Chapter 10
Multiple Factors Influencing the Strategy of Lignin Mycodegradation

Gautam Anand, Sangeeta Yadav, and Dinesh Yadav

1 Introduction

The term "lignin" is derived from the Latin word "lignum," meaning wood. It is the most abundant aromatic compound on earth and is the second (after cellulose) most abundantly present natural polymer. It accounts for 15–30% of the lignocellulosic biomass (Yao et al. 2017). Of all naturally produced organic chemicals, lignin is probably the most recalcitrant. Together with hemicellulose, it forms strong adhesive for cellulose fibers (Grzegorz et al. 2017). This property of lignin is consistent with its biological functions, i.e., to give vascular plants the rigidity and to protect their structural polysaccharides from attack by other organisms. The conversion isolated cellulose or hemicellulose into monosaccharides is known to be a relatively simple process. Many cellulolytic as well as ligninolytic fungi make use of hydrolases which are capable of producing monosaccharides from polysaccharide components of wood. However, when these components are complexed with lignin, they are resistant to hydrolytic breakdown. Therefore, lignin appears to inhibit hydrolytic activity.

G. Anand
Department of Plant Pathology and Microbiology, Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot, Israel

Department of Biotechnology, D.D.U. Gorakhpur University, Gorakhpur, Uttar Pradesh, India

S. Yadav · D. Yadav (✉)
Department of Biotechnology, D.D.U. Gorakhpur University, Gorakhpur, Uttar Pradesh, India

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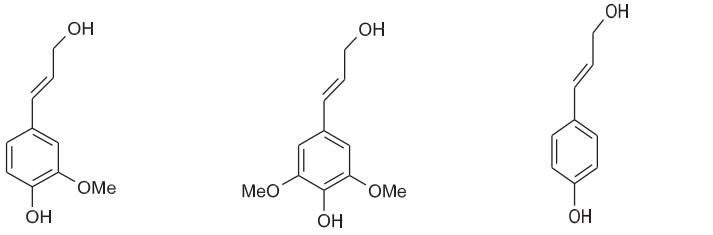
2 Lignin Structure and Biosynthesis

Lignin is a complex, amorphous, three-dimensional aromatic polymer. Lignin is formed in vascular plant cell walls by the oxidative coupling of several related phenylpropanoid precursors: coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol (Fig. 10.1). Because of the different monomers, lignin can be divided into three types: syringyl lignin polymerized by syringyl propane, guaiacyl lignin polymerized by guaiacyl propane, and hydroxy-phenyl lignin polymerized by hydroxy-phenyl propane. Usually, gymnosperm mainly contains guaiacyl (G) lignin; the dicotyledon mainly contains guaiacyl-syringyl (GS) lignin; the monocotyledon mainly contains guaiacyl-syringyl-hydroxy-phenyl (GSH) lignin (Wei and Song 2001). G lignin is chiefly formed through dehydrated oligomerization of coniferyl alcohol, and its structure is homogeneous. Most lignin in softwood belongs to G lignin, which is copolymerized by guaiacyl. GSH lignin is the result of the dehydrated oligomerization of coniferyl alcohol and sinapyl alcohol; the content of lignin is 17–23%. The ratio of syringyl propane to guaiacyl propane is 0.5:0.1; it also contains 7–12% ester groups. *p*-Coumaryl alcohol in it is linked to lignin in the form of ester (Gao and Tang 1996).

Peroxidases or laccases in the plant cell wall oxidize these monomers by one electron, yielding transient resonance-stabilized phenoxyradicals that then polymerize in a variety of configurations (Adler 1977; Higuchi 1990). The molecular weight of lignin is difficult to determine because lignins are highly polydisperse materials (Argyropoulos and Menachem 1997). Lignin is covalently associated with hemicelluloses in the cell wall via numerous types of linkage (Fig. 10.2). Among the most important are ether bonds between the benzylic carbon of lignin and the carbohydrate moiety, ester bonds between the benzylic carbon of lignin and uronic acid residues, and lignin-glycosidic bonds.

Recent studies show that lignin can incorporate many more monolignols than the traditional three basic units (Vanholme et al. 2008), e.g., acetylated lignin units have been identified in non-woody plants (Martínez et al. 2005). In graminaceous plants,



Coniferyl alcohol sinapyl alcohol p-coumaryl alcohol

Fig. 10.1 Three precursors of lignin

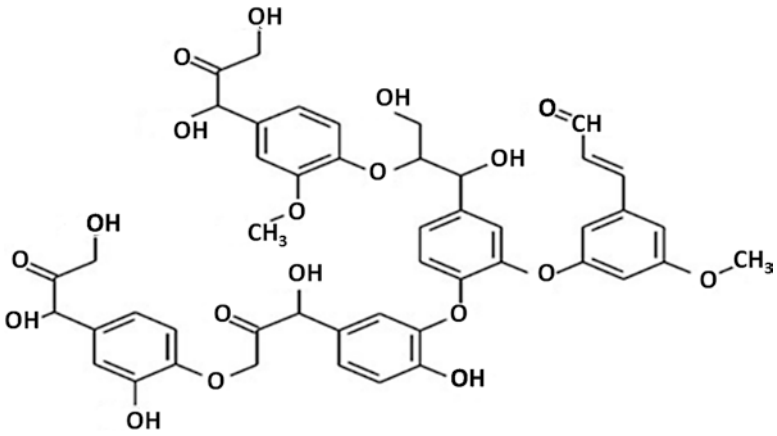


Fig. 10.2 Polymeric structure of lignin

hydroxycinnamic acid residues are frequent in the lignin and are attached to hemicelluloses via ester linkages. The matrix of lignin and hemicellulose encrusts and protects the cellulose of the plant cell wall (Jeffries 1991).

Lignin polymer contains characteristic methoxyl groups, phenolic hydroxyl groups, and some terminal aldehyde group. Most of the phenolic hydroxyl groups are bonded to neighboring phenylpropane units. Carbonyl groups and alcoholic hydroxyl groups are introduced in the final lignin structure during dehydrogenative polymerization process. Also, in some of the wood species substantial alcoholic hydroxyl groups are esterified with *p*-hydroxybenzoic acid or *p*-hydroxycinnamic acid.

Biosynthesis of monolignols is carried through the phenylpropanoid pathway. In the shikimate pathway, glucose obtained by photosynthesis is first converted to shikimic acid. This shikimic acid is converted into the final products of the pathway, i.e., phenylalanine and tyrosine through prephenic acid. Phenylalanine and tyrosine are starting components for the cinnamic acid pathway. After a set of reactions, such as deamination, hydroxylation, methylation, and reduction, three monomers of lignin are finally synthesized (Lin et al. 2003; Geng et al. 2003).

The rate-limiting step, controlling lignin synthesis and the pathway from which carbon can go into lignin biosynthesis is catalyzed by Cinnamoyl CoA reductase (CCR). Reduction of coniferaldehyde, another redox reaction in the lignin synthesis process, is catalyzed by cinnamyl alcohol dehydrogenase (CAD). CAD is located in tissues with precipitation of G lignin. Sinapyl alcohol dehydrogenase (SAD), ferulic acid 5-hydroxylase (F5H), and bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT) are immunolocalized in cells and tissues that have S lignin. It is believed that the last step of redox reactions of different types of lignin

go through different pathways which are catalyzed by different enzymes (Lin et al. 2003). Two-step methylation reaction at the 3′ and 5′ site is an important step in lignin monomer synthesis. This step is carried out by COMT and CCoAOMT (caffeoyl CoA 3-Omethyltransferase) which are methylases on two different substrate levels.

3 Fungal Degradation of Lignocellulose

Ligninolytic fungi are not able to use lignin as their sole source of energy and carbon. Instead, they depend on the more digestible polysaccharides in lignocellulosic substrates, and the primary function of ligninolysis is to expose these polysaccharides so that they can be cleaved by fungal cellulases and hemicellulases. Oxidative coupling of lignin aromatic alcohol monomers creates a complex structure in lignin which is highly recalcitrant to degradation (Wong 2009). By linking to both hemicelluloses and cellulose, lignin acts as a barrier to any solutions or enzymes and prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure. Not surprisingly, of the components of lignocellulosic material, lignin is the most resistant to degradation (Sánchez 2009; Himmel et al. 2007). Although lignin resists attack by most microorganisms, basidiomycetes white-rot fungi are able to degrade lignin efficiently (Wong 2009; Abbas et al. 2005). In most fungi that have been examined, ligninolysis occurs during secondary metabolism, i.e., under nutrient limitation. With this approach, the fungus avoids synthesizing and secreting metabolically expensive ligninolytic agents when substrates more accessible than lignocellulose are present. The limiting nutrient for fungal growth in most woods and soils is probably nitrogen, and most laboratory studies of ligninolytic fungi have been done in nitrogen-limited culture media (Kirk and Farrell 1987). However, a few ligninolytic fungi, e.g., some species of *Bjerkandera,* are ligninolytic even when sufficient nitrogen is present (Kaal et al. 1993).

Lignin degrading fungi produce extracellular enzymes to break lignin. These fungi are widespread and include species from the ascomycetes and basidiomycetes phyla such as white-rot and brown-rot fungi. In addition, a few anaerobic species are also found to be able to degrade cellulose in the gastrointestinal tracts of ruminant animals (Ljungdahl 2008; Yoon et al. 2007). Biomass degradation by these fungi is performed by complex mixtures of cellulases (Bayer et al. 1998), hemicellulases (Ljungdahl 2008), and ligninases (Weng et al. 2008; Sánchez 2009), reflecting the complexity of the materials. In nature, degradation of cellulosic biomass is performed by mixtures of hydrolytic enzymes collectively known as cellulases. Many microorganisms, including fungi and bacteria, have been found to be capable of degrading cellulose and other plant cell wall fibers contributing significantly to the decay of lignocellulosic residues in nature. The degradation of lignin by different fungi is briefly discussed:

4 White-Rot Fungi

White-rot fungi are a heterogeneous group of fungi classified into the basidiomycota. They are most efficient organisms capable of degrading lignin and the related litter. More than 90% of all wood-rotting basidiomycetes are of the white-rot type (Gilbertson 1980). Different white-rot fungi vary considerably in the relative rates at which they attack lignin. Some of them simultaneously attack lignin, hemicellulose, and cellulose, whereas some other white-rot fungi preferentially work on lignin in a selective manner. Their purpose is to decompose the lignin in wood so that they can gain access to the cellulose and hemicelluloses that are embedded in the lignin matrix. Usually, syringyl (S) units of lignin are preferentially degraded, whereas guaiacyl (G) units are more resistant to degradation. Many white-rot fungi colonize cell lumina and cause cell wall erosion. Eroded zones coalesce as decay progresses and large voids filled with mycelium are formed. This type of rot is referred as non-selective or simultaneous rot. Calcium oxalate and MnO_2 accumulate when the decay proceeds (Blanchette 1995). Some white-rot fungi degrade lignin in woody plant cell walls relatively to a higher extent than cellulose, and they are called selective white-rot fungi. In nature, they may cause white-pocket or white-mottled types of rot, e.g., *Phellinus nigrolimitatus* (Blanchette 1995). There are also fungi, e.g., the tree pathogen *Heterobasidion annosum*, that are able to produce both types of attack in the same wood (Eriksson et al. 1990). Typical examples of such fungi are *Ganoderma applanatum* and *Heterobasidion annosum*. Because fungi selectively degrading lignin are considered the most promising fungi for applications in the pulp and paper industry, the search among these fungi has attained a considerable interest. However, the ratio lignins, hemicelluloses, and celluloses 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. Several screening studies to find suitable fungi for biopulping of wood or straw have revealed fungi that, under certain conditions, degrade lignin preferentially to cellulose. Such lignin-selective fungi are, e.g., *P. chrysosporium*, *C. subvermispora* (Otjen et al. 1987; Eriksson et al. 1990), *Pycnoporus cinnabarinus* (Ander and Eriksson 1977), *Phlebia radiata* (Ander and Eriksson 1977), *Phlebia tremellosa (syn. Merulius tremellosus)* (Ander and Eriksson 1977; Eriksson et al. 1990), *Phellinus pini* (Eriksson et al. 1990), and *Dichomitus squalens* (Eriksson et al. 1990).

White-rot fungi produce a number of extracellular enzymes that directly attack lignin, cellulose, and hemicellulose of the plant cell wall to decompose it. These enzymes include laccases and peroxidases, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP). Laccases and peroxidase enzymes can also cause lignin degradation through low molecular weight-free radicals such as OH, depolymerize the phenolic and non-phenolic lignin polymer, and mineralize the insoluble lignin. Basic research on lignin degradation, e.g., its

mechanisms, physiology, enzymology, and molecular biology, has been mainly carried out with the corticoid fungus *P. chrysosporium* (Kirk and Farrell 1987; Eriksson et al. 1990). After more and taxonomically different fungi had been studied in more detail, it was revealed that both the physiological conditions for lignin degradation and the enzyme systems expressed are fungus-specific and differ from those found in *P. chrysosporium*. Differences may be connected to the taxonomic position and/or ecology of the fungi, e.g., substrate specialization (hardwood, softwood, or certain wood species, heartwood or sapwood), the stage of degradation, etc.

5 Brown Rot

Brown-rot fungi are basidiomycetes that degrade wood to yield brown, shrunken specimens that typically exhibit a pattern of cubical cracks and easily disintegrate upon handling. Only a small proportion, roughly 70%, of all wood decay basidiomycete species falls into this group, which occurs most frequently on gymnosperm wood. In contrast to white-rot fungi, brown-rot fungi, such as *Postia placenta*, *Laetiporus portentosus*, *Piptoporus betulinus*, and *Gloeophyllum trabeum*, can degrade wood carbohydrates, but not oxidized lignin. Brown-rot fungi mainly decompose the cellulose and hemicellulose components in wood, but they can also modify the lignin to a limited extent (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. Lignin degradation by brown-rot fungi mainly involves non-enzymatic oxidation reactions producing hydroxyl radicals via Fenton chemistry (Kirk et al. 1991; Kerem et al. 1998, 1999). Brown-rot fungi partially oxidize lignin via aromatic ring demethylation. During this process, the phenolic hydroxyl content of the reaction mixture increases due to partial oxidation and partially due to the addition of new carboxyl and carbonyl groups (Hatakka and Hammel 2011).

Brown-rotted wood is dark, shrink, and 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. During brown rot, the hemicelluloses in wood are degraded most rapidly, after which virtually all of the cellulose is removed, leaving behind a complex, aromatic ring-containing polymer derived from the original lignin. 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 (Kirk 1975).

6 Factors Affecting Lignin Degradation

Lignin degradation, owing to its complex structure and features, is a complicated process. Apart from type of fungi, many different environmental factors influence lignin degradation. The environmental factors affect growth and metabolism of fungi thereby influencing lignin degradation. Moisture content, temperature, pH, aeration, carbon, and nitrogen sources are important parameters that affect fungal growth (Yang et al. 1980; Leisola et al. 1984). Among different microbes, white-rot fungi are the most efficient organisms for lignin degradation. *Basidiomycetes* govern the list among different white-rot fungi. Expression of *P. chrysosporium* genes are strongly influenced by nitrogen and carbon limitation. Regulatory elements present in the promoter regions of genes encoding lignolytic enzymes play an important role in transcriptional activation (Cohen et al. 2001). Under aerobic environment *P. chrysosporium* can reduce 1 g of different separated lignins in 2 days. The fungus is able to degrade lignin efficiently under 30% CO_2 and 10% O_2 concentrations. Addition of 0.12% nitrogen has been shown to increase lignin degradation. The fungus uses lignin, hemicelluloses, and cellulose as substrate. The lignin reduction happens at the end of primary growth by cooperation of other metabolism-like nitrogen (Kang et al. 2013; Leisola et al. 1984; Yang et al. 1980; Hatakka and Hammel 2011; Hatakka 2005). The fungi use oxidation reactions which decrease methoxy, phenolic, and aliphatic content of lignin. These reactions also cleave aromatic rings and form new carbonyl groups. When it comes to mixed microbial population, soft-rot fungi can efficiently degrade lignin. They are better adapted to variations in temperature, pH, and oxygen content (Hamed 2013; Gupta and Verma 2015; Gupta et al. 2016).

pH plays a prominent role in the growth of fungi and it is very difficult to control an optimum pH when the fungi growing on a solid-state substrate. Production of lignolytic gene is influenced by the initial pH of the medium. At pH 4.0–5.0, most of the white-rot fungi grow optimally and also the substrate acidity decreases their growth (Patel and Gupte 2016).

The optimum temperature during biological pre-treatment varies with the type of fungi used. Most of the white-rot ascomycetes fungi grow best around 39 °C while the white-rot basidiomycetes grow optimally around 25 and 30 °C. The heat generated during fungal growth develops temperature gradients in solid-state media. The accumulated heat can inhibit fungal growth and metabolism. Variations in temperature employed are due to the differences in fungal strain, physiology, and type of substrate (Rouches et al. 2016).

Inoculum concentrations have significant role in lignin degradation. The time required for the colonization of the substrate is affected by the type and amount of inoculums. Fungal spores are the commonly used inoculum. Mostly, it is found that larger quantity of inoculum leads to shorter time for colonization of the substrate (Van Kuijk et al. 2015).

High substrate concentrations have to be used for biological pre-treatment to make the process economically viable. When dry substrate is used, it is found that

concentration of inhibitors increases and this may influence the degradation process. Hence, pre-treatment should be carried in conditions to minimize the generation as well as accumulation of inhibitory compounds. Initial moisture content is essential for the establishment of microbial growth in the biomass. Initial moisture content critically affects the fungal growth and enzyme production and significantly affects lignin degradation (Rouches et al. 2016).

Aeration also has significant role in fungal degradation of lignin. Aeration has many functions which are including oxygenation, CO_2 removal, heat dispersion, humidity conservation, and also dispersal of volatile combinations produced during metabolism. It also affects production of enzymes that are required during lignin metabolism. Since lignin degradation is an oxidative process, oxygen availability is important for ligninase activity of white-rot fungi. However, high oxygen content has also been shown to slow down lignin degradation process in *P. chrysosporium*. High aeration could improve delignification rate and hence controlled aeration is essential for improvement of biological pre-treatment (Salvachua et al. 2011; Leisola et al. 1984).

7 Steps in Lignolysis

Lignin degradation by the fungi involves both depolymerization and cleavage of aromatic ring. Extracellular enzyme secreted by fungi oxidizes lignin in the following steps:

1. Oxidation of β–*O*–4 linkages to arylglycerol compounds
2. Aromatic rings cleavage by the α–ketoadipate pathway
3. Formation of cyclic carbonate structures.

8 Enzymes Involved in Lignin Degradation

Lignin does not contain hydrolyzable linkages, which means that the enzymes must be oxidative. Ligninases can be classified as either phenol oxidases (laccase) or heme peroxidases, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) (Martínez et al. 2005). In general, laccases use molecular oxygen as electron acceptors while peroxidases use hydrogen peroxide as a co-substrate. These enzymes can directly attack lignin, cellulose, and hemicellulose of the plant cell wall to decompose it. Some of these enzymes and their isoenzymes gene have been investigated and characterized. For example, the genome of *P. chrysosporium* contains ten LiP and five MnP genes (Martinez et al. 2004). Four laccase genes have been recognized in the plant pathogenic fungus *Rhizoctonia solani* (Wahleithner et al. 1996). Although LiP is able to oxidize the non-phenolic part of lignin (which forms 80–90% of lignin composition), it is absent from many

Table 10.1 Features of fungal lignolytic enzymes

Enzyme	Prosthetic group	Substrate	Mediators	Optimum pH	Reaction
Laccase	Four Cu atoms	Ortho and para-diphenols, aminophenol, polyphenols, polyamines, lignin, aryl diamines	Hydroxybenzotriazole, syringaldazine, phenols, aniline	2.0–10.0	4 benzenediol + O_2 = 4 benzosemiquinone + 2 H_2O
Lignin peroxidase	Heme	Halogenated phenolic compounds, polycyclic aromatic compounds	Veratryl alcohol	1.0–5.0	1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol + H_2O_2 = 3,4-dimethoxybenzaldehyde + 1-(3,4-dimethoxyphenyl) ethane-1,2-diol + H_2O
Manganese peroxidase	Heme	Lignin and other phenolic compounds	Thiols, unsaturated fatty acids, organic acids as chelators, Mn^{3+}	2.5–6.8	2Mn(II) + $2H^+$ + H_2O_2 = 2Mn(III) + $2H_2O$
Versatile peroxidases	Heme	Phenolic and non-phenolic aromatic compounds	Veratryl alcohol, compounds similar to LiP and MnP mediators	3.0–5.0	Donor + H_2O_2 = oxidized Donor + $2H_2O$

lignin degrading fungi (Wang et al. 2008). Also, oxidative ligninolytic enzymes are too large to penetrate into the wood cell wall micropores (Srebotnik et al. 1988). Hence, prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds initiate changes to the lignin structure (Srebotnik et al. 1988; Tanaka et al. 1999). A comparative list of features of fungal lignolytic enzymes is given in Table 10.1.

8.1 Laccase

Laccase is a copper-containing oxidase that uses molecular oxygen to oxidize various aromatic and nonaromatic compounds through a free radical catalyzed reaction mechanism. Most white-rot fungi typically produce laccase (Bollag and Leonowicz 1984), and the enzyme is common also in higher plants and in other fungi. Laccases couple the electron reduction of dioxygen into two molecules of water with the oxidation of a vast variety of substrates, such as phenols, arylamines, anilines,

thiols, and lignins. Four copper ions in their catalytic center mediate the redox process. The oxidation reactions catalyzed by laccases lead to the formation of free radicals which act as intermediate substrates for the enzymes (Thurston 1994; Messerschmidt and Huber 1990; Ferraroni et al. 2007). These mediators can leave the enzyme site and react with a broad range of high-redox potential substrates and thus create non-enzymatic routes of oxidative polymerizing or depolymerizing reactions. Laccases have been found and studied in white-rot fungi, such as *Pleurotus ostreatus* D1 (Pozdnyakova et al. 2006), *Lentinus tigrinus* (Ferraroni et al. 2007), *Cerrena unicolor* strain 137 (Michniewicz et al. 2006), *Trametes* sp. strain AH28-2 (Xiao et al. 2003), *Trametes pubescens* (Shleev et al. 2007), and *Cyathus bulleri* (Salony et al. 2006). Also, ascomycetes such as *Melanocarpus albomyces* (Hakulinen et al. 2006), *Magnaporthe grisea* (Iyer and Chattoo 2003), *Myrothecium verrucaria* 24G-4 (Sulistyaningdyah et al. 2004), and *Neurospora crassa* (Germann et al. 1988) were also found to produce laccases.

8.2 Lignin Peroxidase

Lignin peroxidase (LiP, EC 1.11.1.14) is a glycosylated enzyme containing heme protein with an iron protoporphyrin prosthetic group that requires hydrogen peroxide (H_2O_2) to catalyze the oxidation of non-phenolic lignin units and mineralize the recalcitrant aromatic compounds. LiPs are heme-containing glycoproteins and play a central role in the biodegradation of the cell wall constituent, lignin (Piontek et al. 2001). LiPs catalyze the H_2O_2-dependent oxidative depolymerization of a variety of non-phenolic lignin compounds (diarylpropane), β-*O*-4 non-phenolic lignin model compounds and a wide range of phenolic compounds such as guaiacol, vanillyl alcohol, catechol, syringic acid, and acteosyringone (Wong 2009). Lignin oxidation takes place by electron transfer, non-catalytic cleavages of various bonds and opening up of aromatic ring. Typically for LiP activity, the amino acid residue needed is a tryptophan and is assumed to long-range electron transfer from a protein radical at the surface of the enzyme, which would act as the substrate oxidizer, to the heme cofactor (Ruiz-Dueñas and Martínez 2009). This helps in oxidization of bulky lignin substrate. They have been found in many white-rot fungi such as *Phanerochaete chrysosporium, Trametes versicolor*, and *Bjerkandera* sp. (Kirk and Farrell 1987; Kaal et al. 1993; Orth et al. 1993). LiPs resemble other peroxidases such as the classical, extensively studied enzyme from horseradish, in that they contain ferric heme and operate via a typical peroxidase catalytic cycle (Kirk and Farrell 1987). However, LiPs are more powerful oxidants than typical peroxidases are, and consequently oxidize not only the usual peroxidase substrates such as phenols and anilines, but also a variety of non-phenolic lignin structures and other aromatic ethers that resemble the basic structural unit of lignin. Unlike the other peroxidases like MnP, LiP is able to oxidize non-phenolic aromatic substrates and does not require the participation of mediators due to its unusually high redox potential (Wong 2009).

8.3 Manganese Peroxidases

Manganese peroxidases (MnPs) are extracellular glycoproteins and are secreted in multiple isoforms which contain one molecule of heme as iron protoporphyrin (Asgher et al. 2008). MnPs occur in most white-rot fungi, and are similar to conventional peroxidases, except that manganese (Mn) is essential for the catalysis. The enzyme MnP plays an important role during the initial stages of lignin degradation (Perez and Jeffries 1990). Compared to laccase, MnP causes greater degradation of phenolic lignin due to its higher redox potential with the eventual release of carbon dioxide (Ten Have and Teunissen 2001). MnP catalyzes the peroxide-dependent oxidation of Mn(II) to Mn(III), which is then released from the enzyme surface in complex with oxalate or with other chelators. Chelated Mn (III) complex acts as a reactive low molecular weight, diffusible redox-mediator of phenolic substrates including simple phenols, amines, dyes, phenolic lignin substructures and dimers (Wesenberg et al. 2003; Asgher et al. 2008). In addition, organic acids such as lactate and malonate can chelate Mn^{3+} ion. Since their discovery in *P. chrysosporium,* they have been found in many basidiomycetes such as *Lenzites betulinus*, *Phanerochaete flavido-alba*, and *Panus tigrinus* (Hoshino et al. 2002; de la Rubia et al. 2002; Lisov et al. 2003).

8.4 Versatile Peroxidases

Versatile peroxidase (VP), as the name suggests, has catalytic properties of both LiP and MnP. VPs are glycoproteins with hybrid properties capable of oxidizing typical substrates of other basidiomycetes peroxidases including Mn(II) and also veratryl alcohol (VA), MnP and the typical LiP substrate, respectively (Wesenberg et al. 2003; Asgher et al. 2008). VP was first purified from the genera of fungi *Bjerkandera* and was found to transform lignin even without an external mediator (Moreira et al. 2007). VPs form an attractive ligninolytic enzyme group due to their dual oxidative ability to oxidize Mn(II) and also phenolic and non-phenolic aromatic compounds. Versatile peroxidase possesses a hybrid molecular architecture with several binding sites including Mn^{2+} and is able to oxidize Mn^{2+} like MnP and LiP. However, unlike MnP, VP has the dual ability to oxidize Mn^{2+} in the independent oxidation of simple amines and phenolic monomers. VP can also oxidize a variety of substrates (with high and low redox potentials) including Mn^{2+}, phenolic and non-phenolic lignin dimers, and aromatic alcohols (Perez-Boada et al. 2005; Camarero et al. 1999). This makes versatile peroxidase a better catalyst than MnP and LiP. Similar to the MnP mechanism, Mn(III) is released from VPs and acts as a diffusible oxidizer of phenolic lignin and free phenol substrates. Like other members of heme peroxidases, heme is buried in the interior of the protein and has access to the outer medium through two channels (Heinfling et al. 1998).

9 Measurement of Lignin Degradation

The delignification of woody tissues by fungi can be assessed by microscopy (Blanchette 1991) with selective staining (Srebotnik and Messner 1994); however, these techniques are relatively complex and semiquantitative. Microscopy is useful only when the substrate contains lignified cell walls.

The removal of lignin from lignocellulosic material can be measured by the simple procedure of extracting its low molecular weight components, weighing the leftover woody tissues, degrading the remaining polysaccharide component with strong acid, and then reweighing the leftover lignin, which is referred to as Klason lignin. Measurement of Klason lignin is simple and useful if interfering substances are not present. However, this method is not reliable if it is used on plant tissues that contain high molecular weight components that cannot be are not removed by extraction and acid treatment. Interfering substances may be proteins and tannins (Theander and Westerlund 1993). On the other hand, the Klason procedure is also not accurate when woody tissues contain polymers which are acid-soluble and consequently lost during the hydrolysis of polysaccharides.

Another approach to study fungal ligninolysis is to assess whether the fungus degrades a simpler molecule rather than growth substrate itself being delignified. Substrates of this type can be incorporated into the natural lignocellulosic growth medium, e.g., wood or litter, or they can be used as probes in defined liquid growth media (Kirk 1975, 1978; Srebotnik and Messner 1994). C^{14}-labelled synthetic lignins are most frequently used in this type study. Carbon dioxide produced during degradation of the radiolabelled polymer can be trapped in alkali and determined by scintillation counting. However, this method is expensive and requires expert facilities for radioisotope study.

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

10 Importance of Biodegradation of Lignin

Of all naturally produced organic chemicals, lignin is probably the most recalcitrant. Lignin is the most abundant aromatic compound on earth and is second only to cellulose in its contribution to living terrestrial biomass. Lignin degradation can thus play a major role in improving earth's biofuel resources and also serve as an alternative to harsh technologies used in the paper and pulp industry. When plants die or drop litter, lignified organic carbon is incorporated into the top layer of the

soil. This recalcitrant material has to be broken down and recycled by microorganisms to maintain the earth's carbon cycle. If this process fails, imagine the amount of carbon which is irreversibly sequestered as lignocellulose.

Lignin biodegradation has diverse effects on soil quality. Microbial lignolysis facilitates the formation of humus by promoting the release of aromatic humus precursors such as flavonoids, terpenes, and tannins from the litter (Hudson 1992). Undegraded lignocellulose, has adverse effect on soil fertility by supporting high microbial load. Increasing population of microbes in soil may not only produce phytotoxins but also compete with crop plants for soil nitrogen and other nutrient. By breaking down the most refractory component of litter, ligninolysis thus contributes to the removal of conditions that inhibit crop productivity.

In paper and textile industries, wood chips are often treated with chemical or mechanical forces to separate their fibers. In chemical pulping, strong chemicals, such as soda, and high pressure are applied to remove lignin. The resulting pulp has high strength but the process is polluting and the overall yield is relatively low (40–50%). Mechanical pulping uses mechanical forces to separate wood fibers. In this process, yields are higher (up to 95%) and results in paper with good printing properties. However, the process requires a lot of electrical energy for refining, the fibers have poor strength properties, and high content of lignin causes a tendency to yellowing. Mechanical pulping can be combined with biological pulping to save refining energy (Akhtar et al. 1998). Also fungal treatment of fibers as a substitute for chemical treatment can not only make the process eco-friendly but high yielding as well by removing the hemicellulose content of the fibers.

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