1 Microorganisms and Enzymes Involved in Lignin Degradation Vis-à-vis Production of Nutritionally Rich Animal Feed: An Overview

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Abstract

Lignocellulosics are the major structural component of woody and nonwoody plants and represent a major source of renewable organic matter. The plant cell wall consists of three major polymers: cellulose, hemicellulose, and lignin. Lignocellulose biomass, available in huge quantity, has attracted considerable attention as an alternate resource for pulp and paper, fuel alcohol, chemicals, and protein for food and feed using microbial bioconversion processes. The current industrial activity of lignocellulosic fermentation is limited because of the difficulty in economic bioconversion of these materials to value-added products. Lignin is degraded to different extents by variety of microorganisms including bacteria, actinomycetes, and fungi, of which wood-rotting fungi are the most effective, white-rot fungi in particular. White-rot fungi degrade wood by a simultaneous attack on the lignin, cellulose, and hemicellulose, but few of them are specific lignin degraders. The selective lignin degraders hold a potential role in economically bioconversion of plant residues into cellulose-rich materials for subsequent bioethanol and animal feed production. Different fungi adapt in accordance to conditions existing in the ecosystem and complete their task of carbon recycling of the lignified tissues, and some white-rot fungi have capability to completely mineralize it. It is known that white-rot fungi are able to perform lignin degradation by an array of extracellular oxidative enzymes, the best characterized of which are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. However, the regulation of the production of individual enzymes and lignin degradation is a complex phenomenon.

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Unfortunately, even selected white-rot fungi take long in delignifying the lignocellulosic substrates. Therefore, it is necessary to improve these fungi for their ability to degrade lignin through various conventional and modern approaches. A considerable progress has been made in this direction during the past two decades; LiP, MnP, and laccase genes have been cloned, and an efficient *Agrobacterium*-mediated transformation system has been developed, which will eventually help in successful expression of the desired protein. This chapter presents an overview of diversity of lignin-degrading microorganisms and their enzymes especially in developing animal feed. In addition to that, advances in molecular approaches to enhance the delignification capability of microorganisms are also discussed.

Introduction

The residues from agricultural crops and agro- and forest-based industrial processes are abundantly available worldwide. Most of these agricultural crops and forest by-products are deficient in protein content, and presence of lignin in cell wall limits the digestibility of these materials in ruminants. Therefore, attempts have been made around the world to improve their protein content and digestibility by fermenting them under solid-state fermentation (SSF) conditions, using various microorganisms. Among these, white-rot fungi are considered the most capable of successfully degrading lignin. Most of them are slow colonizers and degrade cellulose and hemicellulose along with the lignin and are called as simultaneous degraders. However, recently a few white rots have been reported, which degrade lignin selectively (Akhtar et al. 1997; Hakala et al. 2004, 2005; Okano et al. 2009; Gupta et al. 2011; Shrivastava et al. 2011). Most of the work on solid-state fermentations (SSF) of agroresidues have been carried out using simultaneous degrader, *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*, the imperfect form), the most studied lignin-degrading fungus (Eriksson et al. 1990; Kuhad et al. 1997; Basu et al. 2002; Kumar et al. 2006; Gassara et al. 2010). However, recently selective lignin-degrading fungi such as *Lentinus edodes*, *Pycnoporus cinnabarinus*, *Crinipellis* sp. RCK-1, *Ceriporiopsis subvermispora,* and *Phlebia brevispora* are being studied for lignin degradation (Okano et al. 2006, 2009; Kuhar et al. 2008; Arora et al. 2011).

Some white-rot fungi are capable of degrading lignin selectively but the enzymes produced by them are too low for commercial purposes. To improve the enzyme production by selective white-rot fungi, various attempts have been made to optimize the culture conditions, and considerable increase in production of laccase, an important lignin-degrading enzyme, has been achieved (Dhawan et al. 2004; Sharma et al. 2005; Bonugli-Santos et al. 2010). For a commercial perspective, there is need to optimize SSF at pilot scale using selective fungi. The ligninolytic degradation ability of the selective degraders can be improved by using traditional mutagenesis and modern molecular techniques like recombinant DNA technology, and there has been a significant progress in this direction (Sharma and Kuhad 2010).

Genome sequence of the well-known lignin-degrading fungus, *P. chrysosporium,* has been unraveled (Martinez et al. 2004). This fungus possesses an impressive array of genes encoding extracellular oxidative enzymes: lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). Ten LiP genes and five MnP genes have been found. Genome searches revealed no conventional laccases. Instead, four multicopper oxidase (MCO) sequences are found clustered

within a 25-kb segment on scaffold 56. Thus, it appears that *P. chrysosporium* does not have the capacity to produce laccase although distantly related multicopper oxidases may have a role in extracellular oxidation. The genome harbors the genetic information to encode more than 240 putative carbohydrate-active enzymes (http://afmb.cnrs-mrs.fr/CAZY/) including 166 glycoside hydrolases, 14 carbohydrate esterases, and 57 glycosyltransferases, comprising at least 69 distinct families.

A number of genes encoding fungal laccases, lac 1, lac 2, lac 3, lac 4, and lac 5 and other important lignin-degrading enzyme(s), have been cloned including those from basidiomycetous fungi such as *Trametes (Coriolus) versicolor, T. villosa, Coriolus hirsutus*, *Rhizoctonia solani, Agaricus bisporus, Phlebia radiata,* Basidiomycete PMI, *P. cinnabarinus, and C. subvermispora* and ascomycetous fungi such as *Cryphonectria parasitica, Aspergillus nidulans, Podospora anserina,* and *Neurospora crassa* (Leonowicz et al. 1999). Recently, cloning and heterologous expression of a novel ligninolytic peroxidase gene from poroid brown-rot fungus *Antrodia cinnamomea* is carried out (Huang et al. 2009).

The ligninolytic enzymes are difficult to express in non-fungal systems. Laccase has been reported to be expressed in *Saccharomyces cerevisiae, Trichoderma reesei, and Aspergillus oryzae*. However, to the best of our knowledge, reports about purification and characterization of the recombinant proteins are scanty. The expression level has been low in most of the systems tested so far. The overexpression using specific vectors and promoters may provide higher yields.

Here we will discuss in detail about the diversity of lignin-degrading microorganisms, their enzymes, applications, and future prospects in developing biotechnological approaches, especially in developing animal feed.

Composition of Lignocellulosic Materials

Lignocellulosics in the form of wood and agricultural residues are virtually inexhaustible, since their production is based on the photosynthetic processes. They are the most abundant renewable natural material in the biosphere, accounting for approximately 50% of the total biomass present in the world ($10–50 \times 10^9$ t) (Sun and Cheng 2002; Nair 2006; Sánchez 2009). Regardless of source, lignocellulosic material contains three types of polymers – i.e., cellulose, hemicellulose, and lignin – that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross-linkages. Cellulose is polymer of glucose with cellobiose as repeating units, and hemicelluloses are macromolecules from different sugars, whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors. The composition and percentages of these polymers vary from one plant species to another. Moreover, the composition within a single plant varies with age, stage of growth, and other conditions. Table 1.1 shows the typical compositions of these three components in hardwoods, softwoods, agricultural residues, and various other lignocellulosic materials.

Lignin-Degrading Microorganism and Their Enzymes

A wide range of microorganisms such as bacteria, actinomycetes, cyanobacteria, and fungi are known for degradation of lignin; however, the level of degradation varies with microorganisms. The degradation and transformation of lignocellulosic wastes is attributed to the metabolism of indigenous microorganisms. Different microbial population dominates at various stages and has distinct roles in degradation of organic matter (Belyaeva and Haynes 2009).

Lignin-Degrading Bacteria

The degradation of wood cell wall by bacteria was not ascertained until the 1980s (Li et al. 2009). Bacteria generally degrade wood slowly, and degradation takes place on wood surfaces with high moisture content. Because of lack of penetrating ability, bacteria usually invade wood cells simultaneously with fungi. They appear to attack both softwoods and hardwoods by first colonizing the

Table 1.1 The contents of cellulose, hemicellulose, and lignin in some wood and common agricultural residues and wastes

	% dry weight		
Lignocellulosic materials	Cellulose	Hemicellulose	Lignin
Hardwoods stems			
Aspen	50.0	28.0	15.0
Beech	47.0	20.0	23.0
Birch	41.0	26.0	25.0
Cottonwood	46.0	19.0	24.0
Oak	48.0	18.0	28.0
Poplar	45.0	19.0	20.0
Red Maple	39.0	33.0	23.0
Softwood stems			
Douglas fir	57.0	8.0	24.0
Eastern hemlock	43.0	10.0	32.0
Jack pine	41.0	10.0	27.0
White pine	44.0	11.0	28.0
Red spruce	43.0	12.0	27.0
White spruce	44.0	10.0	27.0
Agricultural residues			
Bagasse	33.0	30.0	29.0
Barley straw	40.0	20.0	15.0
Corn cob	42.0	39.0	14.0
Cotton stalks	42.0	12.0	15.0
Groundnut shells	38.0	36.0	16.0
Oat straw	41.0	16.0	11.0
Rice straw	32.0	24.0	13.0
Rye straw	37.0	30.0	19.0
Wheat straw	30.0	24.0	18.0
Cotton seed hairs	80–95	5.0–20.0	0
Others			
Grasses	25.0–40.0	35.0–50.0	10.0–30.0
Paper	85.0–99.0	0.0	0–15.0
Sorted refuse	60.0	20.0	20.0
Leaves	15.0–20.0	80.0–85.0	0
Newspaper	40.0–55.0	25.0–40.0	18.0–30.0
Waste papers from chemical pulps	60.0–70.0	10.0–20.0	5.0–10.0
Primary wastewater solids	8.0–15.0	NA	24.0–29.0
Swine waste	6.0	28	NA
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25.0	35.7	6.4
Switch grass	45.0	31.4	12.0

parenchyma cells. After utilizing the cell contents, they may also attack the parenchyma cell walls (Liese 1970). They move into adjacent cells and tracheids with fast disruption of pits (Levy 1975). Different patterns of cell wall decay, cavitation, and tunneling by bacteria have been found both in natural and laboratory environments (Nilsson and Daniel 1983; Daniel et al. 1987).

In contrast, studies in some laboratories have shown that bacteria are unable to degrade lignified

plant cell walls (Schmidt et al. 1987), but they were able to do so after chemical pretreatment of the cells. Efficient bacterial degradation of wood already treated with cellulase-less mutants of *P. chrysosporium* and *Phlebia gigantea* has been observed.

Although bacteria can directly attack fibers, vessels, and tracheids, few species or strains can degrade all the cell wall components (Eriksson et al. 1990). However, some bacteria have been found to degrade lignified wood cells, which were confirmed by ultrastructural investigations (Zimmermann 1990). Recently, cell wall erosion (Sutherland et al. 1979), tunnel formation (Daniel et al. 1987), and removal of lignin by bacteria have also been reported (Krause et al. 2003). Recently, *Paenibacillus* sp. (AY952466), *Aneurinibacillus aneurinilyticus* (AY856831), and *Bacillus* sp. (AY952465) for ITRC S6, IITRC S7, and ITRC S8, respectively, were found capable to effectively degrade the kraft lignin, a major by-product of the chemical pulping process and main contributor to the color and toxicity of effluent (Chandra et al. 2007).

The enzyme produced by bacteria can catalyze the cleavage of interunit linkage of model lignin dimers (Archana and Mahadevan 2002; Li et al. 2009). The contribution of bacteria to the complete biodegradation of lignin in natural environment where fungi are also present is not much known. However, bacteria seem to play a leading role in decomposing lignin in aquatic ecosystem, because wood-degrading bacteria have a wider tolerance of temperature, pH, and oxygen limitations than fungi (Chandra et al. 2007). Molecular evidence for occurrence of lignin-degrading enzymes has been found in *Mycobacterium tuberculosum*, *M. avium, Escherichia coli*, *Caulobacter crescentus*, *Pseudomonas syringae*, *P. aeruginosa, P. putida, Bordetella pertussis*, *Xanthomonas campestris*, *Rhodobacter capsulatus*, *Yersinia pestis*, *Campylobacter jejuni,* and *Aquifex aeolicus* (Alexandre and Zhulin 2000).

Hungate (1966) discussed early examples in which fibrolytic bacteria dosed in to rumen had little effect on rumen digestion. Rumen bacteria are major degraders of plant fiber cell walls by production of enzymes active against structural components of these cell walls (Akin 1993a; Kuhad et al. 1997; Krause et al. 2003). Some of the most extensively studied rumen bacteria include *Fibrobacter succinogenes, Ruminococcus albus,* and *R. flavefaciens*. These bacteria have a complete set of polysaccharide-degrading enzymes and also the ability to adhere to fibers (Stewart and Bryant 1988; Akin 1993a). These species adhere strongly to partially degraded cell walls but erode the components only if they are adjacent and in direct contact with the bacteria (Krause et al. 2003). Often, the plant cell walls are totally degraded, but at other times digestion seems to be interrupted before the hydrolysis is completed. Very recently the emerging role for bacteria in lignin degradation and bio-product formation has been reviewed and elaborated (Bugg et al. 2011). A range of soil bacteria, often aromatic-degrading bacteria, are able to break down lignin. The enzymology of bacterial lignin breakdown is currently not well understood, but extracellular peroxidase and laccase enzymes appear to be involved. There are also reports of aromatic-degrading bacteria isolated from termite guts, though there are conflicting reports on the ability of termite gut microorganisms to break down lignin.

In brief, bacterial strains identified to have activity for lignin breakdown fall into three classes: actinomycetes, α-proteobacteria, and γ-proteobacteria (Bugg et al. 2011). The activity of these lignin-degrading bacteria was much less than that of white-rot fungus *Phanerochaete chrysosporium* but was found comparable to some other lignin-degrading fungi. *P. putida* mt-2 and *R. jostii* RHA1 were found to break down lignocellulose, releasing low molecular weight phenolic products (Ahmad et al. 2010).

The possible involvement of bacteria in lignocellulose breakdown has also emerged from a different line of research. The gut microflora of termites helps to digest the lignocellulose content of wood, but the role of microorganisms in the degradation of lignin has been the subject of debate (Brune 2007). A recent study by Geib et al. (2008) has shown that depolymerization, demethylation, and ring hydroxylation of lignin

occur using gut microflora from *Anoplophora glabripennis* and *Zootermopsis angusticollis* and have suggested that the aerobic reactions required for lignin depolymerization could occur in the foregut, rather than in the largely anaerobic hindgut. The enzymology for bacterial lignin degradation is at present poorly understood, compared with the fungal lignin-degrading enzymes, yet there are indications that bacteria use similar types of extracellular lignin-degrading enzymes to fungi. Lignin-degrading *S. viridosporus* T7A produces several extracellular peroxidases, which have been shown to catalyze oxidative cleavage of β-aryl ether lignin model compounds (Ramachandra et al. 1988). There are also reports of bacterial laccases, which are copper-containing enzymes that utilize oxygen to oxidize a range of phenolic compounds. While the bulky nature and presence of nonphenolic subunits prohibits the action of laccases on the lignin polymer, they have been shown to depolymerize lignin via the oxidation of smaller molecules such as 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) and hydroxybenzotriazole (HBT) (Ten Have and Teunissen 2001; Sánchez 2009). In addition to their role in the degradation of lignin, the broad substrate specificity of laccases permits the modification of lignocelluloses and the potential to create new, environmentally friendly materials. The potential of bacteria in bioconversion of lignocellulosics may further be explored in the future.

Lignin-Degrading Actinomycetes

Actinomycetes are a group of eubacterial microorganisms, also called as actinobacteria, that are commonly found in the soil. The presence of actinomycetes in the soil is indisputable; what is not clear is their role in the ecology of the soil. It has been suggested that actinomycetes play a role in lignocellulose breakdown, but details of the scale and methods by which such breakdown takes place are less known. However, there is a wide range of examples where *Streptomyces* and other actinomycetes have been identified as degrading lignin or lignocellulose. These strains come from a wide variety of sources, including a range of soils, high temperature environments, and termite guts (Adhi et al. 1989; Iqbal et al. 1994; Ruttiman et al. 1998; Tuncer and Ball 2002; Watanabe et al. 2003). Different species of *Streptomyces* have been reported to colonize vessels, fibers, and parenchyma cells. *S. flavovirens* rapidly colonizes the phloem and degrades parenchyma cells as well as thick-walled highly lignified sclereids. In advanced stages of degradation, parenchyma cells were found to be completely destroyed, and sclereids showed evidence of eroded cell walls. Various studies have established that several actinomycetes attack grass lignocellulosics, leading to the partial solubilization of the substrate rather than mineralization (Kuhad et al. 1997).

A wide range of actinomycetes have been shown to possess extracellular peroxidase activity, though the activity was variable (Mercer et al. 1996). Laccase type enzymes have now been found in five actinomycetes: *Streptomyces antibioticus* (Freeman et al. 1993), *S. griseus, S. coelicolor* (Endo et al. 2003a, b), *S. cyaneus* (Arias et al. 2003), and *S. lavendulae* (Suzuki et al. 2003). However, it is not clear how widespread they are in the actinomycetes because there is indirect evidence for the presence of laccases in the actinomycetes, which is based on rather nonspecific substrate reactions (Sjoblad and Bollag 1981). Thus, with the availability of 17 complete genome sequences and six partially completed genome sequences from the actinobacteria, the possibility of identifying candidate laccases from the actinomycetes is possible using a bioinformatics-based approach. However, these sequenced genomes include only one thermophile, *Thermobifida fusca,* which has not been completely annotated (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi; last accessed 4 January 2005).

Peroxidase and polyphenol oxidase activity have been compared between the thermophilic *Streptomyces* isolates and mesophilic *Streptomyces* (Mhlanga 2001). Polyphenol oxidase activity between the two groups was comparable, and peroxidase activity was significantly higher for the thermophiles than for the mesophiles.

Table 1.2 Types of wood-rotting fungi and some characteristics

Type	Family to which wood rot belongs	Texture of wood after attack by wood rot	Components of wood degraded
Soft rot	Ascomycetes and Deuteromycetes	Wood remains wet, spongy, pitted, and brownish. Usually grows on surface of wood and is least specialized among wood rots	Prefer carbohydrates and modify lignins to a limited extent
Brown rot	Basidiomycetes	Wood remains fibrous and brownish	Primarily carbohydrates degraded, leaving behind brownish modified lignin but does not degrade it
White rot	Basidiomycetes and Ascomycetes	Wood remains fibrous and whitish as cellulose mainly is left behind	All components of wood degraded

RU-A01, RU-A03, and RU-A06 also have an advantage over the mesophilic *Streptomyces* strains because they produce lignin peroxidases also found among some of the white-rot fungi and are known to play a role in lignin solubilization (Ball et al. 1989). Peroxidase and polyphenol oxidase extracts from the thermophilic isolates react the same way as the peroxidases and polyphenol oxidases from the mesophilic *Streptomyces* sp. toward the various substrates. In a recent study Huang et al. (2010) demonstrated the change in microbial population while degrading lignin during composting process through quinine profiling and concluded that high lignin degradation at cooling stage might be attributed to the cooperation of mesophilic fungi, actinomycetes, and bacteria.

Lignin-Degrading Fungi

Wood-Rotting Fungi

By colonizing dead or dying tree trunks and stumps, the fungus preferentially utilizes one or the other cell wall constituents, resulting in the wood decay known as wood rot. These include three types of wood rots, i.e., soft rot, brown rot, and white rot, which are based on the component utilized and the color characteristics of the decayed wood (Table 1.2). Among them, only white rots have the potential to completely degrade all three major components of wood, thus making them ecologically most important to study their detailed diversity. These fungi mainly belong to Ascomycetes, Deuteromycetes, or Basidiomycetes group (Dashtban et al. 2009).

Table 1.2 represents diversity of wood-decaying fungi and their ligninolytic system. Soft rot results in degradation of cellulose and hemicellulose, but lignin may only be partially digested. Little is known about the degradation mechanism of lignocellulose by soft-rot fungi in contrast to white- and brown-rot fungi. However, it is clear that some soft-rot fungi can degrade lignin because they erode the secondary cell wall and decreases the content of acid-soluble material (Klason) in angiosperm wood (Sánchez 2009). Ascomycetes and Deuteromycetes (fungi imperfecti) generally cause soft-rot decay of wood (Blanchette 1995). Two forms of soft rot have been described, type I consisting of biconical or cylindrical cavities that are formed within secondary walls while type II refers to an erosion form of degradation (Blanchette 1995).

In contrast to nonselective white-rot fungi, the middle lamella is not attacked by type II soft-rot fungi. Xylariaceous ascomycetes from genera such as Daldinia, Hypoxylon, and Xylaria have earlier often been regarded as white-rot fungi, but nowadays, these fungi are grouped in soft-rot fungi since they cause typical type II soft rot. Ligninolytic peroxidases or laccases of soft-rot fungi may not have the oxidative potential to attack the recalcitrant guaiacyl lignin. On the other hand, syringyl lignin apparently is readily oxidized and mineralized by the enzymes of soft-rot fungi (Nilsson et al. 1989). Soft-rot fungi are particularly prevalent at the early stages of wood

decay and in conditions of high moisture and increased nitrogen content (Blanchette 1995). Wood affected by soft rot may appear wet, spongy, or pitted. There are over 300 species of known soft rots (Kuhad and Singh 1993). Soft rots are either the members of Ascomycetes or Deuteromycetes (Kuhad et al. 1997) or some genera falling under this category are *Chaetomium*, *Paecilomyces,* and *Fusarium* (Rayner and Boddy 1988; Eriksson et al. 1990; Blanchette 1995). Ascomycetes are known to degrade lignin and are responsible for wood decay referred to as "soft rot," a process that is not well understood (Shary et al. 2007).

The fungi capable of degrading cellulose and hemicellulose but are unable to digest the lignin component of wood belong to brown-rot category (Sánchez 2009). In this case, the lignin remains more or less intact and becomes modified with brown and crumbly matrix appearance (Eriksson et al. 1990; Blanchette 1995; Highley and Dashek 1998). Brown-rot fungi mainly decompose the cellulose and hemicellulose components in wood (Eriksson et al. 1990). They have been much less investigated than white-rot fungi in spite of their enormous economic importance in the destruction of wood. Brown-rotted wood is dark, shrinks, and is typically broken into brick-shaped or cubical fragments that easily break down into brown powder (Blanchette 1995). The brown color indicates the presence of modified lignin in wood. Unlike the soft rots, the brown-rot fungi are relatively few in number, comprising only 6% of all wood-rotting Basidiomycetes (Gilbertson 1980; Rayner and Boddy 1988; Kuhad et al. 1997).

Brown-rot fungi produce low molecular weight chelators which are able to penetrate in to the cell wall (Dashtban et al. 2009). *Poria* sp., *Polyporus* sp., *Coprinus* sp., *Gloeophyllum trabeum, Laetiporus sulfurous, Wolfiporia cocos, Piptoporus betulinus,* and *Fomitopsis* sp. are some examples of brown-rot fungi (Buswell and Odier 1987; Rayner and Boddy 1988; Eriksson et al. 1990; Straatsma et al. 1994; Blanchette 1995; Dix and Webster 1995; Machuca and Ferraz 2001; Valaskova and Baldrian 2006; Dashtban et al. 2009; Deswal et al. 2011). The fungal hyphae penetrate from one cell to another through existing pores in wood cell walls early in the decay process. The penetration starts from the cell lumen where the hyphae are in close connection with the S3 layer. In brown rot, the decay process is thought to affect the S2 layer of the wood cell wall first (Eriksson et al. 1990). Brown-rot fungi have a unique mechanism to break down wood polysaccharides. In contrast to white-rot fungi that successively depolymerize cell wall carbohydrates only to the extent that they utilize hydrolysis products in fungal metabolism, brown-rot fungi rapidly depolymerize cellulose and hemicellulose, and degradation products accumulate, since the fungus does not use all the products in the metabolism (Cowling 1961). To some extent, brown-rot fungi have similar degradative capabilities and pathways as white-rot fungi. Both wood-decay mechanisms rely on radical formation, low pH, and the production of organic acids. They cause increased alkali solubility of lignin, and the decay is enhanced by high oxygen tension, all of which indicate a crucial involvement of radicals, especially in the early stages of decay.

White-rot fungi are capable of degrading all the major components of wood (cellulose, hemicellulose, and lignin) (Sánchez 2009). The fungi causing white rots mostly belong to Basidiomycetes (Table 1.3), but some Ascomycetous fungi have been also identified to result wood decay (Eriksson et al. 1990) such as *Xylaria hypoxylon* and *X. polymorpha* commonly known as candle snuff fungus and dead man's finger, respectively, (Deacon 1997); *Ustulina vulgaris* (Kuhad et al. 1997) have been reported to aid in lignin degradation.

White-rot fungi are a heterogeneous group of fungi classified in the Basidiomycota. Different white-rot fungi vary considerably in the relative rates at which they attack lignin and carbohydrates in woody tissues. Some remove lignin more readily than carbohydrates, relative to the original amount of each (Blanchette 1995). Many white-rot fungi colonize cell lumina and cause cell wall erosion. Eroded zones increase as decay progresses, and large voids filled with mycelium are formed. This type of rot is referred to as

Table 1.3 Diversity of wood-decay fungi and their ligninolytic enzymes

S. No.	Wood-decaying fungus	Family	Enzyme(s)	References
1.	*Abortiporus biennis*	Meripilaceae	MnP/Lac	Aggelis et al. (2002)
2.	*Agaricus bisporus*	Agaricaceae	MnP/Lac	Bonnen et al. (1994)
3.	*Agrocybe praecox*	Bolbitiaceae	MnP/Lac	Steffen et al. (2002b)
4.	*Armillaria mellea* (honey fungus, bootlace fungus)	Marasmiaceae	MnP/Lac	Robene-Soustrade et al. (1992)
5.	*Armillaria ostoyae*	Marasmiaceae	MnP/Lac	Robene-Soustrade et al. (1992)
6.	*Auricularia* sp. (wood ear, Jew's-ear)	Auriculariaceae	MnP/Lac	Hofrichter and Fritsche (1997) and Saparrat et al. (2002),
7.	*Bjerkandera adusta*	Hapalopilaceae	LiP/MnP	Pickard et al. (1999), Moreira et al. (2000a, b), and Romero et al. (2007)
8.	*Ceriporiopsis subvermispora*	Hapalopilaceae	MnP/Lac	Lobos et al. (1994), Okano et al. (2005), Mendonça et al. (2008) and Arora et al. (2011)
9.	*Cerrena unicolor*	Polyporaceae	Lac	Gianfreda et al. (1998)
10.	*Cerrena maxima*	Polyporaceae	Lac	Koroleva et al. (2002a) and Elisashvili et al. (2008)
11.	*Chrysonilia sitophila*	–	LiP/MnP	Ferrer et al. (1992)
12.	*Clitocybula dusenii*	Tricholomataceae	MnP/Lac	Nuske et al. (2002)
13.	*Collybia dryophila*	Tricholomataceae	MnP/Lac	Steffen et al. (2002a)
14.	*Coriolopsis gallica*	Polyporaceae	Lac	Pickard et al. (1999)
15.	*Coriolopsis occidentalis*	Polyporaceae	LiP	Nerud and Misurcova (1989)
16.	*Coriolopsis polyzona*	Polyporaceae	MnP/Lac	Nerud et al. (1991) and Elisashvili et al. (2008)
17.	*Coriolopsis rigida*	Polyporaceae	MnP/Lac	Capeleri and Zadrazil (1997) and Saparrat et al. (2002)
18.	*Cyathus bulleri*	Nidulariaceae	LiP/MnP/Lac	Dhawan and Kuhad (2002)
19.	*Cyathus stercoreus*	Nidulariaceae	MnP/Lac	Orth et al. (1993) and Dhawan and Kuhad (2002)
20.	*Daedalea flavida*	–	Lac	Arora and Gill (2001) and, Arora et al. (2002)
21.	*Daedaleopsis confragosa*	Polyporaceae	LiP/MnP	De Jong et al. (1992)
22.	*Dichomitus squalens* (red rot)	Polyporaceae	MnP/Lac	Nerud et al. (1991), Orth et al. (1993) and Arora et al. (2002)
23.	*Fomes fomentarius*	Polyporaceae	LiP	Varela et al. (2000)
24.	*Fomes lignosus*	Polyporaceae	LiP	Huoponen et al. (1990) and Waldner et al. (1988)
25.	*Fomes sclerodermeus*	Polyporaceae	MnP/Lac	Papinutti et al. (2003)
26.	*Ganoderma applanatum*	Ganodermataceae	LiP	Varela et al. (2000)
27.	*Ganoderma australe*	Ganodermataceae	LiP	Varela et al. (2000) and Mendonça et al. (2008)
28.	*Ganoderma lucidum*	Ganodermataceae	LiP/MnP/Lac	Orth et al. (1993), D'Souza et al. (1999), and Han et al. (2005)
29.	*Ganoderma valesiacum*	Ganodermataceae	MnP/Lac	Nerud et al. (1991)
30.	*Grifola frondosa*	Meripilaceae	MnP/Lac	Orth et al. (1993)
31.	*Inonotus hispidus*	Hymenochaetaceae	MnP/Lac	Aggelis et al. (2002)
32.	*Irpex lacteus*	Steccherinaceae	LiP/MnP/Lac	Novotny et al. (2000)

(continued)

Table 1.3 (continued)

S. No.	Wood-decaying fungus	Family	Enzyme(s)	References
33.	*Junghuhnia separabilima*	–	LiP/Lac	Vares et al. (1992)
34.	*Lentinula edodes* (shiitake)	Marasmiaceae	MnP/Lac	Orth et al. (1993), Hatvani and Mecs (2001), Nagai et al. (2003) and Okano et al. (2005)
35.	*Lentinus degener*	Polyporaceae	LiP	Varela et al. (2000)
36.	*Lentinus tigrinus*	Polyporaceae	MnP/Lac	Moreira et al. (2000c) and Aggelis et al. (2002)
37.	*Marasmius quercophilus*	Tricholomataceae	MnP/Lac	Tagger et al. (1998) and Klonowska et al. (2001)
38.	*Mycena galopus*	Tricholomataceae	MnP/Lac	Ghosh et al. (2003)
39.	*Nematoloma frowardii*	Strophariaceae	LiP/MnP/Lac	Hofrichter et al. (1999b) and Nuske et al. (2002)
40.	*Oudemansiella radicata*	Marasmiaceae	LiP/Lac	Hüttermann et al. (1989)
41.	*Panaeolus sphinctrinus*	Bolbitiaceae	MnP/Lac	Heinzkill et al. (1998)
42.	*Panellus stipticus*	Tricholomataceae	MnP/Lac	Aggelis et al. (2002)
43.	*Panus tigrinus*	Tricholomataceae	MnP/Lac	Maltseva et al. (1991) and Fenice et al. (2003)
44.	*Peniophora gigantea*	Peniophoraceae	LiP	Varela et al. (2000)
45.	*Perenniporia medulla-panis*	Polyporaceae	MnP	Orth et al. (1993)
46.	*Phanerochaete sordida*	Phanerochaetaceae	MnP	Moreira et al. (2000c)
47.	*Phanerochaete chrysosporium*	Phanerochaetaceae	LiP/MnP	Glenn et al. (1983), Tien and Kirk (1983), Glenn and Gold (1985), Schoemaker and Leisola (1990), Kaal et al. (1995), Gill and Arora (2003), Kuhar et al. (2008) and Zeng et al. (2010)
48.	*Phanerochaete flavido-alba*	Phanerochaetaceae	LiP/MnP	Hamman et al. (1999) and Varela et al. (2000)
49.	*Phellinus gilvus*	Hymenochaetaceae	MnP/Lac	Capeleri and Zadrazil (1997) and Saparrat et al. (2002)
50.	*Phellinus igniarius*	Hymenochaetaceae	Lac	Szklarz et al. (1989)
51.	*Phellinus pini*	Hymenochaetaceae	LiP/MnP	Blanchette et al. (1989) and Bonnarme and Jeffries (1990)
52.	*Phlebia brevispora*	Meruliaceae	LiP/MnP/Lac	Ruttimann et al. (1992), Arora and Rampal (2002) and Arora et al. (2011)
53.	*Phlebia fascicularia*	Meruliaceae	Lac	Arora and Gill (2001), Arora and Rampal (2002), Arora et al. (2002) and Arora et al. (2011)
54.	*Phlebia floridensis*	Meruliaceae	Lac	Arora and Gill (2001), Arora and Rampal (2002), Arora et al. (2002) and Arora et al. (2011)
55.	*Phlebia ochraceofulva*	Meruliaceae	LiP/Lac	Vares et al. (1993)
56.	*Phlebia radiata*	Meruliaceae	LiP/MnP/Lac	Hatakka et al. (1987), Saloheimo et al. (1989), Niku-Paavola et al. (1990), Moreira et al. (2000a, c), Arora and Gill (2001), Arora and Rampal (2002) and Arora et al. (2011)
57.	*Phlebia subserialis*	Meruliaceae	MnP	Bonnarme and Jeffries (1990)
58.	*Phlebia tremellosa*	Meruliaceae	LiP/MnP/Lac	Ralph et al. (1996), Vares et al. (1994) and Robinson et al. (2001)

(continued)

Table 1.3 (continued)

S. No.	Wood-decaying fungus	Family	Enzyme(s)	References
59.	*Pholiota aegerita*	Bolbitiaceae	Lac	Von Hunolstein et al. (1986)
60.	*Pleurotus eryngii*	Pleurotaceae	LiP/MnP	Orth et al. (1993), Munoz et al. (1997a, b), Heinfling et al. (1998) and Okano et al. (2005)
61.	*Pleurotus florida*	Pleurotaceae	LiP/Lac	Hüttermann et al. (1989) and Das et al. (1999)
62.	*Pleurotus ostreatus* (oyster mushroom)	Pleurotaceae	LiP/MnP/Lac	Waldner et al. (1988), Palmieri et al. (1997), Reddy et al. (2003), Kurt and Buyukalaca (2010) and Shrivastava et al. (2011)
63.	*Pleurotus pulmonarius*	Pleurotaceae	MnP/Lac	Orth et al. (1993), D'Souza et al. (1996) and Valdez et al. (2008)
64.	*Pleurotus sajor-caju*	Pleurotaceae	LiP/MnP/Lac	Hatakka (1994), Chagas and Durrant (2001), Reddy et al. (2003) and Kurt and Buyukalaca (2010)
65.	*Pleurotus sapidus*	Pleurotaceae	MnP/Lac	Orth et al. (1993)
66.	*Polyporus brumalis*	Polyporaceae	LiP/Lac	Hüttermann et al. (1989)
67.	*Polyporus ciliatus*	Polyporaceae	MnP/Lac	Moreira et al. (2000c)
68.	*Polyporus osteiformis* (brown-rot fungus)	Polyporaceae	LiP	Dey et al. (1994)
69.	*Polyporus pinsitus*	Polyporaceae	LiP/Lac	Hüttermann et al. (1989)
70.	*Polyporus platensis*	Polyporaceae	LiP/Lac	Hüttermann et al. (1989)
71.	*Polyporus varius*	Polyporaceae	LiP	Hüttermann et al. (1989)
72.	*Polyporus versicolor*	Polyporaceae	MnP	Orth et al. (1993)
73.	*Pycnoporus cinnabarinus*	Polyporaceae	Lac	Eggert et al. (1996), Lomascolo et al. (2002) and Kuhar et al. (2008)
74.	*Pycnoporus sanguineus*	Polyporaceae	LiP/MnP/Lac	Pointing et al. (2000), Lomascolo et al. (2002) and Capeleri and Zadrazil (1997)
75.	*Rigidoporus lignosus*	Meripilaceae	MnP/Lac	Galliano et al. (1991) and Cambria et al. (2000)
76.	*Stereum hirsutum*	Stereaceae	MnP/Lac	Nerud et al. (1991)
77.	*Stropharia coronilla*	Strophariaceae	MnP/Lac	Steffen et al. (2002b)
78.	*Stropharia rugosoannulata*	Strophariaceae	MnP/Lac	Steffen (2003)
79.	*Trametes cingulata*	Polyporaceae	MnP/Lac	Orth et al. (1993) and Tekere et al. (2001)
80.	*Trametes elegans*	Polyporaceae	MnP/Lac	Tekere et al. (2001)
81.	*Trametes gibbosa*	Polyporaceae	LiP/MnP/Lac	Nerud et al. (1991)
82.	*Trametes hirsuta* (hairy stereum)	Polyporaceae	LiP/MnP/Lac	Nerud et al. (1991) and Koroleva et al. (2002)
83.	*Trametes pocas*	Polyporaceae	MnP/Lac	Tekere et al. (2001)
84.	*Trametes trogii*	Polyporaceae	LiP/MnP/Lac	Levin et al. (2001, 2002)
85.	*Trametes versicolor* (turkey tail)	Polyporaceae	LiP/MnP/Lac	Blanchette et al. (1989), Huoponen et al. (1990), Black and Reddy (1991), Hatakka (1994), Couto et al. (2002), Gill and Arora (2003) and Shrivastava et al. (2011)
86.	*Trametes villosa*	Polyporaceae	MnP	De Jong et al. (1992) and Capeleri and Zadrazil (1997)
87.	*Volvariella volvacea*	Pluteaceae	Lac	Chen et al. (2004) and Akinyele et al. (2011)

nonselective or simultaneous rot (Blanchette 1995). Some white-rot fungi preferentially remove lignin without a substantial loss of cellulose and cause white-pocket or white-mottled type of rot, e.g., *Phellinus nigrolimitatus* (Blanchette 1995). There are fungi that are also able to produce both types of attack in the same wood (Eriksson et al. 1990). Typical examples of such fungi are *Ganoderma applanatum* and *Heterobasidion annosum*. Because selectively lignin degrading fungi are considered the most promising ones for applications in pulp and paper industry and animal feed development, the search for potent selective lignin degraders has attained a considerable interest. However, the ratio of lignin-hemicellulose-cellulose decayed by a selected fungus can differ enormously, and even different strains of the same species, e.g., of *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*, may behave differently on the same kind of wood. The lignin-degrading systems of these fungi are important to study since they are very efficient. *C. subvermispora* may be considered as a model fungus for selective lignin degradation.

Other Ascomycetes have also been described to produce laccase and other lignocellulolytic enzymes, which include *Rhizoctonia solani* (Wahleithner et al. 1996), *Aspergillus nidulans* (Clutterbuck 1972), *Podospora anserina, Neurospora crassa* (Tamaru and Inoue 1989), *Gaeumannomyces graminis* var. *tritici* (Edens et al. 1999), and *Trichoderma reesei* (Nieves et al. 1998). The role of Ascomycetous fungi in wood degradation has to be explored. Basidiomycotina attack either hardwood or softwood, while Ascomycotina probably degrade only hardwood (Kirk and Farrell 1987).

Overlapping habits have been found in the three main ecophysiological groups of fungi such as wood-decaying, mycorrhiza-forming, and litter-decomposing fungi (LDF). Wood-decay fungi such as *Hypholoma* sp. (*Nematoloma* sp.), *Pleurotus* sp., and *Armillaria* sp. are also capable of colonizing soil in contact with wood debris (Dix and Webster 1995). Other litter-decomposing fungi can also grow on straw (e.g., *Stropharia rugosoannulata*) that is favored only by wood-decay fungi (Haselwandter et al. 1990). *Volvariella volvacea* is a litter-decomposing fungus belonging to Pluteaceae family but secretes ligninolytic enzymes (laccase) (Chen et al. 2004). *Agaricus bisporus* is also LDF which secretes laccases and manganese peroxidases (Bonnen et al. 1994). The ability to break down lignin and cellulose enables some of the LDF to function as typical white-rot fungi in soil (Hofrichter 2002). Diversity of all these groups of fungi is broad and hence explains their importance in the carbon cycle (Dix and Webster 1995).

Lignin-Degrading Enzymes

Lignin does not contain hydrolysable linkages, which means that the enzymes must be oxidative in nature to degrade it. Lignin is stereoirregular, which also points to more nonspecific attack compared to many other natural polymers. Lignin degradation by white-rot fungi is an oxidative process, and primarily three enzymes, i.e., manganese peroxidase (MnP), E.C. 1.11.1.13; lignin peroxidase (LiP), E.C. 1.11.1.14; and laccases, E.C. 1.10.3.2, are majorly responsible for lignin degradation (Kuhad et al. 1997). However, there are many other enzymes participating in lignin degradation (Sánchez 2009) (Table 1.4). When wood-degrading fungi colonize wood, their extracellular enzymes are not able to diffuse into intact wood cell walls because the enzymes are too large to penetrate the pores of the wood cell walls. Hydroxyl radicals (•OH), a radical species highly destructive for cellulose and lignin, are proposed as a principal low molecular mass oxidant that erodes wood cell walls to enhance the accessibility of the extracellular enzymes of wood-rot fungi to wood cell wall components (Wood 1994).

LiP and MnP oxidize the substrate by two consecutive one-electron oxidation steps with intermediate cation radical formation. Laccase has broad substrate specificity and oxidizes phenols and lignin substructures with the formation of oxygen radicals. Other enzymes that participate in the lignin degradation processes are H_2O_2-producing enzymes and oxidoreductases, which can be located either intracellularly or produced extracellularly.

Table 1.4 Extracellular ligninolytic enzymes involved in lignin degradation

S. No.	Enzyme	Cofactor	Substrate/mediator	Main effect or reaction
1.	Lignin peroxidase (LiP)	H_2O_2	Veratryl alcohol	Aromatic ring oxidized to cation radical
2.	Manganese peroxidase (MnP)	H_2O_2 Mn^{2+}	Organic acids	Chelators, thiols and unsaturated lipids Mn^{2+} oxidized to Mn^{3+}; further oxidation of phenolic compounds to phenoxy radicals
3.	Laccase (Lac)	O_2	Hydroxybenzotriazole, ABTS	Phenols are oxidized to phenoxyl radicals; mediator radicals
4.	Versatile peroxidases (VP)		H_2O_2	Same or similar compounds as LiP and MnP. Same effect on aromatic and phenolic compounds as LiP and MnP
5.	Aryl alcohol oxidase (AAO)	–	Aromatic alcohols (anisyl, veratryl alcohol)	O_2 reduced to H_2O_2
6.	Cellobiose quinone 1-oxidoreductase (CBQ)	–	–	Cellobiose reduced to o- and p-quinones

The accessibility of cell wall polysaccharides from the plant to microbial enzymes is dictated by the degree to which they are associated with phenolic polymers (Chesson 1981; Kuhad et al. 1997). Several white-rot fungi such as *P. chrysosporium, P. ostreatus, T. versicolor, C. subvermispora,* and *P. cinnabarinus* have been studied in more detail for their ligninolytic system (Table 1.3). Some strains of white-rot fungi produce all the three well-characterized enzymes, while others produce either two or one of them. For details, please see Table 1.3. The model fungus for lignin degradation is *P. chrysosporium;* however, recently certain other lignin-degrading fungi have also been studied for lignin degradation, viz., C. *subvermispora*, *P. radiata*, *P. eryngii, C. bulleri, P. ostreatus, and T. versicolor* (Kirk 1990; Lundell 1993; Martinez et al. 1994; Hatakka 2001; Vasdev et al. 2005; Okano et al. 2009; Shrivastava et al. 2011).

Lignin-degrading enzymes are produced during the secondary metabolism of white rots. Since lignin oxidation does not provide net energy to the fungus, synthesis and secretion of these enzymes is often induced by limited nutrient levels (mostly C or N). Production of LiP and MnP is generally optimal at high oxygen tension but is repressed by agitation in submerged liquid culture, while laccase production is often repressed by agitation; however, it varies from fungus to fungus (Vasdev and Kuhad 1994). Production of laccase by solid-state fermentation has been reported for *P. cinnabarinus*, which was grown on sugarcane bagasse packed in 250-mL columns (Lomascola et al. 2003). Banana waste can also be used as a substrate for laccase production by *Aspergillus* sp. (Shah et al. 2005). *P. sanguineus* produced laccase on three more substrates under SSF conditions which are sago "hampas," rubberwood sawdust, and oil palm from parenchyma tissue (OPFPt) (Vikineswary et al. 2006). More recently production of LiP, MnP, and laccase has been reported using various substrates and organisms, i.e., *P. ostreatus* on wheat straw; *Aspergillus sclerotiorum*, *Cladosporium cladosporioides,* and *Mucor racemosus* in salinity conditions with wheat bran; and *P. chrysosporium* on compost (Robertson et al. 2008; Bonugli-Santos et al. 2010; Zeng et al. 2010). The ligninolytic enzymes have varied properties, and their discussion will be beyond the scope of this chapter; however, some properties are given in Table 1.5.

Lignin-degrading enzymes are essential for lignin degradation; however, for lignin mineralization, the role of some additional enzymes is equally significant. Such auxiliary enzymes (by themselves unable to degrade lignin) are glyoxal oxidase and superoxide dismutase for

Table 1.5 Some properties of MnP, LiP, and Laccase

E.C. No:	MnP 1.11.1.13	LiP 1.11.1.14	Lac 1.10.3.2
Cofactor	Mn(II): H_2O_2	Diarylpropan O_2, H_2O_2	p-Benzendiol: O_2-
Class	Oxidoreductases	Oxidoreductases	Oxidoreductases
Prosthetic group	Heme	Heme	type 1 Cu, type 2 Cu, type 3 Cu
MW (kDa)	32–62.5 (122)	38–47	59–110 (tetramers V 390c)
Glycosylation	N-type	N-type	N-type
Isoforms	Monomers; up to 11	Monomers; up to 15	Mono-, di-, tetramers; several
p*I*	2.8–7.2	3.2–4.7	2.6–4.5
pH range	2.6–4.5	2.0–5.0	2.0–8.5
E° (mV)	1,510	1,450	500–800
C–C cleavage	Yes	Yes	No
H_2O_2 regulated	Yes	Yes	No
Stability	+ + +	+	+ + +
Native mediators	Mn^{2+}; Mn^{3+} Mn^{2+}	VA, 2Cl-14DMB	3-HAA
Specificity	Mn^{2+}	Broad, aromatics, including nonphenolics	Broad, phenolics
Secondary and synthetic mediators	Thiols, unsaturated fatty acids	No	ABTS, HBT, syringaldazine

Modified from Fakoussa and Hofrichter (1999)
ABTS 2′,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate), *VA* veratryl alcohol, *HAA* 3-hydroxy-antranilic acid, *DMB* 2,6-dimethyl-1,4-benzoquinone, *HBT* 1-hydroxybenzotriazole

intracellular production of H_2O_2, a cosubstrate of LiP and MnP, as well as glucose oxidase, aryl alcohol oxidase, and cellobiose dehydrogenase (CDH) involved in feedback circuits and linking ligninolysis with cellulose and hemicellulose degradation in nature (Leonowicz et al. 1999). Lignin has been found to be partly mineralized in cell-free system of lignin-degrading enzyme, with considerably enhanced rates in the presence of co-oxidants such as fatty acids or thiols (Hofrichter et al. 1998).

Lignin Peroxidase (LiP)

LiP (EC 1.11.1.14) is an extracellular heme-containing peroxidase which is dependent on H_2O_2 and has an unusually high redox potential and low optimum pH (Bonugli-Santos et al. 2010), typically showing little specificity toward substrates and degrades a variety of lignin-related compounds (Barr and Aust 1994). It has molecular masses between 38 and 47 kDa (Table 1.5) (Tien et al. 1986). LiP is well known as part of the ligninolytic system both of aphyllophoralic and agaricalic fungi (Glenn et al. 1983; Hatakka et al. 1987; Hofrichter and Fritsche 1997). Lignin peroxidases (LiP) of *P. chrysosporium* are encoded by a family of six closely related genes (Stewart et al. 1992). More than ten heme proteins displaying ligninolytic activity have been detected in the extracellular fluid of cultures of *P. chrysosporium* BKM-F-1767, and they are designated H1–H10 (Rothschild et al. 1997)

LiP preferably oxidizes methoxylated aromatic ring without a free phenolic group, such as the model compound dimethoxybenzene (Kersten et al. 1990). Thus, the cleavage of Cα–Cα bonds is catalyzed preferentially in dimeric nonphenolic lignin model compounds (Kuhad et al. 1997). LiP oxidizes target substrates by two one-electron oxidation steps with intermediate cation radical formation (Sánchez 2009; Dashtban et al. 2010) (Fig. 1.1). The simplest aromatic substrates for LiP are methoxylated benzenes and benzyl alcohols, which have been used extensively by enzymologists to study LiP reaction mechanisms. The H_2O_2-dependent oxidation of veratryl alcohol (3, 4-dimethoxybenzyl alcohol) to veratraldehyde is the basis for the standard assay used to detect LiP

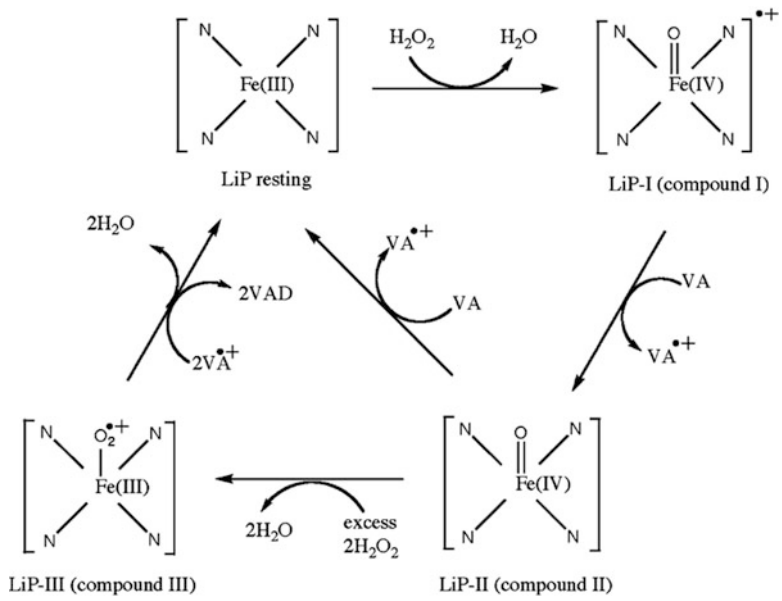

Fig. 1.1 Catalytic cycle of lignin peroxidase (Source: Wong 2009)

in fungal cultures (Kirk 1990). The role of LiP in ligninolysis could be further transformation of lignin fragments which are initially released by MnP. Studies have shown that LiP may not be essential for the attack on lignin, as several highly active WRF and litter-decaying fungi (e.g., *C. subvermispora, Dichomitus squalens, P. tigrinus, R. lignosus*) do not excrete this enzyme (Galliano et al. 1991; Maltseva et al. 1991; Périé and Gold 1991; Hatakka 1994). LiP has been used to mineralize a variety of recalcitrant aromatic compounds, such as three- and four-ring polyaromatic hydrocarbons, polychlorinated biphenyls, and dyes (Chivukula et al. 1995; Gunther et al. 1998; Krcmar and Ulrich 1998). 2-Chloro-1, 4-dimethoxybenzene, a natural metabolite of white-rot fungi, is reported to act as a redox mediator in the LiP-catalyzed oxidations (Teunissen and Field 1998).

Manganese Peroxidase (MnP)

MnP (EC 1.11.1.13), an extracellular heme-containing peroxidase with a requirement for Mn^{2+} as its reducing substrate, was first discovered shortly after LiP from *P. chrysosporium* by Kuwahara et al. (1984) and simultaneously described by Glenn and Gold (1985). MnP is one of the most common lignin-degrading peroxidases produced by the majority of wood-decaying fungi and by many litter-decomposing fungi (Hofrichter 2002). These are glycosylated proteins with an iron protoporphyrin IX (heme) prosthetic group (Glenn and Gold 1985). The molecular weights range between 32 and 62.5 kDa, and these are secreted in multiple isoforms (Table 1.5) (Urzúa et al. 1995; Hofrichter 2002; Boer et al. 2006). MnP oxidizes Mn^{2+} to Mn^{3+}, which in turn oxidizes phenolic structures to phenoxyl radicals (Gold et al. 1989). Mn^{3+} formed is highly reactive and complexes with chelating organic acids such as oxalate or malate (Cui and Dolphin 1990; Kishi et al. 1994), which are produced by *P. radiata* (Galkin et al. 1998; Hofrichter et al. 1999b). With the help of these chelators, Mn^{3+} ions are stabilized and can diffuse into materials such as wood. The redox potential of the MnP-Mn system is lower than that of LiP, and these preferably oxidizes phenolic substrates (Vares 1996). The phenoxyl radicals produced can further react with the eventual release of CO_2.

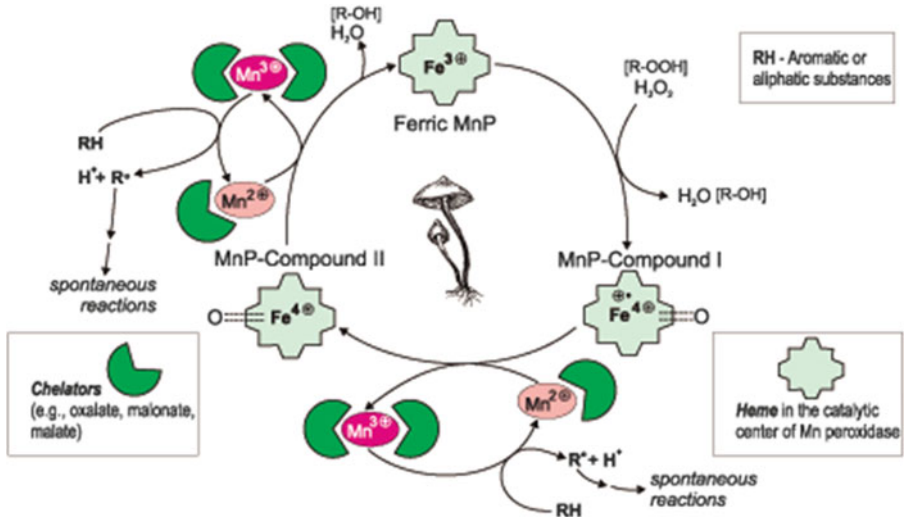

Fig. 1.2 The catalytic cycle of MnP (Redrawn from Wariishi et al. 1989; Hofrichter 2002)

The catalytic cycle of MnP is reviewed in detail by Kuhad et al. (1997), and is very similar to that of LiP, differing only in that compound II is readily reduced by Mn^{2+} to its native form (Fig. 1.2) (Wariishi et al. 1989; Sánchez 2009). The phenoxyl radicals formed subsequently cleave Cα-Cα or alkyl-phenyl bonds causing depolymerization to smaller intermediates including quinones and hydroxyl quinones (Kuhad et al. 1997). Purified or crude MnP has been used in cell-free systems (in vitro) and shown to oxidize not only lignin (Hofrichter et al. 1999a, 2001) but also chlorolignins (Lackner et al. 1991) and synthetic lignin compounds (Hofrichter et al. 1999b, c; Cullen and Kersten 2004).

Laccase

Laccases (EC 1.10.3.2) are widespread in nature, and in fungi they are implicated in pigmentation (Clutterbuck 1972; O'Hara and Timberlake 1989), fruit body formation (Leatham and Stahman 1981), and pathogenicity (Choi et al. 1992), as well as in lignin degradation (Sigoillot et al. 2005; Dashtban et al. 2010). Fungal laccases as part of the ligninolytic enzyme system are produced by almost all wood and litter-transforming Basidiomycetes and some Ascomycetes; however, their levels differ (Claus 2003). This group of N-glycosylated extracellular blue oxidases with molecular mass of 40–90 kDa contains four copper atoms in the active site that are distributed among different binding sites and are classified into three types with differential specific characteristic properties one type 1, one type 2, and two type 3 (Table 1.5) (Reinhammar 1984; Call and Mucke 1997; McGuirl and Dooley 1999; Claus 2003; Claus and Decker 2006). All these copper ions are apparently involved in the catalytic mechanism. Fungal laccases of different origin have shown some divergence in copper atom number and their spectrum character. Type 3 binuclear copper is absent in both L2 from *P. florida* and the laccase from *P. eryngii* (Munoz et al. 1997a, b), while type 1 copper is absent in laccase from *Phellinus ribis* and *P. ostreatus* (Palmieri et al. 1997).

Laccases catalyze the oxidation of a variety of aromatic hydrogen donors with concomitant reduction of oxygen to water (Fig. 1.3). Moreover, laccases do not only oxidize phenolic and methoxyphenolic acids but also decarboxylate them and attack their methoxy groups (demethylation). Until 1990, laccase had been considered to be able to degrade only phenolic compounds (Higuchi 1989). However, Bourbonnais and Paice (1990) reported first time that laccase can

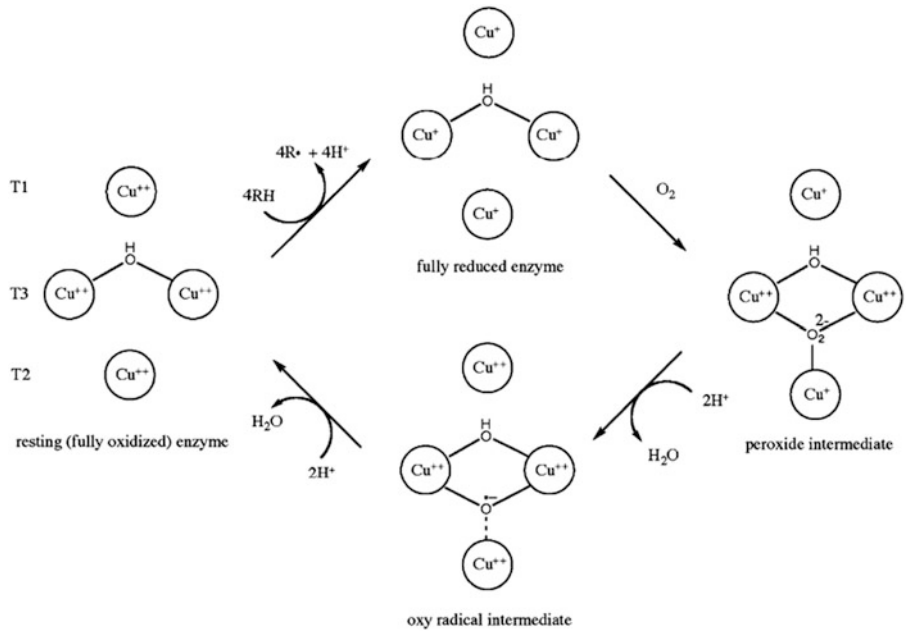

Fig. 1.3 Typical reaction of laccase (Source: Wong 2009)

also oxidize nonphenolic compounds in the presence of a suitable redox mediator such as ABTS 2′,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate). Recently, Riva (2006) has shown the laccase-catalyzed redox cycles for substrate oxidation in the presence and absence of mediators (Fig. 1.4).

Several fungal laccases have been compared for the oxidation of a nonphenolic lignin dimer, 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy) propan-1,3-diol (I), and a phenolic lignin model compound, phenol red, in the presence of the redox mediators 1-hydroxybenzotriazole (1-HBT) and violuric acid (Li et al. 1998). Laccases have been reported to oxidize many recalcitrant substances, such as chlorophenols (Ricotta et al. 1996; Grey et al. 1998; Fahr et al. 1999), polycyclic aromatic hydrocarbons (PAHs) (Majcherczyk et al. 1998; Duran and Esposito 2000; Pointing 2001), lignin-related structures (Bourbonnais et al. 1996), and organophosphorous compounds (Amitai et al. 1998; Couto and Herrera 2006), probably following one of the reactions shown in Fig. 1.4. Recently, laccase production has been successfully reported using distillery spent wash and lignocellulosic biomass by *Aspergillus heteromorphus* (Singh et al. 2010).

However, commercial application of laccase faces major obstacles such as the lack of sufficient enzyme stocks and the cost of redox mediators. Heterologous expression of the enzymes with protein engineering may allow for the cost-effective creation of more robust and active enzyme (Dashtban et al. 2010). The development of molecular techniques applied in the fungus has now allowed the identification of regulators of laccase by methods such as insertional mutagenesis and complementation of mutants (Sharma and Kuhad 2010).

Other Lignin-Degrading Enzymes

For complete decomposition and partial mineralization of plant lignocellulose, additional fungal enzymes are required. On the contrary to cellulose, lignin does not supply a carbon or energy source for the wood-decaying fungi (Kirk and Farrell 1987). Another important enzyme called versatile peroxidase (VP) has been recently recognized that can be regarded as hybrid between MnP and LiP, since it can oxidize not only Mn^{2+}

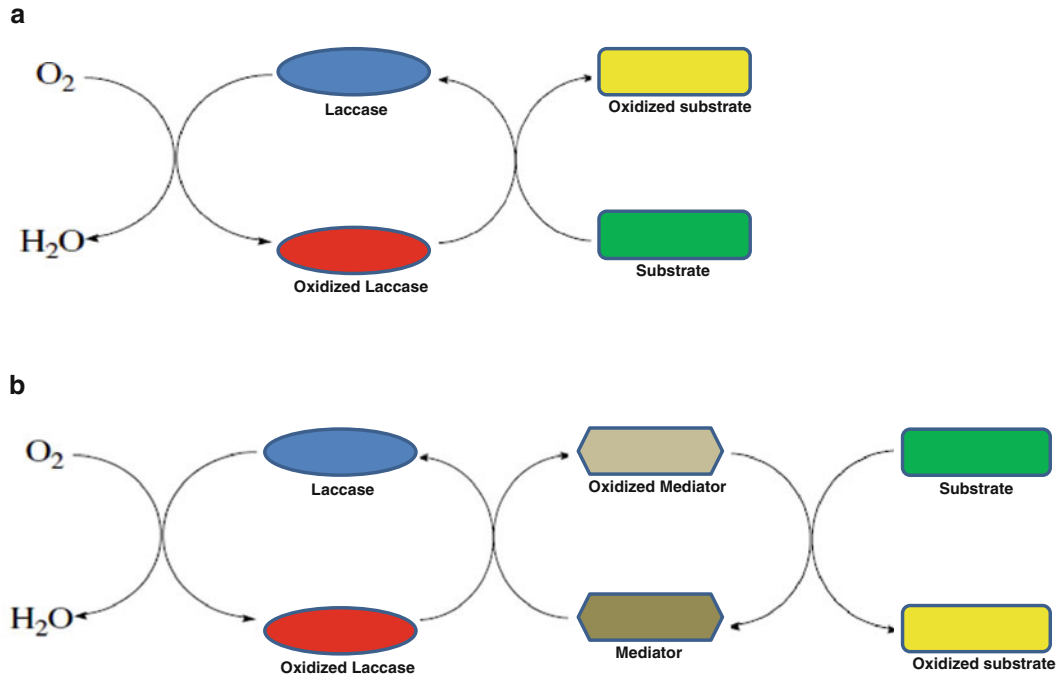

Fig. 1.4 Laccase-catalyzed redox cycles for substrates oxidation in the absence (**a**) or in the presence (**b**) of mediator (Modified from Kunamneni et al. 2008)

but also phenolic and nonphenolic aromatic compounds including dyes. It has been suggested that VPs can oxidize substrate spanning a wide range of potentials, including low- and high-range potentials (Dashtban et al. 2010). This is a result of their hybrid molecular structures which provide multiple binding sites for the substrates (Camarero et al. 1999). VP has been reported in species belonging to *Pleurotus* and *Bjerkandera* (Heinfling et al. 1998; Mester and Field 1998). White-rot fungi possess a variety of oxidative enzymes (Table 1.4), which are capable of generating H_2O_2 (required by peroxidases), through the oxidation of different substrates. Glyoxal oxidase (GLOX; EC 1.2.3.5) and aryl alcohol oxidase (AAO; EC 1.1.3.7) are both extracellular enzymes which were first described by Kersten and Kirk (1987) and later by others (Waldner et al. 1988; Muheim et al. 1990). They use either glyoxal or aromatic alcohols as substrate (Hatakka 2001). In particular AAO is involved in the selective degradation of lignin by *Pleurotus* species (Martinez et al. 1994).

Moreover, intracellular enzymes such as glucose oxidase and pyranose oxidase are also produced by various basidiomycetous fungi, which in turn make H_2O_2 available to the fungus for lignin degradation (Kuhad et al. 1997; Volc et al. 2001). For control of the organic acid metabolism, the mainly intracellular, Mn-containing enzymes oxalate decarboxylase (ODC, EC 4.1.1.2) and oxalate oxidase (EC 1.2.3.4), and the NAD^+-dependent enzyme formate dehydrogenase (EC 1.2.1.2) play important roles in the wood-decaying fungi (Micales 1997; Mäkelä et al. 2002, 2009). One fungal enzyme connecting cellulose decomposition to lignin decay is the extracellular cellobiose dehydrogenase (CDH, EC 1.1.99.18), of which the quinone-reducing cellobiose oxidase is a proteolytical cleavage product (Henriksson et al. 2000). This enzyme was early on described in, e.g., *P. chrysosporium,* and is able to use cellobiose as reducing substrate while donating electrons to quinones and oxidized phenolic intermediates, which may be formed during lignin decomposition (Bao et al. 1993).

Delignification of Lignocellulosic Materials and Their Bioconversion in Digestible and Nutritive Animal Feed

Lignocellulosic materials are the most abundant renewable organic compounds. Since they are major products from agriculture, forestry, urban refuge, and food wastes, tremendous quantities of inexpensive materials are potentially available for use as substrates in fermentation media (Akinyele et al. 2011). Crop residues constitute about 123×10^6 t year^{-1} of this renewable resource, containing approximately 60% cellulose and hemicellulose and 30% lignin on dry mass basis (Bhatnagar et al. 2008). The close association of hemicellulose and cellulose with lignin makes the carbohydrates of plant materials less accessible to microorganisms and enzymes.

Methods to remove lignin partially or completely would offer a means of increasing palatability, digestibility, and nutritional value of plant residues. Several physical treatments, such as pelleting, grinding, steaming, and irradiation as well as physical and chemical treatments, particularly use of ammonia fiber expansion and alkali, have been tested to improve the dry matter digestibility and intake of straw (Walker 1984; Sundstol and Owen 1984; Yadav and Tripathi 1991; Sirohi and Rai 1999; Abdullah et al. 2004; Kristensen et al. 2008; Bals et al. 2010). The practical use of the treatments is limited by safety concern, costs, and serious environmental concerns (Kuhar et al. 2008). The biological treatment may offer a practical and environment friendly alternative to nutritionally upgrade the low-quality roughage. Lignin is degraded to different extents by various microorganisms, of which wood-rot fungi are the most effective, white-rot fungi in particular (Kirk and Farrell 1987; Eriksson et al. 1990; Kuhad et al. 1997; Villas-Boas et al. 2002; Okano et al. 2005, 2009; Shrivastava et al. 2011). The common pattern of attack on plant material by white-rot fungi is simultaneous decay of polysaccharides (cellulose and hemicellulose) and lignin, but preferential degradation of lignin may also occur (Blanchette et al. 1992; Akin et al. 1993b; Kuhad et al. 1997; Eggert et al. 1998; Dhawan and Kuhad 2002; Okano et al. 2005; Kuhar et al. 2008; Shrivastava et al. 2011). Pattern and extent of lignin removal vary, however, for different fungal species and even for strains of a species. Some white-rot fungi selectively degrade lignin without affecting much of the carbohydrates, thus exposing protected and available carbohydrates, which is a prerequisite for animal feed development (Basu et al. 2002).

Considering the availability of the crop residues and grasses, considerable efforts have been made to upgrade their nutritive value (Flachowsky et al. 1999; Basu et al. 2002; Okano et al. 2005; Shrivastava et al. 2011).

Since more than a century, wood delignified by *Ganoderma australe* and other microorganisms was traditionally used as cattle feed in Southern Chile and is called Palo Podrido (Phillippi 1893). Moreover, most of the reported research has dealt with delignification of wood by white-rot fungi, of which *P. chrysosporium* is most studied (Eriksson et al. 1990; Kirk and Cullen 1998; Basu et al. 2002; Kumar and Gomes 2008; Gassara et al. 2010). However, many investigations have been carried out simultaneously to microbially delignify herbaceous plants biomass to improve the utilization of lignocellulosics by ruminating animals. In the similar context a great amount of work has been done at laboratory scale for the utilization of lignocellulosic residues for microbial protein and feed production using fungi such as *Chaetomium cellulolyticum, Aspergillus terreus, Trichoderma viride, Aspergillus niger, Sporotrichum pulverulentum (Phanerochaete chrysosporium), Fusarium oxysporum, T. koningii, Agrocybe aegerita, Ganoderma lucidum, Fusarium concolor, Volvariella volvacea, and Pleurotus ostreatus* (Moo-Young et al. 1979; Chahal et al. 1979; Ek and Eriksson 1975, 1980; Garg and Neelakantan 1982; Hatakka and Pirhonen 1985; Kahlon 1986; Singh et al. 1991; Zadrazil and Puniya 1995; Adamovic et al. 1998; Han et al. 2005; Li et al. 2008; Akinyele et al. 2011).

Research initiated in 1970 at the Central American Research Institute, Guatemala, on utilization of coffee wastes, was later evaluated

in a pilot plant with two 20,000 L fermenters (Aguirre et al. 1976). By growing two fungal strains, *Trichoderma harzianum* and a *Verticillium* sp. under non-sterile conditions, an average 62% reduction of COD of the waste was achieved. The dry mass yield was about 0.4 g g^{-1} carbohydrate, with a net increase of 3–3.5 g of cell mass L^{-1} attained after 24 h. Amino acid analysis of the product revealed a typical fungal protein profile, with limited methionine and high lysine content.

A process for a direct conversion of solid lignocellulosic wastes to protein by the white-rot fungus *Sporotrichum pulverulentum* (*P. chrysosporium*) was developed at the Swedish Pulp and Paper Research Institute (STFI) (Ek and Eriksson 1980). The fungus chosen for the STFI process is particularly well suited for the purpose of protein production from wood components since it produces enzymes for degradation of all the components in lignocellulosic materials. However, the STFI process was not found to be economically feasible on evaluation, since the protein produced could not compete on a cost basis with soybean protein, unless the substrate used in the fermentation had a negative value.

The STFI approach to protein production based on lignocellulosic material was thus changed to a water purification process, in which dissolved substances in the white water from mechanical pulp production were used as substrates (Eriksson et al. 1990). The water purification and protein production process were operated on a 25-m^3 scale using wastewater from a board factory (Ek and Eriksson 1980). The resulting protein was tested in feeding trials at the Swedish Agricultural University, Uppsala. The feeding trials were essentially positive with ruminants, but monogastric animals could not easily digest the fungal cell walls (Thomke et al. 1980). In spite of the positive feeding trials with ruminants, the production of fodder protein by S. *pulverulentum* (*P. chrysosporium*) was economically feasible only if the cost of alternative water purification is taken into account (Eriksson et al. 1990).

The "Waterloo" process for the production of microbial protein from waste biomass was developed at the University of Waterloo, Ontario, Canada (Moo-Young et al. 1979). The process is based on the cellulolytic fungus *Chaetomium cellulolyticum* and utilizes agricultural residues such as straw, corn stoves, bagasse, and forestry residues such as wood sawdust and pulp mill sludges (Moo-Young et al. 1978). The process uses a three-stage operation involving:

1. Thermal and/*or* Chemical Pretreatment of Lignocellulosic substrate
2. Aerobic Fermentation of the Pretreated Material with Nutrient Supplement
3. Recovery of the microbial protein product

The pretreated substrate is fed (2% w/v) continuously (dilution rate of 0.24 h^{-1}) into a 1,000-L fermenter operating at pH 5.5 and 37 °C. Recovery of fungal mycelium is accomplished by filtration, and the dried product contains about 45% crude protein. Nutrition trials conducted with rats, chicks, and piglets indicated that the product is satisfactory as an animal feed protein in terms of its safety, digestibility, and nutritive value (Touchburn et al. 1986). The process employs low-technology operations and efficient mass and energy exchanges between processing streams. Capital and operational expenditures required for the manufacture of fungal protein product are relatively small compared to those for other microbial protein production processes. This could allow for an economic operation of relatively small-size plants in medium-size villages in developing countries (Moo-Young et al. 1978). Agosin and his coworkers (Agosin et al. 1985) found that nonselective degradation of plants by *P. chrysosporium* resulted in small increase in in vitro digestibility by ruminating microorganisms, while other two fungi tested improved the digestibility by 63 and 94%.

A research group at NDRI, Karnal, India, attempted to study the effect of urea treatment and followed by fermentation of urea-pretreated wheat straw by a white-rot fungus, *Coprinus fimetarius* (now called as *C. cinereus*). This treatment, the so-called Karnal process, was characterized by a 30-day ensiling of rice straw sprayed with urea, inoculated with 30% *C. fimetarius,* and treated for 14 days at 35–45 °C (Gupta 1987). Fungal-treated straw was degraded to a lesser extent than untreated and urea-treated straw.

However, this process could not be improved and commercialized.

Zadrazil (1985) evaluated 235 strains of fungi for their ability to delignify wheat straw and reported extreme variations in activity, with substantial influences by temperature and gases (Zadrazil and Puniya 1995). Jung et al. (1992a) reported both increased and decreased in vitro digestibility of oat straw after pretreatment with different fungi. Further work by these workers with *P. chrysosporium* to delignify grass and legume cell walls resulted in loss of more polysaccharides than lignin and did not result in increase in digestibility by rumen microorganisms under their conditions (Jung et al. 1992b). In the similar context, Moyson and Verachtert (1991) reported *Pleurotus pulmonarius* as the best organism for animal feed production while compared with well-studied organisms, i.e., *Pleurotus sajor-caju* and *Lentinus edodes,* due to its potential for degrading higher lignin in comparison to carbohydrate content.

Similarly, Karunanandaa et al. (1992) have tested various fungi and reported increases and decreases in in vitro ruminal digestibilities of corn and rice straw. They further reported the improvement in the digestibility when the fungi selectively used hemicellulose rather than cellulose. Akin et al. (1993b) have also studied the delignification ability of three wild-type fungi and two cellulase-less mutants of *P. chrysosporium* K-3, and among them *C. subvermispora* caused greatest losses in lignin and improved the biodegradation of Bermuda grass over the control substrates.

"Ideal organisms" for converting agricultural crop residues into animal feed should have a strong lignin metabolism with poor degradation of cellulose and hemicellulose, so that biological treatment could lead to produce cellulose-rich material for the utilization of ruminating microflora as an energy source in ruminants (Moyson and Verachtert 1991; Zadrazil et al. 1996; Basu et al. 2002; Okano et al. 2005; 2009; Shrivastava et al. 2011).

Table 1.6 depicts the ability of various white-rot fungi for upgrading fibrous animal feed. It is apparent from the above-mentioned works that white-rot fungi generally require longer duration to maximize the degradation of lignin. The major problem of biological conversion of lignocellulosics into nutritionally improved animal feed is to select microorganisms capable of degrading the lignin selectively. Suitable microorganism should metabolize lignin efficiently and selectively without much degradation of cellulose (Villas-Boas et al. 2002).

Keeping in view the importance of selective lignin degradation and higher digestibility improvement, SSF process was further attempted to be optimized for obtaining the best possible conditions for animal feed production by various workers. Among them, initially, Tripathi and Yadav (1992) optimized SSF of wheat straw into animal feed by *Pleurotus ostreatus* in terms of its cultural and nutrition conditions, i.e., pH, initial moisture, temperature, incubation period, form of inoculum, substrate turning, urea level, etc.; the fungus behaved optimally under the following set of fermentation conditions: initial pH, 5.5; initial moisture, 55%; temperature, 22 °C; period, 21 days; form of inoculum-grain culture (spawn); substrate-turning frequency, once at mid-incubation; urea (nitrogen source) level, 1% (sterile) or 2% (unsterile); single superphosphate (phosphorus + sulfur source), 0.3%; and no addition of free carbohydrates (as whey or molasses). A maximum (10.4%) increase in IVDMD accompanied by a 2.7% degradation of lignin was attained in the optimized SSF under the above conditions. Furthermore Basu et al. (2002) also studied the effect of seed culture on solid-state bioconversion of wheat straw by *Phanerochaete chrysosporium* for animal feed production under statistical designs based on a central composite experimental design. The conditions of the seed culture most suitable for rapid induction of the ligninolytic activity of the fungus were determined. When the seed culture with an initial pH of 5.8 was grown under agitated conditions at 130 rpm in baffled flasks at 38 °C, it was predicted to give lignin degradation of 19.5% and cellulose degradation of 17.8%. A time profile study of the solid-state bioconversion of wheat straw indicated that the highest lignin and lowest cellulose degradation levels occurred on the sixth

Table 1.6 Modification to composition and rumen digestibility of agricultural waste biomass by white-rot fungi

S. No.	Substrate	Fungus	Period of incubation (days)	Relative change in			References
				Lignin (%)	Cellulose (%)	IVDMD (%)	
1.	Ryegrass	*Candida utilis* (NRRL-Y-1084)	3	+3.8	−11.6	+49.2	Grant et al. (1978)
2.	Wheat straw	*Pleurotus ostreatus*	30	−2.8	−14.9	+8.8	Tripathi and Yadav (1992)
3.	Corn straw	*Lentinus edodes*	49	−43	~0	~67	Sermanni et al. (1994)
4.	Hardwood trunks	*L. edodes*	2 years	1	–	427	Suzuki et al. (1995)
5.	Hardwood sawdust, + rice bran	*Pholiota nameko*	100	−30	–	135	Suzuki et al. (1995)
6.	Maize stover	*Cyathus stercoreus*	28	9	17	28	Chen et al. (1995)
7.	Maize stover	*Phanerochaete chrysosporium*	28	48	−29	−24	Chen et al. (1995)
8.	Rice straw	*C. stercoreus*	30	−53	−3	–	Karunanandaa and Varga (1996)
9.	Wheat straw	*Trametes gibbosa*	30	−27	−7	30	Jalc et al. (1996)
10.	Sugarcane bagasse	*Athelia* sp.	30	−17	9	–	Breccia et al. (1997)
11.	Wheat straw	*Peniophora utriculosa*	30	−55	–	36	Capeleri and Zadrazil (1997)
12.	Wheat straw	*Daedalea quercina*	30	−38	−9	43	Jalc et al. (1997)
13.	Wheat straw	*Inonotus dryophilus*	30	26	1	35	Jalc et al. (1997)
14.	Wheat straw	*Pleurotus* sp.	30	−12.82	−7.82	+6.7	Kakkar and Dhanda (1998)
	Paddy straw	*Pleurotus* sp.	10	−11.96	−7.48	−9.24	Kakkar and Dhanda (1998)
15.	Wheat straw	*Phanerochaete chrysosporium*	8	−19.5	−17.8	–	Basu et al. (2002)
16.	Apple pomace	*Candida utilis*	6	−70	−8.3	+8.2	Villas-Boas et al. (2003)
	Apple pomace	*Pleurotus ostreatus*	30	−40	−11.11	+7.0	Villas-Boas et al. (2003)

17.	Sugarcane bagasse	*Lentinus edodes*	16 weeks	−38.09	–	+51.53	Okano et al. (2006)
	Sugarcane bagasse	*Pleurotus eryngii*	16 weeks	+4.76	–	+5.04	Okano et al. (2006)
	Sugarcane bagasse	*Pleurotus salmoneo stramineus*	16 weeks	+10.47	–	+6.36	Okano et al. (2006)
	Sugarcane bagasse	*Ceriporiopsis subvermispora*	16 weeks	−30.48	–	+34.43	Okano et al. (2006)
18.	Wheat straw	*Phanerochaete chrysosporium*	5	−27.0	−29.0	–	Kumar and Gomes (2008)
19.	Wheat straw	*Phanerochaete chrysosporium*	7	−13 to −37	−25 to 50	–	Bhatnagar et al. (2008)
20.	Paddy straw	*Phanerochaete chrysosporium*	60	−39.4	−52.0	+35.13	Sharma and Arora (2010b)
		Ceriporiopsis subvermispora	60	−18.8	−30.8	+29.73	Sharma and Arora (2010b)
		Phlebia brevispora	60	−20.0	−8.7	+36.22	Sharma and Arora (2010b)
		Pleurotus fascicularia	60	−21.0	−10.3	+28.1	Sharma and Arora (2010b)
		Pleurotus floridensis	60	−21.8	−11.4	+25.4	Sharma and Arora (2010b)
		Pleurotus radiata	60	−222.8	−13.4	+34.05	Sharma and Arora (2010b)
21.	Wheat straw	*Pleurotus ostreatus*	10	−20.52	−15.64	+9.5	Shrivastava et al. (2011)
	Wheat straw	*Trametes versicolor*	10	−7.45	−12.18	+6.12	Shrivastava et al. (2011)
22.	Wheat straw	*Pleurotus brevispora*	30	−30.6	–	+66.80	Arora et al. (2011)
		Pleurotus radiata	30	−27.9	–	~+40	Arora et al. (2011)
		Pleurotus floridensis	30	−27.5	–	~+51.16	Arora et al. (2011)
		Ceriporiopsis subvermispora	30	−25.2	–	~+45.35	Arora et al. (2011)
		Pleurotus fascicularia	30	−23.1	–	~ +56.98	Arora et al. (2011)

+ and – signs indicate % increase and decrease, respectively

day of cultivation. The desirability coefficient for this process also passed through a maximum of 0.705 on the sixth day, which was predicted to be the best time for harvesting the batch.

Several strains of white-rot fungi, i.e., *P. chrysosporium*, *T. versicolor*, *C. stercoreus, C. subvermispora*, and *P. cinnabarinus,* have been largely used as model systems to study lignin degradation. To the best of our knowledge the *C. subvermispora* have been found an efficient lignin degrader when tested under laboratory scale. Kakkar and Dhanda (1998) fed *Pleurotus*-fermented wheat and rice straw to buffalo, and fermentation caused an increase in CP up to 80%, lignin reduction up to 12%, and improvement in dry matter intake and nutrient digestibility in animals. Whereas, Okano et al. (2005) fermented wheat straw, bagasse, and Konark oak with *Pleurotus* and *Lentinus* spp. up to 96 days and 4 years, respectively, and reported both increase and decrease in organic matter digestibility. Recently, in Nigeria, Belewu (2006) has converted Masonia tree sawdust and cotton plant by-product (CBP) into feed by *P. sajor-caju*. The lignin content decreased from 44.36 to 25.53% for sawdust and from 20 to 14.2% for CBP. The nitrogen vis-a-vis proteins were also increased significantly, i.e., 0.35 to 1.25% in sawdust and 1.52 to 2.48% in CBP.

Similarly various *Pleurotus* sp. have been tested for bioconversion of citrus bagasse and rice straw into animal feed. Out of four species of *Pleurotus*, *P. ostreatus* 814 was found to be efficient in improving the protein level (Albores et al. 2006), and a scale-up process, using rice straw bales in the open, is under progress. Thus, globally there are constant efforts to develop fastest method, where lignin is preferentially degraded without touching much of the cellulose. Such efficient method eventually will boost biopulping as well as animal feed production technologies. Li et al. (2008) successfully screened *Fusarium concolor* as an efficient organism for selective lignin degradation, and it was found to degrade 13% of lignin with only 7% loss of holocellulose after 5 days. In another distinct attempt, our group characterized the *Pleurotus ostreatus*- and *Trametes versicolor*-fermented wheat straw using in vitro gas production for its metabolizable energy and digestibility (Shrivastava et al. 2011). The 10th day was found to be best to attain the maximum efficiency of SSF based on the nutritive value and selective lignin degradation.

In a very recent report Sharma and Arora (2010a) have optimized conditions for production of lignocellulolytic enzymes by *Phlebia floridensis* during solid-state fermentation of wheat straw along with enhancement of in vitro digestibility, and response surface methodology (RSM)-based experiment was performed. Effect of moisture content, inorganic nitrogen source (NH_4Cl) and malt extract on lignocellulolytic enzymes, changes in chemical constituents, and digestibility of wheat straw was evaluated. With increase in moisture content, laccase production was found to increase up to 34-fold, while Manganese peroxidase was optimally produced in the presence of almost equal amount (50–55 mg g^{-1} of WS) of NH_4Cl and malt extract. These supplements also significantly ($p<0.05$) enhanced the production of CMCase and xylanase. In vitro digestibility was increased by almost 50% with a loss of 27.6 and 14.6% in lignin and total organic matter, respectively. The findings revealed that *P. floridensis* was an efficient organism for lignocellulolytic enzymes production and simultaneous enhancement in in vitro digestibility of wheat straw. However, despite having such kind of studies, complete optimization of the process is yet to be done and is in progress in our laboratory. In another recent report, Arora et al. (2011) tested various fungi for bioconversion process, and among them *Phlebia brevispora* degraded maximum lignin (30%) and enhanced digestibility from 172 to 287 g kg^{-1} along with an increase in antioxidant property of fermented straw.

Only a few processes reached pilot plant scale and none so far to commercial scale. Among them, pilot scale semisolid fermentation of ryegrass straw was carried out in a pilot plant at 100-kg scale using *Candida utilis,* and the fermentation was found to increase protein content, crude fat, and in vitro rumen digestibility (up to 50%) (Grant et al. 1978). For the large-scale

animal feed production in a reactor, Kumar and Gomes (2008) elaborated the chronology of reactors' design from lab to pilot scale for the bioconversion of wheat straw into animal feed using *P. chrysosporium*. Performances of designs were compared between horizontal, fluidized, and vertical based on engineering and bioconversion parameters. During scale-up of solid-state bioconversion process, poor conductivity of lignocellulosic substrate, the metabolic heat generated causing spatial temperature gradient, and mass transfer were figured out as major problems. The staged vertical reactor (design V-G2b) was studied in detail. The response surface generated from the data showed that the maximum value of the desirability coefficient obtained was 0.752 for an inoculum size of 0.35 g/100 g of dry wheat straw, a wheat straw loading of 1.5 kg per stage, and an air flow rate of 15.0 L min^{-1}. The lignin and cellulose degradation achieved was 27 and 29%, respectively. No single reactor design can solve all the problems faced in solid-state bioconversion processes and can provide solutions only to particular problems. Finally, a vertical deep bed reactor was designed with special impellers that ensured the circulation of the solid substrate. The average mixing characteristic time is 3 min. The 1,200-L reactor, a steam generator, sterile air supply system, and seed culture tank together constituted a complete design, and it is capable of stand-alone operation.

Alternatively, various other strategies, involving single or simultaneous organisms and sequential consortia, have been tried (Adamovic et al. 1998; Nigam 1998). Currently, *C. utilis* and *P. ostreatus* have been evaluated for conversion of apple pomace into nutritionally enriched and digestible animal feed, either individually or in sequential order (Villas-Boas et al. 2003). The increase in crude protein obtained almost 100%, which was accompanied by 8.2% rise in the digestibility when apple pomace fermented with *C. utilis* alone. While the crude protein increased by 10.5% (wt dry wt^{-1} of the sample), when substrate was fermented in sequential manner. However, no lignin degradation was observed. More recently, Chi et al. (2007) have cocultured various white-rot fungi to study the degradation of aspen wood. They have reported first time that coculture of *P. ostreatus* with *C. subvermispora* significantly stimulated more laccase production and had shown that cocultivation of wood increases wood degradation and alters the lignin degradation pattern toward a more recalcitrant part of lignin; however, no significant increase in total lignin removal was obtained

Based on the literature, lignin degradation and mineralization largely appears to be exclusive domain of white-rot fungi. This selecting ability of white-rot fungi to degrade lignin preferably from agricultural crop waste and agro-industrial waste could be exploited for their conversion into nutritional-rich animal feed. Based on the large-scale screening program carried to select better delignifier, the most selective fungi were *D. squalens*, *Phlebia* sp., *Ceriporiopsis subvermispora,* and *C. rivulosa* (Otjen et al. 1988; Akhtar et al. 1997; Hakala et al. 2004, 2005; Okano et al. 2009). However, the mechanism of selective degradation of lignin has not been elucidated. Thus, if selective lignin-degrading white-rot fungi are grown under optimized conditions and by manipulating the culture condition or lignin-degrading genes using modern molecular biological tools, it would be possible to tackle the feed problem round the corner of the world.

Molecular Biology of Lignin Degradation

Lignin depolymerization system constitutes multiple isozymes and corresponding genes of laccase, lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and versatile peroxidases (Have and Teunissen 2001). As mentioned earlier, robust investigations have been made on fungal system of lignin degradation, viz., biochemical and biophysical characterization of enzymes and their production. It has been observed in literature that the production of these enzymes from various microbial sources is too low for commercial applications. To overcome this hurdle, molecular cloning and characterization of fungal laccases as well as improvement of the expression level using homologous as well as

heterologous expression systems have been carried out (Kunamneni et al. 2008).

Research has been carried out concerning the molecular genetics of lignin degradation by white-rot fungi, particularly with *P. chrysosporium*. Tien and Tu (1987) were the first to report the cloning of the cDNA-encoding lignin peroxidase H8. Since then much reports have been published about the number, structure, and organization of the *P. chrysosporium* peroxidase genes. However, there is still uncertainty about the exact number and structure of LiP genes (Highley and Dashek 1998). The LiP genes in *P. chrysosporium* have been reported to vary from 5 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 homogenous electrical field (CHEF) electrophoresis, five LiP genes were assigned to a single chromosome. In agreement with the RFLP map, another LiP clone (GLG4) was assigned to the same chromosome as a cbhl cluster (Gaskell et al. 1994). Cullen and Kersten (1992) have shown that at least one MnP gene resides on the same chromosome as do five LiP. Thereafter, Stewart and his team (1996) reported the successful expression of MnP in *Aspergillus oryzae*. Moreover, occurrence of multiple LiPs has been reported in *Bjerkandera adusta*, *C. versicolor,* and *Fomes lignosus* (Huoponen et al. 1990).

Several LiP genes have been characterized from other fungal species, including four *T. versicolor* clones LPGI (Jonsson and Nyman 1992), LPGII (Jonsson and Nyman 1994), VLGI (Black and Reddy 1991), LPGVI, *Bjerkandera adusta* clone LPO-1, and *Phlebia radiata lpg3* (Saloheimo et al. 1989). On the basis of Southern blot hybridization to the *P. chrysosporium* genes, LiP-like sequences also appear to be present in the genomes of *Fomes lignosus* (Huoponen et al. 1990), *P. brevispora,* and *C. subvermispora* (Rajakumar et al. 1996). PCR amplification of LiP-like sequences also suggests the existence of functional LiP genes in *C. subvermispora* and *P. sordida,* species that lack detectable lignin peroxidase activity (Rajakumar et al. 1996). Sugiura et al. (2009) have successfully cloned LiP genes from *Phanerochaete sordida* and their homologous expression system constructed. They used promoter of glyceraldehyde-3-phosphate dehydrogenase to drive the expression of cloned LiP genes.

Similar to LiP, the multiple isozymes of MnP have been reported from *P. chrysosporium.* Alic et al. (1997) have reported four MnP genes from this fungus. There are also numerous reports on the MnPs of white-rot fungi such as *P. ostreatus* (Kamitsuji et al. 2004), *T. versicolor* (Johansson et al. 2002), and others (Manubens et al. 2003; Hakala et al. 2006). MnPs in white-rot fungi have conserved amino acid sequences for metal binding regions, and the nucleotide sequences in those regions can be used as the PCR primers for gene cloning (Kim et al. 2005). More recently, MnP-encoding genes have been successfully cloned and characterized (Yeo et al. 2007; Nagai et al. 2007; Sakamoto et al. 2009).

Multiple sequence alignment reveals features that help to distinguish MnP and LiP; putative Mn^{2+}-biding residues have been identified in MnP genes (Sundaramoorthy et al. 1994). Excluding *T. versicolor,* the MnP gene can be distinguished by the presence of 7–11 amino acid surface loop (Sundaramoorthy et al. 1994) and an extended terminus. The loop contains a fifth disulfide bond, which is not found in LiP genes. Heterologous gene expression of MnP and LiP genes has been reviewed by Pease and Tien (1992). Baculovirus system has been used to produce active recombinant MnP isozyme H4. The recently sequenced enzymes MnPL1 and MnPL2 from cultures of *P. eryngii* exhibit high sequence and structural similarities with LiP from *P. chrysosporium* (Moreira et al. 2005).

Discovery of the ligninolytic peroxidases leads to the isolation of the responsible genes from various white-rot fungi (Gold and Alic 1993; Asada et al. 1995; Johansson and Nyman 1996; Kimura et al. 1990; Lobos et al. 1998). Several regulatory elements have been described in the promoter regions of LiP and MnP. Promoter regions of LiP or MnP genes studied so far contain cAMP response elements (CRE) to notice starvation. Aside from starvation, the presence of Mn^{2+} is essential for MnP gene expression in

P. chrysosporium and *Dichomitus squalens* (Périé and Gold 1991; Brown et al. 1991). The observation is in agreement with the general finding that elevated manganese levels are beneficial for the production of MnP in many white-rot fungi. Fifty-eight putative metal response elements (MRE) were found in *P. chrysosporium* which are similar to a gene that encodes for a mouse metalloprotein (Gold and Alic 1993; Alic et al. 1997). There are two different laccases which have been distinguished on the basis of gene expression: constitutive and inducible (Bollag and Leonowicz 1984; Yaver et al. 1996). Induction of laccase has been observed at the level of transcription and translation upon addition of copper, xylidine, veratric acid, etc. (Bollag and Leonowicz 1984; Palmieri et al. 2000).

Genome sequence of the most intensively studied white rot, *P. chrysosporium,* is now unraveled by Martinez et al. (2004). Since LiP and MnP require extracellular H_2O_2 for their in vivo catalytic activity, likely source for this is the six sequences of copper radical oxidase (cro1 through cro6), glyoxal oxidase GLOX, and extracellular FAD-dependent oxidases (Kersten and Kirk 1987; Kersten et al. 1990; Kersten and Cullen 2007). Genes encoding FAD oxidases in related white-rot fungi include aryl alcohol oxidases (AAO) from *P. eryngii* and pyranose oxidase from *C. versicolor*. Four distinct AAO-like sequences, a pyranose oxidase-like sequence, and a glucose oxidase-like sequence have been identified in the genome data. The precise roles and interactions of these genes in lignin degradation still remain to be determined (Ander and Maezullo 1997). Production of H_2O_2 by AAO through aromatic-aldehyde redox cycling provides a continuous source of oxidative power for lignin degradation. H_2O_2 participates in reactions catalyzed by ligninolytic peroxidases and acts as a precursor of hydroxyl-free radical ($OH^{\bullet}$).

In addition to H_2O_2 production, AAO also prevents the repolymerization of products released from enzymatic degradation of lignin. Genome searches revealed no conventional laccases in *P. chrysosporium*. Instead, four multicopper oxidase (MCO) sequences are found clustered within a 25-kb segment on scaffold 56. Thus, it appears that *P. chrysosporium* does not have the capacity to produce laccases although distantly related multicopper oxidases may have a role in extracellular oxidations. In addition to lignin, *P. chrysosporium* completely degrades all major components of plant cell walls including cellulose and hemicellulose. The genome harbors the genetic information to encode more than 240 putative carbohydrate-active enzymes (Henrissat 1991) (http://afmb.cnrs-mrs.fr/CAZY/) including 166 glycoside hydrolases, 14 carbohydrate esterases, and 57 glycosyltransferases, comprising at least 69 distinct families.

The progress in research work on the transcriptional regulation of peroxidases has been hampered by difficulties in distinguishing closely related genes (Cullen and Kersten 1992). However, it is obvious that LiP genes are transcriptionally regulated and that expression of MnP genes is Mn^{2+} dependent (Brown et al. 1991), although the specificity of the transcripts observed on Northern blots is questionable (Cullen and Kersten 1992).

Versatile peroxidases (VPs) are heme enzymes that combine catalytic properties of lignin peroxidases and manganese peroxidases, being able to oxidize Mn (II) as well as phenolic and nonphenolic aromatic compounds in the absence of mediators. The catalytic process, initiated by hydrogen peroxide, is the same of classical peroxidases with the involvement of two oxidizing equivalents and the formation of the so-called Compound I. This latter state contains an oxoferryl center and an organic cation radical which can be located either on the porphyrin ring or on a protein residue (Pogni et al. 2006).

Two genes encoding VP isoenzymes VPL and VPS1, expressed in liquid and solid-state fermentation cultures, respectively, have been cloned from *P. eryngii* (Ruiz-Dueñas et al. 1999). The deduced amino acid sequences for both isoenzymes were used to build molecular models by homology modeling, taking advantage from sequence identity with *P. chrysosporium* LiP and MnP and *Coprinopsis cinerea* peroxidase (CIP). The structure of the peroxidase from *C. cinereus* (CiP) has been determined in three different space groups and crystalline environments

(Houborg et al. 2003). By combining a homologous recombinant gene expression system and optimization of the culture conditions, hyper overproduction of *Pleurotus ostreatus* versatile peroxidase MnP2 has been achieved (Tsukihara et al. 2006). They have reported 7,300 U L^{-1} of MnP from recombinant strain TM2-18, more than a 30-fold overproduction as compared to the previous reports. Ruiz-Duenas et al. (2001) have reported two versatile peroxidases from *Pleurotus* and *Bjerkandera* spp., which have been cloned, sequenced, and characterized. They have reported to show their high affinity for Mn^{2+}, hydroquinones, and dyes and also oxidize veratryl alcohol, dimethoxybenzene, and lignin dimers. Huang et al. (2009) have cloned and characterized a novel ligninolytic peroxidase gene (ACLnP) from a poroid brown-rot fungus, *Antrodia cinnamomea*. ACLnP that was cloned into vector pQE31 and successfully expressed in *E. coli* strain M15 under the control of the T5 promoter produced a non-glycosylated protein of about 38 kDa, p*I* 5.42.

Literature survey indicates that various strategies have been used to isolate and clone the laccase coding sequences from fungal wild type. The laccase genes have been isolated, cloned, and characterized based on the sequence information of purified protein of *Coriolus hirsutus* (Kojima et al. 1990), *P. ostreatus* (Giardina et al. 1995; Giardina et al. 1999), *T. versicolor* (Jonsson et al. 1995), and *Neurospora crassa* (Germann et al. 1988) by screening of genomic or cDNA libraries using probes designed on the basis of partial amino acid sequence of fungal laccases. To clone laccase genes, similar PCR methods based on conserved Cu-binding regions have also been used (D'Souza et al. 1996; Temp et al. 1999; Ducros et al. 1998; Hoshida et al. 2001). In a novel approach, Joo et al. (2008) have reported to isolate the laccase-specific genomic sequence applying inverse PCR and subsequently, cloned and expressed the laccase in *Pichia pastoris*. The cDNA corresponding fungal laccase comprise of 1,554–1,563 nucleotides encoding a protein of 518–520 amino acids with about 21–23 signal peptide. The gDNA of laccase reported to date has variable number of introns ranging from 10 to 12 interrupting the coding sequence.

Homologous expression systems for extracellular proteins of Basidiomycetes have been reported for several enzymes including laccase (Mayfield et al. 1994; Sollewijn Gelpke et al. 1999; Irie et al. 2001; Ma et al. 2003; Kajita et al. 2004). Similarly, the heterologous expression of fungal genes for laccases has been achieved in filamentous fungi (Saloheimo et al. 1991; Yaver et al. 1996; Cassland and Jönssson 1999; Liu et al. 2003; Record et al. 2002; Kiiskinen et al. 2004; Rodríguez et al. 2008). In this regard, several approaches have also been described in order to successfully express basidiomycetous laccases using yeast (Jönsson et al. 1997; Hoshida et al. 2001; Piscitelli et al. 2005; Jolivalt et al. 2005; Guo et al. 2005; Bohlin et al. 2006; Faraco et al. 2008; Kim et al. 2010; López et al. 2010; Huang et al. 2011). Survey of literature clearly indicates that both homo- and heterologous expression systems have been used in laccase cloning and expression.

Kilaru et al. (2006) reported the homologous expression of *C. cinerea* laccase gene under non-inductive conditions using various homologous and heterologous promoters. They observed that irrespective of promoter used, addition of Cu to the medium increased enzymatic activity by 10–50-fold. However, promoter efficiency for heterologous expression needs extensive evaluation. O'Callaghan et al. (2002) studied optimization of the expression of a *Trametes versicolor* laccase gene in *Pichia pastoris* under shake flask culture. They described the development of a medium that allows convenient pH control of *P. pastoris* without the need for continuous neutralization. Quite recently, López et al. (2010) studied growth kinetics of *Pichia pastoris* and heterologous expression of *Trametes versicolor* laccase under solid-state (SSF) and submerged fermentations (SmF). For the first time, they used polyurethane foam (PUF) for solid-state yeast cultures and observed that this system may be a promising and simple way to produce heterologous proteins with *P. pastoris*. The results showed the strong inhibition of laccase production in the

SmF experiments, compared with the high laccase titer in the SSF experiments. They observed that oxygen mass transfer is more efficient in SSF which is related to the higher area/volume ratio compared with SmF.

We have been working on molecular genetics of the lignin degradation in our laboratory and have reported the efficient and convenient *Agrobacterium*-mediated gene transformation system in fungi (Sharma et al. 2006). We successfully delivered the T-DNA carrying the genes coding for β-glucuronidase (uidA), green fluorescent protein (gfp), and hygromycin phosphotransferase (hpt) to the nuclear genome of lignin-degrading white-rot fungi such as *Phanerochaete chrysosporium, Ganoderma* sp. RCKK-02, *Pycnoporus cinnabarinus, Crinipellis* sp. RCK-1, *Pleurotus sajor-caju,* and fungal isolate BHR-UDSC. This methodology will provide a rapid and reproducible transformation method without external addition of acetosyringone, which could be useful for improving white-rot fungi for their various biotechnological applications (Sharma and Kuhad 2010).

Conclusion

The mechanism of lignin degradation by fungi has been understood to a larger extent; however, knowledge about the mechanism that promotes selective delignification of plant cell wall is limited. This necessitates the need to understand the culture conditions promoting delignification, such as effect of (1) pure oxygen than in presence of air, (2) manganese in the culture medium, (3) nitrogen and its concentration, and (4) certain inducers. Lignocellulose bioconversion by SSF will have an important role in future biotechnologies, and animal feed production will be one of them, mainly because of its favorable economy, larger availability, and ease of on-site operation in agricultural facilities. For faster conversion of lignocellulosic material into digestible and protein-rich animal feed, there is need to design an appropriate bioreactor to run solid-state fermentation by selective lignin-degrading fungi. There is further need to improve the white-rot fungi for their lignin-degrading ability, which could be achieved following rDNA technology.

Despite the good level on knowledge of biochemistry and genetics of microbial degradation, still there are limitations to achieve complete exploitation of plant-based resources to value-added products, especially animal feed. Several aspects of lignin degradation still remain unsolved. Among them is the knowledge about the mechanism of lignin degradation occurring in the environment and to what extent there are different microorganism, especially white-rot fungi, involved in this process. Moreover, the production level from the best known culturable white-rot fungi is low, which is the matter of concern in improving delignification of plant material and eventually economization of the bioconversion process. Thus, to overcome the existing problems, the effort should be to analyze total DNA from various environments and to hunt for the robust ligninolytic gene(s) or biocatalyst(s). If we succeed getting a robust system, which fastens lignin degradation in lesser duration, the problem of animal feed can be solved to a larger extent.

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