15 Fungal Biodegradation of Lignocelluloses

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I. Introduction

Wood and lignified gramineous and other annual plants are generally called lignocellulose because they are composed of the three main natural polymers: cellulose, hemicelluloses and lignin. Lignocellulosic biomass is renewable, and huge amounts of lignocellulose are annually synthesized and degraded in nature. It has been estimated that annual worldwide production of terrestrial biomass is 200×10^{12} kg (Foust et al. 2008). In Earth's carbon cycle, especially in a forest ecosystem, saprotrophic wood-decaying and litter-decomposing fungi perform an essential role. Among them certain basidiomycetes, so-called white-rot fungi, have a special role since they are the only organisms that can efficiently degrade and even mineralize the most recalcitrant natural polymer, lignin.

Cellulose is considered to be one of the most abundant biopolymers on Earth. It is the main constituent of wood, and approximately 40% of the dry weight of most wood species is cellulose, which is located predominantly in the secondary cell wall (Sjöström 1993). Cellulose is a homopolysaccharide composed of β-D-glucopyranoside units which are linearly linked together by (1→4)-glycosidic bonds. Cellulose can be crystalline, sub-crystalline and even amorphous, depending on the tissue source in native plant, or the way that cellulose is isolated (Ding and Himmel 2008). The structural integrity of cellulose is one of the main obstacles of enzymatic hydrolysis of cellulose. For the isolation of cellulose harsh extraction methods involving sequential acid and alkaline treatments are usually employed. Fiber aggregation caused by sample processing and found in isolated cellulose does not necessarily represent the cellulose structure, and the detailed molecular structure of plant cell wall cellulose remains unknown (Ding and Himmel 2008).

Hemicelluloses in wood consist of relatively short, mainly branched heteropolymers of glucose, xylose, galactose, mannose and arabinose as well as uronic acids of glucose, galactose and 4-*O*-methylglucose linked by (1→3)-, (1→6)- and (1→4)-glycosidic bonds. Galactoglucomannans are the principal hemicelluloses in softwoods, which also contain arabinoglucuronoxylan. Xylose-based hemicelluloses are often termed xylans in both softwoods and hardwoods. Depending on hardwood species, the xylan content varies within the limits of 15-30% of the dry

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wood. Acetyl groups are present as substituents particularly in the glucomannans of gymnosperms and the xylans of angiosperms (Sjöström 1993). Hemicelluloses are reported to be linked to lignin through cinnamate acid ester linkages, to cellulose through interchain hydrogen bonding, and to other hemicelluloses via covalent and hydrogen bonds (Decker et al. 2008).

Lignin is a complex, amorphous, three-dimensional aromatic polymer. Lignins are synthesized from the oxidative coupling of *p*-hydroxycinnamyl alcohol monomers, dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (*p*-hydroxyphenyl, H) phenylpropanoid units. The molecular weight of lignin is difficult to determine because lignins are highly polydisperse materials (Argyropoulos and Menachem 1997). New bonding patterns have been described in softwood lignin, e.g. dibenzodioxocin structures (Karhunen et al. 1995). Recent studies show that lignin can incorporate many more monolignols than the traditional three basic units (Vanholme et al. 2008), e.g. acetylated lignin units have been identified in non-woody plants (Martínez et al. 2005). The isolation of native lignin is complicated if possible at all (Buswell and Odier 1987). Isolated lignin usually has a brown color but in sound non-degraded wood it is obviously colorless because the wood of many tree species is almost white, and after attack by white-rot fungi, by definition, the modified lignin in residual wood and cellulose is also white.

Uncertainties in the basic structures of especially lignin but also other components in lignocellulose make fungal biodegradation studies a challenging task. The following properties are important in terms of microbial or enzymatic attack: (1) lignin polymers have compact structures that are insoluble in water and difficult to penetrate by microbes or enzymes, (2) the intermonomeric linkages that account for the rigidity of lignin comprise many kinds of C–C and C–O bonds with the β-aryl ether linkage being the most significant and (3) intermonomeric linkages in lignin are not hydrolyzable. A conclusion from the above items is summarized as follows: (1) polymeric lignin degradation requires extracellular enzymes and/or small molecular weight mediators or factors such as radicals, (2) the lignin degrading system must be unspecific and (3) the enzymes must be oxidative, not hydrolytic.

II. Fungal Degradation of Lignocellulose

A. White-Rot Fungi

White-rot fungi are a heterogeneous group of fungi that usually belong to basidiomycetes, although there are ascomycetous fungi that cause pseudo-white rot (also designated as soft-rot type II), such as fungi belonging to the family Xylariaceae (Blanchette 1995; Liers et al. 2006). Basidiomycetous white-rot and some related litter-decomposing fungi are the only organisms which are capable of mineralizing lignin efficiently (Kirk and Cullen 1998; Hatakka 2001). More than 90% of all wood-rotting basidiomycetes are of the white-rot type (Gilbertson 1980). White-rot fungi are more commonly found on angiosperm than on gymnosperm wood species in nature. Usually syringyl (S) units of lignin are preferentially degraded, whereas guaiacyl (G) units are more resistant to degradation. Many white-rot fungi colonize cell lumina and cause cell wall erosion. Eroded zones coalesce as decay progresses and large voids filled with mycelium are formed. This type of rot is referred as non-selective or simultaneous rot. Calcium oxalate and MnO_2 accumulate when the decay proceeds (Blanchette 1995). *Trametes* (syn. *Coriolus*, *Polyporus*) *versicolor* is a typical simultaneous-rot fungus (Eriksson et al. 1990). Some white-rot fungi degrade lignin in woody plant cell walls relatively to a higher extent than cellulose, and they are called selective white-rot fungi. In nature they may cause white-pocket or white-mottled types of rot, e.g. *Phellinus nigrolimitatus* (Blanchette 1995). There are also fungi, e.g. the tree pathogen *Heterobasidion annosum*, that are able to produce both types of attack in the same wood (Eriksson et al. 1990).

In a screening to find suitable fungi for wood chip pretreatment for biopulping, 90 white-rot fungi were cultivated in spruce (*Picea abies*) wood blocks for 10 weeks, and about 20% of these fungi degraded more lignin than cellulose (Hakala et al. 2004). The selectivity depends on the wood species, cultivation time, temperature and many other things. This work and several other screening studies have shown that e.g. *Ceriporiopsis subvermispora*, *Dichomitus squalens Phanerochaete chrysosporium*, *Phellinus pini*, *Phlebia radiata*, *Phlebia tremellosus* (syn. *Merulius tremellosa)*, *Phlebia subserialis*, *Physisporinus rivulosus*, *Pleurotus eryngii, Pleurotus ostreatus* and *Pycnoporus cinnabarinus* are lignin-selective fungi at least under certain conditions (Eriksson et al. 1990; Akhtar et al. 1998; Hatakka 2001; Hakala et al. 2004).

B. Brown-Rot Fungi

Brown-rot fungi are basidiomycetes that degrade wood to yield brown, shrunken specimens that typically exhibit a pattern of cubical cracks and easily disintegrate upon handling. Only a small proportion – roughly 7% – of all wood decay basidiomycete species falls into this group, which occurs most frequently on gymnosperm wood. Nevertheless, brown-rot fungi are essential biomass recyclers in coniferous forests and are the most important cause of decay in man-made wooden structures (Gilbertson and Ryvarden 1986; Eriksson et al. 1990). Recent phylogenetic analyses based on ribosomal RNA sequences suggest that most brown-rot fungi evolved repeatedly from white-rot fungi, most likely by the selective loss of some biodegradative mechanisms. Six or more separate brown-rot groups, including the frequently studied genera *Gloeophyllum* and *Postia*, have probably arisen independently from the white-rot lineage via loss of some decay capabilities. One possible exception is the white-rot genus *Grifola*, which may have evolved from a brown-rot ancestor (Hibbett and Thorn 2001).

The hallmark of brown-rot is rapid strength loss in the wood before extensive weight loss has occurred. This observation was first explained by Cowling (Cowling 1961), who chemically delignified sweetgum wood that was undergoing fungal decay to obtain holocellulose fractions (i.e. the cellulose and hemicelluloses), and then determined their degree of polymerization by measuring their viscosities after solubilization. The results showed that the brown-rot fungus *Poria monticola* (= *Postia placenta*) decreased the average chain length of the wood polysaccharides about fourfold at only 10% weight loss. Since a principal function of the lignin in wood is to shield the structural polysaccharides from enzymatic attack, this result led to the conclusion that the biodegradative agents responsible for incipient brown-rot are low-molecular-weight species that can penetrate the lignin in sound wood despite its low porosity (Cowling 1961; Koenigs 1974).

During brown rot, the hemicelluloses in wood are degraded most rapidly, after which virtually all of the cellulose is removed, leaving behind a complex, aromatic ring-containing polymer derived from the original lignin. Experiments with *P. placenta* grown on cotton have shown that carbonyl and carboxylic acid groups are introduced into the residual cellulose during decay, which suggests that depolymerization of the polysaccharides involves not only hydrolytic enzymes, but also an oxidative component (Kirk et al. 1991). Analyses of lignin from brown-rotted wood have likewise shown that it becomes oxidized, partially via demethylation of the aromatic rings, which increases the phenolic hydroxyl content, and partially via introduction of new carbonyl and carboxyl groups. Nevertheless, the residual lignin is still polymeric, and most of it appears to remain in situ (Kirk and Adler 1970; Kirk 1975). These findings have led to two conclusions: (1) brown-rot fungi have little capacity to degrade lignin and (2) the low-molecular-weight species they use to initiate decay are oxidants, with oxygen-centered free radicals such as the hydroxyl radical (•OH) being the prime candidates (Koenigs 1974; Eriksson et al. 1990; Hammel et al. 2002).

The apparent persistence of the lignin after extensive brown-rot of wood is consistent with earlier proposals that low-molecular-weight agents that can penetrate the secondary cell wall must play a central role, but is also perplexing because brown-rot fungi clearly produce cellulases and hemicellulases (Herr et al. 1978, Ritschkoff et al. 1994; Mansfield et al. 1998; Machuca and Ferraz 2001; Cohen et al. 2005), which can presumably operate only if some of the lignin is degraded beforehand. Recent work based on nuclear magnetic resonance spectroscopy of dissolved wood samples have clarified the picture somewhat by revealing that many of the intermonomer linkages of lignin actually disappear during brown rot (Yelle et al. 2008). These results suggest that the lignin may be transiently depolymerized, which might facilitate the access of polysaccharide hydrolases.

C. Soft-Rot Fungi

Ascomycetes and mitosporic fungi usually cause soft-rot decay of wood (Nilsson et al. 1989; Blanchette 1995). The decayed wood is brown and soft, and the residue is cracked when dry. Soft-rot fungi form cavities within secondary walls or erosion but then the middle lamella is not attacked. Xylariaceous ascomycetes from genera such as *Daldinia*, *Hypoxylon* and *Xylaria* are grouped with the soft-rot fungi because they cause a typical erosive soft rot. Compared to basidiomycetous fungi, the knowledge about

lignocellulose degradation by ascomycetes is limited, and very little is known about how they degrade lignin. They mainly degrade hardwood, and weight losses up to 53% of birch wood were found within 2 months after decay by the most efficient fungus of this group, *Daldinia concentrica* (Nilsson et al. 1989).

III. Fungal Degradation of Wood Polysaccharides

The ability to degrade plant biomass and cellulose as its major component is widespread in fungi. *Trichoderma reesei* is a mesophilic soft-rot fungus that is extensively used as a source of cellulases and hemicellulases for various applications. It has also long been a model system for the degradation of plant cell wall polysaccharides. It was shown to be an anamorph of the pantropical ascomycete *Hypocrea jecorina*. The sequence data of the whole genome of this fungus has become available recently (Martinez et al. 2008). Thus the cellulolytic systems of *T. reesei* and those of the first studied white-rot basidiomycete, *Phanerochaete chrysosporium* (Martinez et al. 2004), and the first brown-rot basidiomycete, *Poria placenta* (Martinez et al. 2009), the genomes of which are available, give a new basis for comparisons.

The concept of how fungi degrade cellulose (and hemicelluloses) is almost totally based on the enzyme system of *T. reesei*, while only rather incomplete data are available on other ascomycetes. Cellulolytic enzymes of basidiomycetes were recently reviewed (Baldrian and Valášková 2008). The fungal degradation of cellulose is catalyzed by: (1) cellobiohydrolases (CBHs, EC 3.2.1.91) and (2) endoglucanases (EGs EC 3.2.1.4), of which CBHs cleave the polymeric cellulose from reducing or non-reducing ends, and EGs randomly endo-wise along the glucose chain. The cellulolytic system also contains (3) extra- or intracellular β-glucosidases (EC 3.2.1.21), which hydrolyze the resulting cellobiose or cello-oligosaccharides to glucose. The hydrolysis products can also be oxidized by cellobiose dehydrogenase (CDH). In addition, numerous other carbohydrate-active enzymes are produced by fungi. In the carbohydrate-active enzyme database CAZy (http://www.cazy.org) glycoside hydrolases are grouped in as many as 115 families (Cantarel et al. 2009).

Table 15.1 summarizes the characteristics of the sequenced genomes of lignocellulose-degrading basidiomycetes. Taxonomically and ecologically very different fungi have been used as models for lignin and cellulose/hemicellulose degradation, namely the white-rot basidiomycete *P. chrysosporium* and the mitosporic (ascomycete) fungus *T. reesei* (syn. *H. jecorina*), respectively. The latter fungus cannot degrade lignin while the former fungus degrades lignin but in addition also efficiently cellulose. Taking into account the ecological importance of wood-rotting basidiomycetes in carbon cycling and wood cellulose degradation, the databanks contain only surprisingly few genes encoding cellulases of basidiomycetous fungi.

P. chrysosporium has an unexpectedly high number of cellulase encoding genes compared to *T. reesei* (Martinez et al. 2008; Table 15.2). While *T. reesei* has 10 genes encoding cellulolytic enzymes, one CBH1 (Cel7A), and one CBH2 (Cel6), the *P. chrysosporium* genome has seven CBH1 and one CBH2 encoding genes. Only three of these CBHs have been purified and characterized (Uzcategui et al. 1991a, b). Many other ascomycetous fungi such as *Aspergillus nidulans, A. fumigatus* and *A. oryzae*, *Magnaporthe grisea, Neurospora crassa* and *Fusarium graminеum* have a higher number of cellulases, and even 1–5 of the most efficient CBHs (Table 15.2). *T. reesei* has rather many genes encoding hemicellulose-degrading enzymes, 16, and *P. chrysosporium* has 19 (Table 15.1). There are 34–44 hemicellulase encoding genes in the ascomycetes *Aspergillus nidulans*, *A. fumigatus* and *A. oryzae*, *Magnaporthe grisea* and *Fusarium gramineum* genomes, indicating that these saprotrophic and plant pathogenic fungi rely on hemicellulose degradation (see also Chapter 16).

It was originally reported that many brown-rot fungi grow poorly on pure cellulose and that crude cellulases obtained from cultures degrade amorphous cellulose but lack activity on crystalline cellulose, which is relatively recalcitrant to hydrolysis (Nilsson and Ginns 1979; Eriksson et al. 1990). These results have been interpreted to mean that brown-rot fungi lack cellobiohydrolases, the exo-acting enzymes that are required for the operation of a complete, synergistic cellulase system. In agreement with this original picture, almost all cellulases purified from brown-rot fungi are endoglucanases, i.e. enzymes that lack the processive activity characteristic of cellobiohydrolases. However, it is now clear that some brown-rot fungi can utilize crystalline cellulose as a sole carbon source (Cohen et al. 2005; Yoon et al. 2008),

Table 15.1. Characteristics of the sequenced genomes of lignocellulose-degrading basidiomycetes (modified from Lundell et al. 2010)

Fungus	Type of fungus	Genome size[a], Mbp	Number of (putative) genes encoding:					Number of CAZyme modules[b]			Reference
			Proteins	Laccase	LiP	MnP	VP	Glycoside hydrolase	Carbohydrate-binding	Cellulose-binding	
Coprinopsis cinerea (*Coprinus cinereus*)	Litter-decaying	37.5	13 544	17	–[c]	–[c]	–[c]	211	26	1	*C. cinereus* genome homepage (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/MultiHome.html)
Phanerochaete chrysosporium	White-rot	35.1	10 048	–[c]	10	5	–[c]	180	89	46	Vanden Wymelenberg et al. (2006) (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html)
Pleurotus ostreatus	White-rot	34.3	11 603	12	Several	Several	Several	?[e]	?[e]	?[e]	*P. ostreatus* genome homepage (http://genome.jgi-sf.org/PleosPC15_1/PleosPC15_1.home.html)
Postia placenta	Brown-rot	90.9[d]	17 173	2	–[c]	–[c]	–[c]	144	6	–[c]	Martinez et al. (2009) (http://genome.jgi-psf.org/Pospl1/Pospl1.home.html)
Schizophyllum commune	White-rot	38.5	13 181	8	?[e]	?[e]	?[e]	?[e]	?[e]	?[e]	*S. commune* genome homepage (http://genome.jgi-psf.org/Schco1/Schco1.home.html)

[a]Haploid, if not otherwise depicted
[b]Adapted from Martinez et al. (2009)
[c]Not present
[d]Dikaryon
[e]Not yet determined

Table 15.2. The number of genes encoding cellulolytic enzymes in fungi (data adopted from Martinez et al. 2008)

Fungus	Type	CBH1 Cel7A	CBH2 Cel6	EG1 Cel7B	EG2 Cel5	EG3 Cel12	EG4 Cel61	EG5 Cel45	Sum
Aspergillus fumigatus	A	2	1	2	3	3	7	1	19
A. nidulans	A	2	2	1	2	1	9	1	18
A. oryzae	A	2	1	1	2	2	8	0	16
Fusarium gramineum	A	1	0	1	2	2	13	1	23
Hypocrea jecorina (syn. *Trichoderma reesei*)	A	1	1	1	2	1	3	1	10
Magnaporthe grisea	A	3	2	2	2	3	17	1	30
Neurospora crassa	A	2	3	3	1	0	14	1	23
Phanerochaete chrysosporium	B/WR	7	1	2	2	1	14	0	27

Types: *A* ascomycetes, *B* basidiomycetes, *WR* white-rot fungus
Enzymes: *CBH1* exocellobiohydrolase 1, *CBH2* exocellobiohydrolase II, *EG1* endoglucanase I, *EG2* endoglucanase II, *EG3* endoglucanase III, *EG4* glycoside hydrolase family, *EG5* endoglucanase V

which raises the possibility that their non-processive cellulases act in concert with low-molecular-weight oxidants to enable complete substrate degradation.

Although there was one early report that *Coniophora puteana* produces cellobiohydrolases (Schmidhalter and Canevascini 1992), these enzymes should be re-examined because the assay used, cleavage of *p*-nitrophenyl lactoside, is not completely specific. In addition, it has been reported that one endoglucanase from *G. trabeum* has processive activity, as shown by the high ratio of soluble to insoluble reducing sugars it produced from crystalline cellulose (Cohen et al. 2005). Additional work is needed to check this conclusion, because measurements of insoluble reducing sugars are technically challenging and require careful replication (Irwin et al. 1993; Medve et al. 1998). Even if future work confirms some exceptions such as these, it appears that the cellulolytic systems of brown-rot fungi are generally less complex than those of white-rot fungi. In this connection, it is noteworthy that the genome of *P. placenta* apparently encodes neither cellobiohydrolases nor endoglucanases with cellulose-binding modules (Martinez et al. 2009).

IV. Fungal Degradation of Lignin

A. White-Rot and Brown-Rot Fungi

Lignin is a large macromolecular and heterogeneous polymer the degradation of which is difficult due to its chemical structure, e.g. it does not contain by definition any hydrolysable linkages (Kirk and Cullen 1998). The enzymes must be therefore oxidative (i.e., oxidoreductases), and due to heterogeneity of the polymer and many types of linkages (C–C, C–O), also nonspecific. Lignin is also stereo-irregular, thus differing from e.g. cellulose or hemicelluloses.

Due to difficulties in the isolation and analysis of lignin preparations, various lignin model compounds, e.g. dimeric β-O-4 model compounds and synthetic lignin (dehydrogenation polymerizate of coniferyl alcohol or other lignin precursors, DHP) are commonly used in microbiological and enzymatic studies. These model systems do not contain any linkages to wood polysaccharides. All lignin preparations have disadvantages in reproducibility of preparation, or exhibit altered structure or molecular weight compared to natural lignin (Buswell and Odier 1987). One of the most reliable methods to determine fungal lignin-degrading ability is based on ^{14}C-lignins (Haider and Trojanowski 1975; Kirk et al. 1975; Kirk et al. 1978). Usually the evolution of $^{14}CO_2$ from ^{14}C-DHP or other ^{14}C-(lignin)-lignocelluloses is monitored. These studies have given information on the suitable conditions for lignin degradation by white-rot fungi such as *Phanerochaete chrysosporium*, *Phlebia radiata* and many others (Kirk 1975; Hatakka and Uusi-Rauva 1983; Hatakka et al. 1983; Kirk and Farrell 1987; Hatakka 2001), as well as by litter-decomposing basidiomycetous fungi (*Stropharia* spp., *Agrocybe praecox*; Steffen et al. 2000). Dimeric lignin model compounds attached to e.g. polyethylene glycol (Kawai et al. 1995), thus mimicking polymeric lignin, have allowed the use of efficient analytical tools (e.g. NMR) for more defined characterization of degraded lignin structures.

Solubilization (formation of water-soluble lignin fragments) and mineralization (evolution of $^{14}CO_2$) of

^{14}C-labelled natural and synthetic lignins have been demonstrated for various white-rot fungi (Hatakka and Uusi-Rauva 1983; Hatakka 2001). A high mineralization of ^{14}C-(ring)-DHP was observed in the case of *Phlebia radiata* that released up to 71% $^{14}CO_2$ from ^{14}C-DHP in 37 days when grown under optimal conditions for lignin degradation, i.e. under 100% oxygen atmosphere in a low nutrient nitrogen liquid medium (Hatakka et al. 1983). *Phlebia* sp. Nf b19 (former name *Nematoloma frowardii* b19, Hildén et al. 2008) caused even higher mineralization (75%) of ^{14}C-DHP during growth on wheat straw while only a small percentage of the initial radioactivity (6%) was incorporated into the residual straw and the fungal biomass (Hofrichter et al. 1999b). Usually only the end-product of lignin degradation, $^{14}CO_2$, is determined. However, in the case of certain fungi or under special conditions, the rate-limiting steps may be the reactions after the initial attack on polymeric lignin, in which case soluble oligomers may be detectable. The variety of different ligninolytic enzymes and the isoenzymes and isoforms produced by the fungus may also influence the production of $^{14}CO_2$.

1. Ligninolytic Peroxidases of White-Rot Fungi

The extracellular enzymes involved in lignin degradation are peroxidases and laccases, with their accessory enzymes (Hatakka 1994; Kirk and Cullen 1998; Hammel and Cullen 2008). Peroxidases include: (1) lignin peroxidases (LiPs, "ligninases", EC 1.11.1.14) and (2) manganese peroxidases (MnPs, "Mn-dependent peroxidases", EC 1.11.1.13), which were discovered in the early 1980s, and (3) versatile peroxidases (VPs, EC 1.11.1.16), which were found in the 1990s (Martínez 2002) and apparently represent hybrids of LiPs and MnPs. Fungal oxidoreductases are listed in the FOLy database (http://foly.esi.univ-mrs.fr; Levasseur et al. 2008).

The plant and microbial peroxidase superfamily covers three classes of peroxidase families of which class I includes intracellular prokaryotic peroxidases, class II consists of secretory fungal peroxidases, i.e. lignin peroxidases (LiPs), manganese peroxidases (MnPs) and versatile peroxidases (VPs), while class III includes secretory plant peroxidases (Morgenstern et al. 2008). The analysis of the genes suggests that the class II sequences constitute a monophyletic gene family, and that they diversified extensively in the basidiomycetes. LiPs evidently arose only once in the Polyporales, which harbors many white-rot taxa, whereas MnPs and VPs are more widespread and may have multiple origins. The phlebioid clade of Polyporales includes such fungi as *Phanerochaete chrysosporium* and *Phlebia radiata*, which are well known producers of LiP. The *Phanerochaete chrysosporium* whole genome has ten *lip* genes and five *mnp* genes (Table 15.1), of which two were previously unknown (Martinez et al. 2004), and the corresponding proteins were not characterized. The data suggest that *P. chrysosporium* also has a versatile peroxidase gene the sequence of which shares residues common to both *mnp*s and *lip*s. Phylogenetic comparisons between some well-known peroxidases show that white-rot and litter-decomposing fungi exhibit a continuum of variability in their peroxidases (Lundell et al. 2010). Even a single fungal species can produce several different peroxidases as exemplified by the white-rot basidiomycetes *P. radiata* (Hildén et al. 2005) and *Physisporinus rivulosus* (Hakala et al. 2006).

The fungal peroxidases LiPs, MnPs and VPs are all heme-containing glycoproteins which require hydrogen peroxide as an oxidant and have heme (protoporphyrin IX) as their prosthetic group coordinated by two highly conserved histidine residues (Martínez 2002). The heme cofactor is located in an internal cavity connected by two access channels. The main channel is used by hydrogen peroxide and the second is the site where MnP and VP oxidize Mn^{2+} to Mn^{3+} (Ruiz-Dueñas et al. 2009). Typically for LiP activity, the amino acid residue needed is a tryptophan, Trp171 in the isozyme LiPA (LiP H8) of *P. chrysosporium*. Tryptophan exposed on the LiP protein surface is conserved in LiP sequences and also in VPs (Martínez 2002; Pérez-Boada et al. 2005). It is assumed that it takes part in long-range electron transfer (LRET) from a protein radical at the surface of the enzyme, which would act as the substrate oxidizer, to the heme cofactor (Ruiz-Dueñas and Martínez 2009). This could allow the enzyme to oxidize bulky substrates such as polymeric lignin that cannot directly contact the oxidized heme in the active centre of LiP or VP.

Most white-rot fungi secrete several isoenzymes into their cultivation medium (Hatakka 1994). The molecular weight of the LiP, MnP and VP varies between 35–48 kDa, 38–62 kDa and 42–45 kDa, respectively. Lignin-modifying peroxidases have typically acidic p*I* values of 3.0–4.0 (Hatakka 2001), while also neutral MnPs have been detected from litter-decomposing fungi (Steffen et al. 2002).

LiP oxidizes non-phenolic lignin substructures by abstracting one electron and generating aryl cation radicals that then decompose chemically (Kirk and Farrell 1987). Reactions of LiP using a variety of lignin model compounds and synthetic lignin have thoroughly been studied, catalytic mechanisms elucidated and the enzyme's capability for C_α–C_β bond cleavage, ring opening

and other reactions demonstrated (Kirk and Farrell 1987). MnP oxidizes Mn(II) to Mn(III) which then oxidizes phenolic rings to phenoxy radicals, leading to the decomposition of the structures (Gold et al. 2000). Studies with white-rot fungi have shown that the expression of MnP is more common than that of LiP (Orth et al. 1993; Hatakka 1994; Vares et al. 1995; Hofrichter 2002; Rainio, Maijala, Hatakka et al., unpublished data). VP has been reported so far only from *Pleurotus* spp. and *Bjerkandera* spp. (Ruiz-Dueñas and Martínez 2009). Crystal structures of substrate binding site mutants of MnP indicate that there is only one major Mn-binding site (Sundaramoorthy et al. 1997). This proposed site consists of a heme propionate, three acidic ligands and two water molecules.

The characteristics and potential applications of MnPs and VPs were extensively studied in the 1990s and frequently reviewed (Hatakka 2001; Hofrichter 2002; Martínez 2002; Ruiz-Dueñas and Martínez 2009), while LiPs were less studied. MnP has an important role in the depolymerization of lignin and chlorolignin as well as in the demethylation of lignin and bleaching of pulp (Hatakka 2001). Moreover, the enzyme mediates initial steps in the degradation of high-molecular-weight lignin (Perez and Jeffries 1992). MnP oxidizes Mn(II) to Mn(III) that is stabilized by organic acids such as oxalate, malate, lactate or malonate via chelation (Kishi et al. 1994; Kirk and Cullen 1998). Chelated Mn(III) in turn oxidizes various compounds, including lignin. In the presence of unsaturated fatty acids, it contributes to lipid peroxidation, and the formed peroxyl radicals may act as selective oxidants. Significant mineralization of synthetic ^{14}C-labelled lignin (DHP), up to 16% of the applied ^{14}C as evolved $^{14}CO_2$, was measured in an in vitro system (without fungal mycelium) consisting of a mixture of MnP, linoleic acid, Mn and H_2O_2 (Hofrichter et al. 1999a; Kapich et al. 1999).

In addition, some accessory enzymes are involved in lignin degradation. These enzymes include H_2O_2-generating enzymes such as aryl alcohol oxidase (AAO, EC 1.1.3.7), glyoxal oxidase (GLOX), and pyranose-2 oxidase (EC 1.1.3.10; Hatakka 2001; Kersten and Cullen 2007).

2. Peroxidases of Brown-Rot Fungi?

The components most often cited as missing in brown-rot fungi are the secreted enzymes generally thought to have a key role in delignification by white-rot fungi. As far as ligninolytic heme-containing peroxidases are concerned, the situation appears straightforward so far. The currently known lignin and versatile peroxidases (LiP, VP) of white-rot fungi, i.e. those enzymes that can cleave non-phenolic lignin structures directly, all contain an exposed tryptophan required for catalysis (Martínez 2002) and no brown-rot fungus has yet been shown to produce a peroxidase with this essential residue. Similarly, manganese peroxidases (MnPs), which oxidize Mn(II) to the ligninolytic agent Mn(III), all contain an essential manganese-binding site comprised of acidic amino acid residues (Martínez 2002), and no brown-rot fungus peroxidase has been found with this property. Additional white-rot peroxidases that may have a ligninolytic function have been described recently (Martínez 2002; Miki et al. 2009) but none of them has a brown-rot counterpart so far. Perhaps most pertinent, no currently known type of ligninolytic peroxidase is encoded in the *P. placenta* genome (Martinez et al. 2009).

3. Laccases

Laccase (EC 1.10.3.2, *p*-diphenol:oxygen oxidoreductase) has been studied since the 1880s when it was first described in the lacquer tree (Thurston 1994; Baldrian 2006). Laccase is a copper-containing oxidase that utilizes molecular oxygen as oxidant and also oxidizes phenolic rings to phenoxy radicals (though the redox potential is somewhat lower than that of peroxidases; Thurston 1994; Baldrian 2006). Most white-rot fungi typically produce laccase (Käärik 1965; Bollag and Leonowicz 1984), and the enzyme is common also in higher plants and in other fungi, and even in bacteria and insects laccase-like multicopper oxidases have been described (Giardina et al. 2010).

Laccases belong to a superfamily of multicopper oxidases (MCOs; Hoegger et al. 2006), forming a phylogenetically divergent group of so called "blue oxidases" that all contain four copper atoms per mol of enzyme, arranged into three metallocentres between three structural domains formed by a single polypeptide of about 500 amino acids in length (Ducros et al. 1998; Bertrand et al. 2002; Piontek et al. 2002; Lundell et al. 2010). Phylogenetically, multicopper oxidases may be classified as true fungal laccases and separated from other laccase-like enzymes (Lundell et al. 2010). In fungi laccases or laccase-like copper enzymes can be involved in lignin degradation, but in fungal physiology laccases may also have other functions, for example, in pigmentation, fruiting body formation, sporulation, pathogenicity and detoxification (Thurston 1994). The crystal

structures of ascomycete and basidiomycete laccases were solved for enzymes of *Melanocarpus albomyces* (Hakulinen et al. 2002) as well as *Coprinus cinereus* (Ducros et al. 1998), *Trametes versicolor* (Piontek et al. 2002) and *Lentinus tigrinus* (Ferraroni et al. 2007), respectively.

Laccases catalyze the oxidation of a wide range of organic substrates, such as phenols, aromatic amines and heterocyclic compounds, usually restricted by low redox potential characteristics (< 0.8 V). They catalyze the complete four-electron reduction of dioxygen (O_2) to water with concomitant electron withdrawal from the reducing organic substrate compounds (Thurston 1994; Call and Mücke 1997). Laccase catalyzes the cleavage of the C_α–C_β bond in phenolic β-1 and β-O-4 lignin model dimers by oxidizing C_α-carbon and by splitting the aryl–alkyl bond (Eriksson et al. 1990). Due to its rather low redox potential, it is not able to directly oxidize non-phenolic lignin units, which have a high redox potential (> 1.2 V). Laccases can, however, oxidize monomeric lignin-like phenols and anilines of redox potentials ranging from 0.5 V to even 1.9 V for one electron abstraction, and the efficiency of oxidation correlates to the redox potential but also to steric properties of the enzyme (Tadesse et al. 2008).

In native wood, less than 20% of all lignin units are phenolic (Higuchi 1990), and as a consequence, laccases are incapable of depolymerizing macromolecular lignins. In the presence of oxidation mediator compounds (redox mediators), such as 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate, (ABTS; Bourbonnais and Paice 1990) or organic N-OH compounds such as 1-hydroxybenzotriazole (HBT), which were discovered in the 1990s by Call and others (Call and Mücke 1997), laccase-aided oxidation of non-phenolic compounds and even delignification is promoted. There have been attempts to find possible natural mediators (Eggert et al. 1996), but except in one case (3-hydroxyanthranilic acid from *Pycnoporus cinnabarinus*), no such compound has been identified. Synthetic laccase mediators have widely been investigated for various applications in the pulp and paper/forest sector, the most common being HBT and violuric acid (Call and Mücke 1997; Widsten and Kandelbauer 2008). There is a vigorous search for safer and cheaper mediator molecules, e.g. among naturally occurring aromatic compounds. Syringaldehyde and other related compounds have shown promising results as charge-transfer mediators in laccase catalyzed reactions (Kawai et al. 2004; Camarero et al. 2005; Nousiainen et al. 2009). In nature, hardwood lignin could be a source of syringyl compounds, but even if they would act as redox mediators in nature, in softwood, these substructures are not present. Although white-rot fungi typically prefer hardwood species, many of them grow and degrade also softwood and its lignin.

More than 100 laccases have been purified from fungi and more or less characterized in detail (Baldrian 2006). Typically, laccases are inducible enzymes with pH optima between 3.0 and 5.7. Laccases of some soil inhabiting basidiomycetes have higher pH optima (pH 7.0), e.g. those of *Rhizoctonia praticola* (Bollag and Leonowicz 1984) and *Coprinus cinereus* (Schneider et al. 1999). The optimal temperature may be as high as 75°C as found using the laccase of the litter-decomposing fungus *Marasmius quercophilus* (Dedeyan et al. 2000). Laccases from white-rot fungi usually have molecular masses of 60–80 kDa, acidic p*I*s (2.5–5.0) and are glycosylated (Thurston 1994).

Laccases have long been proposed to have a role in lignin biodegradation (Eriksson et al. 1990) but the exact role of laccase in wood and non-wood (grass) lignin degradation is still unclear. Although laccase is very common in wood-rotting white-rot fungi, it was confirmed that the whole genome of *P. chrysosporium* (a model white-rot fungus) does not contain laccase-encoding genes, although some other multicopper oxidase encoding genes have been identified in its genome. The role of laccase in lignin degradation becomes even more questionable, since the whole genome of the dung-dwelling, non-lignin-degrading basidiomycete *C. cinerea* contains several (17!) laccase-encoding genes (Table 15.1). Moreover, the recent whole-genome sequence analysis of *Poria* (*Postia*) *placenta* indicated the presence of true laccases also in a cellulolytic brown-rot fungus (Martinez et al. 2009).

Nevertheless, laccases are apparently rare in brown-rot fungi, as shown by the long usage of the Bavendamm reaction to differentiate between white-rot from brown-rot basidiomycetes (Bavendamm 1928). This test depends on the development of a color reaction after application of a phenol oxidase substrate such as gallic acid to the mycelium, and most brown-rot fungi give a negative result. However, recent work has revealed the presence in the growth medium of some brown-rot fungi of uncharacterized substances that oxidize laccase substrates, and the genomes of some of these fungi harbour laccase-like gene sequences (D'Souza et al. 1996; Lee et al. 2004). Furthermore, at least one brown-rot fungus, *P. placenta*, clearly expresses a true laccase on wood, as shown by peptide fingerprinting of extracted proteins and by heterologous expression of the responsible gene (Wei et al. 2010).

Even when laccases are present in brown-rot fungi, it does not necessarily follow that this decay type involves enzyme-catalyzed delignification of the substrate. Although there is a long association in the literature between fungal ligninolysis and the presence of laccases, there is little evidence

that these enzymes can truly depolymerize lignin directly. Instead, they rather cause additional polymerization when tested on model lignins in vitro, unless suitable redox mediators are present (Rochefort et al. 2004). It may be that natural laccase mediators do occur and simply remain to be discovered (perhaps even in the case of brown-rotters), or that laccases actually have no role in fungal ligninolysis, perhaps being involved instead in melanin formation or in the unspecific detoxification of phenols via polymerization (Bollag et al. 1988; Galhaup and Haltrich 2001). Alternatively, the laccases of brown-rot fungi may participate in the generation of biodegradative oxygen-centered radicals, as discussed below (Wei et al. 2010).

Thus the present genomic information strongly suggests that the role of laccase in fungal physiology, that is in the fruiting body and spore formation as well as pigmentation and detoxifying of phenolic compounds derived from lignin, and in other similar reactions, may be in nature more important than the participation of laccase in lignin biodegradation. Even if its role in lignin biodegradation is not clear, this enzyme has undoubtedly more potential than other fungal oxidoreductases in various industrial applications (Widsten and Kandelbauer 2008), and because its heterologous production in industrial fungal hosts has been developed, its use on a relatively large scale is possible even now (Yaver et al. 1996; Berka et al. 1997; see also Chapters 14, 21).

4. Role of Small Oxidants in Incipient Decay

The chemical changes that occur in lignin and cellulose during brown-rot, in combination with the apparent lack of ligninolytic enzymes or complete cellulase systems in these fungi, suggest the involvement of small oxidants in decay. The best-known and perhaps most likely candidate is Fenton reagent, i.e. the oxidant that is produced when Fe^{2+} reacts with H_2O_2 (Fig. 15.1; Koenigs 1974). The Fenton oxidant is generally depicted as the hydroxyl radical (•OH), although it is possible that the actual species is a similarly reactive iron–oxygen complex in which the iron has a formal charge of +4 or +5 (Halliwell and Gutteridge 1999). Essentially, all a fungus requires for the production of extracellular Fenton reagent is a mechanism to reduce extracellular Fe^{3+} to Fe^{2+}, because Fe^{2+} auto-oxidizes in most biological

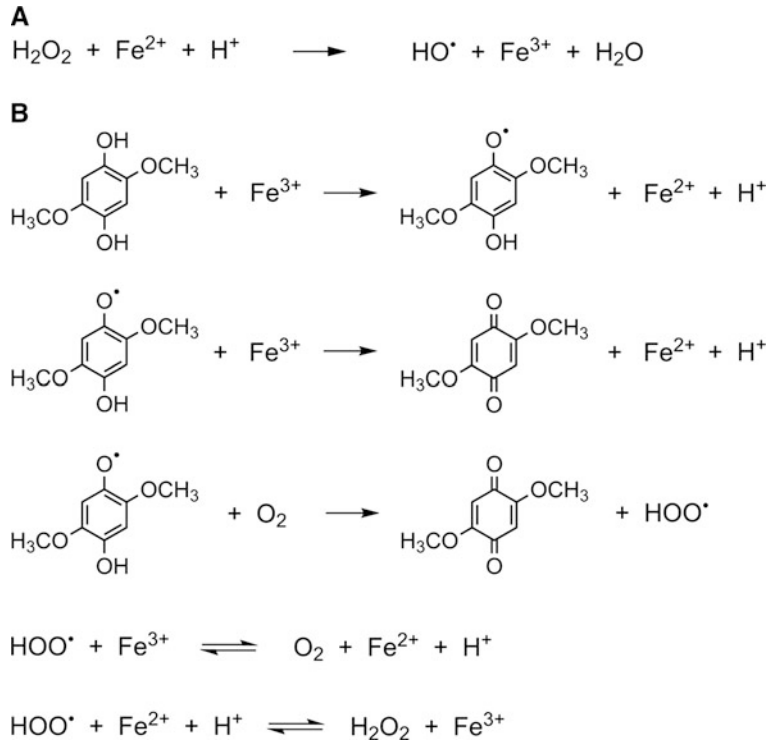

Fig. 15.1. Extracellular free radical reactions proposed to have a role in incipient brown-rot. **A** The Fenton reaction. **B** Hydroquinone-dependent processes that produce the Fe^{2+} and H_2O_2 required for Fenton chemistry

environments to produce the perhydroxyl radical/superoxide acid–base pair ($\bullet OOH/O_2\bullet^-$), which rapidly dismutates or oxidizes Fe^{2+} to produce H_2O_2. Alternatively, Fenton chemistry will occur if the fungus has a system that generates extracellular $\bullet OOH/O_2\bullet^-$, because these radicals are sufficiently reducing to convert most forms of Fe^{3+} to Fe^{2+} (Fig. 15.1; Halliwell and Gutteridge 1999).

Fenton reagent is the strongest oxidant that can occur in water, and reacts non-selectively with virtually any organic compound it meets. It abstracts hydrogens from aliphatic structures such as polysaccharides, thus depolymerizing them and introducing oxygen functional groups, in agreement with the chemical changes that have been observed in brown-rotted cellulose. Fenton reagent also oxidizes aromatic rings, causing ring hydroxylation and scission of adjacent aliphatic structures, which may explain why brown-rot fungi are apparently capable of some ligninolysis (Hammel et al. 2002). In the process of these reactions, an array of new substrate-derived oxidants is produced, especially alkoxyl (•OR) and peroxyl (•OOR) radicals, which are less reactive than Fenton regent but able to cause additional lignocellulose oxidation (Hammel et al. 2002). Several hypotheses have been advanced to explain how brown-rot fungi initiate Fenton chemistry.

5. Hydroquinones

Work over the past decade has shown that extracellular hydroquinones are produced by diverse, phylogenetically distinct brown-rot fungi, including *G. trabeum*, *P. placenta* and *Serpula lacrymans*. In *G. trabeum*, both 2,5-dimethoxyhydroquinone (2,5-DMHQ) and 4,5-dimethoxycatechol occur, whereas *P. placenta* and *S. lacrymans* appear to produce only 2,5-DMHQ (Kerem et al. 1999; Paszczynski et al. 1999; Shimokawa et al. 2004; Suzuki et al. 2006). The significance of this finding is that hydroquinones bearing electron-donating substituents (such as methoxyls) auto-oxidize rapidly in the presence of most Fe^{3+} salts, thus generating Fe^{2+} and semiquinone radicals. Some of the resulting semiquinones then reduce additional Fe^{3+}, or react further with O_2 to produce quinones and $\bullet OOH/O_2\bullet^-$. This last species then dismutates or oxidizes some of the Fe^{2+} to produce H_2O_2 (Fig. 15.1). The Fe^{2+} and H_2O_2 thus generated then undergo the Fenton reaction, as described above.

Some studies done with decaying wood support a role for hydroquinone-driven Fenton chemistry in incipient brown-rot. The hydroquinones are present in wood undergoing decay by *G. trabeum* and *P. placenta*, and are maintained in the reduced state despite their tendency to oxidize rapidly when removed from the wood (Suzuki et al. 2006; Wei et al. 2010). This result indicates that the hydroquinones must be present in a steady state, i.e. that they are continuously oxidized and regenerated in the wood. Computer modeling of these reactions has provided evidence that the quantity of Fenton reagent produced via this redox chemistry is large enough for the hydroquinones to have a role in polysaccharide scission during incipient decay by *G. trabeum*. However, the quantity appears insufficient to account for all of the cleavage that occurs, and thus other process must also contribute (Suzuki et al. 2006). An additional difficulty is that some brown-rot fungi, including *P. placenta*, produce large quantities of oxalic acid, a strong Fe^{3+} chelator that makes the reduction of Fe^{3+} by 2,5-DMHQ thermodynamically infeasible (Green et al. 1991; Park et al. 1997). In this case, it appears that *P. placenta* laccases rather than Fe^{3+} are the initial oxidants of 2,5-DMHQ. The resulting semiquinone radicals then react with O_2 to produce $\bullet OOH/O_2\bullet^-$, a species which, unlike 2,5-DMHQ, is sufficiently reducing to convert Fe^{3+} to Fe^{2+} in the presence of a high oxalate concentration, thus generating all components needed for a complete Fenton system (Wei et al. 2010).

6. Cellobiose Dehydrogenases

Cellobiose dehydrogenases (CDHs, EC1.1.99.18) are heme- and flavin-containing oxidoreductases that many fungi secrete. They catalyze the oxidation of cellobiose and some other sugars with concomitant reduction of an electron acceptor, molecular oxygen originally being considered the physiological substrate. Subsequent work with the enzyme from the white-rot fungus *P. chrysosporium* showed: (1) some Fe^{3+} salts are better electron acceptors, (2) the Fe^{2+} thus formed auto-oxidizes to produce the H_2O_2 precursor $\bullet OOH/O_2\bullet^-$ and (3) a complete Fenton system can thus be produced. Most of the work on CDH-catalyzed Fenton chemistry has focused on white-rot fungi and the possible role of reactive oxygen species in their decay processes (Henriksson et al. 2000). Genes encoding CDH have been described in the white-rot fungi *P. chrysosporium*, *Pycnoporus cinnabarinus*, *Trametes versicolor* and *Ceriporiopsis subvermispora*. In the last fungus it has been speculated that the poor degradation of cellulose is due to its lack of cellobiohydrolases or and CDHs. Recently, a CDH from C. *subvermispora* was purified and characterized (Harreither et al.

2009). The fungus produces it in later stages of cellulose degradation, and therefore it was concluded that CDH does not participate in lignin degradation but it could help the weak cellulolytic system of this fungus, lacking cellobiohydrolases, to degrade cellulose at a late stage of growth on cellulose. Thus, even now the exact role of this enzyme is not known.

This mechanism may also be relevant to the brown-rot fungus *Coniophora puteana*, which produces a CDH (Hyde and Wood 1997), but it must be absent in *P. placenta* because no CDH is encoded in this genome (Martinez et al. 2009). One problem with a role for CDH in Fenton chemistry is that it is no stronger a reductant than are hydroquinones such as 2,5-DMHQ (Hyde and Wood 1997), and thus like them it cannot reduce Fe^{3+} efficiently in the presence of high oxalate concentrations. In addition, there is a "chicken or egg" problem with CDH as a source of biodegradative oxidants – presumably, these low-molecular-weight species are needed during incipient decay to release sugars for the fungus to grow on, yet the sugars are required beforehand as electron donors for CDH to operate.

7. Redox-Active Glycopeptides

Nearly 20 years ago, wood-grown cultures of *G. trabeum* were reported to produce extracellular Fe^{2+}-containing glycopeptides that reduced O_2 to $\bullet OOH/O_2\bullet^-$ and also used H_2O_2 to produce a Fenton system. These substances also utilized Fe^{3+}, provided a reductant such as NADH or ascorbate was included in the reactions. The glycopeptides appeared somewhat heterogeneous, as shown by their chromatographic properties, but had a sufficiently low-molecular-weight mass range of 1–5 kDa to suggest they could have a role in incipient brown rot (Enoki et al. 1992). Subsequent work showed that similar substances were produced by a variety of brown- and white-rot fungi (Enoki et al. 1997; Tanaka et al. 2007). Recently, genes that apparently encode these peptides were also identified in the white-rotter *P. chrysosporium* (Tanaka et al. 2007), and it is interesting that similar genes are present and expressed by the brown-rotter *P. placenta* (Martinez et al. 2009).

However, so far some features of these substances are difficult to reconcile with a biodegradative role. First, the inferred molecular masses of the encoded peptides are around 14 kDa, much larger than reported for the substances that were first isolated from colonized wood. Indeed, the possibility has not been ruled out that some of the originally reported glycopeptides might simply be heterogeneous mixtures of partially degraded extracellular fungal proteins. Second, as with other proposed fungal Fenton systems, a reductant is needed to return the bound iron to its active ferrous state. Since the physiological reductant is unlikely to be either NADH or ascorbate, a key part of the biodegradative system remains unidentified. Finally, it is not clear what advantage is conferred by having the iron bound to a glycopeptide, since Fenton chemistry occurs equally well with iron salts bound to much simpler chelators such as oxalate. One possibility worth investigating is that the Fenton systems involving glycopeptide-bound iron might oxidize polysaccharide or lignin structures more selectively than simple Fenton systems do. Another is that the ferric glycopeptides might be able to undergo reduction by donors that do not work with simpler Fe^{3+} chelates. In this connection, it is interesting that the *G. trabeum* glycopeptide was reported to undergo reduction by cellobiose (Wang and Gao 2003), but very few details of this reaction were provided. Additional work is clearly needed with these potentially relevant substances.

B. Soft-Rot Fungi

Ascomycetes are usually thought to degrade mainly carbohydrates in soil, forest litter and compost, but they may also degrade lignin in these environments (Rodriguez et al. 1996; Regalado et al. 1997; Tuomela et al. 2000; Kluczek-Turpeinen et al. 2003). Thus, some ascomycetes/deuteromycetes ("molds") were found to be able to mineralize grass lignins (Haider and Trojanowski 1975) and e.g. *Penicillium chrysogenum*, *Fusarium oxysporum* and *F. solani* (Rodriguez et al. 1996) mineralized in 28 days up to 27% of a ^{14}C-labelled lignin prepared from milled wheat straw. Unlike model white-rot basidiomycetes such as *Phanerochaete chrysosporium* and *Phlebia* spp., which degrade lignin during secondary metabolism (Kirk and Farrell 1987; Hatakka 2001), the degradation by molds was maximal during primary metabolism (Regalado et al. 1997).

Pine wood was degraded very little, only showing 2.5% weight loss (Nilsson et al. 1989). The high concentration of guaiacyl units in the middle lamella of coniferous wood may cause its resistance to decay by soft-rot fungi because they may not have the oxidative potential to attack the more condensed recalcitrant guaiacyl lignin. In contrast, syringyl lignin apparently is readily oxidized and mineralized by soft-rot fungi (Nilsson et al. 1989). Usually these fungi, e.g. *P. chrysogenum* and *F. proliferatum*, mineralize less than 10% of the applied ^{14}C-labelled guaiacyl type

synthetic lignin (DHP; Rodriguez et al. 1994; Regalado et al. 1997). *Paecilomyces inflatus* mineralized 6.5% of a synthetic $^{14}C_{\beta}$-labelled lignin in 12 weeks during solid-state cultivation of the fungus in autoclaved compost and 15.5% was converted into water-soluble fragments (Kluczek-Turpeinen et al. 2003). Two wood inhabiting fungi, *Xylaria hypoxylon* and *X. polymorpha*, when growing on beech wood meal mineralized 9% of the same DHP as above, and the major fraction (65.5%) was polymerized into water and dioxin insoluble material (Liers et al. 2006). These fungi formed large lignocellulose fragments unlike the basidiomycetous white-rot fungus *Bjerkandera adusta*, which released smaller lignocellulose fragments. This might have connections to the finding that *Xylaria* spp. produced high levels of hydrolytic enzymes, like esterase and xylanases.

There are no reliable reports on the presence of ligninolytic heme peroxidases in ascomycetes. The genome of the efficient cellulose degrading fungus *Hypocrea jecorina* (*Trichoderma reesei*) does not contain genes encoding laccase or ligninolytic peroxidases (Martinez et al. 2008). Laccase activities in lignin-degrading ascomycetes such as *Xylaria* spp. (Liers et al. 2006) and *P. inflatus* (Kluczek-Turpeinen et al. 2003) have usually been low. Laccase, aryl-alcohol oxidase and superoxide radicals were detected in liquid cultures of *F. proliferatum*, but neither MnP nor LiP were present (Regalado et al. 1999). Interestingly, in the ascomycete *Petriellidium fusoideum*, the specific inhibition of hydroxyl radical production was found to decrease the mineralization of ^{14}C-labelled synthetic lignin (DHP; Gonzales et al. 2002).

V. Biopulping as an Example of Potential Applications of White-Rot Fungi

Fungal or enzymatic treatment of wood or wood chips combined with mechanical or chemical pulping is a process known in a broad sense as biopulping, or more precisely as biomechanical, or biochemical pulping, depending on the pulping process which the biological process is combined with (Akhtar et al. 1998; Ferraz et al. 2008). In chemical pulping, such as soda or Kraft pulping, strong chemicals and high pressure are applied to remove lignin. Chemical pulping produces pulp with high strength but the process is polluting, hemicelluloses are also removed and the overall yield is relatively low (40–50%). Mechanical pulping uses mechanical forces to separate wood fibers, and the yields are higher (up to 95%); it results in paper with good printing properties – newspaper is typically manufactured from mechanical pulp – but the process requires a lot of electrical energy for refining, the fibers have poor strength properties, and the high content of lignin causes a tendency to high color reversion (yellowing). Biological (fungal) treatment is usually combined with mechanical pulping to save refining energy (Akhtar et al. 1998), and its compatibility is not good with alkaline (Kraft) pulping, as the most useful white-rot fungi produce oxalic acid and other organic acids when growing in wood or straw (Galkin et al. 1998; Hofrichter et al. 1999b; Mäkelä et al. 2002; Hakala et al. 2005), and the neutralization of these acids causes a need for extra alkali.

The use a lignin-degrading white-rot fungus to delignify wood is not a new idea as reviewed by Akhtar et al. (1998) and Gramss (1992). Already in the 1940s, Luthardt in East Germany started comprehensive studies to prepare softened myco-wood ("Mykoholz") for the production of pencils using white-rot fungi (e.g. *Kuehneromyces mutabilis*, *Trametes versicolor*; summarized by Luthardt 1969, 2005). In 1957 a United States pulp and paper company screened wood-rotting fungi for delignification, and in 1972, it was found that treatment (cultivation) with a common white-rot fungus, *Rigidoporus ulmarius*, reduced the electrical energy needed for papermaking and produced stronger fibers from aspen wood chips. At the same time a pioneering work was published from the Swedish Forest Products Laboratory (STFI; Ander and Eriksson 1977). This screening identified some promising biopulping fungi from which also cellulaseless mutants were produced. These fungi were *Phlebia radiata*, *Pycnoporus cinnabarinus* and *Sporotrichum pulverulentum* (an anamorph of *P. chrysosporium*). For biopulping experiments and lignin biodegradation studies, the researchers from the United States used a strain of the same fungus, *P. chrysosporium* Burdsall. The next generation of biopulping research was started in the Forest Products Laboratory, USDA (Madison, Wis., USA) in 1987 by a large international biopulping consortium (Akhtar et al. 1998).

A key factor in successful biopulping is the use of a competitive and fast-growing but at the same time efficiently lignin-degrading fungus. Extensive screenings of white-rot fungi (Otjen and Blanchette 1987; Blanchette et al. 1988, 1992) resulted in the selection of the highly effective species *Ceriporiopsis subvermispora*, and also fungi of the genus *Phlebia* have been frequently

studied for biopulping purposes, e.g. strains of *P. tremellosa*, *P. subserialis* and *P. brevispora*. *C. subvermispora* was efficient during both hardwood and softwood treatment, and since then, it has been a target for lively research, and its lignin and cellulose degrading enzymes have been thoroughly studied (Lobos et al. 2001; Heidorne et al. 2006).

In fungal treatment of lignocelluloses, it would be useful to regulate the relationship of the degradation of polysaccharides versus lignin. To better understand the regulation of lignocellulose degradation, *Phlebia radiata* was grown under different conditions and the degradation and mineralization of ^{14}C-labelled tobacco cellulose, ^{14}C-labelled wheat-straw hemicelluloses and ^{14}C-(lignin)-labelled wheat straw was monitored. The results indicate that the degradation of lignin and hemicelluloses proceeds under similar conditions while the degradation of cellulose occurred under different conditions (Cho et al. 2009). For the acceleration of lignin degradation and the simultaneous repression of fungal attack on cellulose cultivation of the fungus in an oxygen atmosphere, addition of a small amount of glucose and supplementation of the medium with vanillic acid has been suggested.

In a study (Hakala et al. 2004) to find suitable biopulping fungi for the treatment of Norway spruce (*Picea abies*), about 300 white-rot fungi were initially screened on agar plate tests, and about 90 well-growing fungi were then cultivated for 10 weeks on spruce wood blocks. About 15 species of these pre-selected fungi degraded more lignin than cellulose (Hakala et al. 2004); some fungi such as *Phellinus viticola* even converted almost three times more lignin than cellulose. However, the weight losses were usually rather low, indicating slow growth. The most promising fungus in this screening was *Physisporinus rivulosus*, which was able to grow and degrade wood also at rather high temperature (37°C). *P. rivulosus* produces readily MnP and secretes also oxalate (Hakala et al. 2005, 2006). Both *C. subvermispora* and *P. rivulosus* caused losses of Klason lignin and an increase in acid-soluble lignin when growing in spruce wood. Two MnP isoenzymes were found in *P. rivulosus* and they are differentially regulated (Hakala et al. 2005, 2006).

Recently the so-far most intensive biopulping studies, both regarding technological and mechanistic aspects, have been started in Brazil (Ferraz et al. 2008) with the aim to treat *Eucalyptus grandis* and *Pinus taeda* with *C. subvermispora*. Mill-scale pilot studies at the 50-t scale have shown which kind of practical difficulties biopulping must overcome. The same level of energy savings as at laboratory scale were possible to attain at mill-scale, i.e. 18–27%, depending on the mechanical pulping process and freenesss. There is, however, still a need for more resistant and competitive fungal species, since the main drawback has been contamination of wood-chip piles with other fungi ("molds"). Earlier studies by the biopulping consortium (Akhtar et al. 1998) showed that the extent of lignin removal is not related to energy savings in biomechanical pulping, but Ferraz and others (Ferraz et al. 2008) have shown that there are good correlations between biopulping effects and intense lignin depolymerization observed during the initial stages of wood biotreatment by selective white-rot fungi.

The dominant oxidative enzyme that the potentially most applicable biopulping fungi produce on wood chips is (again) MnP (Hatakka et al. 2003; Maijala et al. 2008; Cunha et al. 2010). For industrial purposes, fungal biopulping is still considered too slow and technically demanding, and the direct application of enzymes on wood chips has become a more attractive alternative. When Scots pine (*Pinus sylvestris*) wood chips were treated with an MnP–lipid peroxidation system, 11% less energy was consumed while fiber strength and optical quality were maintained (Maijala et al. 2008). The treatment also increased the number of carboxyl groups on the surface of wood fibers while the total number of carboxyl groups was not affected. This indicates that the enzyme may act only on the surface of wood.

Most white-rot fungi readily produce oxalate during growth on lignocelluloses such as straw (Galkin et al. 1998; Hofrichter et al. 1999), and *Physisporinus rivulosus* started to secrete oxalate on the fifth day when growing on spruce wood (Hakala et al. 2004). Oxalate may directly contribute biopulping, namely it was found that esterification of oxalate produced by the fungus to the fibers may improve fiber saturation and correlates well with energy savings in biomechanical pulping (Hunt et al. 2004). Finally oxalate may have also many other roles in wood-rotting fungi (Mäkelä et al. 2002, 2009). In addition to MnP and oxalate, white-rot fungi secrete also an array of other oxidative and hydrolytic enzymes into the surrounding wood matrix, which all may be involved in the alteration of the microstructure of woody cell walls.

VI. Overview of Fungal Lignin Degradation and Outlook

Despite intensive research, especially on fungal oxidoreductases, it is still not clear in detail how lignin-degrading white-rot fungi actually attack lignin. The peroxidases involved in lignin degradation are rather well-known and their role in lignin degradation is generally accepted, while the role of laccase is not certain (Hatakka 2001; Hammel and Cullen 2008). Analogously to the degradation of polysaccharides (cellulose, hemicelluloses), it is assumed that lignin fragments produced by the peroxidase attack are taken up by the fungal hyphae and metabolized intracellularly. This process requires active uptake of lignin fragments through the fungal cell wall and membranes, which means that the fungus must possess transport mechanisms (Shary et al. 2008). This assumption is supported by the capability of the model fungus *P. chrysosporium* to metabolize aromatic compounds and de novo to synthesize veratryl alcohol (Lundquist and Kirk 1978). It was found at the end of the 1970s that mycelial pellets of *Sporotrichum pulverulentum* (the anamorph of *P. chrysosporium*) metabolize vanillic acid supplied to the cultivation medium, and several intermediates were analyzed from the extracellular medium (Ander et al. 1980). The fungus most probably utilizes intracellular or membrane bound cytochrome P450 enzymes, since it has as many as 154 cytochrome P450 encoding genes in its genome (Martinez et al. 2004). However, only a few transporters were upregulated under ligninolytic conditions (Shary et al. 2008). The uptake of lignin fragments may be an inefficient process. Apparently the lignin fragments cannot provide the fungus enough carbon and energy for growth since white-rot fungi cannot grow on polymeric lignin as a sole carbon source, i.e. lignin degradation by *P. chrysosporium* is a co-metabolic process associated with secondary metabolism and not linked to the growth of the fungus (Kirk and Farrell 1987).

All lignin-degrading white-rot fungi studied so far are apparently able to produce MnP (Hofrichter 2002). Laccase is also produced by almost all white-rot fungi, but not by the model fungus *P. chrysosporium*. This makes the role of laccase difficult to understand. A minor subset of white-rot fungi produce LiP or VP, the enzymes that can directly attack lignin (Ruiz-Dueñas and Martínez 2009). Thus the majority of lignin-degrading fungi seem to utilize a combination of MnP and laccase (Hatakka 1994). This distribution was apparent also in a recent screening of about 50 lignin-degrading white-rot and litter-decomposing fungi, which were efficient degraders of wood lignin on wood blocks (Hakala et al. 2004) or in other test systems. MnP was the most common ligninolytic peroxidase, being even more commonly expressed by the fungi than laccase, but only about half of the species, such as those belonging to the genera *Cerrena*, *Phlebia*, *Pleurotus*, *Phanerochaete* and *Trametes* bleached the dyes Azure B and Reactive Black 5 indicative for LiP and/or VP production (Rainio, Maijala, Hatakka et al., unpublished data). As pointed out earlier it was concluded that, among more than 150 agaricomycetes, LiPs evidently arose only once in the Polyporales, whereas MnPs and VPs are more widespread and may have multiple origins (Morgenstern et al. 2008). The production of MnP is apparently distinctive to basidiomycetous fungi, since there are no serious reports on bacteria, yeasts and molds, nor mycorrhiza-forming basidiomycetes producing MnP (Hofrichter 2002). Studies claiming the contrary (i.e. production of "true" ligninolytic peroxidases by asco- and deuteromycetes, bacteria and other organisms) should be regarded with suspicion because they may either rely on incorrectly identified fungi or suffer from incorrect/insufficient enzyme assays (examples of such maybe misleading reports are Ferrer et al. 1992; Kanayama et al. 2002; Bermek et al. 2004). Last but not least, VP has only been reported from *Pleurotus* spp. and *Bjerkandera* spp.

The widespread production of MnP by white-rot fungi suggests its key role in lignin degradation. It was proposed that lignin can be mineralized outside the cell by an "enzymatic combustion" mechanism based on MnP acting as a radical pump (Hofrichter et al. 1999a; Hatakka 2001; Fig. 15.2). The most common way of attack on the lignin polymer would occur by oxidized and chelated manganese (Mn^{3+}-*di*-oxalate chelates; Fig. 15.2). The suggested mechanism involves indirect oxygenation of phenolic rings (ether peroxide formation), spontaneous ring opening to produce muconic acid derivatives and decarboxylation of the formed carboxyl groups to carbon dioxide (Hofrichter 2002). This mechanism does not necessarily produce small aromatic lignin fragments since the aromatic rings are gradually eroded outside the fungal cell.

According to the traditional model of lignin degradation, the action of extracellular heme peroxidase through one-electron and hydrogen abstractions from lignin units (the key step in the degradative process) produces unstable aryl cation radical intermediates, which undergo different reactions including breakdown of C_α–C_β and C4–ether linkages releasing the corresponding aromatic aldehydes (vanillin in the case of guaiacyl units) that can be intracellularly mineralized (Kirk and Farrell 1987; Ruiz-Dueñas and Martínez 2009). It should be kept in mind here that vanillin is toxic to the fungus already at low concentrations and probably oxidized and polymerized by peroxidases (Ander et al. 1980). In the case of *P. chrysosporium* LiP, lignin attack requires the presence of veratryl alcohol, probably as an enzyme-bound mediator, and hydrogen peroxide is mainly generated by glyoxal oxidase. In this traditional model concept there is no role for MnP (or laccase), and there is still the question how fungi that do not produce LiP (or VP) can also mineralize

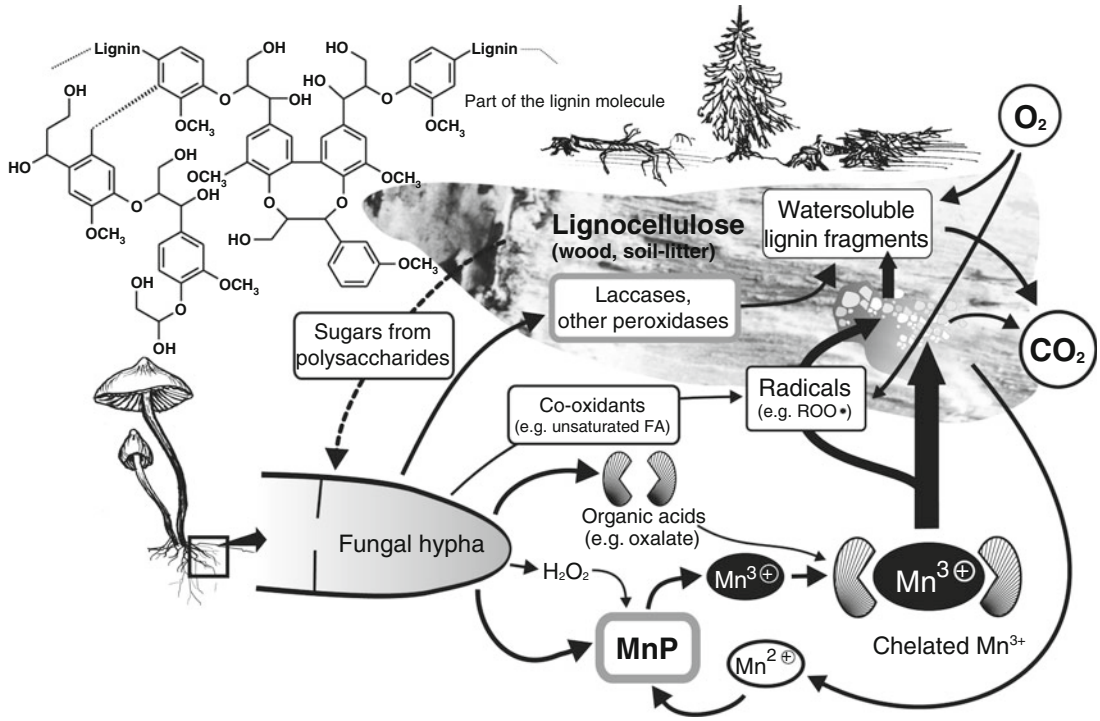

Fig. 15.2. Proposed mechanism of lignin degradation based on the radical-mediated reactions initiated by manganese peroxidase (MnP) acting as a radical pump (modified according to Fritsche and Hofrichter 2004)

lignin. If lignin fragments were (preferably) metabolized intracellularly, at least some energy and carbon should be gained from lignin for the fungus, and the fungus should be able to grow on lignin.

It is possible that white-rot fungi use both mechanisms to varying extents so that their role is important in fungi producing LiP and/or VP, and these fungi have an efficient metabolism of aromatic acids (e.g. vanillin) with necessary membrane transport mechanisms, while in other fungi the role of MnP is more significant. MnP-promoted lipid peroxidation causes the gradual extracellular breakdown of polymeric lignin (Bao et al. 1994), ring opening and formation of muconic acid residues, which can be decarboxylated by MnP, resulting in the production of carbon dioxide (Hofrichter et al. 1999a; Kapich et al. 1999, 2005; Hofrichter 2002).

The evidence is good that the earliest stage of brown-rot is oxidative and relatively non-selective, but there are also some differences between brown-rot species in the relative extents to which they modify lignin and cellulose (Irbe et al. 2001; Filley et al. 2002; Niemenmaa et al. 2008), and the only other clear unifying feature in this decay type is that the fungi fail to mineralize much of the lignin. Given the diverse phylogenetic origins of brown-rot, one question is whether it has arisen repeatedly simply via the loss of ligninolytic enzymes that are essential for white-rot. If this is the case, there is presumably an underlying suite of oxidative components that are able by themselves to attack lignocellulose, albeit less efficiently where the lignin is concerned, and it should then theoretically be possible to convert any white-rot fungus to a brown-rot fungus by knocking out components of its ligninolytic apparatus. Alternatively, some brown-rot fungi may have evolved new decay mechanisms that were not already present in the white-rot lineages whence they arose. Presently, we have too little information to draw firm conclusions, but it is interesting that some proposed biodegradative components, e.g. cellobiose dehydrogenases and redox-active glycopeptides, have been reported in both decay types, whereas oxidant-generating hydroquinones have been found so far only in brown-rot fungi.

Despite all the progress of the past three decades, many questions regarding lignocellulose biodegradation by fungi are still open. Thus, more effort will be necessary to elucidate above all the regulation of ligninolysis both on molecular and enzymatic levels. Not least this will be the prerequisite for the further development of biopulping technologies and other applications of wood-rot fungi and their biocatalytic systems.

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