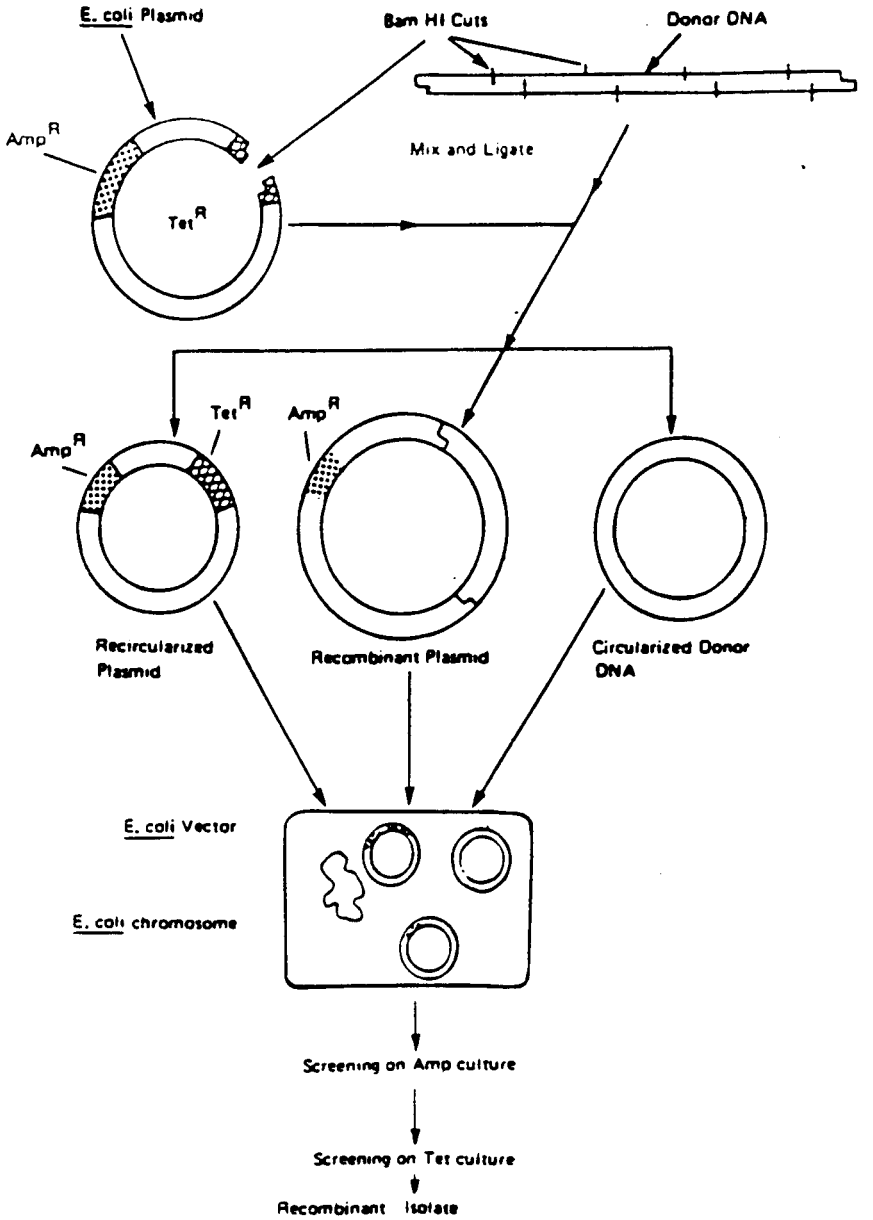

Figure 2.3 Generalized foreign gene insertion. Individual steps for making plasmid receptive to gene insertion and antibiotic screening for selection of properly transformed cells (reprinted from Kammermeyer and Clark (1989), by courtesy of Marcel Dekker, Inc)

2.4 Summary

Because lignocelluloytic enzymes are of commercial value to the pulp and paper industry (Zadrazil and Reninge, 1988) and the agricultural community (Vander Meer *et al.*. 1987), an inexpensive and readily available supply of these enzymes is highly desirable. Mechanistic studies also require large quantities of these enzymes-

Limiting amounts of lignocelluloytic enzymes are produced by wood decay fungi, which impede their commercial use and also prevent true assessment studies of various potential applications. Biotechnology techniques such as cloning, mutation and overproduction of lignocellulolytic enzymes have helped to overcome these limitations.

An example of this approach is that of Williams *et al.* (1991), who applied biotechnological techniques to enhance production of polyphenol oxidase by the white-rot fungus *Trametes versicolor.* Polyphenol oxidase converts *o*-diphenois to *o*-diquinones and oligomerizes syringic acid. Because *T. versicolor* can be 'batch cultured' (Fahraeus and Reinhammar, 1967), overproduction and enhanced secretion of these enzymes by molecular genetics, for example, recombinant DNA technology, are feasible. Briefly, the technology involves isolation of fungal genomic DNA, restriction endonuclease treatment of the genomic DNA, ligation of the restriction fragments into a plasmid, and transformation of *E. coli* with the recombinant plasmid (Williams *et al.,* 1991) (Figure 2.3). This technology should be applicable to the overproduction of more commercially important cellulases and ligninases (Desoretz, 1993). In this connection, Kammermeyer and Clark (1989) published an extensive monograph regarding foreign gene insertion and cell transformation. Recently, MnP was successfully expressed in *Aspergillus oryzae* (Stewart *et al.,* 1996). The recombinant MnP is secreted into culture medium in active form, which will provide large quantities of pure enzyme suitable for mechanistic studies.

References

ALIC, M. and GOLD, M. (1991) Genetics and molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium.* In: Bennett J. and Lasue, L., eds. *More Gene Manipulations in Fungi.* New York: Academic Press, pp. 319–341.

ALIC, M., KORNEGAY, J. R., PRIBNOW, D. and GOLD, M. H. (1989) Transformation by complementation of an adenine auxotroph of the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **55**, 406–411.

AYERS, A. R., AYERS, S. B. and ERIKSSON, K. E. (1978) Cellobiose oxidase, purification and partial characterization of a hemoprotein from *Sporotrichum pulverulentum. Eur. J. Biochem.* **90**, 171–181.

AZEVEDO, M., FELIPE, M., ASTOLFI-FILHO, S. and RADFORD, A. (1990) Cloning, sequence and homologies of the *cbh-1* (exoglucanase) gene of *Humicola grisea* var. *thermoidea. J. Gen. Microbiol.* **136**, 2569–2576.

BACKA, S., GRIRER, J., REITBERGER, T. and NILSSON, T. (1992) Hydroxyl radical activity in brown-rot fungi by a new chemiluminescence method. *Holzforschung* **46**, 61–67.

BECH-ANDERSON, J. (1987) *Production, Function and Neutralization of Oxalic Acid Produced by the Dry Rot Fungus and Other Brown-Rot Fungi,* Document IRG/WP/1330, International Research Group on Wood Preservation.

BLANCHETTE, R. A., ABAD, A. R., FARRELL, R. L. and LEATHERS, T. D. (1989) Detection of lignin peroxidase and xylanases by immunocytochemical labelling in wood decayed by basidiomycetes. *Appl. Environ. Microbiol.* **55**, 1457–1465.

BOURBONNAIS, R. and PAICE, M. G. (1988) Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju. Biochem. J.* **255**, 445–450.

BROWN, J. A., ALIC, M. and GOLD, M. H. (1991) Manganese peroxidase gene transcription in *Phanerochaete chrysosporium*: activation by manganese. *J. Bacteriol.* **173**, 4101–4106.

BUSWELL, J. A. and ODIER, E. (1987) Lignin biodegradation. *CRC Crit. Rev. Biotechnol.* 6, 1–60.

CHANG, M. M., CHOU, T. C. and TSAO, G. T. (1981) Structure pretreatment and hydrolysis of cellulose. In: Ziechter, A., ed., *Advances in Biochemical Engineering*, Berlin: Springer-Verlag, pp. 15–42.

COVERT, S., BOLDUC, J. and CULLEN, D. (1992a) Genomic organization of a cellulase gene family in *Phanerochaete chrysosporium. Curr. Genet.* 22, 407–413.

COVERT, S. F., WYMELENGERG, A. V. and CULLEN, D. (1992b) Structure, organization and transcription of a cellobiohydrolase gene cluster from *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 58, 2168–2175.

COWLING, E. B. (1961) *Comparative Biochemistry of the Decay of Sweetgum Sapwood by White-Rot and Brown-Rot Fungi*, Tech. Bull. No. 1258, Washington, DC.: US Department of Agriculture.

COWLING, E. B. and BROWN W. (1969) Structural features of cellulosic materials in relation to enzymatic hydrolysis. In: Hajny, G. J. and Reese, E. T., eds, *Cellulases and their Applications, Advanced Chemical Series* 95, 152–187.

CULLEN, D. and KERSTEN, P. (1992) Fungal enzymes in lignocellulose degradation. In: Kinghorn, R. and Turner, G., eds. *Applied Molecular Genetics of Filamentous Fungi*, London: Chapman and Hall, pp. 100–131.

CULLEN, D. and KERSTEN, P. J. (1996) Enzymology and molecular biology of lignin degradation. In: Bramble, R. and Marzluf, G., eds, *The Mycota III*, Berlin: Springer-Verlag, pp. 297–314.

DANIEL, G., NILSSON T. and PETTERSON, B. (1989) Intra- and extracellular localization of lignin peroxidase during the degradation of solid wood and wood fragments by *Phanerochaete chrysosporium* by using transmission electron microscopy and immuno-gold labelling, *Appl. Environ. Microbiol.* 55, 871–881.

DANIEL, G., PETTERSON, B., NILSSON, T. and VOLC, J. (1990) Use of immunogold cytochemistry to detect Mn(II)-dependent and lignin peroxidases in wood degraded by the white rot fungi *Phanerochaete chrysosporium* and *Lentinula edodes. Can. J. Bot.* 68, 920–933.

DESHPANDE, V., ERIKSSON, K. E. and PETTERSSON, B. (1978) Production, purification and partial characterization of 1,4-β-glucosidase enzymes from *Sporotrichum pulverulentum. Eur. J. Biochem.* 90, 191–198.

DESORETZ, C. G. (1993) Overproduction of lignin peroxidase by *Phanerochaete chrysosporium* (BKM-F-1767). *Appl. Environ. Microbiol.* 59, 1919–1926.

DEY, S., MAITI, T. K. and BHATTACHACHARYYA, B. C. (1991) Lignin peroxidase production by a brown-rot fungus *Polyporous ostriformes. J. Ferment. Bioeng.* 72, 402–404.

ENOKI, A., TANAKA, H. and FUSE, G. (1989) Relationship between degradation of wood and production of H_2O_2-producing one-electron oxidases by brown-rot fungi. *Wood Sci. Technol.* 23, 1–12.

ENOKI, A., YOSHIOKA, S., TANAKA, H. and FUSE, G. (1990) *Extracellular H_2O_2-Producing and One-Electron Oxidation System of Brown-Rot Fungi*. Document IRG/WP/1445, International Research Group on Wood Preservation.

ENOKI, A., FUSE, G. and TANAKA, H. (1991) *Extracellular H_2O_2-Producing and H_2O_2-Reducing Compounds of Wood Decay Fungi*, Document IRG/WP/1516, International Research Group on Wood Preservation.

ERIKSSON, K. E. (1978) Enzyme mechanisms involved in cellulose hydrolysis by the rot fungus *Sporotrichum pulverulentum. Biotechnol. Bioeng.* 20, 317–332.

ERIKSSON, K. E. (1981) Microbial degradation of cellulose and lignin. *Proceedings of International Symposium on Wood and Pulping Chemistry*, Stockholm, June, Vol. 3, pp. 60–65.

ERIKSSON, K. E. and GOODELL, B. (1974) Pleiotropic mutants of the wood-rotting fungus *Polyporus adustus* lacking cellulase, mannanase and xylanase. *Can. J. Microbiol.* 20, 371–378.

ERIKSSON, K. E. and HAMP, S. G. (1978) Regulation of endo-1,4-β-glucanase production in *Sporotrichum pulverulentum*. *Eur. J. Biochem.* **90**, 183–190.

ERIKSSON, K. E. and PETTERSSON, B. (1975a) Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. 1. Separation, purification and physico-chemical characterization of five endo-1,4-β-glucanases. *Eur. J. Biochem.* **51**, 193–206.

ERIKSSON, K. E. and PETTERSSON, B. (1975b) Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum* (*Chrysosporium lignorum*) for the breakdown of cellulose. 3. Purification and physico-chemical characterization of an exo-1,4-β-glucanase. *Eur. J. Biochem.* **51**, 213–218.

FAHRAEUS, G. and REINHAMMAR, B. (1967) Large-scale production and purification of laccase from cultures of *Polyporus versicolor* and some properties of laccase A. *Acta Chem. Scand.* **21**, 2367–2378.

FLOURNOY, D. S. (1994) Chemical changes in wood components and cotton cellulose as a result of brown-rot: is Fenton chemistry involved? In: *Biodeterioration Research* Vol. 4, New York: Plenum Press, pp. 257–294.

FORNEY, L. J., REDDY, C. A. and PANKRATZ, H. S. (1982) Ultrastructural localization of hydrogen peroxide production in ligninolytic *Phanerochaete chrysosporium* cells. *Appl. Environ. Microbiol.* **44**, 732–736.

FUKUZUMI, T. (1987) Ligninolytic enzymes of *Pleuortis sajorcoya*. In: Odier, E., ed., *Lignin: Enzymic and Microbial Degradation*, Paris: Institute National de la Recherche Agronomique, pp. 137–142.

GALLAGHER, I. M. and EVANS, C. S. (1990) Immunogold-cytochemical labelling of β-glucosidase in the white-rot fungus *Coriolus versicolor*. *Appl. Microb. Biotechnol.* **32**, 588–593.

GALLIANO H., GAS, G. and BOUDET, A. (1988) Biodegradation of *Hevea brasiliensis* lignocellulose by *Rigidoporus lignosus*: influence of culture conditions and involvement of oxidizing enzymes. *Plant Physiol. Biochem.* **26**, 619–627.

GARCIA, S., LATGE, J. P., PREVOST, M. C. and LEISOLA, M. (1987) Wood degradation by white rot fungi: cytochemical studies using lignin peroxidase–immunoglobulin–gold complexes. *Appl. Environ. Microbiol.* **53**, 2384–2387.

GASKELL, J., DIEPERINK, E. and CULLEN, D. (1991) Genomic organization of lignin peroxidase genes of *Phanerochaete chrysosporium*. *Nucl. Acids Res.* **19**, 599–603.

GASKELL, J., STEWART, P., KERSTEN, P. J., COVERT, S. F., REISER, J. and CULLEN, D. (1994) Establishment of genetic linkage by allele-specific polymerase chain reaction application to the lignin peroxidase gene family of *Phanerochaete chrysosporium*. *Biotechnology* **12**, 1372–1375.

GLUMOFF, T., HARVEY, P., MOLINARI, S., GOBLE, M., FRANK, G., PALMER, J., SMIT, J. and LEISOLA, M. (1990) Lignin peroxidase from *Phanerochaete chrysosporium*, molecular and kinetic characterization of isozymes. *Eur. J. Biochem.* **187**, 515–520.

GODFREY, B. J., MAYFIELD, M. B., BROWN, J. A. and GOLD, M. H. (1990) Characterization of a gene encoding a manganese peroxidase from *Phanerochaete chrysosporium*. *Gene* **93**, 119–124.

GREEN, F., CLAUSEN, C. A., MICALES, J. A., HIGHLEY, T. L. and WOLTER, K. E. (1989) Carbohydrate-degrading complex of the brown-rot fungus *Poria placenta*: purification of β-1,4-xylanase. *Holzforschung* **43**, 25–31.

GREEN, F. III, LARSEN, M. J., WINANDY, J. E. and HIGHLEY, T. L. (1991a) Role of oxalic acid in incipient brown-rot decay. *Mat. u. Org.* **26** (3), 191–213.

GREEN, F. III, CLAUSEN, C. A., LARSEN, M. J. and HIGHLEY, T. L. (1991b) Ultrastructural characterization of the hyphal sheath of *Postia placenta* by selective removal and immunogold labelling. In: *Proceedings, 8th International Biodeterioration and Biodegradation symposium*, pp. 530–532.

GREEN, F. III, CLAUSEN, C., LARSEN, M. and HIGHLEY, T. L. (1992a) Immunoscanning electron microscopic localization of extracellular polysaccharidases within the fibrillar sheath of the brown-rot fungus *Postia placenta*. *Can. J. Bot.* **38**, 898–904.

GREEN, F., LARSEN, M. J., HACKNEY, J. M., CLAUSEN, C. A. and HIGHLEY, T. L. (1992b) Acid-mediated depolymerization of cellulose during incipient brown-rot decay by *Postia placenta*. In: Kuwahar, M. and Shimada, M., eds, *Biotechnology in Pulp and Paper Industry*, 5th International Conference, Tokyo, Japan: Uni Publishers.

HAIDER, K. and TROJANOWSKI, J. (1980) A comparison of the degradation of ^{14}C-labelled DHP and corn stalk lignins by micro- and macrofungi and by bacteria. In: Kirk, T. K., Higuchi, T. and Chang, H. M., eds, *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications*, Vol. I, Boca Raton, FL: CRC Press, p. 111.

HALLIWELL, G. (1965) Catalytic decomposition of cellulose under biological conditions. *Biochem. J.* **95**, 35–40.

HALLIWELL, B. and GUTTERIDGE, J. M. C. (1988) Iron as a biological pro-oxidant. *ISI Atlas of Science, Biochemistry* **1**, 48–52.

HARVEY, P. J., SCHOEMAKER, H. E. and PALMER, J. M. (1986) Lignin degradation by wood degrading fungi, Document IRG/WP/1310, International Research Group on Wood Preservation.

HERR, D., BAUMER, F. and DELLWEG, H. (1978) Purification and properties of an extracellular endo-1,4-β-glucanase from *Lenzites trabea*. *Arch. Microbiol.* **117**, 287–292.

HIGHLEY, T. L. (1973) Influence of carbon source on cellulase activity of white-rot and brown-rot fungi. *Wood Fiber* **5**, 50–58.

HIGHLEY, T. L. (1976) Hemicellulases of white- and brown-rot fungi in relation to host preferences. *Mat. u. Org.* **11**, 33–46.

HIGHLEY, T. L. (1977) Requirements for cellulose degradation by a brown-rot fungus. *Mat. u. Org.* **12**, 25–36.

HIGHLEY, T. L. (1982) Is extracellular hydrogen peroxide involved in cellulose degradation by brown-rot fungi? *Mat. u. Org.* **17**, 205–214.

HIGHLEY, T. L. and FLOURNOY, D. S. (1994) Decomposition of cellulose by brown-rot fungi. In: Garg, K. L., Gerg, N. and Mukerji, K. G., eds, *Recent Advances in Biodeterioration and Biodegradation*, Vol. II, Naya Prokash, 206, Bidhan Serani, Calcutta-6, India, pp. 191–221.

HIGHLEY, T. L., WOLTER, K. E. and EVANS, F. (1981) Polysaccharide-degrading complex produced in wood and in liquid media by the brown-rot fungus, *Poria placenta*. *Wood Fiber* **13**, 265–274.

HIGHLEY, T. L., MURMANIS, L. L. and PALMER, J. G. (1985) Micromorphology of degradation in western hemlock and sweetgum by the brown-rot fungus *Poria placenta*. *Holzforschung* **39**, 73–78.

HUOPONEN, K., OLLIKKA, P., KALIN, M., WALTHER, I., MANTSALA, P. and REISER, J. (1990) Characterization of lignin peroxidase-encoding genes from lignin-degrading basidiomycetes. *Gene* **89**, 145–150.

HYDE, S. M. and WOOD, P. M. (1995) A model for attack at a distance from the hyphae based on studies with the brown-rot *Coniophora puteana*. Document IRG/95/10104, International Research Group on Wood Preservation.

ILLMAN, B. L., MEINHOLTZ, D. C. and HIGHLEY, T. L. (1989a) Manganese as a probe of fungal degradation of wood. In: *Biodeterioration Research II*, New York: Plenum Press, pp. 485–496.

ILLMAN, B. L., MEINHOLTZ, D. C. and HIGHLEY, T. L. (1989b) Oxygen free radical detection in wood colonized by the brown-rot fungus, *Postia placenta*. In: *Biodeterioration Research II*, New York: Plenum Press, pp. 497–509.

ISHIHARA, M., SHIMIZU, K. and ISHIHARA, T. (1978) Hemicellulases of brown rotting fungus, *Tyromyces palustris*. III. Partial purification and mode of action of an extracellular xylanase. *Mokuzai Gakkaishi* **24**, 108–115.

JEFFRIES, T. W., CHOI, S. and KIRK, T. K. (1981) Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **42**, 290–296.

JELLISON, J., CHANDHOKE, V., GOODELL, B. and FEKETE, F. (1991) The isolation and immunology of iron-binding compounds produced by *Gloeophyllum trabeum*. *Appl. Microbiol. Biotechnol.* 35, 805–809.

KAMMERMEYER, K. and CLARK, V. L. (1989) *Genetic Engineering Fundamentals, An Introduction to Principles and Applications*, New York: Marcel Dekker.

KEILICH, G., BAILEY, P. and LIESE, M. (1970) Enzymatic degradation of cellulose, cellulose derivatives and hemicelluloses in relation to the fungal decay of wood. *Wood Sci. Technol.* **4**, 273–283.

KERSTEN, P. (1990) Glyoxal oxidase of *Phanerochaete chrysosporium*: its characterization and activation by lignin peroxidase. *Proc. Natl. Acad. Sci. USA* **87**, 2936–2940.

KERSTEN, P. J. and CULLEN, D. (1993) Cloning and characterization of cDNA encoding glyoxal oxidase, a H_2O_2-producing enzyme from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. *Proc. Natl. Acad. Sci. USA* **90**, 7411–7413.

KERSTEN, P. J., WITEK, C., WYMELENBERG, A. V. and CULLEN, D. (1995) *Phanerochaete chrysosporium* glyoxal oxidase is encoded by two allelic variants: structure, genomic organization and heterologous expression of glxl and glx2, *J. Bacteriol.* **177**, 6106–6110.

KING, N. J. (1966) The extracellular enzymes of *Coniophora cerebella*. *Biochem. J.* **100**, 784–792.

KIRK, T. K. (1975) Effects of the brown-rot fungus, *Lenzites trabea*, on lignin in spruce wood. *Holzforschung* **29**, 99–107.

KIRK, T. K. (1981) Toward elucidating the mechanism of action of the ligninolytic system in basidiomycetes. In: Hollaender, A., Rabson, R., Rogers, A., San Pietro, A., Valentine, R. and Wolfe, R., eds, *Trends in the Biology of Fermentations*, New York: Plenum Press, pp. 131–149.

KIRK, T. K. and ADLER, E. (1970) Methoxyl-deficient structural elements in lignin of sweetgum decayed by a brown-rot fungus. *Acta Chem. Scand.* **24**, 3379–3390.

KIRK, T. K. and CHANG, H-M. (1974) Decomposition of lignin by white-rot fungi, I. Isolation of heavily degraded lignins from decayed spruce. *Holzforschung* **28**, 217–222.

KIRK, T. K. and CHANG, H-M. (1975) Decomposition of lignin by white-rot fungi, II. Characterization of heavily degraded lignins from decayed spruce. *Holzforschung* **29**, 56–64.

KIRK, T. K. and COWLING, E. B. (1984) Biological decomposition of solid wood. In: Rowell, R. M., ed, *Chemistry of Solid Wood*. Advances in Chemistry Series, 207, Washington, DC: American Chemical Society Press, pp. 455–487.

KIRK, T. K. and FENN, P. (1982) Formation and action of the ligninolytic system in basidiomycetes. In: Frankland, J. C., Hedger, J. N. and Swift, M. J., eds, *Decomposer Basidiomycetes*, British Mycological Society Symposium 4, Cambridge University Press, pp. 67–90.

KIRK, T. K. and HAMMEL, K. C. (1992) What is the primary agent of lignin degradation in white rot fungi. In: Kurwaloa, M. and Shida, M., eds. *Biotechnology in Pulp and Paper Industry*, Proceedings of 5th International Conference in Biotechnology in Pulp and Paper Industry, Japan: Uni Publishers, pp. 535–540.

KIRK, T. K. and HIGHLEY, T. L. (1973) Quantitative changes in structural components of conifer woods during decay by white- and brown-rot fungi. *Phytopathology* **63**, 1338–1342.

KIRK, T. K., SCHULTZ, E., CONNORS, W. J., LORENZ, L. F. and ZEIKUS, J. G. (1978) Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. *Arch. Microbiol.* **117**, 277–285.

KIRK, T. K., HIGHLEY, T. L., IBACH, R. and MOZUCH, M. D. (1989) *Identification of Terminal Structures in Cellulose Degraded by the Brown-Rot Fungus* Postia placenta,

Document IRG/WP/1389, International Research Group on Wood Preservation.

KIRK, T. K., IBACH, R. E., MOZUCH, M. D., CONNER, A. H. and HIGHLEY, T. L. (1991) Characterization of cotton cellulose depolymerized by a brown-rot fungus, by acid or by chemical oxidants. *Holzforschung* 45, 239–244.

KOCKERT, G. (1991) Restriction fragment length polymorphism in plants and its implications. In: *Subcellular Biochemistry: Plant Genetic Engineering*, Vol. 17, New York: Plenum Press, pp. 167–190.

KOENIGS, J. W. (1972a) Effects of hydrogen peroxide on cellulose and on its susceptibility to cellulase. *Mat. u. Org.* 7, 133–147.

KOENIGS, J. W. (1972b) Production of extracellular hydrogen peroxide by wood-rotting fungi. *Phytopathology* 62, 100–110.

KOENIGS, J. W. (1974a) Hydrogen peroxide and iron: a proposed system for decomposition of wood by brown-rot basidiomycetes. *Wood Fiber Sci.* 6, 66–80.

KOENIGS, J. W. (1974b) Production of hydrogen peroxide by wood-rotting fungi in wood and its correlation with weight loss, depolymerization and pH changes. *Arch. Microbiol.* 99, 129–145.

KOENIGS, J. W. (1975) Hydrogen peroxide and iron: a microbial cellulolytic system? *Biotechnology and Bioengineering Symposium*, No. 5, pp. 151–159.

KUAN, I-C., CAI, D. and TIEN, M. (1991) The lignin-degrading system of the wood-destroying fungus *Phanerochaete chrysosporium*. In: Pell, E. and Steffen, K., eds, *Active Oxygen/Oxidative Stress and Plant Metabolism*, Rockville, MD: American Society of Plant Physiologists, pp. 180–192.

KUREK, B. and KERSTEN, P. J. (1995) Physiological regulation of glyoxal oxidase from *Phanerochaete chrysosporium* by peroxidase systems. *Enzyme Microb. Technol.* 17, 751–756.

LEATHAM, G. and KIRK, T. K. (1983) Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes. *FEMS Microbiol. Lett.* 16, 65–67.

LU, J., GOODELL, B., LIU, J., ENOKI, A., JELLISON, J. and FEKETE, F. (1994) *The Role of Oxygen and Oxygen Radicals in One-Electron Oxidation Reactions Mediated by Low-Molecular Weight Compounds Isolated from* Gloeophyllum trabeum, Document IRG/WP95-1457, International Research Group on Wood Preservation.

MERRILL, W. and COWLING, E. B. (1966) Rate of nitrogen in wood deterioration: amounts and distribution of nitrogen in tree stems. *Can. J. Bot.* 44, 1555–1580.

MOUKHA, S. M., WOSTEN, H. A. B., ASTHER, M. and WESSELS, J. G. H. (1993) *In situ* localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. *J. Gen. Microbiol.* 139, 969–978.

MUHEIM, A., WALDNER, R., LEISOLA, M. S. A. and FIECHTER, A. (1990) An extracellular aryl alcohol oxidase from the white rot fungus *Bjerkendera adusta*. *Enzyme Microb. Technol.* 12, 204–209.

NAKATSUBO, F., REID, I. D. and KIRK, T. K. (1982) Incorporation of $^{18}O_2$ and absence of stereospecificity in primary product formation during fungal metabolism of a lignin model compound. *Biochem. Biophys. Acta* 719, 284–291.

NIKU-PAAVOLA, M. L., KARHUNEN, E., SALOLA, P. and RAUNIO, V. (1988) Lignolytic enzymes of the white-rot fungus *Phlebia radicata*. *Biochem. J.* 254, 877–884.

ODIER, E., MOZUCH, M. D., KALYANARAMAN, B. and KIRK, T. K. (1988) Ligninase-mediated phenoxy radical formation and polymerization unaffected by cellobiose: quinone oxidoreductase. *Biochemica* 70, 847–852.

PEASE, E. A., ANDRAWIS, A. and TIEN, M. (1989) Manganese-dependent peroxidase from *Phanerochaete chrysosporium*: primary structure deduced from cDNA sequence. *J. Biol. Chem.* 264, 13531–13535.

PERIE, F. H. and GOLD, M. H. (1991) Manganese regulation of manganese peroxidase expression and lignin degradation by the white-rot fungus *Dichomitus squalens*. *Appl. Environ. Microbiol.* 57, 2240–2245.

PHILLIP, B., DAN, D. C. and FINK, H. P. (1981) Acid and enzymatic hydrolysis of cellulose in relation to its physical structure. *Proceedings of International Symposium on Wood and Pulping Chemistry*, Stockholm, pp. 79–83.

POLACK, J. M. and PRIESTLY, J. V. (1992) *Electron Microscopic Immunocytochemistry Principles and Technologies*, Oxford: Oxford University Press.

PRIBNOW, D., MAYVIELD, M. B., NIPPER, V. J., BROWN, J. A. and GOLD, M. H. (1989) Characterization of a cDNA encoding a manganese peroxidase, from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *J. Biol. Chem.* **264**, 5036–5040.

PRYOR, W. A. (1976) *Free Radicals in Biology*, Vol. I, San Francisco, London: Academic Press.

RAEDER, U., THOMPSON, W. and BRODA, P. (1989) RFLP-based genetic map of *Phanerochaete chrysosporium* ME446: lignin peroxidase genes occur in clusters. *Mol. Microbiol.* **3**, 911–918.

RUEL, K. and JOSELEAU, J. P. (1991) Involvement of an extracellular glucan sheath during degradation of *Populus* wood by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **57**, 374–384.

RUEL, K., ODIER, E. and JOSELEAU, J.-P. (1989) Observation of wood cell wall degradation by immunocytochemical techniques. In: Kirk, T. K. and Chang, H.-M., eds, *Biotechnology in Pulp and Paper Manufacture*, Boston: Butterworth-Heinemann, pp. 83–97.

SALOHEIMO, M., BARAJAS, V., NIKIO-PAAVOLA, M. and KNOWLES, J. (1989) A lignin peroxide-encoding complementary DNA from the white-rot fungus *Phlebia radiata*: characterization and expression in *Trichoderma reesei*. *Gene* **85**, 343–351.

SCHMIDT, C. J., WHITTEN, B. K. and NICHOLAS, D. D. (1981) A proposed role for oxalic acid in nonenzymatic wood decay by brown-rot fungi. *Proc. Am. Wood Pres. Assoc.* **77**, 157–164.

SHIMADA, M. (1993) Biochemical mechanisms for the biodegradation of wood. *Curr. Jap. Mat. Res.* **II**, 207–222.

SHOEMAKER, S., SLCHWEICKART, V., LADNER, M., GELFAND, D., KWOK, S., MYAMBO, K. and INNIS, M. (1983) Molecular cloning of exo-cellobiohydrolase I derived from *Trichoderma reesei* strain L27. *Bio/Technology* **1**, 691–696.

SIMS, P., JAMES, C. and BRODA, P. (1988) The identification, molecular cloning and characterisation of a gene from *Phanerochaete chrysosporium* that shows strong homology to the exocellobiohydrolase I gene from *Trichoderma reesei*. *Gene* **74**, 411–422.

SREBOTNIK, E., MESSNER, K., FOISNER, R. and PETTERSON, B. (1988) Ultrasturctural localization of ligninase of *Phanerochaete chrysosporium* by immunogold labelling. *Curr. Microbiol.* **16**, 221–227.

STEWART, P., KERSTEN, P., VANDEN WYMELENBERG, A., GASKELL, J. and CULLEN, D. (1992) The lignin peroxidase gene family of *Phanerochaete chrysosporium*: complex regulation by carbon and nitrogen limitation, and the identification of a second dimorphic chromosome. *J. Bacteriol.* **174**, 5036–5042.

STEWART, P., WHITWAM, R. E., KERSTEN, P. J., CULLEN, D. and TIEN, M. (1996) Efficient expression of *Phanerochaete chrysosporium* manganese peroxidase gene in *Aspergillus oxyzae*. *Appl. Environ. Microbiol.* **62**, 860–864.

TANAKA, N., AKAMATSU, Y., HATTORI, T. and SHIMADA, M. (1994) Effect of oxalic acid on the oxidative breakdown of cellulose by the Fenton reaction. *Wood Res.* **81**, 8–10.

TIEN, M. and TU, C. P. D. (1987) Cloning and sequencing of a cDNA for a ligninase from *Phanerochaete chrysosporium*. *Nature* **326**, 520–523.

UEMURA, S., MITSURO, M. and JELLISON, J. (1993) Differential responses of wood-rot fungi cellulases towards antibodies against *Trichoderma viride* cellobiohydrolase I. *Appl. Microbiol. Biotechnol.* **39**, 788–794.

UZCATEGUI, E., RUIZ, A., MONTESINO, R., JOHANSSON, G. and PETTERSSON, G. (1991) The 1,4-β-D-glucan cellobiohydrolases from *Phanerochaete chrysosporium* I. A

system of synergistically acting enzymes homologous to *Trichoderma reesei*. *J. Biotechnol.* **19**, 271–286.

VANDER MEER, J. M., RIJKES, B. A. and FERIARNTI, M. P. (1987) Degradation of lignocelluloses. In: *Ruminants and or Industrial Processes*, Amsterdam: Elsevier Science Publishers.

VENESS, R. G. and EVANS, C. S. (1989) The role of hydrogen peroxide in the degradation of crystalline cellulose by basidiomycete fungi. *J. Gen. Microbiol.* 135, 2799–2806.

WAINWRIGHT, M. (1992) *An Introduction to Fungal Biotechnology*, New York: Wiley and Sons.

WESTERMARK, U. and ERIKSSON, K. E. (1974) Cellobiose: quinone oxidoreductase, a new wood-degrading enzyme from white-rot fungi. *Acta Chem. Scand.* **B28**, 209–214.

WILLIAMS, A. L., WILLIAMS, A. C., MOORE, N. L. and DASHEK, W. V. (1991) Biotechnology of wood deteriorating enzymes synthesized and secreted by *Coriolus versicolor*, a white-rot basidiomycete. In: Rossmore, H. W., ed, *Biodeterioration and Biodegradation*, London: Elsevier Applied Science, pp. 547–549.

ZADRAZIL, F. and RENINGER, P. (1988) *Treatment of Lignocelluloses With White-Rot Fungi*, Amsterdam: Elsevier Science Publishers.

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