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REVIEW

Microbial enzyme systems for lignin degradation and their transcriptional regulation

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Abstract **Lignocellulosic biomass is the most abundant renewable resource in nature and has received considerable attention as one of the most promising alternatives to oil resources for the provision of energy and certain raw materials. The phenolic polymer lignin is the second most abundant constituent of this biomass resource and has been shown to have the potential to be converted into industrially important aromatic chemicals after degradation. However, due to its chemical and structural nature, it exhibits high resistance toward mechanical, chemical, and biological degradation, and this causes a major obstacle for achieving efficient conversion of lignocellulosic biomass. In nature, lignin-degrading microorganisms have evolved unique extracellular enzyme systems to decompose lignin using radical mediated oxidative reactions. These microorganisms produce a set of different combinations of enzymes with multiple isozymes and isoforms by responding to various environmental stimuli such as nutrient availability, oxygen concentration and temperature, which are thought to enable effective decomposition of the lignin in lignocellulosic biomass. In this review, we present an overview of the microbial ligninolytic enzyme systems including general molecular aspects, structural features, and systematic differences in each microorganism. We also describe the gene expression pattern and the transcriptional regulation mechanisms of each ligninolytic enzyme with current data.**

Keywords lignocellulose biorefinery, lignin degradation, lignin peroxidases, manganese peroxidases, versatile peroxidases, laccases

Introduction

Ever since the Industrial Revolution during the late 18th and early 19th centuries, humans have been using fossil fuels, such as coal, petroleum and natural gas, as major energy sources for the development of socio-economic activities. While the standard of living has been significantly improved as a result of this period, mass consumption of fossil fuels has led to a dramatic increase in the atmospheric concentration of greenhouse gases (Beedlow et al., 2004), and concerns about global warming and climate changes have become realistic in recent years. For example, the period from 1983 to 2012 was reported to be the warmest 30-years during the last 1400 years, and many extreme weather and climate events including heat waves, severe droughts, and tropical cyclones, have been increasingly observed since 1950 (Bindoff et al., 2013). Moreover, although petroleum is still being used as one of the most important feedstock for mankind providing a number of different chemical raw materials through oil refinery processing (Roddy, 2013), it is a finite and non-renewable resource that is continuously being depleted over time. It is therefore imperative to explore and develop alternatives to fossil fuel resources for sustainable development of the economy and society. Against these backdrops, utilization of renewable biomass resources for the production of fuels, energy, and valuable chemicals has attracted increasing interest over the decades (Ohara, 2003; Kamm and Kamm, 2004; Ragauskas et al., 2006; Hatti-Kaul et al., 2007; FitzPatrick et al., 2010).

Among various forms of bioresources, plant biomass (lignocellulosic biomass) has been identified as the most abundant renewable resource on earth. It has been estimated that approximately 170×10^9–200×10^9 tons of the biomass is formed annually through photosynthesis, and thus it plays an important role in the global carbon cycle as a major carbon

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sink (Lieth, 1975; Pauly and Keegstra, 2008). Lignocellulose is mainly found in nature as the major structural component of the plant cell wall. The three main components of this biomass resource are polysaccharides cellulose and hemicelluose, and an aromatic polymer lignin, which are tightly packed together in the cell wall structure (Lynd et al., 2002). Because of its abundance, lignocellulose has become recognized as one of the most promising alternatives to oil resources for the provision of energy and raw materials (Kamm and Kamm, 2004). Although there are several forms of alternative renewable energy sources, such as wind, water, and solar power, lignocellulosic biomass is the only sustainable organic resource that can be converted to both energy and various value-added products (Kamm and Kamm, 2004; Zhang, 2008). For example, lignocellulosic biomass can be converted to energy sources such as biofuels by hydrolyzing the polysaccharide components into fermentable sugars with downstream fermentation processing (Hahn-Hägerdal etal., 2006). Bioethanol derived from lignocellulose can be used as transportation fuel and is now considered to be one of the most promising alternatives to petrol (Antoni et al., 2007). Moreover, small lignocellulose-derived molecules such as monomeric sugars and phenolic compounds could serve as raw materials for the production of various value-added products via biological- and chemical-conversions (Octave and Thomas, 2009; FitzPatrick et al., 2010; Menon and Rao, 2012). In 2004, the US Department of Energy selected the top 12 building block chemicals that can be produced from lignocellulose from more than 300 candidates (Peterson, 2004; Bozell and Petersen, 2010). The selected chemicals were shown to have the potential to be converted to a number of high-value chemicals or materials, and many researchers have used this report as their guideline to develop a conversion route for effective utilization of lignocellulosic biomass (Dusselier et al., 2014). The integrated utilization of biomass resources is also attractive from the point of view of the reduction of carbon dioxide (CO_2) emissions due to the carbon neutral nature of the resources. This is because CO_2 that arises during the use of products derived from plant biomass can be absorbed from the atmosphere by plants throughout their growth (Liu et al., 2012). Therefore effective utilization of lignocellulosic biomass has been considered to be one of the key technologies for the conservation of the environment and the sustainable development of humans providing a cure for resource and energy problems.

To initiate the production of useful materials from lignocellulosic feedstocks, decomposition of the major polymeric components into appropriate molecular formats, such as monomeric sugars, is necessary. However, due to its structural nature, lignocellulose exhibits high resistance toward chemical and biological degradation, and this is a major obstacle for establishing an efficient biorefining process (Himmel et al., 2007). In nature, many microorganisms including both fungi and bacteria are known to produce a wide variety of enzymes that are involved in the degradation of lignocellulosic materials (Lynd et al., 2002; Ruiz-Dueñas and Martínez, 2009; Bugg et al., 2011b; Paliwal et al., 2012). They utilize lignocellulose as energy and carbon sources through specific bioconversion systems and metabolic pathways for their growth, and thus play an important role in the global carbon cycle. Since enzyme reaction can be performed under environmentally friendly conditions, the use of microbial bioconversion systems for the production of useful materials is key to attaining an economically and environmentally feasible biorefinery process for lignocellulose. Consequently, new studies aimed at understanding lignocellulose biodegradation and bioconversion processes have come to the forefront of biomass research.

In this review, we will give an overview of the structural component of lignocellulosic biomass and lignin-degrading enzyme systems in microorganisms including white-rot fungi and bacteria with recent progress in enzymatic and genomic data analysis. Subsequently, gene expression profiles and transcriptional regulation of the lignin-degrading enzyme production in the microorganisms will be reviewed in order to understand their strategy for lignin degradation.

Plant cell wall polysaccharides and lignin

Lignocellulosic materials are derived from wood, grass, agricultural residues, and forestry wastes in nature. The approximate composition of the three major components in the plant cell wall are: 35%–50% cellulose, 20%–35% hemicellulose, and 5%–30% lignin (Lynd et al., 2002). These components are tightly packed together in the secondary cell wall of the plant cell to form a rigid and recalcitrant structure (Somerville et al., 2004).

Cellulose is the most abundant carbohydrate on earth, and is a homopolysaccharide consisting of D-glucose units that are linked by β-1,4-glycosidic bonds. Each glucose unit is rotated 180° relative to its two neighbors, thus forming linear chains. Individual cellulose chains are associated to each other by hydrogen bonds and Van der Waals forces to form a highly crystalline structure, called microfibrils (Gardner and Blackwell, 1974; Kolpak and Blackwell, 1976). One of the most important features of this polysaccharide is the crystalline nature of its cellulose molecules, which makes it strongly resistant to enzymatic and chemical degradation. The highly crystalline regions of cellulose in the microfibrils are separated by less ordered amorphous regions and the crystallinity of cellulose varies from 50% to 90% depending on the source (Hon, 1994).

Hemicellulose is a group of heteropolysaccharides with a lower molecular weight than cellulose. It consists of pentoses (D-xylose, L-arabinose), hexoses (D-mannose, D-glucose, D-galactose), and sugar acids (4-*O*-methyl-glucronic and D-galacturonic acid). These monomeric sugars are linked together by β-1,4- and occasionally by β-1,3-glycosidic bonds to form the backbone of hemicelluloses (Pauly et al.,

2013). Since hemicellulose has branches with short lateral chains, it does not form a crystalline structure like cellulose. Therefore its hydrolysis can be performed relatively easily; however, because of the heterogenetic nature of the hemicellulose their enzymatic degradation requires a variety of degrading-enzymes. The major hemicellulose in hardwood is glucuronoxylan, whereas glucomannan is predominant in softwood (Saha, 2003). Xylan is one of the most common hemicelluloses which consists of a backbone of β-1,4-linked D-xylopyranosyl residues substituted with L-arabinosyl, 4-*O*-methyl-glucuronosyl, and acetyl side chains. The most important biological function of hemicelluloses is reinforcing the cell wall structure by cross-linking cellulose microfibrils and lignin via direct interactions (Saha, 2003).

Lignin is a phenolic heteropolymer with a complex three-dimensional structure composed of phenylpropane units joined together by a variety of different ether or carbon-carbon bonds (Boerjan et al., 2003; Ralph et al., 2004) (Fig. 1). This polymer is synthesized through enzyme-mediated oxidative radical coupling of three main *p*-hydroxycinnamyl alcohols (monolignols) with different degrees of methoxyl substituents on their aromatic ring, including *p*-coumaryl (non-methoxylated), coniferyl (monomethoxylated), and sinapyl alcohol (dimethoxylated) (Fig. 2) (Boerjan et al., 2003; Ralph et al., 2004). The corresponding phenylpropanoid units in the lignin polymer are designated as *p*-hydrophenyl (H), guaiacyl (G), and syringyl (S) units, respectively. It has been shown that relative abundance of the main lignin monomer units varies among plant type, tissue, cell type, and developmental stage. In general, softwood lignins (gymnosperm lignins) are mainly made up of G units with very low levels of H units (G-type lignins), whereas hardwood lignins (angiosperm lignins) comprise of almost equal amounts of G units and S units (GS-type

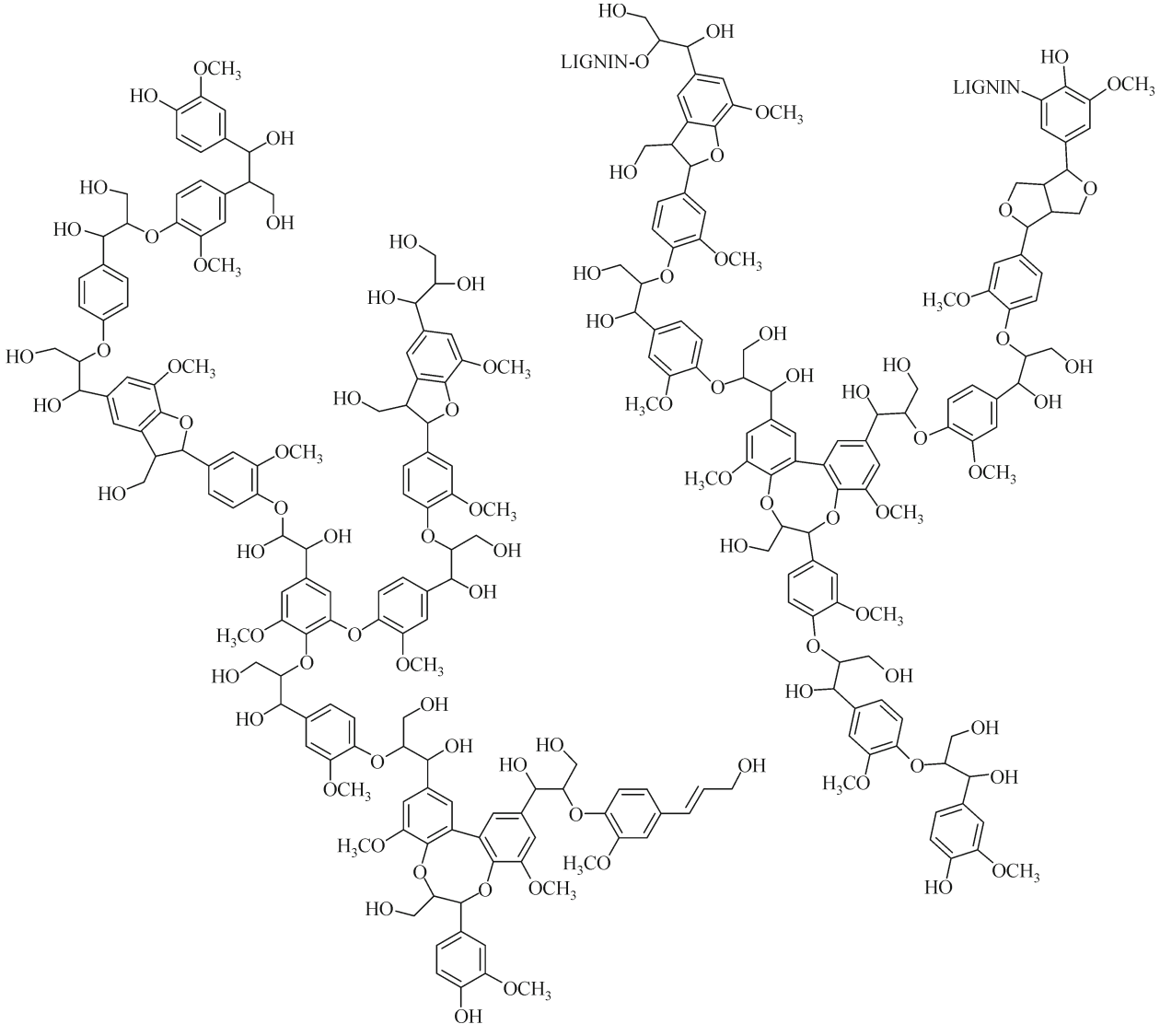

Figure 1 Structural model of softwood lignin. Schematic representation of a structural model of softwood lignin proposed by Adler (Adler, 1977) (adapted from (Crestini et al., 2010)).

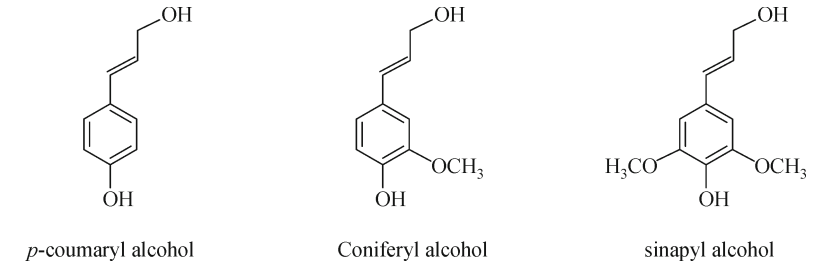

Figure 2 Chemical structure of the primary lignin monomers. Chemical structure of the three main monolignols *p*-coumaryl, coniferyl, and sinapyl alcohol is shown.

lignins). Grass lignins (monocot lignins) contain all the lignin monomer units in variable quantities; in which G and S units are the major constituents, and are classified as GSH-type lignin (Boerjan et al., 2003). Lignin polymerization takes place via oxidative-dehydrogenation of the monolignols mediated by endogenous plant peroxidases and/or laccases generating monolignol radicals. The generated radical species cause radical coupling reactions of the monomer units as well as the cross-coupling of the growing lignin oligomer/polymer units to form a complex three-dimensional molecular network. Because the radical species formed during lignin biosynthesis are resonance stabilized to generate various sites with enhanced single electron density, a variety of different ether and carbon-carbon bonds are formed during lignin biosynthesis. The most frequent inter-unit linkage found in the lignin structure is the β-*O*-4 (β-aryl ether) linkage. Other major linkages include β-5 (phenylcoumaran), β-β (resinol) 5-5 (biphenyl), 5-*O*-4 (diaryl ether) and β-1 (diphenyl ethane) linkages (Fig. 3). While the β-*O*-4 linkages can be broken down under harsh acidic or alkaline conditions, the other C-C linkages exhibit high resistance to both chemical and biological degradation (Boerjan et al., 2003; Ralph et al., 2004; Crestini et al., 2011), which makes lignin the most recalcitrant component of lignocellulose. Lignin gives strength and rigidity to the plant cell wall through chemical bonding to hemicellulose, in which the polysaccharides components are embedded by lignin (Balakshin Mikhail et al., 2007). In addition, it also provides the cells with impermeability to enable the transportation of water and solutes through the vascular system and resistance against oxidative stress and microbial attack by forming a barrier protecting the polysaccharides from the environments (Pérez et al., 2002; Crestini et al., 2010).

Since lignin is the only widely available aromatic renewable polymer on earth, it is considered as one of the most promising potential feedstocks for the production of renewable aromatic chemicals (Tuck et al., 2012; Ragauskas et al., 2014). However, due to its recalcitrant nature, effective conversion of lignin into value-added products is very challenging and this also causes a major obstacle in the

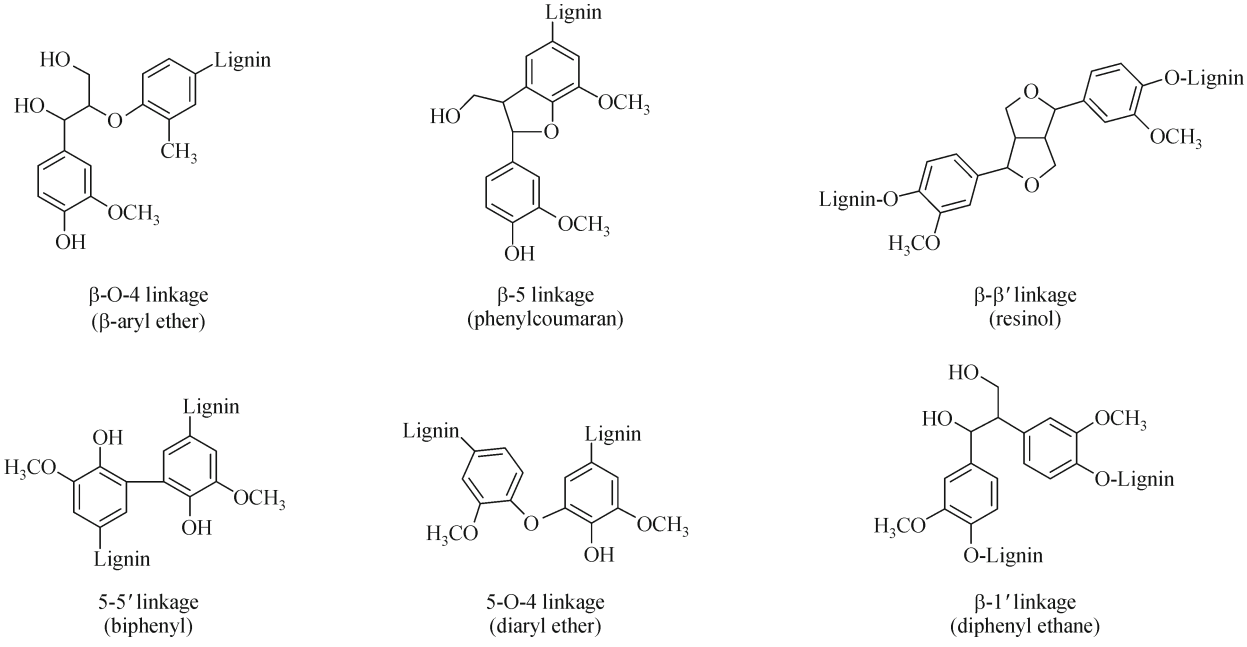

Figure 3 Common inter-unit linkages found in lignin polymer. Major structural units in the lignin polymer are shown (adapted from (Crestini et al., 2010).

biodegradation of lignocellulosic polysaccharides for biorefining (Hamelinck et al., 2005; Chen and Dixon 2007; Gupta et al., 2011; Zeng et al., 2014). Consequently, despite its excellent molecular characteristics, the use of lignin is still limited; most lignin is burned for power generation, and its valorization processes are substantially less developed compared to those for the polysaccharide component (Doherty et al., 2011; Azadi et al., 2013). However, total valorization of lignocellulosic biomass is necessary to lead to improvements in overall economics and sustainability for integrated biorefining in the future (Ragauskas et al., 2006). To utilize lignin as a raw material for the production of high-value products, a number of different approaches for its degradation have been studied; including physical, chemical, and biological methods (Kleinert and Barth 2008; Gasser et al., 2012; Azadi et al., 2013). In the following section we will summarize the biological machinery involved in lignin degradation.

Lignin-degrading microorganisms and lignin degrading enzyme machinery

Lignin-degrading microorganisms

In nature, many microorganisms including both fungi (e.g. *Trichoderma*, *Aspergillus*, *Humicola*, *Fusarium*, *Penicillium*, and *Phanerochaete*) and bacteria (e.g. *Streptomyces*, *Bacillus*, *Thermomonospora*, *Clostridium* and *Cellulomonas*) are known to produce a wide variety of enzymes that are involved in the degradation of plant cell wall polysaccharides (Lynd et al., 2002). On the other hand, only a limited group of microorganisms, especially a group of filamentous fungi belonging to the phylum of basidiomycota, has been shown to possess the ability to efficiently depolymerize lignin (Hatakka, 2001; Paliwal et al., 2012). These microorganisms can be found in fallen trees and forest litter where degradation of plant material takes place and plays a pivotal role in the recycling of carbon in the land ecosystem. Several studies indicate that lignin biodegradation is a slow process taking a number of years to complete (Kirk and Farrell, 1987; Hatakka, 2001). Lignin decomposition generally occurs in aerobic conditions and many oxidative enzymes, which generate highly reactive and non-specific free radicals, have been shown to be involved in this process.

Wood-rotting basidiomycetous fungi are generally classified into two distinct groups mainly based on the macroscopic appearances of the decayed wood: white-rot fungi and brown-rot fungi (Schwarze et al., 2000; Hatakka, 2001; Martínez et al., 2005). Among the lignin-degrading microorganisms white-rot fungi have been recognized as the most efficient lignin degraders, and they are the only known microorganisms that have the ability to efficiently depolymerize and mineralize large lignin molecules to water and carbon dioxide (Kirk and Farrell, 1987; Eriksson, 1990). The most popular white-rot fungi include *Phanerochaete chrysosporium, Pycnoporus cinnabarinus, Pleurotus ostreatus, Pleurotus eryngii, Phlebia subserialis*, *Trametes versicolor*, *Heterobasidion annosum* and *Irpex lacteus* (Hatakka, 2001; Paliwal et al., 2012). Although many white-rot fungi are able to degrade all the plant cell wall components simultaneously, some white-rot fungi, such as *Ceriporiopsis subvermispora*, preferentially decompose lignin without a substantial loss of cellulose and hemicellulose (Otjen et al., 1987; Blanchette Robert et al., 1992; Akin et al., 1996; Fackler et al., 2006). These white-rot fungi are referred to as selective lignin degrading fungi. White-rot fungi give the decayed wood a bleached white fibrous appearance, in which cellulose molecules are enriched, because of selective lignin decomposition during the wood rotting process (Hatakka, 2001; Martínez et al., 2005). Lignin-degrading enzyme systems in white-rot fungi have been extensively studied during the last three decades to develop their biotechnological application in the field of biopulping, biobleaching, bioremediation and biorefinery (Wesenberg et al., 2003; Bajpai, 2004; Bajpai et al., 2006; Raj et al., 2007; Asgher et al., 2008; Cañas and Camarero, 2010; Osma et al., 2010; Wan and Li, 2012).

In contrast to white-rot fungi, brown-rot fungi mainly degrade the plant cell wall polysaccharides but they also have the ability to modify lignin structure through the action of oxygen-derived free radicals, such as the hydroxyl radical ($\cdot$OH) generated by the extracellular Fenton-type reaction: $Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + \cdot OH$ (Eriksson 1990; Goodell et al., 1997; Hatakka, 2001; Arantes et al., 2012). The resultant brown-rotted lignin has been found to undergo extensive oxidative demethylation as well as significant side chain oxidation, and a slight depolymerization (Yelle et al., 2008; Koenig et al., 2010; Martínez et al., 2011; Arantes et al., 2012). Since the major part of modified lignin fragments still remain in the wood structure, the decayed wood exhibits a brown color and it forms a cubical structure with cracks and clefts on the surface as a result of cellulose degradation (Blanchette, 1995; Arantes et al., 2012). Brown-rot fungi represent less than 10% of the wood-rotting basidiomycetes (Gilbertson 1980) but they are widely spread in nature and cause wood decay mainly in softwoods (Martínez et al., 2005). Well-known examples of these brown-rotting fungi include *Gloeophyllum trabeum*, *Laetiporus portentosus*, *Fomitopsis palustris, Piptoporus betulinus, Postia placenta,* and *Serpula lacrymans* (Hatakka, 2001; Martínez et al., 2005)

In addition to white-rot fungi, some bacterial species, particularly filamentous bacteria belonging to the genus of *Streptomycesare* and *Nocardia*, have been reported to solubilize and modify lignin structure (Zimmermann, 1990; Bugg et al., 2011b). While bacteria are thought to possess a limited lignin degrading ability and to play a major role in the mineralization process of low-molecular weight compounds produced from lignin by fungi in soil (Masai et al., 2007),

recent studies indicate that several species are able to effectively break down lignin to release low molecular weight phenolic products (Ahmad et al., 2010; Ahmad et al., 2011; Roberts et al., 2011). The bacterial species reported to produce lignin-degrading activity fall into three classes of bacteria: actinomyces (*Rhodococcus* and *Nocardia*), α-proteobacteria (*Sphingobium*), and γ-proteobacteria (*Pseudomonas*) (Bugg et al., 2011b; Paliwal et al., 2012). Examples of lignin degrading bacteria include *Streptomyces viridosporus* T7A, *Pseudomonas putida* mt-2, and *Rhodococcus jostii* RHA1 (Ramachandra et al., 1988; Ahmad et al., 2010). Generally, non-filamentous bacteria are not able to decompose large lignin molecules, but some species have the ability to degrade low-molecular weight lignin derivatives through specific catabolic pathways. Bacterial catabolic pathways for lignin-derived aromatic compounds have been extensively studied in *Sphingobium* sp. strain SYK-6 and reviewed in several papers (Masai et al., 2007; Bugg et al., 2011a; Paliwal et al., 2012).

Lignin-degrading enzyme machinery

Over the past three decades, significant progress has been made in elucidating the molecular machinery involved in the biological degradation of lignin in-wood-rotting fungi and bacteria. Extensive studies of the model lignin degrader *P. chrysosporium*, as well as other white-rot fungi, has led to the identification of extracellular oxidative enzymes which play a key role in lignin decomposition. These oxidative machineries include four major groups of enzymes: lignin peroxidases (LiP, EC1.11.1.14), manganese peroxidases (MnP, EC 1.11.1.13), versatile peroxidases (VP, EC1.11.1.16), and laccases (Lac, EC 1.10.3.2). In addition to these oxidative enzymes, several accessory enzymes including glyoxal oxidases (GLOX: EC 1.1.3.-) (Kersten and Kirk, 1987; Kersten, 1990) and alcohol oxidases (AAO: EC 1.1.3.7) (Guillén et al., 1992), which provide hydrogen peroxide to the peroxidase reactions, have been reported to play a role during lignin degradation. Moreover, recent studies of bacterial lignin degradation systems have discovered the involvement of a new class of enzyme, Dye-decolorizing peroxidases (DyP: EC 1.11.1.19) (Ahmad et al., 2011), in lignin degradation suggesting wide diversity in the lignin degrading enzymes found in nature. Due to the structural and chemical complexity of lignin molecules, ligninolytic microorganisms generally produce an array of extracellular oxidases. However, characteristics of each enzyme system, such as the type of major enzyme component and physiologic conditions for the enzyme productions, vary significantly between ligninolytic microorganisms. Thus, it has been observed that some white-rot fungi produce several different classes of oxidative enzymes while others produce only one or two of them (Lundell et al., 2010; Floudas et al., 2012). Microbial enzymes involved in decomposition of lignin will be discussed in the following sections.

Lignin peroxidases

Lignin peroxidase (LiP) was first discovered in 1983 (Tien and Kirk, 1983) in *P. chrysosporium* and further characterized as a true lignase because of its high redox-potential. In addition to its high redox-potential nature, LiPs generally have a very low pH optimum near pH 3.0, which makes them distinctive from the other peroxidases (Kirk and Farrell, 1987; Tien and Kirk, 1988; Eriksson 1990; Cullen, 1997). Lignin peroxidases containing a heme glycoprotein belonging to the class II secreted fungal heme peroxidase family of the plant peroxidase family, in which bacterial, fungal and plant peroxidases are classified into three subfamilies (Welinder et al., 1992). LiPs have the ability to oxidize a wide range of aromatic compounds unsusceptible to the action of other peroxidases including lignin and lignin analogous compounds by one-electron oxidation mechanisms under the presence of H_2O_2 (Paliwal et al., 2012). One of the most important aspects of LiPs in the lignin degradation is that this type of enzyme can oxidize not only phenolic units but also non-phenolic lignin units, which share up to 90% of the lignin structure (Hammel et al., 1993). Therefore, LiPs have been considered to play an important role in enzymatic lignin degradation in natural environments. Reactions catalyzed by LiPs include the Cα-Cβ cleavage of propyl side chains in lignin and lignin model compounds; the hydroxylation of benzylic methylene groups; the oxidation of benzyl alcohols to corresponding aldehyde or ketones; phenol oxidation and aromatic cleavage of non-phenolic lignin model compounds (Wong, 2009; Paliwal et al., 2012).

The catalytic cycle of LiPs is similar to that of typical heme-containing peroxidases, such as horseradish peroxidase, and consists of a three step reaction (Fig. 4). In the first step, the resting state of the ferric enzyme [Fe(III)] reacts with H_2O_2 to form a Compound I oxo-ferryl intermediate [Fe(IV) = $O\cdot^{+}$] by two-electron oxidation with hydrogen peroxide cleavage. Compound I subsequently oxidizes electron donor substrates by one-electron oxidation forming Compound II [Fe(IV) = O] and a substrate cation radical. Finally, Compound II again oxidizes the substrate by subtracting one electron to return the enzyme to the native resting state completing one catalytic cycle (Kirk and Farrell, 1987; Conesa et al., 2002; Martínez, 2002). In the presence of an excess of H_2O_2 and the absence of a reducing substrate, the LiP-Compound II intermediate is also known to react with H_2O_2 to form a catalytically inactivated form of the enzyme, designated as Compound III which exists as a ferric-superoxo complex [Fe(III)$O\cdot_2^{-}$]·. Compound III can be returned to the resting form by spontaneous auto oxidation or by oxidation with a veratryl alcohol cation through displacement of superoxide from the active site (Valli et al., 1990; Wariishi and Gold, 1990).

While LiPs have similar catalytic cycle to typical heme peroxidases, this enzyme is characterized by its unique ability to oxidize high redox potential substrates, which enable oxidation of non-phenolic lignin substrates. Analysis of the

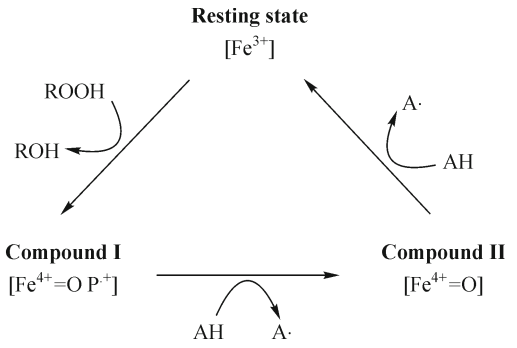

Figure 4 Typical catalytic cycle of general heme peroxidases. The catalytic cycle of general heme peroxidases consist of three step reactions including two-electron oxidation of the resting ferric enzyme [Fe(III)] by hydroxyperoxide to yield Compound I oxo-ferryl intermediate [Fe(VI) = OP·+]. Compound I subsequently oxidizes electron donor substrates (AH) by one-electron oxidation forming Compound II [Fe(VI) = O] and a substrate cation radical (A·). Finally, Compound II oxidizes the substrate by subtracting one electron to return to the native resting state (Dunford 1999).

crystal structure of ligninolytic peroxidases (Edwards et al., 1993; Poulos et al., 1993; Choinowski et al., 1999) revealed two important structural aspects that differentiate lignin peroxidase from the other heme peroxidases (Fig. 5). The first difference was observed in the molecular structure around the heme pocket region. In lignin peroxidases the Nε2 of the side chain of a histidine residue (the so called proximal histidine) which is located away from the heme iron, formed a significantly longer hydrogen bond than is formed by the other low-redox peroxidases. The length of this hydrogen Fe-Nε2 bond affects the electron deficiency of iron and consequently increases the redox potential of the oxo-ferryl complex (Conesa et al., 2002; Martínez, 2002). The second difference was the presence of specific binding sites for oxidation of specific substrates, including non-phenolic aromatics (Conesa et al., 2002; Martínez, 2002). On the LiP protein surface, there is the main channel that enables both hydrogen peroxide and reducing substrates to access the heme cofactor for LiP mediated enzyme reaction (Poulos et al., 1993). Although small substrate molecules, such as small phenolic compounds, can enter the protein to transfer electrons directly to the heme cofactor, a large bulky lignin polymer is not able to access the main channel. Therefore LiPs have developed the ability to oxidize these compounds on the protein surface using a mechanism called a long-range electron transfer system (Doyle et al., 1998; Ruiz-Dueñas and Martínez, 2009). These enzymes use an exposed tryptophan on the protein surface to form a tryptophanyl-free radical via a reaction with heme Compound I, where they can directly interact with the lignin polymer. Therefore these substrates are oxidized at the enzyme surface, and electrons are transferred to the heme through long-range electron transfer mechanisms (Ruiz-Dueñas and Martínez, 2009).

Lignin peroxidases are usually secreted as sets of multiple isozymes and isoforms in *P. chrysosporium* and other white-rot fungi (Gaskell et al., 1994). Interestingly, some of the white-rot fungi (e.g. *C. subvermispora*, *Dichomitus squalens*, *Panus tigrinus*, *Rigidoporus lignosus*) have been reported not to produce any extracellular lignin peroxidase even though they show significant lignin depolymerizing activity (Galliano et al., 1991; Périé and Gold, 1991; Golovleva et al., 1993; Lobos et al., 1994; Hatakka, 2001). This suggests that lignin peroxidases are not always related to efficient degradation of the lignin polymer and each microorganism has evolved their own strategy for efficient utilization of lignocellulosic materials.

Manganese peroxidases

Manganese peroxidase was also discovered in the culture fluid of *P. chrysosporium* in the mid-1980s (Gold et al., 1984; Kuwahara et al., 1984; Paszczynski et al., 1985). Manganese peroxidases are a heme-containing glycoprotein belonging to the class II peroxidase family that catalyzes the oxidation of Mn^{2+} to Mn^{3+} using H_2O_2 as an oxidant (Paszczynski et al., 1985). The generated Mn^{3+} is a strong, readily-diffusible oxidizer but it is quite unstable in aqueous solution. Consequently, Mn^{3+} can be stabilized by chelation with organic acids such as oxalate and malonate forming a Mn^{3+}-chelator complex, which acts as a small diffusible oxidizer for lignin oxidation (Glenn and Gold, 1985; Glenn et al., 1986). Indeed, many MnP producing white-rot fungi secrete organic acids, mainly oxalate, as a secondary metabolite, and physiologic concentration of oxalate in the *P. chrysosporium* culture filtrate has shown to be enough to stimulate MnP activity (Kuan et al., 1993; Kishi et al., 1994). The generated Mn^{3+}-chelator complex can oxidize a variety of phenolic substrates including monomeric phenols and phenolic lignin structures via one-electron oxidation of the substrates to produce a phenoxy-radical intermediate, which finally leads to the decomposition of the compounds (Wong, 2009). However, after stabilization by the chelator molecule, the electron potential of the Mn^{3+}-complex is lowered in comparison to the non-chelated Mn^{3+} cation and acts as a mild oxidant. Thus it cannot directly attack the dominant non-phenolic structures in the lignin polymer, unlike lignin peroxidases. However, it has been proven that MnPs also contribute to the oxidation of non-phenolic lignin structures in the presence of a second mediator through the formation of highly reactive radical species (Wariishi et al., 1989; Reddy et al., 2003). The enzymatically generated Mn^{3+} has been shown to oxidize both thiols (e.g. glutathione) and unsaturated lipid (e.g. linoleic acid) to thiyl and lipid peroxyl radicals respectively. The radicals formed are able to oxidize a variety of non-phenolic compounds via hydrogen abstraction mechanisms to form a benzylic radical. The resultant radical undergoes non-enzymatic reactions to produce the degradation compounds (Reddy et al., 2003). The MnP-lipid system

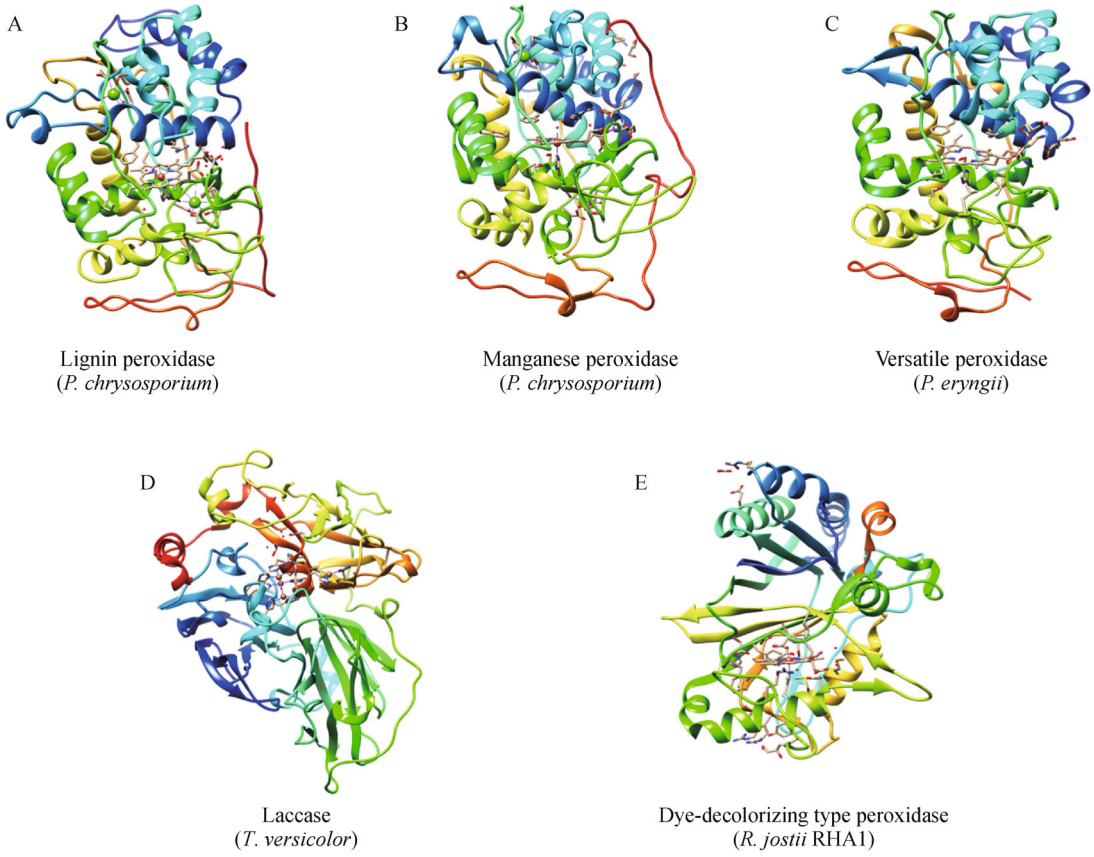

Figure 5 Three-dimensional structures of ligninolytic enzymes. 3D structures of the ligninolytic enzymes are shown. (A) A lignin peroxidase from *P. chrysosporium*; Protein Data Bank (PDB) ID: 1LGA (Poulos et al., 1993). (B) A manganese peroxidase from *P. chrysosporium*; 3M5Q (Sundaramoorthy et al., 2010). (C) A versatile peroxidase from *P. erynjii*; PDB ID: 3FM1 (Perez-Boada et al., 2005). (D) A laccase from *T. versicolor*; PDB ID: 1GYC (Piontek et al., 2002). (E) A Dye-decolorizing type peroxidase from *R. jostii*; PDB ID: 3QNR (Roberts et al., 2011).

has been reported to catalyze the decomposition of Cα-Cβ and β-aryl ether bonds of non-phenolic β-*O*-4 lignin models (Bao et al., 1994). To support the involvement of the MnP-lipid oxidation system in lignin degradation, several peroxidizable unsaturated fatty acids were detected from wood decaying culture of *C. subvermispora* (Gutiérrez et al., 2002). Furthermore, significant upregulation of the putative fatty acid desaturase-encoding genes, which were possibly involved in the production of these unsaturated lipids, was observed in this fungus during their growth in aspen wood culture conditions, together with the upregulation of MnP genes (Fernandez-Fueyo et al., 2012). These results suggest that unsaturated fatty acids probably mediate the oxidation reaction of non-phenolic lignin structures under physiologic conditions.

The catalytic cycle of MnPs is also similar to that of the typical plant heme peroxidase (Fig. 4), in which the native ferric state of MnP reacts with hydrogen peroxide to form a Compound I oxo-ferryl intermediate. However, MnPs are unique among peroxidases in utilizing Mn^{2+} as its primary reducing substrate, which it then oxidizes to form Compound II and Mn^{3+}. The resulting Compound II performs another round of Mn^{2+} oxidation before returning to the native resting state, completing its catalytic cycle (Wariishi et al., 1988). It has been reported that MnP Compound II also forms Compound III in the presence of an excess amount of hydrogen peroxide resulting in heme bleaching and irreversible inactivation of this enzyme (Wong, 2009).

Comparative structural analysis of manganese peroxidases and lignin peroxidases highlights similarities in their entire structure and the heme active site environment (Sundaramoorthy et al., 1994; Martínez, 2002) (Fig. 5). However, manganese peroxidases do not have the surface exposed tryptophan residue for long-range electron transfer that is found in lignin peroxidases (Martínez, 2002). Instead, manganese peroxidases possesses a Mn^{2+} binding site on their surface that is composed of two or three acidic amino acid residues (Sundaramoorthy et al., 1994; Sundaramoorthy et al., 1997), which enable both high-efficiency oxidation of Mn^{2+} substrate and Compound II reduction. This binding site is located near the heme cofactor, which enables direct electron transfer to one of the heme propionates, the function of which has been confirmed by site-directed mutagenesis (Kishi et al., 1996; Whitwam et al., 1997).

Comparative genome analysis of 31 different fungal strains has shown that manganese peroxidases are the most common ligninolytic enzyme produced by almost all white-rot fungi (Floudas et al., 2012) suggesting that manganese peroxidases play a more important role in the fungal lignin degradation system than lignin peroxidases. Manganese peroxidases are generally secreted as a set of multiple isozymes and isoforms in the fungi, in a similar way to lignin peroxidases. There are five manganese peroxidase-encoding genes that have been detected in the genome of *P. chrysosporium* and 13 that have been found in the genome of *C. subvermispora*, respectively (Floudas et al., 2012). Studies of the lignin-degrading enzyme system in the white-rot fungus *Phlebia radiata* showed that there are two different types of manganese peroxidase which belong to phylogenetically different subfamilies in the class II fungal peroxidases; a group of manganese peroxidase with long C-terminal extension including the typical Mn^{2+}-oxidizing peroxidases (long manganese peroxidases) and a group of the enzyme without the C-terminal extension (short manganese peroxidase) (Hildénet al., 2005). While both groups of the enzymes show Mn^{2+}-oxidizing activity, the latter group is reported to be able to oxidize phenols, amines and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) in the absence of Mn^{2+}, suggesting functional diversity of manganese peroxidases in the fungal lignin degradation system (Lundell et al., 2010). Also, with regards to substrate specificity, the short manganese peroxidases are similar to the third group of the class II fungal peroxidases, the versatile peroxidases, which will be summarized in the following section. In addition to these groups, two sub-types of manganese peroxidases, which have an extra-long C terminus structure found in *D. squalens* manganese peroxidase (Li et al., 1999) and a slightly modified Mn^{2+} binding site lacking one or two of the conserved acidic amino acids found in *Agrocybe praecox* manganese peroxidase (Hildénet al., 2014), have been reported. From the genome analysis, it has been indicated that the old lignin peroxidase and manganese peroxidase ancestor does not contain the Mn^{2+} binding site and the surface tryptophan for long-range electron transfer. Recently the first crystal structure of the short manganese peroxidase was determined during an intensive characterization of ligninolytic peroxidase in *P. ostreatus* (Fernández-Fueyo et al., 2014b).

Versatile peroxidases

Versatile peroxidases (VPs) are the third class of the fungal peroxidase family first discovered in *P. eryngii* (Martínez et al., 1996). Historically, this class of enzyme was identified as a manganese peroxidase, which exhibited oxidizing activities on some aromatic substrates in a similar manner to lignin peroxidases (Martínez, 2002). Latterly, they were characterized as versatile peroxidases and have been identified from the *Bjerkandera* species, and the *Pleurotus* species (Heinfling et al., 1998; Mester and Field, 1998). Extensive research of the enzymatic properties of these enzymes has revealed unique catalytic properties. Versatile peroxidases have been shown to act in a bifunctional manner similar to both MnPs and LiPs showing hybrid catalytic functions (Ruiz-Dueñas et al., 2001; Martínez, 2002; Moreira et al., 2005; Pérez-Boada et al., 2005; Ruiz-Dueñas and Martínez, 2009; Morales et al., 2012). Therefore, VPs are able to directly oxidize Mn^{2+} as well as high redox potential methoxybenzenes, and aromatic compounds including both phenolic and non-phenolic lignin models. Interestingly, VPs show different pH optima for the two different types of enzyme activities; pH5.0 was determined as optimum for Mn^{2+} oxidation and pH3.0 was characterized for aromatic compounds oxidation (Pérez-Boada et al., 2005; Ertan et al., 2012; Pozdnyakova et al., 2013; Fernández-Fueyo et al., 2014b). These optimum pH values correspond to those observed for typical MnPs and LiPs, respectively (Paliwal et al., 2012). VPs have also been reported to catalyze the two different types of oxidation reactions following the molecular mechanisms that have been described in the previous sections for MnPs and LiPs. It has been reported that the VP from *P. eryngii* exhibits a catalytic efficiency for Mn^{2+} oxidation that is comparable to that observed for a typical MnPs (Ruiz-Dueñas et al., 2007). On the other hand, its catalytic efficiency toward veratryl alcohol is reported to be significantly lower than reported for *P. chrysosporium* LiPs (Ruiz-Duñas et al., 2009a) .

The molecular structures of VPs share common features with the other fungal ligninolytic heme peroxidases in terms of overall structure and the characteristic heme binding environment (Fig. 5). Analysis of the crystal structural of *P. eryngii* VP revealed that its entire structure is more similar to the LiPs than the MnPs from *P. chrysosporium* (Pérez-Boada et al., 2005; Ruiz-Dueñas and Martínez, 2009). In addition to these features, VPs have revealed to possess a LiP-MnP hybrid molecular structure, in which the amino acids corresponding to the surface tryptophan residue involved in the long-range electron transfer found in the LiPs and the three acidic amino acid residues for Mn^{2+} binding observed in MnPs have been conserved (Banci et al., 2003; Pérez-Boada et al., 2005; Pogni et al., 2006; Morales et al., 2012). The basic features of the catalytic cycle for VPs are similar to that of the other fungal heme peroxidases, in that they are initiated by the formation of Compound I with two-electron oxidation of the resting enzyme followed by the two consecutive one-electron oxidations of the substrates to return the enzyme to the resting state (Fig. 4). In the presence of Mn^{2+}, VPs can use this ion as the electron donor to run its catalytic cycle via the formation of typical Compound I and II (Pérez-Boada et al., 2005). However, because of the hybrid nature of VPs, the involvement of another catalytic cycle has also been reported during their catalytic process. This extended cycle includes the formation of two additional intermediates called Complex I_B (containing Fe^{4+}-oxo-ferryl intermediate and tryptophan radical) generated from Complex I, and Complex II_B (containing Fe^{3+} and tryptophan) derived from Complex II (Pérez-Boada et al., 2005; Pogni et al., 2006). These

intermediates have the potential to oxidize the high-redox potential aromatic compounds by a long-range electron transfer mechanism using the tryptophan radical on the protein surface as a catalytic site (Pogni et al., 2006; Ruiz-Dueñas et al., 2009b). Consequently, multiple reaction sites could be involved in the VP mediated oxidation reaction depending on the substrates available and environmental pH conditions. Because of these unique catalytic properties, VPs have attracted great interest in the biotechnological application field.

According to the recent whole-genome analysis in basidiomycetous fungi, it has been indicated that only limited members of the white-rot fungi group produce versatile peroxidases with small numbers of isozymes (Floudas et al., 2012). So far, VPs have been identified in the genome of *B. adusta* (1VP), *D. squalens* (3VPs), *Ganoderma* sp. (2VPs), *P. ostreatus* (4VPs), and *T. versicolor* (1VP) (Kersten and Cullen 2014). The original versatile peroxidase producer *P. eryngii* has been characterized to secrete at least two VP isozymes into the culture medium, which only differ in three amino acid residues showing 99% identity (Ruiz-Dueñas et al., 1999). Among the available genome sequences, *P. ostreatus* has been shown to have the highest number of putative VP-encoding genes in its genome (Floudas et al., 2012; Kersten and Cullen, 2014). Intensive molecular characterization of the class II heme peroxidase system including four putative VPs and five putative MnPs has only recently been performed in *P. ostreatus* (Fernández-Fueyo et al., 2014b). This analysis showed significant differences in their pH and temperature stabilities among the heterologously expressed VPs and one of the *in silico* predicted VPs has been reclassified as a MnP according to its catalytic properties. Most importantly, successful oxidative degradation of non-lignolytic model compounds with the β-*O*-4 ether structure and ^{14}C-labeled synthetic lignin were demonstrated by applying the heterologously expressed VP1, which provides direct evidence of the functional involvement of versatile peroxidases in lignin degradation.

Laccases

Laccases (benzenediol:oxygen oxidoreductase, E.C. 1.10.3.2) are multicopper oxidases that catalyze the one-electron oxidation of various phenolic substrates with concomitant reduction of oxygen to water (Wong, 2009) (Fig. 6). Laccase was first identified from the sap of the Japanese lacquer tree *Rhus vernicifera* (Yoshida, 1883). Since this discovery laccases have been isolated from a wide variety of organisms including plants (Mayer and Staples 2002), insects (Kramer et al., 2001), some bacteria (Claus, 2003), and various ascomycetous and basidiomycetous fungi (Baldrian, 2006) (Dwivedi et al., 2011). Among the laccase-producing organisms, white-rot basidiomycetous fungi are generally

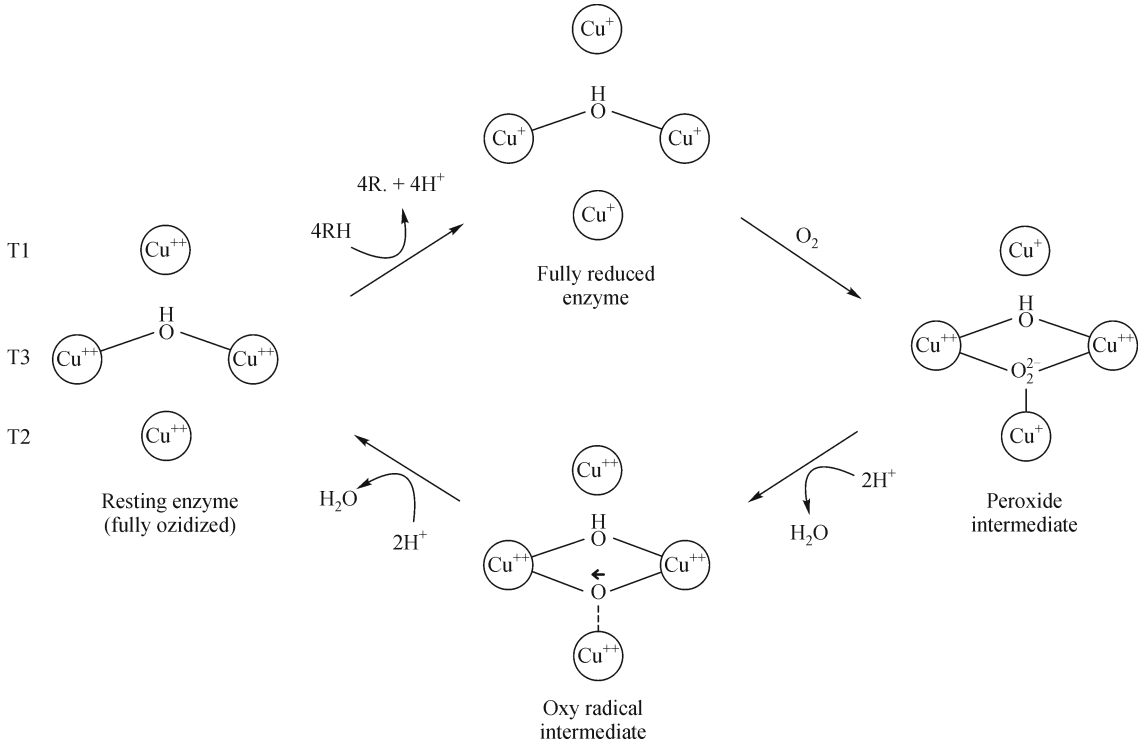

Figure 6 Catalytic cycle of laccases. A schematic representation of the catalytic site and the catalytic cycle of laccases, showing the mechanism of reduction and oxidation of the enzyme copper sites. A substrate reduces the T1 site in the resting enzyme, which then transfers the electron to the trinuclear site resulting in a fully reduced state of the enzyme. Thereafter, reduction of one oxygen molecule occurs at the trinuclear center through the formation of oxygen-bound intermediates, peroxy-intermediates and native-intermediates, with two sequential electron transfer resulting in a formation of two molecules of water (Reproduced from (Wong, 2009))

found to produce high levels of laccase activity, and *P. cinnabarinus* has been shown to produce more than 1g per litter of laccases into the culture medium (Levasseur et al., 2014). As is expected from the wide distribution of laccases in the living world, laccases have shown to be involved in various biological processes that include lignin biosynthesis in the plant cell wall (Boerjan et al., 2003), sclerotization of cuticle in the epidermis in insects (Kramer et al., 2001), morphogenesis, stress resistance and pigmentation, copper and iron homeostasis, detoxification of the living environment and lignin biodegradation in bacteria and fungi (Williamson et al., 1998; Enguita et al., 2003; Stoj and Kosman 2003; Baldrian 2006; Sharma et al., 2007). Laccases have been reported to catalyze oxidation of a wide-variety of compounds including substituted phenols, aminophenols and aromatic thiols, some inorganic ions, and importantly several phenolic lignin compounds (Baldrian 2006; Wong 2009; Giardina et al., 2010). After one-electron oxidation by laccases, the phenolic substrates turn into phenoxy radicals, which can spontaneously cause radical polymerization as is observed during the lignin biosynthesis or random molecular rearrangement with several types of cleavage reactions. From studies using the β-1 lignin structure model compound, it has been reported that the cleavage reactions including alkyl-alkyl cleavage, Cα oxidation, Cα-Cβ cleavages, and aromatic ring cleavages can occur during the oxidation reaction mediated by laccases (Wong, 2009), suggesting that microbial laccases play a role in lignin-degradation to some extent.

In ligninolytic fungi, laccases are generally produced as an extracellular monomeric glycoprotein with multiple isozymes (Baldrian, 2006). Each laccase molecule contains four copper atoms designated as type-1 (T1, one copper atom), type-2 (T2, one copper atom), and type-3 (T-3, two copper atoms) in their active site (Claus, 2004) (Fig. 6). The catalytic cycle of laccase is started from the resting state, in which all four copper atoms are in the 2^+ oxidation state (Malkin and Malmström 1970; Wong, 2009) (Dooley et al., 1979). The T1 copper has been show to act as the primary electron acceptor, which mediates one-electron oxidations of reducing substrates with a generation of free radicals. After four cycles of the one-electron oxidation, the captured electrons are then transferred to the trinuclear site formed by the T2 and T3 copper atoms resulting in a fully reduced state of the enzyme. Finally reduction of one oxygen molecule occurs at the trinuclear center through the formation of oxygen-bound intermediates, peroxy-intermediates and native-intermediates, with two sequential electron transfer resulting in a formation of two molecules of water (Gianfreda et al., 1999; Wong 2009; Giardina et al., 2010) (Fig. 6). It is generally accepted that molecular environment of the copper atom in the T1 copper site affects electrochemical potential of laccases, which define its reactivity toward different reducing substrates (Yaropolov et al., 1994; Morozova et al., 2007). Laccases can be classified into three groups according to the redox potential of the T1 site: Low (0.4–0.5 V), medium (0.5–0.6 V) or high (0.7–0.8 V) redox potential laccase (Xu et al., 1996). Because of their high reactivity toward a wide range of substrates, high redox potential laccases have been found attractive for various industrial applications. Due to their broad substrate specificity, laccases are of great industrial interest and have been used in paper and wood processing and in the textile industry (Rodríguez Couto and Toca Herrera, 2006; Husain and Husain, 2007; Kunamneni et al., 2008; Cañas and Camarero, 2010; Osma et al., 2010; Shraddha et al., 2011).

On the other hand, due to relatively low redox potential of laccases compared with the ligninolytic peroxidases (0.8-1.4V), it has been reported that they cannot directly attack non-phenolic units in the lignin molecules. However, as some ligninolytic fungi, such as the *Trametes* and *Pycnoporus* species have been shown to use laccases as the main ligninolytic enzymes in their lignin-degrading system (Eggert et al., 1997), laccases are also able to attack the non-phenolic lignin structures under the presence of certain compounds, so-called mediators. The mediator molecules are the compounds that can act as an electron carrier between the enzyme and the final substrate (Bourbonnais and Paice, 1990), and the biocatalytic system using the combination of laccase and mediators is called the laccase-mediator system (LMS) (Cañas and Camarero, 2010). To support the involvement of the LMS in lignin degradation in nature, 3-hydroxyanthranilic acid (3-HAA), a secondary metabolite produced by *P. cinnabarinus,* was shown to mediate the oxidation of the non-phenolic compounds by laccases (Eggert et al., 1996). So far, over 100 synthetic chemical compounds have been reported to act as a mediator (Call and Mücke, 1997; Cañas and Camarero, 2010), in which 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 1-hydroxybenzotriazole (HBT) have been widely used for oxidation of non-phenolic compounds (Bourbonnais and Paice, 1990; Cañas and Camarero, 2010). In addition to the synthetic mediators, some naturally-occurring phenolic compounds related to lignin polymer, have also been reported to function as an effective mediator (Camarero et al., 2005). These compounds include syringaldehyde, acetosyringone, sinapic acid, vanillin, and *p*-coumaric acid, which significantly expand the oxidation ability of laccases. These compounds have recently been successfully applied to LMS for various applications including delignification of paper pulp and Eucalyptus wood chips (Fillat et al., 2010; Andreu and Vidal, 2011; Rico et al., 2014). These recent reports suggest that laccases also play a role in the degradation of non-phenolic lignin structures using these natural mediators during the natural ligninolysis process.

In wood-rotting fungi, laccases are generally produced as a multiple isozyme but their enzymatic properties including molecular weight, optimum conditions, and redox-potentials, are significantly different from each other (Baldrian, 2006). On the other hand, bacteria have been reported to produce only a few laccase isozymes and they were assumed to be

mostly intracellular or spore-bound (Claus, 2004; Santhanam et al., 2011). Interestingly, while laccase-encoding genes have been identified in the genome of nearly all wood-rotting fungi (Floudas et al., 2012; Riley et al., 2014), some basidiomycete including the model ligninolytic microorganism *P. chrysosporium* does not possess any conventional laccase genes (Martinez et al., 2004) suggesting strategic diversity in the biological lignin degradation system. Recent genome analysis suggest that white-rot fungi seem to have more laccase encoding genes in their genome compared to brown rot-fungi, where the maximum number of the putative genes were predicted as 16 in the white-rot fungus *Ganoderma* sp. and six in the brown-rot fungus *C. puteana* (Riley et al., 2014). In contrast to fungal laccase research, relatively few studies have been done for bacterial laccases. However, several laccases with unusual characteristics, such as high thermo-resistance, have been identified from bacteria (Miyazaki, 2005), and are becoming recognized as an important potential enzyme source for future biotechnological application. Ausec et al. (Ausec et al., 2011) recently performed a bioinformatic survey on over 2000 completed and draft genome and metagenomic data sets to identify putative laccase-like enzymes. As a result of this survey more than 1200 putative laccase-like genes have been retrieved from chromosomes and plasmids of diverse bacteria including anaerobic bacteria, autotrophs and alkaliphiles. In the case of bacterial genome, two to four of the laccase-encoding genes have generally been predicted in the genome and one to four of them have been identified on plasmid DNA (Ausec et al., 2011). These results suggest great potential of bacteria as a source of novel laccases for future biotechnological applications.

Dye-decolorizing peroxidases

Dye-decolorizing peroxidases (DyP) are members of a novel heme peroxidase family (DyP-type peroxidase family), which is distinct from the general plant and animal heme peroxidase family (Welinder et al., 1992; Sugano, 2009). The first DyP was discovered in 1995 from the basidiomycete *Thanatephorus cucumeris* and was reported to be able to decolorize 18 types of dyes including anthraquinone dyes, which are a poor substrate for the plant and animal peroxidases (Kim et al., 1995; Sugano, 2009). Later study including purification and characterization of a DyP-type peroxidase (Dec1) from this fungus revealed that this enzyme is a heme-containing protein with different substrate specificity for the typical heme-peroxidases belonging to the plant peroxidase family (Kim and Shoda, 1999). Although some white-rot fungi have been shown to produce DyP-type peroxidases (Kim et al., 1995; Faraco et al., 2006; Salvachúaet al., 2013), it has been reported that DyP-type peroxidases are prominently identified from the genome of bacteria, and only a small number are found in fungi and higher eukaryote (Colpa et al., 2014). Therefore, the authors have suggested that this peroxidase family should be renamed into the superfamily of bacterial peroxidases (Colpa et al., 2014). Meanwhile, based on their molecular structure, DyP-type peroxidases have also been categorized into a new protein superfamily called the CDE superfamily (Goblirsch et al., 2011). The DyP-type peroxidases family is further divided into four different subfamilies (A to D) based on phylogenetic analysis, subcellular localization, and the origin of the enzyme (Ogola et al., 2009; Sugano, 2009).

The involvement of DyP-type peroxidases in the biological lignin-degradation process was first characterized for DypB from *R. jostii* RHA1 during studies aiming to isolate bacterial lignin-degraders (Roberts et al., 2011). Bioinformatic analysis of the genome sequence of *R. jostii* RHA1 revealed the presence of two unannotated putative peroxidases, and this finding led to the identification of the novel lignin-degrading enzyme DypB, which was the first detailed characterization of a recombinant bacterial lignin peroxidase (Ahmad et al., 2010; Ahmad et al., 2011; Roberts et al., 2011; Taylor et al., 2012). DypB has been shown to degrade Kraft lignin especially in the presence of Mn^{2+} ion, and β-aryl ether lignin model compounds with Cα-Cβ bound cleavage (Ahmad et al., 2011; Roberts et al., 2011). Notably, this discovery opens a new door for the research of bacterial lignin-degradation systems and suggests a new role for bacteria in the natural lignin degradation process.

The catalytic cycle of the DyP-type peroxidases have been proposed to be similar to that of the typical plant heme-peroxidases, although their molecular structures are significantly different (Sugano et al., 2007; Sugano, 2009; Roberts et al., 2011; Yoshida et al., 2011) (Fig. 5). Therefore, the resting state of the enzyme reacts with H_2O_2 to form Compound I followed by two one-electron oxidation of the reducing substrate to return the enzyme to the resting state through the formation of Compound II (Sugano et al., 2007; Sugano, 2009; Roberts et al., 2011) (Fig. 4). However, although details of the catalytic cycles still need to be investigated, recent studies show several differences between the Dyp-type peroxidases and the other peroxidases. It has been reported that Dec1 from *T. cucumeris* forms an unusually stable Compound I intermediate which measured a half-life of ~9 min and returned to the resting state without a detectable formation of Compound II (Sugano et al., 2007; Sugano, 2009). The formation of similar intermediates with long half-life times has been observed for DypB from *R. jostii* but the nature of the stable compound is unclear (Roberts et al., 2011). Interestingly, it was found that DypA from *R. jostii*, which has been characterized to not be involved in lignin degradation, generates an intermediate with the features of compound II, suggesting variations in the catalytic cycle of DyP-type peroxidases (Roberts et al., 2011).

X-ray structural analysis of DyP-type enzymes revealed a major structural difference between DyP-peroxidases and the other heme-peroxidases. While the plant heme-peroxidases composed of mainly α-helical structures, Dyp-peroxidases comprise of two copies of a ferredoxin-like domain that contain α-helices and anti-parallel β-sheets (Brown et al.,

2012) (Fig. 5). The heme cofactor is sandwiched in between the two domains forming catalytic center like that observed for the class II fungal peroxidases. However, DyP-peroxidases do not have the typical heme binding motifs found in the plant peroxidases, which are characterized by the two histidine residues called distal and proximal histidine, they have been shown to possess only peroximal histidine in their structure (Zubieta et al., 2007; Colpa et al., 2014). Instead of the distal histidine residues, DyP-peroxidases have been shown to contain a GXXDG motif in their primary sequence, which has been suggested to function as a part of the distal heme binding site (Sugano, 2009). Indeed, the conserved aspartate residue in this motif has been reported to play an essential role in the catalytic activity of the DyP-type peroxidase Dec1, which belongs to the subfamily D (Sugano et al., 2007). By contrast, mutation in the equivalent amino acid residue in some DyP-peroxidases belonging to the distinct subfamilies, A and B, have been reported to have only minor effects on their catalytic efficiencies (Liu et al., 2011; Singh et al., 2012). These results suggest that the distal asparagine is not essential for peroxidase activity in all DyP-peroxidases and it functions differently in the different types of DyP enzymes.

Detailed structural analysis and mutation analysis have been performed for the lignin-degrading DypB from *R. jostii*. It has been reported that the distal face of the heme binding pocket is composed of three residues including two conserved Asp153 and Arg244, and a non-conserved Asn246 (Singh et al., 2012). Mutation analysis of the three distal residues revealed that substitution of Asp153 and Asn246 with alanine resulted in only small effect on the enzyme reactivity with H_2O_2 showing similar levels of k_{cat}/K_M values with the WT enzymes (Singh et al., 2012). By contrast substitution of Arg244 with leucine abolished the peroxidase activity, indicating that only Arg244 is essential for peroxidase activity in DypB. The authors suggested that Arg244 act as the acid-base catalyst in the formation of Compound I in DypB (Singh et al., 2012). The structural data also revealed the presence of the channel that enabled to hydrogen peroxide to access the heme-catalytic center and a potential binding site for Mn^{2+} to perform electron transfer to the heme-cofactor. Interestingly, a hydrophobic groove, which could act as a potential binding site for substrates such as kraft lignin, was also predicted on the surface of the DypB (Singh et al., 2012). This potential substrate binding site including a tryptophan residue that might be involved in the long-range electron transfer, suggesting that DypB uses this electron-transfer mechanism for the oxidation of bulky substrates, as it has been observed during the oxidation reaction of LiPs and VPs (Doyle et al., 1998; Pogni et al., 2006; Ruiz-Dueñas and Martínez, 2009; Ruiz-Dueñas et al., 2009b). More recently, it has been revealed that a DyP-peroxidase from the fungus *Auricularia auricula-judae* actually mediates the long-range electron transfer oxidation using the surface exposed tryptophan and leucine residues (Strittmatter et al., 2013). These results also strongly suggest the possible involvement of DyP-type peroxidases in lignin degradation in nature.

Regulation of ligninolytic enzyme expression in white-rot fungi

As mentioned above, white-rot fungi generally produce a set of extracellular oxidative enzymes, which are encoded by a number of different gene families with multiple isoforms during their lignin-degrading process. Early studies have established culture conditions for high-level production of the ligninolytic enzymes in *P. chrysosporium* (see the review (Singh and Chen, 2008) and references there-in). These studies revealed that production of ligninolytic enzymes occurs during its secondary metabolism and are mainly triggered by limited nutrient levels including carbon and nitrogen limitations (Jeffries et al., 1981; Kirk et al., 1986). However, several other studies also indicate that the expression patterns of ligninolytic enzymes greatly vary depending on the microorganisms, types of the enzyme, and enzyme isoforms, in response to the various environmental and intracellular conditions. Production of ligninolytic enzymes is regulated at the transcriptional level and the involvement of several transcription factors and specific promoter sequences has been demonstrated in the regulation of ligninolytic enzyme production in white-rot fungi. In the following section, we will summarize the information available regarding the regulation of gene expression for ligninolytic enzymes in white-rot fungi.

Regulation of lignin peroxidase production

The regulation of lignin peroxidases has been extensively studied in *P. chrysosporium*. Many early studies have shown that expression of lignin peroxidases is induced by nutrient limitation, in which carbon, nitrogen, and sulfur limitations have been reported to trigger *lip* gene expression in liquid culture conditions (Kirk and Farrell, 1987; Stewart and Cullen, 1999; Kersten and Cullen, 2007). While *P. chrysosporium* possesses 10 different lignin peroxidase genes in its genome, each of the *lip* genes are regulated differently in response to the balance of carbon and nitrogen present in the culture medium, showing different patterns of transcript formations (Holzbaur et al., 1988; Stewart et al., 1992; Reiser et al., 1993; Stewart and Cullen, 1999). For example, it has been reported that in *P. chrysosporium* BKM-F-1767, *lipA* and *lipB* were moderately expressed in both nitrogen- and carbon-limited conditions, while *lipD* was highly transcribed in only carbon-limited conditions. The expression of *lipC* showed an opposite expression pattern to that of *lipD,* which was highly expressed under nitrogen-limited conditions, and these traits were recently further confirmed by transcriptomic analysis (Stewart and Cullen, 1999; Vanden Wymelenberg et al., 2010). In addition to the nutrient limitation, oxidative stress caused by the generation of reactive oxygen species in

an oxygenated culture (Belinky et al., 2003), certain wavelength of lights (Ramírez et al., 2010), and culture substrates (Bogan et al., 1996) have been found to affect the expression of *lip* genes. Transcriptome expression profiling reportedly showed that expression of *lipA* and *lipH* were significantly upregulated in the media containing ball-milled aspen compared to glucose culture, as was the expression of some cellulase genes (Vanden Wymelenberg et al., 2010; Fernandez-Fueyo et al., 2012). These results suggest the involvement of the multiple signaling/regulatory mechanisms in the transcriptional regulation of lignin peroxidase encoding-genes. At this moment, there is only limited information available for transcriptional regulation of *lip* genes and the structure of their promoter region in other white-rot fungi, therefore there is a strong need for further analysis to be undertaken.

Regulation of manganese peroxidase expression

The expression of manganese peroxidases is also known to be regulated by the limitation of carbon and nitrogen sources, although their expression patterns significantly differ for each isozyme as observed in *lip* gene expression. In addition to the nutrient limitation, transcription of *mnp* has also been shown to be induced by high Mn^{2+} ion levels in the culture, oxidative and chemical stress, and heat shock (Brown et al., 1993; Li et al., 1995) in *P. chrysosporium* and other white-rot fungi (Collins et al., 1999). Differential transcriptional response of *mnp* genes by Mn^{2+} addition has been reported in *P. ostreatus* (Cohen et al., 2001), *C. subvermispora* (Tello et al., 2000; Manubens et al., 2003), and *T. versicolor* (Collins et al., 1999; Johansson et al., 2002; Kim et al., 2005), in which only a limited number of *mnp* genes are shown to be induced by Mn^{2+} addition. Copper has also been reported to affect *mnp* gene expression in *C. subvermispora*, in which *mnp1* and *mnp2* showed upregulation in response to an increasing amount of copper in the culture medium (Manubens et al., 2003). A similar effect upon the addition of copper has been reported in *T. torgii* (Levin et al., 2002) and *Stereum hirsutum* (Mouso et al., 2003), suggesting an involvement of metal response regulation in *mnp* expression. Interestingly, some *mnp* genes in *P. ostreatus* (Kamitsuji et al., 2005) and *T. torgi* (Collins et al., 1999) have been reported to be unaffected by high nitrogen conditions, even though some *mnp* isozymes seemed to be affected. Similarly, expression of two *mnp* genes (*pr-mnp*2 and *pr-mnp3*) from *Physisporinus rivulosus* was reported to be induced under carbon sufficient concentrations, but surprisingly they were repressed by the addition of Mn^{2+} (Hildén et al., 2005). In addition to these responses, aromatic compounds (veratryl alcohol) and lignocellulosic materials (spruce sawdust) have also shown to induce expression of *mnp* genes in *P. rivulosus* (Hakala et al., 2006). Similarly, the results of transcriptome analysis have revealed specific upregulation of certain manganese peroxidases in *C. subvermispora* (Fernandez-Fueyo et al., 2012). However, no significant increase in transcript levels of *mnp* genes was observed in a medium containing cellulose (Vanden Wymelenberg et al., 2009) ball-milled aspen (Fernandez-Fueyo et al., 2012) compared to a glucose culture, and only the nutrient limitation was shown to induce high levels expression of *mnp1* and *mnp2* genes in *P. chrysosporium* (Vanden Wymelenberg et al., 2009). These results suggest that the biosynthesis of MnPs in the natural wood decaying process is modulated by complex mechanisms involving several environmental factors.

While expression of some *mnp* genes is stimulated by high Mn^{2+} concentration, indirect involvement of Mn^{2+} in the regulation of LiP expression has been reported in *Bjerkandera* sp. strain BOS55 and *P. chrysosporium* (Mester et al., 1995). It has been suggested that high levels of Mn^{2+} in culture media causes inhibition of veratryl alcohol biosynthesis, which results in a decreased expression of lignin peroxidases. This finding suggests possible regulatory cross talk between the ligninolytic enzyme production systems. Recently another physiologic function of Mn^{2+} in the production of manganese peroxidase was proposed in *C. subvermispora* (Mancilla et al., 2010). The authors suggested that, in addition to the transcriptional stimulation function of Mn^{2+}, it also influences secretion of MnP protein into the culture medium.

Several studies have revealed that the 5′-upstream region of the ligninolytic enzymes contain several putative *cis*-acting elements, such as the CCAAT-boxes, metal responsible elements (MREs), cAMP response elements (CRE), heat shock elements, and a binding site for activator protein 2 (AP-2)(Godfrey et al., 1990; Dhawale and Lane, 1993; Gold and Alic, 1993; Janusz et al., 2013). However, their involvement in the regulation of ligninolytic enzyme expression is still unclear. Instead, a *cis*-acting region responsible for manganese regulation has been discovered in the promoter region of the *mnp1* gene from *P. chrysosporium* OGC101 by detailed promoter analysis using a reporter gene (Ma et al., 2001; Ma et al., 2004). The identified 48-bp region was found to include at least two *cis*-acting elements with different functions; (i) an element involved in repression of *mnp1* expression in the absence of Mn^{2+} and (ii) an Mn^{2+}-responsive element responsible for Mn^{2+} mediated expression of *mnp1*.

In addition to these putative *cis*-acting elements, the involvement of a metal responsible transcription factor in the regulation of *mnp* gene expression has been suggested in *C. subvermispora* (Alvarez et al., 2009). The activation protein of *cup1* expression (ACE1) was originally isolated from *Saccharomyces cerevisiae* as a copper-responsible transcriptional activator for metallothionein-encoding gene *cup1* (Thiele, 1988). An ACE1 ortholog in basidiomycete was first identified in *P. chrysosporium* and its specific binding to the promoter region of a multicopper oxidase encoding gene *mco1* was demonstrated (Canessa et al., 2008). Lately, another ACE1 ortholog was investigated in *C. subvermispora* as a possible transcription factor for copper mediated

upregulation of a laccase (*lcs*) and two manganese peroxidase genes (*mnp1* and *mnp2*) and its binding ability to the *lcs* and *mnp2* promoter region was demonstrated (Alvarez et al., 2009). These results suggest that ACE1-mediated metal response is involved in the regulation of manganese peroxidase expression in white-rot fungi.

Regulation of versatile peroxidase expression

Versatile peroxidases have been mainly identified from the *Pleurotus* and *Bjerkandera* species. It has been reported that the *Pleurotus* species does not produce ligninolytic peroxidase activity in a synthetic medium, unlike *P. chrysosporium*, but that they can produce the activity from either a nitrogen-rich peptone medium or a lignocellulose-containing medium (Martínez et al., 1996; Ruiz-Dueñas et al., 1999). The production of high levels of laccases and MnPs in the presence of a high concentration of peptone have also been reported for some white-rot fungi including the *Pleurotus* and *Trametes* species (Levin et al., 2002). It has been stated that expression of versatile peroxidase encoding-genes (*vpls*) is also regulated at the transcriptional level, and the peptone dependent expression of a *vpl* gene has been demonstrated by Northern blot analysis (Ruiz-Duenas et al., 1999). In addition, expression of the *vpl1* in *P. eryngii* has also been shown to be regulated by Mn^{2+} levels and oxidative stress, even though several *vpl* isozymes show differential expression patterns to the same environmental stimuli as is observed for LiPs and MnPs expression in other white-rot fungi. While oxidative stress has been shown to induce the expression of *vpl1* in a similar manner with *lip* and *mnp* genes, supplementation of Mn^{2+} has been shown to repress *vpl1* transcription. However, in contrast to the repressing effect of Mn^{2+}, supplementation of the cation into *vpl* expressing mycelium was found to cause stabilization of the *vpl1* mRNA transcripts (Ruiz-Duenas et al., 1999). This suggests that Mn^{2+} might play a multiple role during the biosynthesis of ligninolytic peroxidases in filamentous fungi as it has been observed that efficient secretion of MnPs requires the presence of Mn^{2+} ions in the culture medium in *C. subvermispora* (Mancilla et al., 2010).

Mn^{2+} mediated repression of versatile peroxidase expression has also been reported in *P. ostreatus* (Cohen et al., 2002; Salame et al., 2012b). The authors hypothesized that because versatile peroxidases possess evolutionary intermediate features between lignin peroxidase and manganese peroxidase, this *vpl* might retain the transcriptional regulation patterns of lignin peroxidases as it has been reported that *lip* expression in some white-rot fungi is inhibited by Mn^{2+} mediated repression mechanisms (Salame et al., 2012a). To support complex regulatory mechanisms of *vpl* genes, the presence of several *cis*-acting elements including MRE, the CCAAT-box, AP2 binding site and the carbon catabolite repression elements for a carbon catabolite repressor CreA (Dowzer and Kelly, 1991; Drysdale et al., 1993) have been revealed on the promoter region of the versatile peroxidase genes (Ruiz-Duenas et al., 1999).

Differential expression of *vpl* genes in *P. osteratus* in a lignocellulose containing medium have recently been analyzed using qRT-PCR based expression profiling by changing the incubation temperature and pH (Fernández-Fueyo et al., 2014a). It was found that expression of all versatile peroxidase-encoding genes (*vp1-3*) show downregulation in response to any alteration in either temperature or pH from the standard conditions (25°C and pH 5.5). Interestingly, transient upregulation of *vpls* was observed after 6h and 24h of incubation at 37°C. These results suggest that pH regulation and heat shock response might play a role in the regulation of versatile peroxidase expression. This result also corresponds to the existence of heat shock elements (HSE) in the promoter region of these *vpl* genes.

Regulation of laccase expression

White-rot fungi secrete several laccase isozymes into the culture medium in response to intracellular and extracellular environmental changes. While laccases in white-rot fungi play an important role in their lignin-degrading enzyme system, they are also involved in diverse cellular processes (Baldrian, 2006). Therefore, a wide range of factors including nutrient levels, culture conditions, the presence of inducing compounds and the developmental stage, have been shown to affect laccase gene expression levels. A significant amount of research has demonstrated that the expression of laccase genes is regulated at the transcriptional levels, and that metal ions, various aromatic compounds, nitrogen and carbon sources can all regulate laccase gene expression (Piscitelli et al., 2011).

The most significant features of laccase expression in white-rot fungi are its inductive expression responses toward metal ions and certain aromatic compounds; copper mediated expression of laccase mRNA has been shown in many fungi including *T. versicolor* (Collins and Dobson, 1997), *C. subvermispora* (Alvarez et al., 2009), *P. ostreatus* (Palmieri et al., 2000), *P. sajor-caju* (Soden and Dobson, 2001) and *T. pubescens* (Galhaup et al., 2002). In addition to copper, Cd^{2+}, Ag^{+} and Mn^{2+} ions have also been reported to induce laccase transcription in some fungi (Soden and Dobson, 2001; Manubens et al., 2007). Analysis of the promoter region of the metal inducible laccases results in the identification of several metal response elements and ACE1-like transcriptional regulatory elements (Giardina et al., 2010; Piscitelli et al., 2011; Janusz et al., 2013), suggesting an involvement of several metal response factors in these regulatory mechanisms. As described above, recently, a metal responsive transcription factor ACE1 has been identified in *C. subvermispora* and its specific binding to the nucleotide motifs on the laccase-encoding *lcs* promoter region was demonstrated *in vitro* (Alvarez et al., 2009). These results suggest the possible involvement of ACE1 in the copper induction of fungal laccases.

In addition to the metal ions, aromatic compounds have also been reported to induce laccase expression. Among the various aromatic compounds, 2,5-Xylidine, ferulic acid, or veratric acid have been added to the culture medium to enhance laccase production levels (Giardina et al., 2010), with 2,5-Xylidine the most widely reported potent inducer (Elisashvili and Kachlishvili, 2009). However, the inducing effect of each aromatic compound varies depending on the microorganism. In addition to these inducers, 2,6-dimethoxyphenol, vanillic acid, catechol, and pyrogallol can also be used to induce laccase expression (Elisashvili and Kachlishvili, 2009). In agreement with the aromatic compound mediated activation of laccase expression, the presence of a xenobiotic response element (XRE) has been identified in the promoter region of laccase genes from *T. versicolor* (Collins and Dobson, 1997), *P. sajor-caju* (Soden and Dobson, 2003), *P. ostreatus* (Faraco et al., 2003), and *Trametes* sp. AH28-2 (Xiao et al., 2006).

The effect of the carbon and nitrogen source on the production of laccases has been investigated for many white-rot fungi (Elisashvili and Kachlishvili, 2009) and several differences in the expression patterns of the heme peroxidases have been reported. In some fungi, such as *Trametes* sp. AH28-2 (Xiao et al., 2006) and *T. pubescens* (Galhaup et al., 2002), the expression of laccases was shown to be subject to carbon catabolite repression, in which laccase production was repressed even in the presence of inducing compounds. Nitrogen levels in the culture medium were also reported to affect laccase expression levels, however its effects are significantly different depending on the specific genes involved and the different fungal species (Mansur et al., 1998; Soden and Dobson, 2001; Colao et al., 2003). For example, while only upregulation of two *lac* genes was observed in nitrogen-non limited conditions in the basidiomycete-62 (CECT 20197) (Mansur et al., 1998), four laccase genes in *P. sajor-caju* were shown to respond differently to nitrogen concentrations (Soden and Dobson, 2001). To support the involvement of carbon catabolite regulation and nitrogen regulation, a number of consensus sequences for CreA and the nitrogen repression factor NIT2 (Xiao and Marzluf, 1996) have been detected in the promoter region of laccase encoding genes from different species (Janusz et al., 2013). As summarized here, the transcriptional regulation of laccases is mediated by several regulation mechanisms to form a complex gene regulation circuit.

Conclusions

Since the discovery of fungal ligninolytic enzymes, a great number of studies have been made on the isolation of novel ligninolytic microorganisms, cloning and characterization of novel ligninolytic enzymes, and molecular structural analysis. Furthermore, recent omics-driven data analysis has revealed that there are a number of differences in the microbial lignin degrading systems and their regulation, revealing a huge diversity in the microbial ligninolytic enzyme systems as reviewed in this paper. However, despite the significant progress in this research area, there is as yet no commercial biocatalytic process for lignin utilization, in part due to a lack of efficient enzyme production systems and the difficulty in controlling the radical mediated lignin degradation process (Bugg et al., 2011a). Therefore, further studies are necessary toward the understanding of the catalytic properties of the ligninolytic enzymes, the regulation of the gene expression, and the molecular mechanisms controlling lignin-degrading reaction under natural lignin-degrading conditions. These insights will serve as an important basis for engineering microorganisms that exhibit strong lignin-degrading activity and that express the tailor-made enzyme cocktails required in different industrial applications with emerging synthetic biology technologies, or it may lead to the design of an industrial process for the production of various high-value aromatic chemicals from lignin substrates. Moreover, total valorization of lignocellulosic biomass may lead to improvement in the overall economics and sustainability of an integrated biorefinery and thus the development of an effective utilization process for lignin is the key for future biorefinery research (Ragauskas et al., 2014). Recently, it has been suggested by using fungal genome and molecular clock analysis that the appearance of the origin of lignin degradation enzymes might have coincided with the sharp decrease in the rate of organic carbon burial around the end of Carboniferous period (Floudas et al., 2012). This would suggest that the evolution of one enzyme and a group of microorganisms could change an era, showing the great potential of lignin degrading enzymes. In turn, we could change the world from a non-renewable oil-based society to a biorefinery-based sustainable society by effectively exploiting microbial ligninolytic systems and lignocellulosic biomass resources in the future.

Compliance with ethics guidelines

Takanori Furukawa, Fatai Olumide Bello, and Louise Horsfall declare that they have no conflict of interest. This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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