CHAPTER 8

Biological Lignin Degradation

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8.1 Historical Perspective for Lignin Biodegradation Studies

The initial studies on microbial degradation of lignin were intimately associated with the availability of the first relevant information on the chemical structure of this complex polymer, obtained during the 1950s and 1960s. This information was acquired by two complementary approaches: (i) pioneering studies of its *in vitro* and *in vivo* biosynthesis from *p*-hydroxycinnamyl alcohol precursors (monolignols) by Freudenberg and others[1] and (ii) the first analyses of low molecular-mass lignin fractions (dilignols included), and products from chemical degradation (depolymerization) of different lignins.[2,3]

The above studies first revealed that phenolic monolignols give rise to largely etherified (non-phenolic) lignin, after one-electron oxidation by plant peroxidases (or laccases) and random coupling of the phenoxy radicals formed, often involving the C_4 position. Moreover, they taught us to use (i) non-phenolic *in vitro* synthesized lignin (methylated dehydrogenation polymer, DHP)[4] and (ii) non-phenolic model dimers representing the main interunit linkages in lignin (such as β-O-4′ ethers),[5] for demonstration of microbial and enzymatic ligninolysis. These studies often incorporated ^{14}C-labeling to DHP, other lignin preparations, or model dimers for easier

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depolymerization/breakdown detection and identification of the degradation products.

Subsequently, using the tools made available by lignin chemistry, microbiologists and biochemists in the 1970s and 1980s (i) demonstrated that some fungi, such as *Phanerochaete chrysosporium*, are able to depolymerize and fully degrade (mineralize) ^{14}C-lignin to $^{14}CO_2$ (and H_2O) in pure culture,[4,6] (ii) optimized growth parameters (such as C and N sources, vitamin requirement, O_2 supply, shaking, *etc.*) for maximal ligninolysis in the fungal cultures (using ^{14}C-DHP),[7,8] and finally (iii) were able to discover and isolate from these cultures the first lignin-degrading enzyme, a high redox-potential heme peroxidase from *P. chrysosporium* (called lignin peroxidase, LiP) degrading both polymeric lignin and non-phenolic model dimers, as shown using ^{14}C-labeling.[9–11]

The rationale of searching for high redox-potential oxidoreductases in lignin biodegradation studies is nicely illustrated by a comparison between lignin polymerization by vascular plants and its biodegradation by basidiomycetous fungi, two parallel processes characterized by an initial enzymatic oxidation step (the abstraction of one electron from a phenylpropanoid monomer/unit to form a reactive radical) followed by a variety of random radical reactions (going through coupling or bond cleavage, respectively).[12] The main difference between the two processes is the inability of generic plant peroxidases and laccases to act on the non-phenolic polymer once monolignols were polymerized. Otherwise, these low redox-potential enzymes would simultaneously cause lignin polymerization and depolymerization in the plant cell-wall. Catalyzing the latter reaction is a unique ability of the specialized fungal peroxidases.

8.2 Fungal Degradation of Lignin: A Complex Multi-enzymatic Process

Although different basidiomycetes in the orders Agaricales, Boletales, Corticiales, and Russulales, among others, are able to decay lignified materials, the model ligninolytic organism *P. chrysosporium*,[13] and most of the lignin-degrading fungi (as shown by ^{14}C-lignin mineralization in liquid cultures or by significantly reduced initial lignin content in solid wood cultures) are wood-rotting species from the order Polyporales.[14] The ability to degrade lignin was an essential evolutionary trait for recycling the large amounts of carbon fixed by plants after land colonization (currently representing over 100 billion tons C per year),[15] which was acquired by ancestral basidiomycetes in the later Carboniferous period.[16] Nowadays, lignin-degrading organisms and their enzymes, whose extracellular nature often implies quite stable proteins, can play an important role for the sustainable use of plant resources in lignocellulose biorefineries where lignin removal is often required for the production of biobased fuels and chemicals.[17,18]

Lignin degradation was defined as an enzymatic ''combustion''[19] involving different oxidative enzymes (oxidoreductases). In addition to LiP, to date only

reported in Polyporales,[20] the enzymes acting synergistically for lignin degradation by basidiomycetes include:[13,21] (i) two other peroxidases of the same peroxidase-catalase superfamily where LiP is included,[22] namely manganese peroxidase (MnP), discovered in *P. chrysosporium* nearly simultaneously with LiP[23] and recently classified into two different subfamilies (called short and long MnPs),[24] and versatile peroxidase (VP, Figure 8.1A), first reported in *Pleurotus eryngii* (Agaricales)[25–27] and *Bjerkandera adusta* (Polyporales);[28] (ii) oxidases from the copper-radical oxidase (CRO) superfamily,[29] such as glyoxal oxidase (GLX), and oxidases from the glucose-methanol-choline oxidase/dehydrogenase (GMC) superfamily,[30] such as aryl-alcohol oxidase (AAO, Figure 8.1C), methanol oxidase (MOX), pyranose 2-oxidase (P2O) and glucose oxidase (GOX), all of them providing H_2O_2 to peroxidases, among other roles; (iii) phenol-oxidizing laccases (Figure 8.1B) of the multi-copper oxidase (MCO) superfamily;[31–33] and probably other extracellular oxidative enzymes mentioned below (Table 8.1).

The above oxidoreductases make use of different cofactors directly involved in the redox reaction, such as heme in peroxidases (Figure 8.1A and D), flavin in GMC oxidases (Figure 8.1C), and one or several copper atoms in CRO and laccases (Figure 8.1B), respectively. These cofactors are oxidized (activated) by different oxygen species, such as O_2 in oxidases (yielding as reduced product H_2O_2 in GMC oxidases, and H_2O in laccases) and H_2O_2 in peroxidases (yielding H_2O as a byproduct). Then, electrons are recovered during the oxidation of different substrates, such as non-phenolic (by LiP and VP) and phenolic (by laccases, VP, and members of the short MnP subfamily) aromatics (lignin units included), Mn^{2+} (by MnP and VP; Mn^{3+} being a diffusing oxidizer of phenolic structures), benzylic (by AAO) and other alcohols (by MOX), different sugars (by other GMCs, such as P2O and GOX), and glyoxal and related aldehydes (by GLX), among others.[30] It is important to mention that VP, as previously demonstrated for LiP,[9] catalyzes lignin (^{14}C-DHP) depolymerization (in the presence of veratryl alcohol) and direct breakdown of non-phenolic (β-O-4′ ether) lignin model dimers.[34] The catalytic versatility of VP, oxidizing the typical substrates of LiP, MnP, and generic peroxidases (such as plant horseradish peroxidase and fungal *Coprinopsis cinerea* [syn. *Arthromyces ramosus nomen nudum*] peroxidase), is due to a hybrid molecular architecture combining the corresponding substrate oxidation sites (see below).[35]

More recently, two new peroxidase types have been described in wood-rotting basidiomycetes:[36] (i) the so-called dye-decolorizing peroxidases (DyP, Figure 8.1D) in the CDE superfamily including chlorite dismutase, DyP and EfeB (an *Escherichia coli* protein putatively involved in iron uptake);[37] and (ii) the unspecific peroxygenases (UPO) in the heme-thiolate peroxidase superfamily, which also includes the classical chloroperoxidase from *Leptoxyphium fumago* (syn. *Caldariomyces fumago*).[38] UPO is characterized by the presence of a cysteine residue as the fifth ligand of the heme iron, while a histidine is present in the other peroxidases. Its main activity is substrate oxygenation (hydroxylation) in reactions similar to those catalyzed by cytochrome P450 monooxygenases but without requiring an auxiliary enzyme

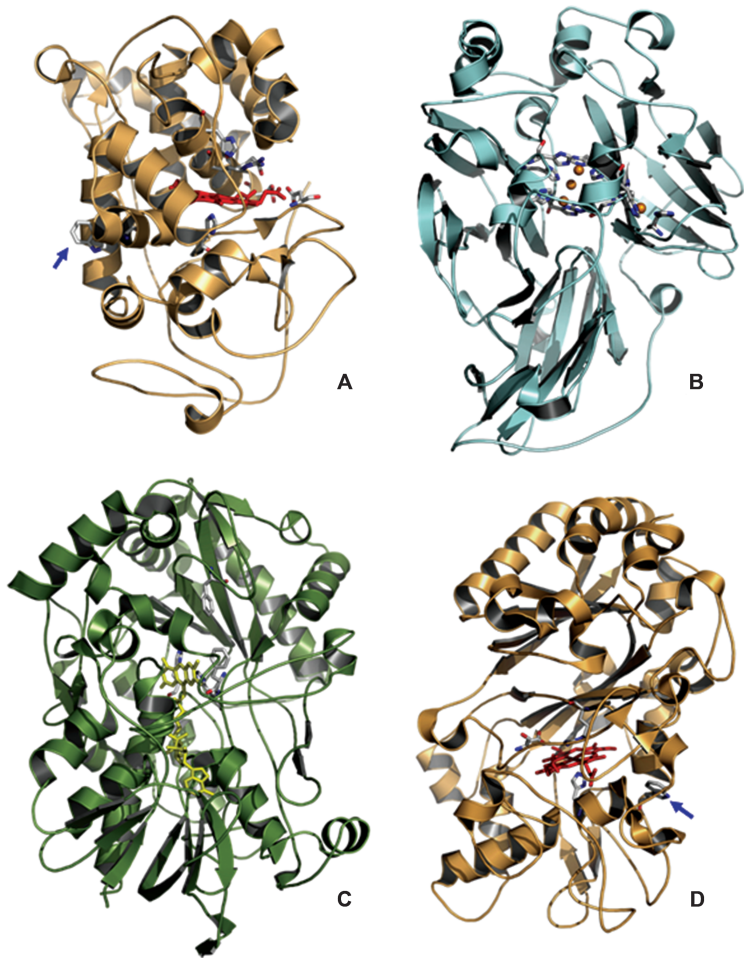

Figure 8.1 General structure of enzyme representatives from classical and new (heme, copper and flavin-containing) oxidoreductase families involved in degradation of lignin and lignin products. (A) *Pleurotus eryngii* VP (PDB 3FJW). (B) *Pycnoporus cinnabarinus* laccase (PDB 2XYB). (C) *P. eryngii* AAO (PDB 3FIM). (D) *Auricularia auricula-judae* DyP (PDB 4W7J). Ribbon representations showing cofactors (heme/FAD and copper ions represented as red and yellow sticks and orange spheres, respectively) and some amino-acid residues (Corey/Pauling/Koltun, CPK, colored sticks) relevant for catalysis such as: (i) His ligand of heme iron in A and D; (ii) His/Arg and Asp/Arg residues involved in activation by H_2O_2 in A and D, respectively; (iii) two Glu and one Asp residues forming the Mn^{2+}-binding site in A; (iv) catalytic Trp in A and D (blue arrows); (v) two His and two Phe active-site residues in C; and (vi) ten His and one Cys residues coordinating four copper ions in B.

Table 8.1 Summary of oxidative extracellular enzymes involved in lignin biodegradation.[a]

Family	Super-family	Cofactor (catalytic residue)	Cosubstrate	Coproduct	Oxidized substrate	Possible mediators	Oxidation electron number	Reported source
LiP	PC	Heme (& Trp radical)	H_2O_2	H_2O	Non-phenolic lignin	VA (no mediators for non-phenolic dimers & soluble lignins)	1 Electron	Polyporales
MnP	PC	Heme	H_2O_2	H_2O	Mn^{2+} (& phenolic lignin for short MnPs)	Mn^{2+} (phenolic lignin) & unsaturated lipids (non-phenolic lignin)	1 Electron	Basidiomycetes
VP	PC	Heme (& Trp radical)	H_2O_2	H_2O	Mn^{2+} & non-phenolic/phenolic lignin	VA, Mn^{2+}, & unsaturated lipids (and no mediators) as described for LiP/MnP	1 Electron	Basidiomycetes
DyP	CDE	Heme (& Trp radical)	H_2O_2	H_2O	Phenolic lignin (Mn^{2+} for some of them)	No (Mn^{2+} for some of them)	1 Electron	Fungi & bacteria
UPO	HTP	Heme	H_2O_2	H_2O	Aromatic & aliphatic compounds (hetero-atoms included)	No	2 Electrons & oxygen transfer	Fungi
Lac	MCO	$4Cu^{2+}$	O_2	H_2O	Phenolic lignin & lignin products	Synthetic (*e.g.* ABTS, HBT & VLA) and phenolic compounds	1 Electron	Fungi & bacteria

Table 8.1 (*Continued*)

Family	Super-family	Cofactor (catalytic residue)	Cosubstrate	Coproduct	Oxidized substrate	Possible mediators	Oxidation electron number	Reported source
GLX	CRO	$1Cu^{2+}$ (& Tyr radical)	O_2	H_2O_2	C_1–C_3 (often substituted) aldehydes	No	2 Electrons	Basidiomycetes
AAO	GMC	FAD	O_2	H_2O_2	Aromatic (& aliphatic polyunsaturated) primary alcohols	No	2 Electrons	Fungi
MOX	GMC	FAD	O_2	H_2O_2	Short-chain primary alcohols	No	2 Electrons	Fungi
P2O	GMC	FAD	O_2	H_2O_2	Glucose (& other aldopyranoses)	No	2 Electrons	Fungi
GOX	GMC	FAD	O_2	H_2O_2	Glucose (& other aldopyranoses)	No	2 Electrons	Fungi

[a]Abbreviations: AAO, aryl-alcohol oxidase; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); CDE, chlorite dismutase, DyP and *E. coli* EfeB (superfamily); CRO, copper-radical oxidase (superfamily); GLX, Glyoxal oxidase; GOX, glucose oxidase; HBT, 1-hydroxybenzo-triazole; HTP, heme-thiolate peroxidase (superfamily); Lac, laccase; MCO, multi-copper oxidase (superfamily); MOX, methanol oxidase (also known as alcohol oxidase); PC, peroxidase-catalase (superfamily); P2O, pyranose 2-oxidase; UPO, unspecific peroxygenase; VA, veratryl alcohol; and VLA, violuric acid.

nor a source of reducing power (a characteristic of the highest biotechnological interest).[39] Fungal DyPs have extremely low, if any, activity on non-phenolic lignin model dimers, although they are able to oxidize the phenolic ones, in agreement with their activity on simple phenols and dyes.[40,41] Recently, the first fungal DyP being able to oxidize Mn^{2+} (a reaction characterizing fungal MnP and VP) has been described,[42] as previously reported for some bacterial DyPs mentioned below. In contrast, UPO is able to break down non-phenolic dimers although the reaction takes places *via* the corresponding phenolic dimers formed by C_4 dealkylation,[43] instead of *via* an aromatic cation radical as in the case of LiP and VP.

8.3 Long-range Electron Transfer (LRET) Characterizes Ligninolytic Peroxidases

A key finding to understand the oxidative biodegradation of lignin was the discovery of the ability of LiP and VP to delocalize one oxidation equivalent from the buried heme cofactor to a tryptophan residue exposed to the solvent (Figure 8.1A, arrow). This surface residue is able to interact with the bulky lignin polymer, oxidize it and transfer the electrons to the activated heme cofactor using a LRET pathway.[44] The tryptophanyl radical formed in these ligninolytic peroxidases was directly detected (by EPR) first in *P. eryngii* VP,[45] including its two-electron (compound I) and one-electron oxidized (compound II) transient states,[46] and later in a *P. chrysosporium* LiP variant and a *C. cinerea* peroxidase engineered to mimic LiP.[47]

The catalytic nature of this surface residue (Trp171 in *P. chrysosporium* LiP and Trp164 in *P. eryngii* VP) had been demonstrated by directed mutagenesis: (i) first with veratryl alcohol, the typical non-phenolic substrate for laboratory studies with these ligninolytic peroxidases,[48,49] and (ii) later with a non-phenolic β-O-4′ model tetramer.[50] Similar steady-state studies cannot be performed with polymeric lignin due to solubility limitations and difficulties in following lignin substrate modification during short reaction times to estimate kinetic constants. However, it has been recently possible to follow direct electron transfer between (water-soluble) lignosulfonates and VP from the ''peroxidase side'' (*i.e.* estimating the kinetic constants for enzyme reduction by lignin under stopped-flow conditions).[51] Moreover, using methylated lignosulfonates it was demonstrated that no electron transfer from non-phenolic lignin is produced when the catalytic tryptophan has been removed (VP W164S mutated variant) confirming the direct involvement of this surface residue in the oxidation of non-phenolic lignin (which represents the main moiety in natural lignins).[52]

Therefore, the catalytic cycle of classical peroxidases must be expanded for ligninolytic peroxidases (LiP and VP), as shown in Figure 8.2 for VP. In this way, compound I, a $Fe^{4+}=O$ and porphyrin cation radical complex formed by two-electron oxidation of the resting enzyme by H_2O_2, would be in equilibrium with another form where one electron has been abstracted from the catalytic

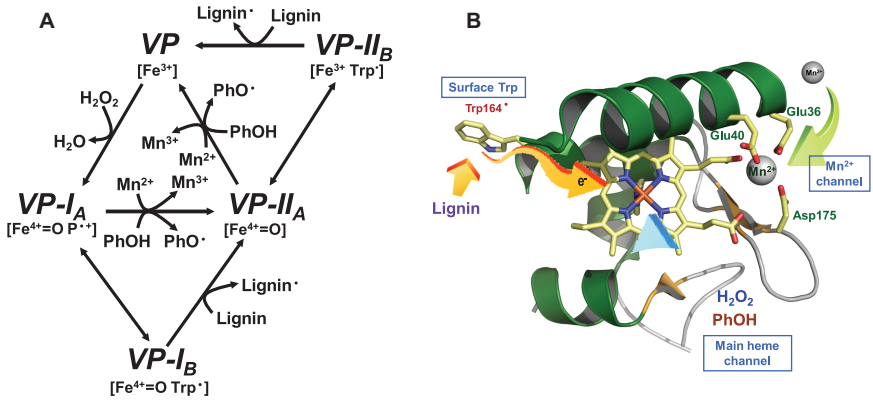

Figure 8.2 VP catalytic cycle and sites. (A) The peroxidase "classical" cycle (central triangle) includes two-electron activation of the resting enzyme (VP, containing Fe^{3+} heme) to compound I (a $Fe^{4+}=O$ and porphyrin cation radical complex) by H_2O_2, followed by one-electron oxidations of two substrate molecules by compounds I and II (the latter with reduced porphyrin after the first substrate oxidation). In VP (and also LiP) this central cycle must be expanded to include two forms of both compound I (VP-IA and VP-IB) and compound II (VP-IIA and VP-IIB) where one electron has been partially abstracted from the catalytic tryptophan by the activated heme. Adapted from Sáez-Jiménez *et al.*[51] (B) The resulting tryptophanyl radical at the protein surface will be able to oxidize the bulky lignin molecule, using a LRET pathway to transfer the electron to the heme. In contrast, Mn^{2+} and some phenols (PhOH) are oxidized in direct contact with the heme, which they reach using a Mn^{2+} channel (formed by three acidic residues) or the main heme access channel (which is also used by H_2O_2 for enzyme activation), respectively.

tryptophan (compounds I_A and I_B in Figure 8.2A). A similar situation would be produced for the partially reduced compound II, formed after oxidation of a first substrate molecule (compounds II_A and II_B in Figure 8.2A). As illustrated in Figure 8.2B, representing the VP catalytic sites, lignin will be oxidized at the surface tryptophan (left-hand side), and the abstracted electron will travel to the heme by LRET and later (after reduction of both compounds I_B and II_B) to the H_2O_2 cosubstrate, which will reach the heme through the main access channel. In contrast, Mn^{2+} and some phenols will transfer electrons directly to the activated heme (compounds I_A and I_B) using a specific Mn^{2+} channel formed by three acidic residues and one heme propionate (Figure 8.2B, right-hand side) or the main heme access channel (bottom), respectively.

The above catalytic tryptophan seems to be present in all LiP and VP, as shown by genomic screening.[16,20] An interesting exception is the *Trametes cervina* LiP that has a surface tyrosine residue,[53] which is activated in the first enzyme cycle forming an adduct with a non-phenolic aromatic compound involved in ligninolysis.[54] Interestingly, LRET from a radical-forming surface aromatic residue is not an exclusive characteristic of LiP and VP, and recently a similar pathway from a surface tryptophan residue (Figure 8.1D,

arrow) has been demonstrated for oxidation of bulky dye substrates by fungal[55–57] and bacterial DyP.[58]

8.4 Indirect Degradation of Lignin by Other Fungal Oxidoreductases

After its definition as an enzymatic ''combustion'',[19] considerable controversy arose concerning the relative importance of the different oxidoreductases involved in lignin biodegradation.[59–61] Difficulties in (i) studying lignin decay under environmentally-relevant conditions and (ii) using complex (and insoluble) lignin as enzyme substrate made it difficult to provide a definitive answer to this important question. In this context, the involvement of enzymes being unable to directly oxidize veratryl alcohol and/or non-phenolic lignin model dimers was suggested. These enzymes, often acting in the presence of redox mediators or generating new oxidizers, include laccases, MnP, bacterial oxidoreductases discussed below,[13,44a,62] and maybe some still unidentified enzymes,[63] together with LiP and VP being able to directly oxidize non-phenolic lignin models.

Laccases from bacteria, ascomycetes, and basidiomycetes have progressively increasing redox potentials but, even the latter ones, only oxidize phenolic lignin model dimers,[64] whereas they are unable to directly oxidize non-phenolic dimers for which the role of laccases in lignin biodegradation is yet controversial.[65] However, some of the early studies for implementing the use of oxidoreductases in the pulp and paper sector (for chlorine-free bleaching) discovered that fungal laccases are able to oxidize veratryl alcohol and remove lignin from paper pulp in the presence of synthetic redox mediators.[66,67] These synthetic compounds can be substituted by ''natural'' ones, including lignin-derived phenols,[68,69] with advantages due to their potentially low cost and environmental friendliness. Degradation of non-phenolic aromatics can be also achieved *via* lipid peroxidation by laccases in the presence of natural mediators,[70] similarly to that demonstrated for MnP (see below). In addition, laccases can generate Mn^{3+} from Mn^{2+} during the oxidation of hidroquinones,[71] or in the presence of some organic acids.[72] One of the most popular natural phenolic mediators is methyl syringate that is formulated by Novozymes (Bagsvaerd, Denmark), together with a commercial laccase from the ascomycete *Myceliophthora thermophila*, for industrially-feasible large-scale delignification.[73] From a more basic point of view, it is interesting that some of these phenols have been proposed as the natural mediators of laccases in nature,[74] although more evidence on the importance of laccase-mediator systems in natural ecosystems would be required.

The situation is similar for the new fungal DyPs since, as mentioned above, their characteristic activity is restricted to phenolic lignin-related compounds, in addition to dyes. Therefore, its reported action improving wheat straw saccharification[75] most probably takes place *via* the phenolic moiety (constituting a more significant fraction in grass than in wood lignin) and/or

using simple phenols (eventually present in the substrate) as redox mediators. The more recently described fungal UPO has a unique mechanism to degrade non-phenolic lignin model dimers described above (based on initial C_4 dealkylation) but no significant action on the lignin polymer is expected.[43]

Concerning MnP, early studies reported its action on lignin in the presence of added thiols,[76] or even in the absence of them,[77] although the latter result is not conclusive since the phenolic content of the lignin used was not reported. More significant are the MnP reactions in the presence of unsaturated lipids (*e.g.* unsaturated fatty acids).[78] Under these conditions, Mn^{3+} initiates lipid peroxidation reactions, and the reactive lipid radicals formed are able to break down non-phenolic lignin model dimers,[79] polycyclic aromatic hydrocarbons, and other recalcitrant compounds.[80] Although the availability of unsaturated fatty acids during fungal decay of wood is a matter of controversy, genomic (including gene duplication discussed below), secretomic, and transcriptomic studies with *Ceriporiopsis subvermispora* suggest that MnP-induced lipid peroxidation could be involved in the selective lignin degradation caused by this model white-rot fungus.[81] Moreover, it has been reported that linoleic acid (and Mn^{2+}) supplementation stimulate lignin degradation by this basidiomycete.[82] Since *C. subvermispora* holds seven laccase genes, whose transcript levels were significantly up-regulated in media containing ball-milled wood, [81] laccase could also be involved in lignin degradation in cooperation with MnP as aforementioned.

8.5 Key Enzymes in Lignin Degradation as Revealed by Genomic Analyses

The most recent evidence on the relative involvement of different basidiomycete oxidoreductases (including peroxidases, laccases, and other oxidases) in lignin degradation comes from the availability of fungal genomes, most of them sequenced at the JGI of the US Department of Energy (http://jgi.doe.gov). An analysis of all the basidiomycete genomes sequenced by Floudas *et al.*[16] and others[20,30,83,84] reveals that some relevant oxidoreductase genes were strongly duplicated or completely lost (two central mechanisms in fungal evolution) paralleling changes in basidiomycete lifestyle, such as the transition from primary white-rot to secondary brown-rot decay, as described below.[16]

Concerning peroxidases, the above genomic surveys reveal that all the typical lignin-degrading basidiomycetes (causing the so-called white-rot decay due to the whitish color of partially delignified wood) include (often duplicated) genes of at least one of the generally-known as ligninolytic peroxidase families (*i.e.* LiP, MnP, or VP) in their genomes. In contrast, these genes are completely absent from all the sequenced cellulose-degrading species (causing the so-called brown-rot decay due to the brownish color of lignin-enriched wood). This correlation has been challenged by Riley *et al.*[85] adducing the absence of these peroxidase genes in *Botryobasidium botryosum* (Cantharellales), *Jaapia argillacea* (Jaapiales), and *Schizophyllum commune* (Agaricales). However, these three species are very poor wood-rotters and,

most probably, function associated to other white-rot fungi or as plant parasites or ectomycorrhizal symbionts.[86]

In the case of the DyP genes, the correlation is less remarkable since they are present in a few brown-rot genomes, but the average number per genome is significantly higher in the white-rot species. On the other hand, MCO genes are widespread in basidiomycete genomes. Nevertheless, laccases *sensu stricto* seem to be specific of Agaricomycetes, with multiple duplications late in evolution, according to substrate use (lifestyle).[87] Interestingly, genes of at least one GMC family are present in all the basidiomycete genomes analyzed, with MOX apparently being the most abundant oxidase in brown-rot species, while in the white-rot species they are generally accompanied by high AAO gene numbers. Regarding heme-thiolate peroxidase genes (where UPO is included), there is no a correlation between their presence and the fungal lifestyle. They have been identified in white rot, brown rot, and other basidiomycete species with different nutritional strategies.[16,20]

In summary, the genomic evidence strongly supports the central role of LiP, VP, and MnP in ligninolysis by white-rot basidiomycetes, the most efficient lignin degraders in nature. These results are in agreement with (i) the ability of the two former enzymes to degrade the main non-phenolic moiety of lignin and (ii) the role of Mn^{3+} from MnP and VP action promoting formation of strong oxidizers (two aspects already described above). The genomic distribution of other oxidoreductases genes, such as laccase and DyP genes, suggests participation in the two wood decay patterns, although their higher duplication rates points to a more important contribution to the white-rot decay. This contribution could include oxidative degradation of lignin-derived compounds in white-rot decay (the broad variety of laccases within a species might be interpreted as an adaptation to the ever changing pattern of multiple aromatic compounds released during the decay of lignocellulose) and promotion of redox-cycling reactions contributing to the hydroxyl radical generation characterizing brown-rot decay.[88,89] The presence of heme-thiolate peroxidase genes in most of the fungi analyzed, and the variability observed in the active site and heme-access channel architectures of the homology models obtained for their deduced amino acid sequences, suggest distinct catalytic functions and substrate specificities. This makes their action on lignin unclear, as previously described based on the analysis of the unique catalytic mechanism of *Agrocybe aegerita* UPO.[20] Finally, the wide distribution of oxidases in the different wood-rotting basidiomycete genomes is in agreement with the important role of H_2O_2 as the peroxidase oxidizing substrate (in white-rot decay) and as the precursor of hydroxyl radical (in brown-rot decay).[30]

8.6 Enzymatic Degradation of Lignin and Lignin Products by Bacteria

Bacterial degradation of lignin emerged as a hot topic during recent years.[90–92] However, some of the main pieces of evidence on the bacterial

capability to degrade sound wood (including its lignin fraction) were reported nearly 30 years ago.[93] The most relevant ones were transmission electron microscopy (and light microscopy) images showing bacterial decay of solid wood with characteristic erosions near the cell-wall lumen, and noteworthy tunnels inside the different cell-wall layers.[94,95] Tunneling and erosion bacteria are also especially relevant in buried and waterlogged wood.[96] Studies on wood-decaying bacteria in pure culture are scarce,[97,98] but their ability to mineralize ^{14}C-labeled lignin was reported as a proof of ligninolytic ability.[94]

Recently, enzymes potentially involved in degradation of lignin or lignin (derived) products have been isolated and characterized from different bacteria and, taking advantage of the large genomic information currently available, related genes have been identified in many other prokaryotic species.[99–101] Some of these enzymes are related to the fungal enzymes mentioned above, including bacterial laccases and DyPs (Section 8.7), while others have no well characterized counterparts in basidiomycetes or other fungi, such as those of the *Sphingobium* β-etherase pathway, described in Section 8.8.

Bacterial laccases are similar to fungal laccases, albeit with lower redox potentials and usually lower catalytic efficiencies on typical laccase substrates,[102] and as found also in fungi they seem to play a variety of functions in prokaryotes.[103,104] For example, one of the first bacterial laccases to be crystallized and fully characterized was CotA,[105,106] which forms part of the *Bacillus subtilis* spore and seems responsible for its brownish pigmentation. As in the case of DyPs discussed below, a significant advantage of bacterial laccases (and related MCOs) is their easier heterologous expression compared with the fungal ones. Some laccases of biotechnological interest are the highly thermostable small laccase of *Streptomyces coelicolor*,[107] and the *Bacillus licheniformis* laccase, which has been reported as an alternative to the commercial laccases of fungal origin.[108] The laccases of *S. coelicolor* and other actinobacteria, such as *Amycolatopsis* sp. whose DyP is mentioned below, are being investigated for lignin degradation and their crystal structures solved in complex with a non-phenolic model dimer that these enzymes oxidize in the presence of redox mediators.[109] However, only oxidation at C_α (with formation of a ketone derivative) was obtained, by contrast to the breakdown of non-phenolic lignin model dimers obtained with fungal laccases in the presence of mediators.[110]

8.7 Bacterial DyPs and Lignin Degradation

At the end of the 1980s, a so-called ''actinomycete lignin peroxidase'' (ALiP) was reported from *Streptomyces viridosporus* T7A,[111] but a retraction on its claimed ability to oxidize veratryl alcohol was published a few years later,[112] and ALiP was finally shown to be a misidentification.[113] Interestingly, the renewed interest in bacterial degradation of lignin seen during recent

years[90–92] includes literature on bacterial DyPs presented as the bacterial "ligninases",[99,114–120] as discussed below.

First, it is important to consider that high redox-potential enzymes are required to oxidize the non-phenolic lignin polymer. The Fe^{3+}/Fe^{2+} redox potentials available (between −0.260 and −0.040 V, all values at pH 7 for comparison) for four bacterial DyPs (from *Amycolatopsis* sp., *Bacillus subtilis*, *Pseudomonas putida*, and *Thermomonospora curvata*)[114,121–123] show values similar to that of the generic peroxidase of *C. cinerea* (−0.183 V)[124] and slightly lower than those of ligninolytic peroxidases (between −0.137 and +0.050 V),[125,126] with all of them being significantly higher than those of plant peroxidases.[127,128] However, more and more precise information, including the potential of rate-limiting compound-II/Fe^{3+} reduction,[129] is required to better understand the ability of bacterial (and other) DyPs to oxidize lignin related compounds.

Second, different pieces of evidence were provided to support the description of bacterial DyPs as lignin-degrading enzymes. Unfortunately, none of them fully meet the requirements described in Section 8.1 to demonstrate ligninolytic activity (*i.e.* breakdown and depolymerization of non-phenolic lignin model compounds and methylated lignin, respectively). The claimed activity of *Rhodococcus jostii* on lignin,[99] was based on (i) a spectrophotometric assay using nitrated lignin,[130] which lacks chemical validation of the used substrate and released product(s), and (ii) the disappearance of an unidentified peak in the chromatographic analysis of unextracted wheat straw treated with the enzyme, whose origin cannot be established. Moreover, the enzyme degrades phenolic lignin model dimers and kraft lignin,[115] a technical lignin that often has >70% phenolic units,[131] but no reaction on a non-phenolic dimer is reported.

In a similar way, oxidation of kraft lignin and release of a phenolic compound from a complex lignocellulosic substrate have been reported for *Pseudomonas fluorescens* DyP.[116] Finally, although the claimed lignin-degrading capabilities of *Thermobifida fusca* DyP[119,120] are also based on oxidation of a phenolic lignin model dimer, oxidation of a non-phenolic β-O-4′ ether dimer has been reported for a *B. subtilis* DyP.[117] However, the enzyme/dimer ratio used in the latter reaction was 28 : 1 ($g\,g^{-1}$) revealing a practically null enzymatic activity.

Interestingly, enzymatic oxidation of Mn^{2+} to Mn^{3+} has been reported for the *R. jostii*, *P. putida*, and *P. fluorescens* DyPs.[115,116,132] However, only the *Amycolatopsis* sp. DyP[114] has kinetic constants for this metal cation similar to those reported for *P. ostreatus* DyP, the only eukaryotic DyP oxidizing Mn^{2+} to date, and fungal MnP and VP.[42] Mn^{2+}-oxidation enhances the enzymatic activity of these DyPs since Mn^{3+}, chelated by organic acids, is an efficient oxidizer of phenolic compounds including phenolic lignin model dimers, and could act on non-phenolic lignin *via* lipid peroxidation reactions, as aforementioned for basidiomycete MnP. In contrast with that reported for fungal MnP and VP, where a specific Mn^{2+}-oxidation site is conserved near one of the heme propionates,[35] the Mn^{2+}-oxidation sites in DyPs are to be

definitively identified, although several crystal structures of DyP-Mn^{2+} complexes have been solved.[114,115]

Mn^{2+} and O_2 dependent decarboxylation of 4-methoxymandelic acid has been reported for the *Amycolatopsis* DyP in absence of H_2O_2,[114] while the same reaction is catalyzed by the peroxidase activity of *T. curvata* DyP (in the absence of Mn^{2+}).[123] However, although 4-methoxymandelic acid is a very poor substrate of *P. chrysosporium* LiP (4% rate compared with veratryl alcohol),[133,134] its transformation by the *T. curvata* DyP would be several orders of magnitude lower (considering a 16-h incubation period) in agreement with the lack of DyP activity with other non-phenolic models. On the other hand, the reaction reported for *Amycolatopsis* DyP is difficult to explain since, although O_2 could be required for a non-enzymatic oxidation step (*e.g.* veratraldehyde is not formed by LiP/VP in anaerobiosis since the cation radical needs to react with O_2), the peroxidase cannot be activated in the absence of H_2O_2, either added or generated in the reaction mixture (maybe the required Mn^{2+} forms H_2O_2 in an unknown reaction). Interestingly, site-directly mutagenesis and EPR studies have shown that *T. curvata* DyP has a surface tryptophan involved in substrate oxidation,[58] as previously described for fungal LiP, VP, and DyP (see above). The exposed tryptophanyl radical formed after enzyme activation by H_2O_2 would be responsible for oxidation of bulky dyes by bacterial and fungal DyPs, and could also be involved in the oxidation of phenolic lignin (such as technical lignins) and oligomeric lignin-degradation compounds by these peroxidases.

8.8 Stereoselectivity in Lignin Decay: The Exception that Proves the Rule

The β-etherase pathway of *Sphingobium* SYK-6 (an α-proteobacterium isolated from waterlogged sludge from a paper pulp industry) represents a unique example of enzyme selectivity in lignin biodegradation routes.[135,136] The heterogeneity of the lignin polymer (synthesized from three different monolignols forming a variety of linkages) is increased by the existence of two chiral centers at the C_α and C_β positions of every unit side-chain.[137] Due to the enzyme selectivity paradigm, a variety of enzymes adapted to the different lignin substructures were foreseen in early studies.[138] However, lignin-degrading fungi had developed the opposite strategy, *i.e.* unspecific attack on the benzenic ring by LiP (and VP) forming cation radicals (or phenoxy radicals from minor phenolic units by phenol-oxidizing enzymes)[139] and subsequent bond cleavage.[21]

The β-etherase pathway of *Sphingobium* sp. represents a noteworthy exception to the above degradation strategy since the three successive steps in the breakdown of enantiomers of β-O-4′ phenolic dilignols are catalyzed by stereospecific dehydrogenases (introducing a C_α keto group), glutathione-S-transferases (substituting the ether guaiacyl group), and glutathione lyases (releasing the monomeric ketone), respectively.[140] Obviously, such

stereospecific attack by intracellular oxidoreductases is possible for the decay of simple (dimeric) lignin-derived compounds but not for the whole polymer degradation, where unspecific oxidation is the rule due to the insoluble nature of lignin and the large number of different substructures formed by random coupling in biosynthesis.[137]

A similar pathway has been reported in a marine *Novosphingobium* strain.[141] The huge amount of undegraded organic carbon (~400 million tons C per year) transported by rivers from land ecosystems to sea, with lignin-derived phenolic compounds as a major fraction, justify the existence of an active catabolism of these compounds in marine sediments. More detailed information on the above enzymes is provided in Chapter 9 by Masai *et al*.

8.9 Lignin-degrading Enzymes in Lignocellulose Biorefineries

A sustainable bioeconomy must be based on lignocellulosic feedstocks, from agricultural and forest crops and wastes, to overcome the inevitable exhaustion of crude oil and reverse the alarming trend of climate change, due to release of carbon dioxide of petrochemical origin. Lignin removal is required to have access to polysaccharides in the production of a variety of sugar-derived biofuels and chemicals in the lignocellulose biorefineries.[18,142] Moreover, the biorefinery economy requires an urgent valorization of the so-called technical lignins,[143–145] including the lignin fraction from biofuel[146] and cellulose[147] production, as a source of aromatic chemicals,[148] polymers,[149] and other value-added products.[146]

Biotechnology already represents a central part of the current lignocellulose biorefinery, providing enzyme cocktails for the hydrolysis of polysaccharides to simple sugars and yeast strains for their subsequent fermentation into ethanol. In addition to the hydrolytic and recently-discovered oxidative enzymes for polysaccharide breakdown,[150,151] the different oxidoreductases (peroxidases and laccases) contributing to natural degradation of lignin and lignin products are also required in future lignocellulose biorefineries to improve the process sustainability and product diversity, with the use of laccases being particularly relevant for most of the targeted processes.[18,62,152]

In bioethanol production, the use of laccases is being considered for both delignification and detoxification applications.[153,154] First, laccase was suggested for detoxifying steam-exploded feedstocks by repolymerizing the phenols released that exert a negative impact in the fermentation step.[155,156] Interestingly, recent studies have shown that laccase-mediator systems can be successfully adapted as a pretreatment for delignifying woody and non-woody lignocellulosic feedstocks, resulting in improved saccharification and fermentation yields without a chemical pretreatment.[157–159]

These oxidative enzymes (laccase-mediator systems included) can be also used for environmentally-friendly upgrading of technical lignins from the

biorefinery industries. As already noticed in early studies,[160] repolymerization often predominates during enzymatic treatment of lignin (in liquid media), due to the natural tendency of the aromatic radicals released to condensate, forming new linkages. In general, polymerization predominates when laccase alone is used, while more degradation products are often observed using the laccase-mediator system.[161] Applications based on condensation reactions are considered for (i) production of enzymatic ''adhesives'' to substitute resins in fiber and particle boards, with or without the addition of lignin products together with the enzymes (in the latter case condensation/adhesion reactions are due to the remaining lignin in the lignocellulosic material),[162,163] (ii) modification of different lignocellulosic materials by enzymatic grafting,[164,165] or (iii) enzymatically-aided recovery of lignin from process liquors.[166] However, applications also include the enzymatic modification of water-soluble commercial lignins to improve their dispersability properties[167] or their molecular weight in the production of plastizisers,[168] and the enzymatic introduction of functional groups of interest,[169] together with the use of lignin monomers discussed below.

The renewed interest in lignin-degrading bacteria,[90–92] is in great measure related to their eventual use to obtain chemicals of interest from lignin wastes taking advantage of the available tools for systems biology and metabolic engineering in model bacterial species. Some technical and waste lignins are often largely depolymerized during their industrial processing resulting in high phenolic preparations, such as kraft lignin (nowadays the main lignin byproduct worldwide). Therefore, they can be used as carbon and energy source by certain bacterial populations degrading phenolic compounds, whose metabolism can be tailored for the production of target molecules in a consolidated bioprocess for the biological revalorization of lignin in biorefineries.[101,170–172]

However, waste lignins from milder biomass pretreatments cannot be easily metabolized by bacteria due to their higher etherification and polymerization degrees. Therefore, an attractive biotechnological approach, together with chemical depolymerization methods,[173,174] consist in combining the natural fungal and bacterial degradation pathways.[21,175] In land ecosystems, basidiomycetes perform the initial attack on native lignin in dead wood while bacteria (and other fungi) largely act on the phenolic degradation products for their mineralization and incorporation to the soil humus. In this way, selected bacterial cultures can act as a microbial ''sink'' for the lignin products released during a previous depolymerization step by fungal enzymes with two important advantages: (i) the rapid bacterial growth will prevent the repolymerization tendency in lignin biodegradation and (ii) the whole transformation can be redirected to the product(s) of interest by *ad hoc* engineering of the bacterial metabolism.[176] Considering the slow fungal growth on lignocellulosic wastes, and their inability to use lignin as a carbon and energy source, crude fungal enzymes would be the choice in the above depolymerization step to develop an industrially-feasible process. However, the application of synthetic biology tools to biomass conversion[177] could

permit in the near future to incorporate lignin-degrading genes into bacterial hosts, and/or to design fast-growing lignin-degrading fungi for the lignocellulose biorefineries.[178]

Finally, it is possible to mention that some already known and new/engineered oxidative enzymes from the basidiomycetes involved in lignin degradation, from laccases[110,179,180] to peroxidases and peroxygenases,[38,39,181] also have great potential in green chemistry reactions for the environmentally-friendly and selective synthesis of a series of added-value organic chemicals of renewable or petrochemical origin.[182,183] Production of specialty chemicals and active pharmaceutical ingredients are typical examples of these enzymatic biotransformations.[184–186] However, the current tendency for lowering the price of enzymes (as shown with cellulases for lignocellulose biorefineries) makes fungal oxidoreductases attractive biocatalysts for some bulk chemistry reactions, *e.g.* in the production of renewable chemical building blocks.[187–189]

More detailed information on microbial upgrading of lignin compounds is provided in Chapter 11 by Eltis *et al.*

8.10 Conclusion

Fungi of the order Polyporales (and a few other basidiomycetes) are the main lignin degraders in nature being able to depolymerize and mineralize the largely non-phenolic polymer present in sound wood. Peroxidases of the LiP, VP, and MnP families are the key enzymes in lignin attack, as shown by genomic evidence revealing duplication of the corresponding genes in all the sequenced genomes of white-rot (ligninolytic) basidiomycetes and their loss in all the brown-rot (cellulolytic) fungal genomes.

With this purpose, fungi developed a unique strategy based on formation of a reactive tryptophanyl radical at the surface of LiP and VP proteins, which can directly interact with the bulky lignin polymer and transfer electrons to the heme cofactor. Additional oxidoreductases act synergistically with the above peroxidases, including H_2O_2-providing oxidases, and phenol-oxidizing DyPs and laccases (the latter largely investigated for delignification and lignin valorization applications due to easier production, stability, and use of O_2 as final electron acceptor).

Recent years have seen a renewed interest on the bacterial degradation of lignin, with prokaryotic DyPs being presented as the new ''ligninases''. Although DyPs (and bacterial MCOs) can oxidize phenolic compounds (and some of them also Mn^{2+}), break down phenolic lignin model dimers, and degrade kraft lignin (and the DyP redox potential is not very far from those of LiP and VP), no definitive evidence has been provided to date on their ability to degrade unmodified (non-phenolic) lignin, as LiP and VP do. However, the discovery of LRET pathways in bacterial and fungal DyPs suggests that they could oxidize phenolic lignin (and oligomeric lignin degradation products) at the protein surface.

Lignin ''catabolism'' in forest ecosystems, including the synergistic action of fungi depolymerizing native lignin and bacteria acting on lignin products, is inspiring new strategies for the simultaneous utilization of plant carbohydrates and lignin in the production of renewable chemicals and fuels, a key aspect for the global economy of lignocellulose biorefineries.

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