Chapter 19
Fungal Enzymes for Bioremediation of Xenobiotic Compounds

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19.1 Introduction

Xenobiotics are natural or synthetic organic compounds foreign to an organism that are potentially toxic and entail negative ecological or physiological consequences, in the form of pollution and disease, respectively (Olicon-Hernandez et al. 2017). Industry and agriculture are two major sources of environmentally prevalent xenobiotics which include fertilizers, insecticides, pesticides, dyes, plastics, and hydrocarbon derivatives (Sharma et al. 2018). Chemically, the vast majority of these compounds are aromatic with one or more frequently substituted phenyl functional groups. Many of these xenobiotic compounds are of major health and ecological concern as they are frequently carcinogenic and teratogenic, thereby disrupting development and reproductive capabilities in humans, fish, fish-eating birds, and other animals. A serious environmental and health issue is the accumulation of persistent, toxic chemical pollutants requiring new cost-effective and efficient ways to tackle the growing threat of environmental toxicity in the modernized world.

The majority of xenobiotic compounds can be decomposed or modified by microbes (Cameron et al. 2000). Bioremediation utilizes the metabolic potential of biological organisms to degrade or transform hazardous compounds in the environment into less toxic or nontoxic forms (Watanabe 2001; Yadav et al. 2019a, b). In particular, the use of fungi, referred to as mycoremediation, has attained widespread

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attention. Although bacteria are also versatile decomposers of xenobiotic pollutants, certain fungal strains are able to tolerate higher levels of pollutants. In some cases, such as the degradation of polyaromatic hydrocarbons (PAHs), bacteria can metabolize them and utilize them as carbon and energy sources, but they cannot mineralize them completely the way fungi can (Mougin et al. 2009). Fungi are capable of metabolizing a wider range of pollutants than their prokaryotic counterparts due to both extra- and intracellular degradation mechanisms and the powerful, nonspecific nature of the enzymes involved in both processes (Christian et al. 2005a). Additionally, the enzymes in question are tolerant of an active under diverse conditions including broad pH ranges, making fungi and their enzymes desirable for bioremediation (Verma and Madamwar 2002) (Rastegari et al. 2019; Yadav et al. 2017, 2018).

Filamentous fungi, particularly from the group called white rot fungi and mainly from *Basidiomycetes*, demonstrate a striking ability for oxidative decomposition of lignin, a recalcitrant component of wood composed of polyphenols (Mougin et al. 2009). These enzymes were evolved by such fungi to assist in the breakdown and detoxification of potentially hazardous by-products resulting from the decomposition of wood and other organic matter (Morel et al. 2013). The presence of these enzymes in many species of white rot fungi has been confirmed using comparative genomics to identify the so-called xenome, which consists of genes involved in xenobiotic detoxification. In the last two decades, these enzymes have been extensively adapted for bioremediation of pollutants in a low-cost, eco-friendly manner.

Bioremediation involves environments toxic to the survival of biological systems and despite fungal robustness and innate degradation infrastructure, there is a limit to the organisms' tolerance of xenobiotic-induced environmental toxicity. Frequently, the polluted areas are too nutrient-poor to support microbial growth. Moreover, the fungi capable of detoxifying a pollutant may be sensitive to other pollutants in the environment (Mougin et al. 2009). To circumvent these problems, one option is to utilize isolated fungal enzymes or enzyme mixtures. The use of fungal enzymes rather than complete fungal populations is also a more eco-friendly approach in contrast to using live microbes as it allows for the mitigation of any adverse environmental impact resulting from the introduction of novel species (Sharma et al. 2018; Kour et al. 2019; Rana et al. 2019a, b). This is especially desirable as release of genetically modified fungi or other organisms is contingent on both acceptance by regulatory bodies like EPA and the general public (Ang et al. 2005).

The most prominent groups of enzymes utilized in xenobiotic bioremediation transformations are oxidoreductases: peroxidases, laccases, and oxygenases (Sharma et al. 2018). Oxidoreductases can detoxify compounds by catalyzing oxidative coupling reactions using oxidizing agents to support the reactions. Laccases (LACs) and CYP monooxygenases (P450s) use molecular oxygen as the electron acceptor, while peroxidases use hydrogen peroxide to oxidize the substrates; both reactions result in the formation of water as a by-product. Peroxidases are heme-containing proteins, and the major types of peroxidases involved in detoxification processes in fungi are manganese peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidases (VP) (Doddapaneni et al. 2005). Xenobiotic detoxification

enzymes are produced by a great diversity of fungi, but many are often produced by a large physiological group called the white rot fungi. These fungi actively degrade lignin – a large, complex, aromatic-containing networked polymer, creating a bleached appearance in the host, hence the name white rot fungi (Pointing 2001). Given the complexity of lignin and the structural motifs common to both lignin and various xenobiotics, these enzymes are well suited for xenobiotic detoxification (Fig. 19.1). A few genera of fungi are used extensively in bioremediation processes; these include *Trametes, Pleurotus, Phanerochaete* spp., etc.

In this chapter, we will review the utility of fungal enzymes in bioremediation processes. In particular, we focus on the detoxification of organic xenobiotics by oxidoreductase enzymes including the extracellular peroxidases and laccases as well as the intracellular CYP450.

19.2 Sources of Xenobiotic Pollutants

Synthetic dyes released from paper, textile, plastic, cosmetic, food, and drug industries can be toxic and carcinogenic (Asgher et al. 2008; Levin et al. 2005). Polycyclic aromatic hydrocarbons (PAHs) like anthracene, pyrene, benzopyrene, and naphthalene are toxic, potentially carcinogenic xenobiotics that are produced from fuel combustion, gas plants, and industrial applications. Endocrine disrupting chemicals (EDCs) include alkylphenols such as nonylphenol and octylphenol as well as biphenyls such as bisphenol A (BPA), stilbene, and genistein estrogens. These are pharmaceutically active compounds that disrupt endocrine homeostasis in animals and are of grave concern to human health (Asgher et al. 2008). Other sources of xenobiotics include bleach plant effluents from paper and pulp industry, which contain toxic polychlorinated phenols (PCPs) and other organic compounds used for bleaching, and include dyes like azo dye and crystal violet (D'Souza et al. 2006). Chlorinated aromatic compounds like dichloro- and trichlorophenol and derivatives such as DDT, chlordane, and lindane are used in pesticides.

19.3 Metabolism of Xenobiotic Pollutants by Fungi

Bioremediation through fungi may be achieved by direct metabolism, whereby a fungus may completely degrade a xenobiotic compound to innocuous end products like carbon dioxide and other simple inorganic compounds (Mougin et al. 2009). This is particularly likely under nutrient-limiting conditions when the substrate compound could serve the carbon and/or energy needs of the organism. While this metabolic pathway is preferred as the toxic compound is eliminated or nearly so, the more common method of detoxification is co-metabolism. In this process, a cosubstrate is used to transform a xenobiotic compound without utilizing the compound for growth or energy needs. Generally, this only results in minor changes in the structure of the pollutant. For instance, substrate-free radicals may be generated

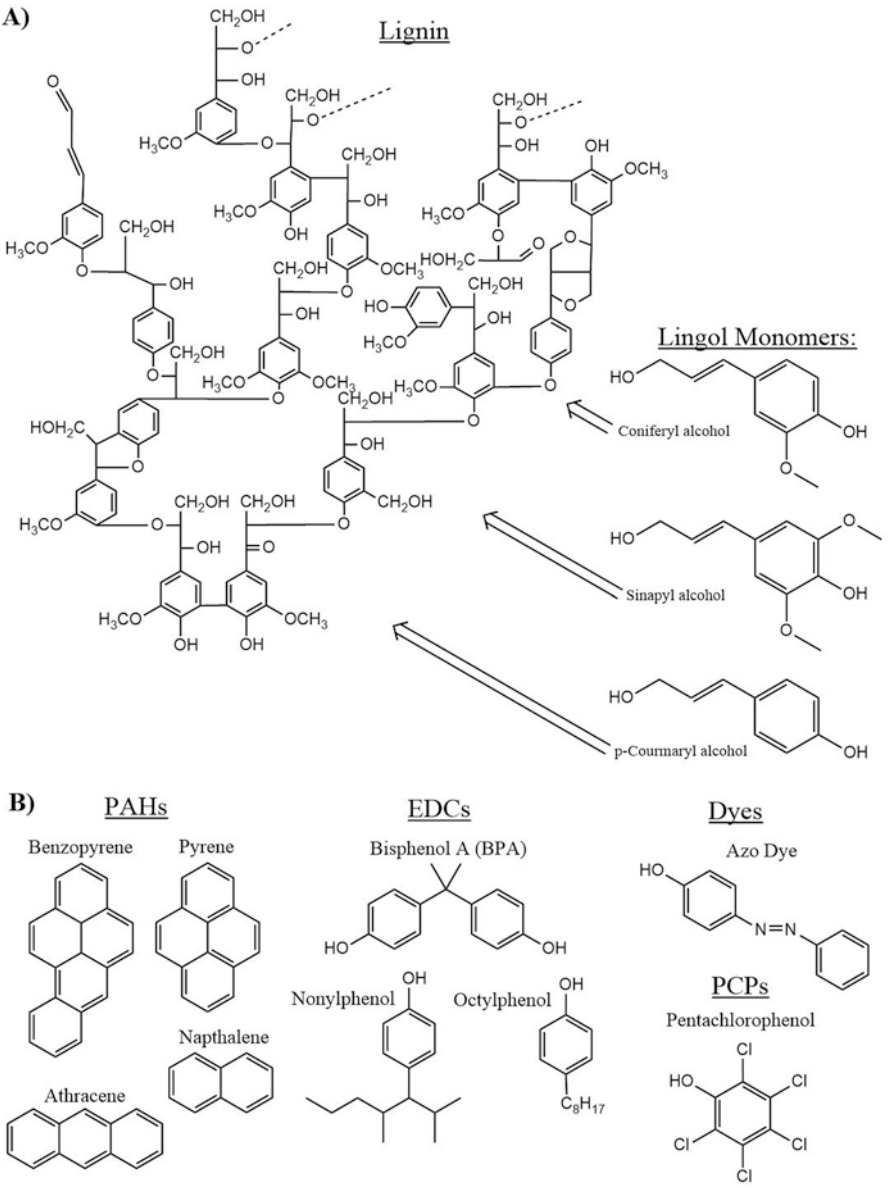

Fig. 19.1 The relationship and commonalities between organically produced lignin and varied xenobiotic chemical structures. (**a**) The structure of lignin according to Laurichesse and Averous (Laurichesse and Avérous 2014) and the monomeric alcohols which polymerize to form the cross-linked complex. (**b**) Common xenobiotic classes and associated structures as lignin model compounds targeted by lignin-degrading enzymes. Alkylated phenolic structures are common to both EDCs and lignin, as is the geminal phenyl motif of BPA. Nitrogen is not commonly present in lignin structures, nor are fused polycyclic aromatics nor polychlorinated phenols making lignin-degrading enzymatic degradation of these compounds less obvious

which subsequently cross-link the substrates with themselves or with environmental structures such as soil components, thereby forming adducts with decreased bioavailability and toxicity (Bollag 1992). Alternatively, these modified compounds could be further metabolized by other organisms leading to further detoxification. A third detoxification pathway involves conjugation or oligomerization of a pollutant. In this process, the resulting products of enzymatic action have larger and more complex structures than the parent compounds, but the structural modification or aggregation reduces the bioavailability of the compound, thereby negating its biological potency. In conjugation, typically, the compound may be methylated, acetylated, alkylated, or conjugated with sugars and amino acids and subsequently excreted or sequestered into storage structures. In oligomerization, a xenobiotic is coupled with a second molecule of the same or different xenobiotic following oxidation, aggregating into structures that become less bioactive.

19.4 Fungal Enzymes in Xenobiotic Remediation

The most commonly studied enzymes involved in mycoremediation all catalyze oxidoreduction reactions in the transformation of xenobiotics (Sharma et al. 2018). Among these enzymes are some active extracellularly and other which are intracellularly involved in the transformation and subsequent detoxification of xenobiotics. Within these oxidoreduction-catalyzing enzymes, some such as laccases (LACs) and CYP monooxygenases (P450s) use molecular oxygen as an eventual electron acceptor, while peroxidases use hydrogen peroxide as electron acceptors in their respective reaction pathways. One specific class of enzyme common to many xenobiotic degradation mechanisms are the heme peroxidases of which the most extensively involved and studied are manganese peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidases (VP) (Doddapaneni et al. 2005). The relatively lower substrate specificity of the extracellular enzymes in oxidative degradation allows them to target a wider variety of xenobiotic compounds and is as such of great interest in bioremediation processes (Harms et al. 2011). As with many secreted proteins, these peroxidases and laccases are glycosylated, at times heavily; for instance, certain laccases show 16% sugar content (Salony et al. 2006). Many fungal metabolites bearing diverse functional groups can serve as redox mediators for the oxidative function of these enzymes and can enhance their activity (Asgher et al. 2008). These include veratryl alcohol, N-hydroxyacetanilide, and acetosyringone to name a few. Mediators are particularly important when the substrates are too large to be accommodated into the active site of the enzyme (Mougin et al. 2009). The toxic xenobiotics or the products formed following their metabolism by extracellular enzymes can be further detoxified by intracellular enzymes including CYPs that are ubiquitously present in all organisms (Morel et al. 2013). These enzymes can catalyze various reactions including dealkylation, hydroxylation, and sulfoxidation and frequently function by inserting molecular oxygen into various substrates.

19.4.1 Laccase

LACs, originally identified as ligninolytic enzymes, are present in ascomycetes, basidiomycetes, and deuteromycetes and are commonly found in gene families (Harms et al. 2011). Laccases are secreted blue multicopper oxidases that catalyze single-electron oxidation of phenolic and other aromatic substrates to create free radicals with the concomitant reduction of molecular oxygen to water. The radical products cross-link by self-coupling or cross-coupling to form less toxic polymers; during this process they may undergo further decomposition reactions such as decarboxylation, dechlorination, and demethoxylation. Following LAC-dependent oxidation events, metabolites commonly exhibit oxidative coupling or radical polymerization resulting in compounds of greater molecular masses than the parent compound (Junghanns et al. 2005) which potentially may further negate the biological effects of such foreign compounds (Tsutsumi et al. 2001). The use of abundantly available oxygen as an oxidizing agent with the formation of water as a harmless by-product makes laccases a desirable bioremediation enzyme. Compared to peroxidases, different laccases can tolerate a wider pH range (2–10) and have broader substrate specificities (Xu 1996). The broad substrate range of laccases encompasses various xenobiotic compounds contaminating soil and water, i.e., PAHs, organophosphorus insecticides, and toxic dyes which include aromatic compounds like phenols, trichlorophenols, aminophenols (anilines), phenylenediamine derivatives, and benzenethiols (Amitai et al. 1998; Kues 2015; Sharma et al. 2018; Xu 1996; Yadav et al. 2016).

The potential of LACs in xenobiotic remediation has long since been a point of interest and investigation. Laccases from *Trametes versicolor* and *Pleurotus ostreatus* have been used in the detoxification of PCBs (polychlorinated biphenyls) which were used as insulators in electrical equipment until they were banned in 1979 for their high toxicity (Keum and Li 2004). Laccases facilitate substrate oligomerization and dechlorination, leading to detoxification of these compounds. LACs catalyze the initial oxidation of polycyclic aromatic hydrocarbons (PAHs), beginning their extracellular degradation (Pozdnyakova et al. 2018b). A LAC isolated from *Coriolopsis gallica* oxidizes the PAHs carbazole, N-ethylcarbazole, and dibenzothiophene in concert with proper mediators such as 1-hydroxybenzotriazole and 2.2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (Viswanath et al. 2014). When free laccase was applied to PAH-contaminated soil, 15 PAHs including anthracene and benzopyrene were degraded, demonstrating the potential of direct enzyme application for bioremediation (Wu et al. 2008). Covalently immobilized laccases from *Trametes versicolor* were shown to efficiently degrade the PAHs, anthracene, naphthalene, and phenanthrene (Bautista et al. 2015). Multiple studies revealed the ability of laccases from *Pleurotus ostreatus, Trametes versicolor*, and other strains to effectively degrade BPA and other endocrine disruptors like alkylphenols. The detoxification of these compounds by laccases may be brought about by oligomerization or mineralization to carbon dioxide, and this action was enhanced by supplementation with mediators (Macellaro and Pezzella 2014;

Margot et al. 2013; Zhang et al. 2015). Laccases from *Pycnoporus sanguineus, Myceliophthora thermophila, Trametes trogii*, and *Ceriporiopsis subvermispora* effectively metabolized the toxic dyes like bromophenol blue, methyl violet, and malachite green and other dyes used in the textile industry (Antosova et al. 2018; Chmelova and Ondrejovic 2016; Mougin et al. 2009). Synthetic laccases are also used in the paper and pulp industries to bleach paper and clothes (Sharma et al. 2018). The addition of mediator compounds can dramatically enhance the detoxification of substrates as witnessed in the transformation of halogenated pesticides by laccases from the fungus, *Coriolopsis gallica* (Torres-Duarte et al. 2009); in this study, acetosyringone and syringaldehyde proved to be the most effective mediators. Finally, laccases from *Trametes versicolor* have been used to oxidize the pharmaceutical drugs, diclofenac and mefenamic acid in municipal wastewater (Margot et al. 2013). Beyond naturally occurring LACs, some researchers have explored the possibility of engineering LACs with the intention of improving transformational efficiency and demonstrating transient expression by means of directed evolution (Camarero et al. 2012; Gu et al. 2014; Mate and Alcalde 2015; Theerachat et al. 2012; Wong et al. 2013b).

19.4.2 Peroxidase

The ligninolytic peroxidases of interest in bioremediation carry high redox potentials (>1.4 V) and catalyze the oxidative breakdown of lignin and other compounds with aromatic ring structures using hydrogen peroxide as a cosubstrate and with the help of certain mediator compounds like veratryl alcohol (Piontek et al. 2001; Sharma et al. 2018). These peroxidases are glycosylated secreted proteins carrying an iron protoporphyrin (heme) ring at the catalytic center. Peroxidases can oxidize and generate phenolic radicals which may aggregate and precipitate. There are several groups of secreted peroxidases in fungi:

19.4.2.1 Lignin Peroxidase

LiPs are heme-containing enzymes which can metabolize various aromatic compounds many of which are generally refractory to breakdown, with pH optima in the acidic range (2–5) (Shrivastava et al. 2005). Since the 1983 discovery of *P. chrysosporium*, LiP in extracellular media, isozyme forms among various basidiomycetes have been isolated ranging in weight from 38 kDa to 43 kDa (Falade et al. 2017). LiPs have been shown to completely oxidize both methylated lignin model and non-methylated lignin model compounds as well as PAHs (Kadri et al. 2017). Through powerful, nonspecific catalytic transformative activity, the aptly named LiP is capable of direct transformation of up to 90% of lignin structural components (Falade et al. 2017).

LiPs are commonly produced by *Phanerochaete* and *Trametes* spp. As demonstrated by LiP isolated from *P. chrysosporium* which transforms the PAHs benzo[a]pyrene, anthracene, 1-methylanthracene, 2-methylanthracene, 9-methylanthracene, fluoranthene, acenaphthene, and dibenzothiophene, these enzymes possess broad substrate ranges (Pozdnyakova 2012). LiPs also demonstrate expanded substrate ranges in the presence of veratryl alcohol which increases oxidation of weak or terminal LiP substrates. With the advantageous kinetics conferred by mediators like veratryl alcohol, LiP transforms most aromatic compounds with an ionization potential less than 8 eV. LiP also has the unique ability to cleave esters in non-phenolic aromatics, thereby further demonstrating the significance of LiPs to bioremediation efforts of diverse, aromatic-containing xenobiotics (Pozdnyakova 2012).

19.4.2.2 Manganese Peroxidase

MnPs are also heme-containing secreted enzymes which function under relatively less acidic conditions (pH 4–7) than those of LiP (Asgher et al. 2008). As a heme peroxidase, MnPs share much of their characteristics and mechanism with LiPs (Deshmukh et al. 2016). MnP appears not to occur in the same large gene families characteristic of other ligninolytic enzymes such as LAC (Torres-Farrada et al. 2017) and is only found in *Basidiomycota* (Harms et al. 2011). MnP is capable of aromatic ring cleavage within monoamino-dinitrotoluene and chlorophenol derivatives (Harms et al. 2011). The oxidative potential of MnP relies on the oxidation of Mn^{2+} to Mn^{3+} by the enzyme followed by the indirect, single-electron oxidation of subsequent substrates as Mn^{3+} is reduced, thereby reverting to Mn^{2+}.

Due to the indirect mechanism, the substrate range is broad, and the extent of transformation of resulting metabolites is near complete. MnP oxidizes phenols, aromatic amines, and dye compounds as well as mineralizes CO_2 from various quinones – common products of PAH radical polymerization – by ring fission transformations. MnPs isolated from *Anthracophyllum discolor* clearly demonstrate this PAH-substrate promiscuity as they are able to oxidize pyrene, anthracene, fluoranthene, and phenanthrene as well as various derivatives of these compounds (Pozdnyakova 2012). A MnP from the white rot fungus, *Trametes*, displayed a strong ability to degrade azo and indigo dyes as well as PAHs (Zhang et al. 2016). Another MnP from *Peniophora incarnata* not only displayed the potential to break down the PAH anthracene, but this ability was transferable by heterologous expression in yeast, signifying a bioremediatory potential. MnP from *Ganoderma lucidum* used as cross-linked enzyme aggregates efficiently degraded the endocrine disrupting nonylphenol and triclosan (Bilal et al. 2017a).

MnP is of great interest in bioremediation efforts as it has been shown to be stable under adverse conditions; however, a complicating factor of its use in remediation effort and applications is the mechanistic need for a suitable chelator (Bogan et al. 1996). Such chelators are commonly organic acids such as oxalic or malonic

acid derivatives (Kadri et al. 2017). These compounds complex with Mn^{3+}, enabling the oxidation of substrate lignin model compounds. In addition to a mechanistic dependence on chelators, MnP activity significantly increases in the presence of redox mediators such as Tween 80. With the added effect of Tween 80, MnP has been shown to transform compounds with ionization potentials of 8.2 eV. Despite the powerful oxidative potential and resistance to adverse conditions of MnP, the application of these enzymes in biotechnical pursuits is complicated by not only chelator requirements and redox mediator reliance for elevated efficiency but also by LAC-dependent initiation of Mn^{3+} complexing (Schlosser and Hofer 2002). No Mn^{3+} complexing was observed in in vitro mixtures of semi-purified MnP, Mn^{2+}, and oxalate or malonate when H_2O_2 sources were excluded. However, the addition of LAC stimulated Mn^{3+} complexing and ultimate MnP-stimulated substrate oxidation. In response to PAH-polluted media, MnP secretion by *Fomes* is very high, reaching concentrations of approximately 1299 U/L after 21 days of xenobiotic exposure (Godoy et al. 2016).

19.4.2.3 Versatile Peroxidase

Versatile peroxidases (VP) are a hybrid between LiPs and MnPs as they contain a heme group and oxidize $Mn^{2+}$to Mn^{3+}, inducing indirect oxidations, as well as oxidize phenolic and non-phenolic substrates; VPs conjoin mechanisms and substrate ranges between these two enzymes (Kues 2015; Pozdnyakova 2012). VPs have thus far only been identified in *Basidiomycetes* (Harms et al. 2011). In the initial characterization of the first identified VP – specifically, PS1 isolated from *Pleurotus eryngii* – it was shown to possess both the Mn oxidation domain of MnPs and the aromatic substrate oxidation center (AS) of LiPs (Camarero et al. 1999). Furthermore, PS1 and subsequently characterized VPs have been shown to actually retain LiP- or MnP-like enzymatic activity in conditions that would inactivate LiPs or MnPs, respectively. Little is understood about the role of VPs in the mediation of xenobiotic transformation; however, it is known that VP production is induced by the presence of PAH pollutants (Pozdnyakova et al. 2018b). In addition to PAHs, VPs can also degrade polyhalogenated aromatic pesticides containing diverse functional groups as demonstrated by VP isolated from *Bjerkandera adusta* which successfully transformed dichlorophen (an antimicrobial polycyclic), bromoxynil (a nitrile herbicide), and pentachlorophenol (PCP) – a potent pesticide (Davila-Vazquez et al. 2005). Given the hybridization of LiP and MnP substrate ranges, they have the potential to be a major component of the subsequent remediation of many chemically diverse xenobiotics. Due to their broad substrate range and various transformation mechanisms, VPs warrant further characterization and investigation regarding their potential application in bioremediation and biotechnical efforts. Examples of fungal laccases and peroxidases employed for xenobiotic detoxification are presented in Table 19.1.

Table 19.1 Details of microbes producing laccase and peroxidase in the bioremediation of xenobiotics

Enzyme source	Xenobiotic	Compounds	Mediator	Reference
Laccases				
Trametes versicolor	PAH	Anthracene, benzopyrene	ABTS	Dodor et al. (2004)
T. versicolor, Pleurotus ostreatus	PCB	Hydroxy PCBs	TEMPO	Keum and Li (2004)
P. ostreatus	Insecticides, nerve agents	VX, Russian VX, diisopropyl-Amiton	ABTS	Amitai et al. 1998)
P. pulmonarius	Toxins	Aflatoxin B_1	ABTS, AS, SA	Loi et al. (2016)
T. sanguineus	Endocrine disruptor, PAH	Bisphenol A, benzopyrene, phenanthrene	ABTS	Balcazar-Lopez et al. (2016)
Echinodontium taxodii	Azo dyes	Brilliant Violet 5R, Direct Red 5B, Direct Black 38	Lignin derivatives	Han et al. (2014)
Clavariopsis aquatic	Endocrine disruptor	Nonylphenol	ABTS	Junghanns et al. (2005)
Peroxidases				
Trametes spp.	Dyes, PAH	Indigo, anthraquinone, azo, triphenylmethane, fluorene, fluoranthene, pyrene, phenanthrene, anthracene	–	Zhang et al. (2016)
Peniophora incarnata	PAH	Anthracene	–	Lee et al. (2016)
Irpex lacteus	Dyes	Azo, indigo dyes	–	Qin et al. (2014)
Phanerochaete chrysosporium	PCB	2,4-dichlorophenol	–	Chen et al. (2011)
Penicillium chrochloron	Dyes	Cotton blue	–	Shedbalkar et al. (2008)

ABTS 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid, *AS* acetosyringone, *PAH* polyaromatic hydrocarbons, *PCB* polychlorinated biphenyls, *TEMPO* 2,2,6,6-tetramethylpiperidine-N-oxyl radical, *SA* syringaldehyde

19.4.3 Cytochrome P450 Monooxygenase

Many fungi also possess intracellular transformation enzymatic machinery capable of further degradation of xenobiotics (Syed and Yadav 2012). They belong to the larger group of oxygenases that are the principal intracellular enzymes involved in the aerobic degradation of aromatic xenobiotics using oxygen (Sharma et al. 2018). These are also heme-containing enzymes that add one or more oxygens to destabilize aromatic rings to break down and even solubilize the compound. Oxygenases could catalyze the addition of one oxygen molecule (monooxygenase) or two molecules (dioxygenase) to the substrate. Many halogenated herbicides, fungicides, and pesticides are detoxified using oxygenases, and the best-studied enzyme belongs to the cytochrome P450 enzyme family, which utilized NADPH as a cofactor to catalyze redox reactions (Doddapaneni et al. 2005).

P450s are very distinct from other xenobiotic-degrading fungal enzymes as it is active intracellularly (Deshmukh et al. 2016). Furthermore, CYP450 are found in massive gene families with multiple subfamilies as discovered in the genome of *P. chrysosporium* with at least 149 isozymes (Doddapaneni et al. 2005; Olicon-Hernandez et al. 2017; Syed et al. 2011). Such gene families have been identified in ascomycetes, basidiomycetes, mucoromycetes, and chytridiomycetes (Harms et al. 2011). As monooxygenases, P450 incorporates a single atom from molecular oxygen into the substrate while reducing the remaining oxygen to water. Compared to CYPs involved in primary metabolism, CYPs mediating detoxification processes are generally less specific (Cresnar and Petric 2011). P450 monooxygenases are a major component in the degradation of various xenobiotics as they are quite nonspecific and demonstrate very powerful enzymatic activity in the transformations of such diverse chemical motifs. However, as these are intracellularly active proteins, their application in fungal enzyme isolation and immobilization is further complicated.

Within each class of P450s, multiple subfamilies exist sharing general function and some sequence identity. Many class-II subfamilies are involved in biosynthetic pathways and homeostatic processes. The subfamily CYP51 has been indicated in maintaining cell wall integrity while CYP61 (a fungi-specific subfamily) is responsible for spore outer wall formation. Various Class II P450s transform PAHs, alkyl phenols (APs), alkanes, and polychlorinated dibenzo-p-dioxins (PCDDs) (Harms et al. 2011; Kues 2015). CYPs from the basidiomycete *Phanerochaete chrysosporium* have been shown to catabolize PAHs including anthracene and the endocrine disrupting alkylphenols (Hirosue et al. 2011; Syed et al. 2011). Among Class II P450 subfamilies, CYP57 has been shown to detoxify pisatin, a heterocyclic, aromatic-containing fungus growth inhibitor produced in pea plants upon microbial attack. Furthermore, CYP53 has been shown to degrade and detoxify benzoate derivatives within multiple species from both the *Ascomycota* and *Basidiomycota* phyla. Additionally, CYP504 is known to degrade phenylacetate derivatives. The significance of Class II P450 activity is further emphasized in observed biodegradation by non-ligninolytic fungi such as *Scopulariopsis brevicaulis* which was shown to completely transform anthracene (a tricyclic PAH), producing the same major metabolite – 9,10-anthraquinone – as ligninolytic fungi such as the basidiomycete *Fomes,* despite the absence of extracellular enzymes (LAC, LiP, and MnP) demonstrating the versatility and potency of Class II P450-dependent intracellular transformation pathways (Godoy et al. 2016). CYPs have been tapped to detoxify a variety of pharmaceutical compounds, including antibiotics, anti-inflammatories, and β-blockers (Olicon-Hernandez et al. 2017). Naproxen is an environmentally prevalent pharmaceutical pollutant and has even invaded drinking water systems due to overuse in treating human and animal diseases. CYPs were shown to detoxify this drug through demethylation and hydroxylation (Aracagok et al. 2017). A fundamental limitation in utilizing CYP enzymes in cell-free systems is the fact that CYP functions in intracellular networks that also involve other enzymes (Haroune et al. 2017). Shotgun proteomics of proteins in response to the PAH, anthracene in *Penicillium oxalicum*, revealed regulation of hundreds of proteins, and intracellular metabolism of such xenobiotics typically follows two distinct phases with the CYPs working in conjunction with other enzymes like epoxide hydrolases and transferases (Lucero Camacho-Morales et al. 2018).

19.4.4 Unspecific Peroxygenase

UPOs are secreted hybrid enzymes which combine the functionalities of heme peroxidases and P450 monooxygenases (Karich et al. 2017). Among this class of peroxygenases, two common structural motifs have been observed: short UPOs are approximately 29 kDa, while long UPOs are approximately 44 kDa. Short UPOs are found across all fungal phyla, but long UPOs are exclusive to ascomycetes and basidiomycetes. Both short and long UPOs have immense substrate ranges, acting in association with hydrogen peroxide as a cofactor, a marketed improvement in simplicity from the nuanced requirements of MnP. Furthermore, 41 of EPA-listed priority pollutants have been shown to be transformed by UPOs, and more than 300 other aromatic, poly- and heterocyclic, and aliphatic substrates have been identified thus far. Despite the recent findings regarding xenobiotic substrates, the physiological role of UPOs remains to be identified (Olicon-Hernandez et al. 2017); however, Karich et al. theorized that UPOs and P450s may work in harmony with UPOs crudely transforming xenobiotics extracellularly to reduce negative biological effects, while P450s "fine-tune" the resulting metabolites so that they may be further transformed and rendered inert within the cell or even consumed as carbon sources due to the incredibly diverse functionality of these enzymes. Thus far, present understanding explains that UPOs' substrate transformations are limited by steric hindrance, bioavailability and potential substrate solubility, or strong inactivation of an aromatic ring by electron withdrawing groups (Karich et al. 2017). Despite these regulatory factors in UPOs' substrate specificity, UPO isolated from *Agrocybe aegerita* demonstrated significant transformation of naphthalene, phenol, anisole, toluene, ethylbenzene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, benzo[a]anthracene, 1,2-diphenylhydrazine, benzidine, and 2,4-dimethylphenol as well as various phthalate esters, nitroarenes, and polychlorinated benzenes (Karich et al. 2017). Among these known substrates exist several previously only thought to be transformed by P450s or MnPs and LiPs, again demonstrating the remarkable potential of these enzymes. Such versatile enzymes in extracellular degradation processes carry great implications for biotechnical development of xenobiotic mitigation strategies.

19.5 Mechanisms of Xenobiotic Detoxification by Fungal Enzymes

Fungal extracellular xenobiotic degradation occurs in two conserved steps (Rao et al. 2010). Firstly, the hydrolytic system targets macromolecules for degradation by hydrolase activity. The hydrolyzed substrates are then further transformed by the ligninolytic system comprised of nonspecific oxidative enzymes. The enzymatic mechanisms we focus on in this section are the oxidoreductases whose mechanisms are well researched (Fig. 19.2).

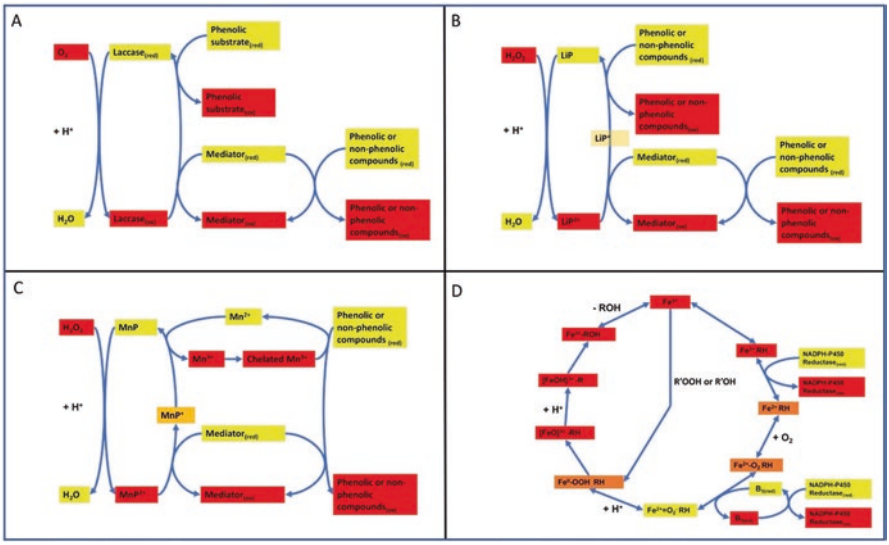

Fig. 19.2 Simplified mechanism of fungal enzymes used in bioremediation. (**a**) Laccase enzymatic cycle. Mediators include veratry alcohol (VA), Tween 80, and other small organic molecules. (**b**) Lignin peroxidase enzymatic cycle. Both oxidized states of LiP are able to oxidize substrates and mediators. Mediator molecules are used when the substrates cannot access enzyme active site. (**c**) Manganese peroxidase enzymatic cycle. MnP preferentially oxidizes Mn^{2+} in the first redox reaction but can also oxidize other mediators. Mn^{2+} oxidation is highly specific for the second redox reaction which restores the base state. (**d**) CytP450 enzymatic cycle. The reduction of heme-bound molecular oxygen is either directly catalyzed by the reductase coenzyme or indirectly via the use of cytochrome B5 as a redox mediator. (Adapted from Kues (2015) and Guengerich (2001b))

19.5.1 Laccases

Laccases are multicopper oxidases with low substrate specificity. Within basidiomycetes these families range from 5 to 17 isozymes (Yang et al. 2017). Within LAC isozymes, enzymatic copper involvement is conserved with four copper atoms at +2 oxidation states in the resting enzyme. The four copper atoms are characterized as T1, T2, and T3 – which is binuclear. The T1 copper facilitates substrate oxidation, while T2 and T3 copper atoms store the resulting electrons which then convert diatomic oxygen to water. They catalyze coupled redox reaction between a substrate and molecular oxygen resulting in the formation of a radical cation and water, respectively. The type I copper is found in a wide cavity of the enzyme surface, which allows it to bind to many different types of substrates (Su et al. 2018).

The significance of LACs in the remediation of xenobiotics is only amplified by laccase-mediator systems (LMSs) which act as electron transfer chains and thereby

further broaden the substrate range as well as increase kinetic favorability of transformation (Wong 2013a). LMSs share the common mechanism of facilitating the indirect oxidation of a substrate following the primary oxidation event of the mediator by LAC. The enzymatic cycle of laccases can be simplified as a four-electron abstraction from the substrate by the oxidized enzyme followed by the reduction of molecular oxygen to water to regenerate the active enzyme (Fig. 19.2a). The enzyme primarily exists in the resting oxidized state (RO) with each of the copper atoms oxidized. The catalytic reaction is initiated by the donation to the type I copper of four electrons from suitable substrates (reductants). Three electrons are transferred through a conserved His-Cys-His sequence of amino acids to the trinuclear cluster (TNC) consisting of the type II copper and the two type III copper atoms, resulting in the fully reduced enzyme. The electrons are used to reduce molecular oxygen which binds to the TNC, forming a peroxide intermediate (PI). The reduction then proceeds by donation of a hydrogen by a nearby glutamic acid residue resulting in the cleavage of the oxygen-oxygen bond and the native intermediate (NI) state of the enzyme. This state can directly proceed to the fully reduced state in the presence of large amounts of suitable substrates, producing two water molecules or proceed to the fully oxidized state with loss of a single water molecule in the absence of significant levels of reductants (Jones and Solomon 2015).

Laccases tend to accommodate substrates with relatively low redox potentials such as phenolic compounds which are oxidized to phenoxyl radicals which may undergo coupling reactions or isomerization to form quinones. Laccases are generally unable to directly oxidize compounds with high redox potentials or steric hindrance. Instead, such compounds are oxidized via the laccase-mediator system which involves oxidation of micromolecular organics which can then oxidize target compounds through redox reactions leading to cleavage of bonds (Su et al. 2018).

19.5.2 Peroxidases

Of the various peroxidases discussed herein, only the general mechanism of lignin peroxidase and manganese-dependent peroxidases has been elucidated. The shared mechanism of these two types of peroxidases involves the oxidation of the enzyme by two electron abstractions by the H_2O_2 cosubstrate followed by two one-electron transfer steps where the oxidized enzyme abstracts an electron from the substrate (Mougin et al. 2009).

19.5.2.1 Lignin Peroxidases

In LiPs, Fe(III) in the heme ring is coordinated with four heme tetrapyrrole nitrogens and to a histidine residue. LiP has been shown to fold into a globular profile measuring about $50 \times 40 \times 40$; this form is subdivided into proximal and distal

domains relative to the heme group. Two small molecular channels allow for heme accessibility despite its fixed position within the protein's tertiary structure. LiPs are capable of cleaving C_α-C_β bonds as well as bonds between aryl C_α (Kadri et al. 2017). LiP is further differentiated from other peroxidases by the optimal pH of approximately 3.0 (Falade et al. 2017). Lignin peroxidases are activated by a two-electron oxidation of the native enzyme by the cosubstrate H_2O_2 (Mougin et al. 2009). This results in compound I which is reduced by the substrate as part of a one-electron redox reaction to compound II. Compound II then abstracts a second electron from the substrate resulting in regeneration of the native enzyme. The activated forms of the enzyme have a high redox potential allowing it to oxidize compounds such as lignin that other peroxidases are unable to transform. LiPs act through three different mechanisms based on the availability of its heme cofactor toward the substrate. First, LiPs act directly on certain phenolic and non-phenolic compounds which can access the heme group. This can lead to breakage of carbon-carbon bonds in substrates leading to conformational changes (Fig. 19.2b). The second lignin peroxidase mechanism is indirect, acting through a redox mediator to oxidize compounds that cannot access the heme group (Fig. 19.2b). Lignin peroxidase oxidizes mediators such as veratryl alcohol (VA) to a cation radical (VA^+) which then oxidizes compounds through a redox reaction. The third mechanism occurs through further reactions of VA. The cation radical oxidizes organic acids into anion radicals which act as reductant. These radicals can also reduce molecular oxygen to a dioxygen anion which acts as a reductant. Through the reduction of ferrous ions, this dioxygen anion can also reduce hydrogen peroxide to a hydroxyl radical which can oxidize compounds through the non-enzymatic Fenton's reaction (Christian et al. 2005b).

19.5.2.2 Manganese Peroxidase

Manganese peroxidases, also known as manganese-dependent peroxidases, are oxidized to a two-electron-deficient state (compound I) and restore its basal oxidation state by two single-electron abstracting steps (Christian et al. 2005b). The first step which reduces the compound I to a single-electron-deficient state (compound II) is not very specific and can use either Mn^{2+} or small compounds such as VA as reductants. The second step which restores the basal oxidation state of the enzyme is highly specific to Mn^{2+} (Fig. 19.2c). Both of the activated enzyme states oxidize Mn^{2+} to Mn^{3+} which acts as a nonspecific small, diffusible redox mediator. Mn^{3+} are chelated to carboxylic acids such as oxalic acid and are thus able to cause one-electron abstraction oxidation of various compounds (Fig. 19.2c). They can also react with carboxylic acids to produce radicals such as VA+ and superoxide. Manganese peroxidases are also able to act indirectly as reductors. This is done through oxidation of hydroquinone to semiquinone radicals which reduce highly oxidized compounds. This generates a quinone which is then reduced back to hydroquinone by quinone reductase enzyme.

19.5.3 Cytochrome P450

Cytochrome P450 enzymes are part of a superfamily with remarkable variation. Due to this variation within the CYP superfamily, the reactions associated with P450 enzymes are classified based on reactionary motifs and various protein involvements into ten distinct classes, three of which have been observed in fungi: class II, class VIII, and class IX (Cresnar and Petric 2011). Most commonly, CYP450 enzymes act as monooxygenases that incorporate an atom of oxygen into substrates (van den Brink et al. 1998).

P450 enzymes therefore serve as versatile catalysts for region-specific and stereospecific oxidation resulting in hydroxylation, heteroatom oxygenation, dealkylation, and epoxidation of C=C bonds (Deshmukh et al. 2016; Olicon-Hernandez et al. 2017). However, the basis of all these reactions can be summarized as being

$$\mathrm{XH} + \mathrm{NAD(P)H} + \mathrm{H^+} + \mathrm{O_2} \rightarrow \mathrm{XOH} + \mathrm{NAD(P)^+} + \mathrm{H_2O}$$

with R-H as the substrate of cytochrome P450. Among these transformations, NADH or NADPH frequently acts as an electron donor to the second oxygen as it is reduced to water (Kues 2015). The reaction does not usually produce a phenol but instead an oxide which isomerizes to the more favorable phenolic conformation (Christian et al. 2005b). The phenolic form is activated toward further enzyme-catalyzed reactions, resulting in trans-dihydrodiols (Tongpim and Pickard 1999).

Much like the peroxidases, P450 enzymes are heme-based, with their catalytic active site containing a heme-bound iron atom (Guengerich 2001a). The substrate first binds to the heme iron Fe^{3+} which is reduced by an accessory enzyme, NADPH reductase (Fig. 19.2d). This reduced iron Fe^{2+} then binds with molecular oxygen. The accessory enzyme then reduces molecular oxygen which is then protonated. This leads to cleavage of the O-O bond, causing the protonated oxygen to be released as H_2O. The resulting FeO^{3+}complex radicalizes the substrate by proton (or electron abstraction). The radical then accepts the hydroxyl group (or oxygen atom in case of electron abstraction) before being released, simultaneously returning the iron to its base state. There exists a shunt pathway in which a peroxide is used as cosubstrate instead of molecular oxygen, skipping the need for the reductase coenzyme (Fig. 19.2d).

The monooxygenation mechanism of P450 is not limited to carbons but can also apply to heteroatoms such as nitrogen, sulfur, phosphorus, and iodine. In these cases, the oxygen transfer is more complicated, with the oxygen being added to the substrate after two successive electron transfer steps.

19.6 Strategies and Considerations in Xenobiotic Bioremediation

Either fungi or enzymes isolated from fungi could be used for the detoxification of xenobiotic compounds. Some fungal phyla possess more versatile degradation systems relative to bacterial strains (Pozdnyakova et al. 2018a). The fundamental limitations in using isolated fungal enzymes include productivity as well as stability and retention of activity (Sharma et al. 2018). Laccases, for example, have an acidic pH optimum that may not be available in effluent soils and waters, presenting a major limitation to their use. Furthermore, cost of application, intolerance of enzymes to high levels of cosubstrate hydrogen peroxide, and issues with enzyme reusability have hampered the application of these enzymes in the field (Nicell and Wright 1997). Once applied to sites for remediation, the enzymes are also under threat of denaturation and destruction by physical and chemical forces and by the action of microbes and their enzymes. Besides, excessive exposure of heme-containing proteins to oxidative species can lead to inactivation and subsequent degradation of the protein (Valderrama et al. 2002). Indeed, these limitations are so critical that they have delayed or limited large-scale application of these enzymes in bioremediation processes (Ayala et al. 2008).

One approach to the identification of potential fungal strains with significant remediation potential and biotechnical application is to isolate populations from xenobiotic-polluted environments (Godoy et al. 2016). Doing so results, in part, in fungi with innate tolerance to the environmentally present pollutants. The pollutant-resistant detoxifying fungi often produce biodegradative enzymes that are too limited in amount to isolate and utilize. To scale up production of these enzymes, genetic engineering could be used to overexpress fungal enzymes in fungi, plants, or other organisms that could colonize the polluted substratum. Alternatively, enzymes could be mass-extracted from such organisms by heterologous expression and utilized for bioremediation. This is a cost-effective strategy that not only allows purification of large amounts of enzyme with stability and activity, the purification process of recombinant proteins is also simpler as they can be isolated using cleavable tags (Alcalde et al. 2006). Furthermore, the stability, activity, and other features of an enzyme can also be enhanced using genetic and enzyme engineering approaches. By introducing mutations using strategies like DNA shuffling, error-prone PCR, and site-directed mutagenesis (Dua et al. 2002), enzyme engineering could be accomplished, where a change in protein sequence from mutations results in a possible change in enzyme structure or regulation with improved traits including increased stability and activity; increased xenobiotic substrate specificity or wider substrate range; tolerance to a wider range of pH, temperature, and stress conditions; and decreased susceptibility to proteases in the natural environment (Rayu et al. 2012). One such mutated laccase protein from *Pleurotus ostreatus* identified in a screen gained greater enzymatic activity and higher stability at acidic pH, widening its pH tolerance in detoxifying toxic industrial dyes (Miele et al. 2010).

Similarly, directed evolution of a laccase resulted in 3.5-fold increase in acetonitrile catabolism while tolerating relatively high concentrations of it (Alcalde et al. 2005).

As enzymes are sensitive to physical and chemical environmental changes, various strategies have been evolved to fortify and preserve their structure and function while exposed to adverse conditions. Enzyme immobilization in carbohydrate-based matrices has been shown to be effective in prolonging the half-life and enhancing the stability and catalytic function of the remediative enzymes. Enzymes can be immobilized to solid support systems for more effective use in bioremediation. Enzyme immobilization can be done by covalent linkage or adsorption of enzymes onto solid surfaces like glass, affinity tag-bearing beads or nylon membranes, suspension in polymeric gels like chitosan, gelatin and encapsulation in solid matrices such as sodium alginate and could also accompany enzyme cross-linking using glutaraldehyde (Diano et al. 2007; Koyani and Vazquez-Duhalt 2016; Sirisha et al. 2016). Immobilization has been shown to improve the stability, catalytic function, and longevity of enzymatic function of the fungal enzymes and additionally protects the enzymes from proteolytic degradation (Asgher et al. 2007; Cheng et al. 2007; Jing and Kjellerup 2018). This is brought about in part by the increased resistance to physical, chemical, and biological denaturing agents afforded by the immobilization. Additionally, immobilized enzymes can be recovered and reused, thus economizing the process. Laccases immobilized on glass beads from *Trametes* retained 90% of their activity and showed greater resistance to proteases (Bilal et al. 2017b; Dodor et al. 2004). Similar benefits have been observed for other fungal enzymes like MnPs (Bilal and Asgher 2016). Enzymatic nanoreactors with dendritic copolymers bearing glycosidic groups anchoring laccases not only showed improved enzymatic activity but also increased thermostability (Gitsov et al. 2008). Similarly, vault nanoparticles with hollow cores covalently anchoring multiple MnP enzymes showed enhanced stability while displaying a threefold increase in phenol degradation (Wang et al. 2015).

The addition of cofactors, cosubstrates, and mediators is important to drive the action of the enzymes in bioremediation scenarios; this is particularly important for recalcitrant pollutants. Additionally, the presence of mediator compounds can enhance enzyme activity. The fungal metabolites, veratryl alcohol and 2-chloro-1,4-dimethoxybenzene, can stimulate LiP enzyme activity, and this is particularly useful when treating recalcitrant substrates (Asgher et al. 2008), while acetohydroxamic acid serves as an excellent mediator for laccases (Minussi et al. 2007). In some cases, the addition of glucose as a carbon source to fungal cultures also enhanced the detoxification process (Asgher et al. 2008).

Marine fungi are adapted to much harsher conditions than terrestrial fungi. Their enzymes are able to withstand high salinity and concentration of phenolic compounds. Certain marine fungi have shown bioremediation potential for water-soluble crude oil fractions between 0.01 and 0.25 mg/mL (Deshmukh et al. 2016). Other extremophilic fungi such as *Pestalotiopsis palmarum* are able to survive in high concentration of extra-heavy crude oil and salt while producing oxidative exoenzymes (laccases and lignin peroxidases) which degrade the maltene and asphaltene fractions of oil for use as a carbon and energy source (Naranjo-Briceno et al. 2013).

The isolation of enzymes from marine organisms can select for traits such as heat and cold tolerance and salt and pressure tolerance (Lima and Porto 2016).

Recently, non-protein enzyme mimics or next-generation artificial enzymes in nanoparticles or nanocomposites have been used to replace enzymes in bioremediation processes (Gao and Yan 2016). Desired for their low cost and higher stability, these structures display enzyme-like properties under physiological conditions. Nanozymes, although lacking an active site, bind substrates specifically and catalyze their transformation. The Fe_3O_4-based magnetic nanoparticles mimic peroxidases and can degrade toxic dye compounds like methylene blue (Wu et al. 2015). Carbon-based nanomaterials made of graphene oxide (GO) also display peroxidase-like activity (Ma et al. 2017). Similarly, a guanosine monophosphate (GMP) coordinated copper nanocomposite mimicked laccases in being able to degrade phenolic compounds including hydroquinone and naphthol (Liang et al. 2017).

Although it is desirable to identify and isolate detoxification enzymes in polluted environments, a basic handicap is the ability to culture these microbial species. Only a handful of microbes are culturable, and these may not include the microbes that make the enzyme of interest. This problem is circumvented by recent developments in metagenomics which can identify potential detoxification enzyme-coding genes. These genes could be expressed in the heterologous system to mass-produce the enzymes of interest for bioremediation. Clues to the functionalities of these genes could be revealed by metatranscriptomic and metaproteomic approaches, which could also reveal biochemical pathways and synthetic pathways for xenobiotic-transforming enzyme production.

In silico approaches can be employed to understand the evolution of xenobiotic detoxification by phylogenetic analyses. More recently, bioinformatic analysis in the form of molecular docking tools has been developed to predict pollutant substrates of the detoxification enzymes. This approach has been particularly useful for laccases which have broad substrate specificity; in one study, laccase enzyme structures were screened against a database of toxic compounds to identify putative substrates (Suresh et al. 2008). The study found that 30% of the studied compounds that were recognized as environmental pollutants could be potentially metabolized by fungal laccases. Similar approaches could be employed for other enzymes with known structures to test if they could be used to detoxify pollutants of interest.

19.7 Conclusions and Future Perspectives

Increased global industrialization has presented many challenges including the production of ecotoxic industrial wastes that also present health threats as xenobiotic compounds. Fungi have specialized enzymes that are highly efficient in detoxifying these pollutants. Harnessing the power of these enzymes is proving to be an effective strategy for the bioremediation of the xenobiotic compounds. In particular, oxidoreductase enzymes including laccase, peroxidase, and oxygenases like CYP from various fungal species are employed to detoxify a wide range of pollutants.

In addition to bioremediation by detoxification, enzymes can also be employed for the detection and quantification of xenobiotics in the environment. White rot fungi (WRF) are a versatile group of microbes that are capable of oxidative detoxification of a variety of chemical pollutants and xenobiotic compounds that are environmentally harmful. These organisms and their enzymes have the ability to not only tolerate the xenobiotics but in many cases also metabolize or help sequester them. The limitation in biodegradation of xenobiotic compounds is frequently the initial stages of degradation. The oxidoreductase enzymes are all extracellular and specialized for less specific activity in initial stages of xenobiotic metabolism and, as such, as ideal candidates for detoxification of these compounds.

Development of heterologous expression systems and industrial scale expression is still a limitation. Limitations of enzyme quantity and stability are being overcome by the adoption of genetic engineering technology and enzyme immobilization approaches. Fungal enzymes can also be modified for enhanced catalytic activity in addition to thermostability to maximize the efficiency of the detoxification process. Creation of enzymes with increased redox potentials, especially in those such as laccases which have a lower redox potential, could widen the substrate range. The utility of laccases and CYPs may be limited by the availability of molecular oxygen. Development of peroxidases to target those substrates could be one possible approach. Present bioengineering tactics are focused on developing superior enzymes for the bioremediation of known pollutants, but such approaches may be extended to target novel pollutants, not known to be biodegradable. Alternatives to fungal enzymes in the form of nanozymes are also being explored.

An inherent limitation in the use of single enzymes in bioremediation systems is the fact that detoxification processes are often multi-step pathways, requiring the action of multiple enzymes in a sequence. The ability to use fungal enzymes in synthetic pathways for biotransformation of toxic pollutants into economically valuable compounds would be a great future direction. Since fungal-fungal and fungal-bacterial consortia have been employed for remediation of xenobiotics like PAHs, a similar strategy with enzymes could follow suit.

Structural studies of enzymes as well as directed evolution can facilitate changes in structure that could substantially enhance activity and xenobiotic metabolism. Better structural understanding of laccases as well as other enzymes could lead to minimization of the use of redox mediators, especially in in situ bioremediation scenarios in environmental settings, where the massive release of these eco-unfriendly compounds may be undesirable. The solving of crystal structures of fungal detoxification enzymes enables molecular docking analyses to predict the ability to metabolize various xenobiotic substrates. Similarly, the application of enzymes also requires a fine understanding of soil structure and soil-water interactions as these can significantly affect enzyme activity. Recent advances in this area are likely to benefit soil modeling studies and assessment of enzyme function for future bioremediation projects. Adopting a bioprospecting-like approach to find new strains of fungi with superior ligninolytic enzymes in environments like rainforests could be a promising way forward in continued efforts to combat environmental pollutants of industrial origin in cost-effective and environmentally sustainable tactics.

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