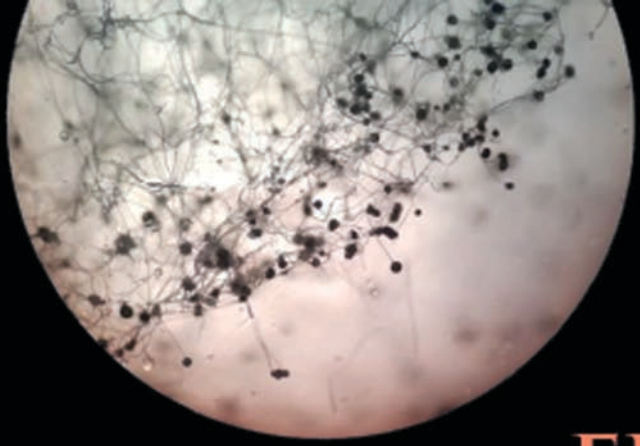
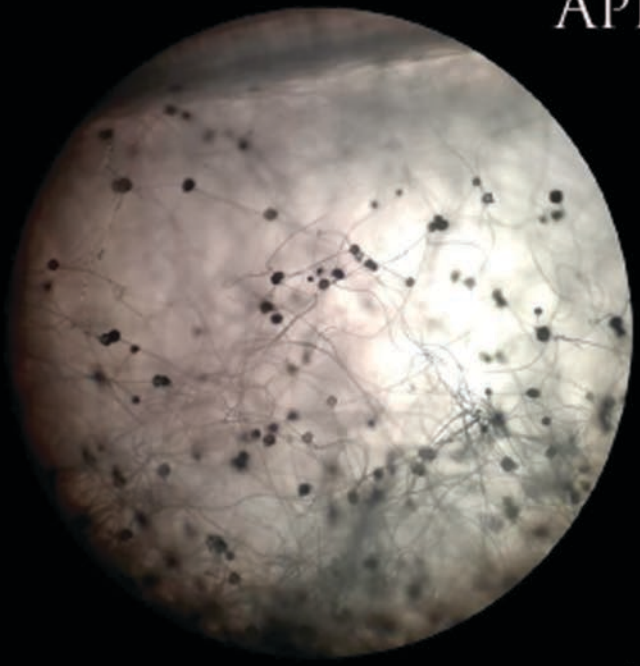


FUNGAL BIOREMEDIATION

Fundamentals and Applications



Editors
Araceli Tomasini
H. Hugo León-Santiesteban

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Fundamentals and Applications

Editors

Araceli Tomasini

Departamento de Biotecnología
Universidad Autónoma Metropolitana-Iztapalapa
Ciudad de Mexico
Mexico

H. Hugo León-Santiesteban

Departamento de Energía
Universidad Autónoma Metropolitana-Azcapotzalco
Ciudad de Mexico
Mexico

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Preface

The large number of people that inhabit our planet and all their activities have caused severe environmental pollution problems and disruption of the ecological balance. The air, ground, and water are polluted at different levels depending on the geographic zone. Some of these pollutants are compounds of natural origin or xenobiotics. Xenobiotics are human-made compounds for a specific use. Many researchers have dedicated themselves to studying and understanding the biodegradation of the pollutants, with the goal of proposing processes for their mitigation. These processes may be biological and physicochemical; and each of them has advantages and disadvantages. One of the main problems that prevent institutions and businesses from applying these processes is that the cost is very high without giving anything in return economically. Biological processes, also known as bioremediation, are less expensive to apply than the physicochemical, since they involve the use of living organisms, such as plants, bacteria, fungi, and/or algae.

We know that nature has her own ways of eliminating organic waste. One of the main actors involved in these processes are the filamentous fungi. However, the large number and diversity of compounds that are disposed of into the environment has overcome the natural capacity of these organisms.

In this book, studies in fungal bioremediation conducted by experts are mentioned. Studies of fungi bioremediation began since the second half of the last century. And there have been many advancements in different topics, such as the isolation of xenobiotic-degrading fungi, degradation mechanisms, enzyme involved in degradation, molecular studies, proteomic studies, microscopy studies, and application technologies.

This book shows the newest advancements in the field of fungal bioremediation, and is mainly addressed to grad students, as well as to professionals and researchers. The book has three parts: fundamentals, applications, and technologies and tools. Chapters were written by leading researchers who are doing work on fungi and bioremediation. In the first part, the characteristics of filamentous fungi and their role in the environmental remediation are briefly described. The main enzymes that participate in the processes of degradation of lignin and toxic compounds are also described. Cutting edge knowledge on fungal enzymes involved in the degradation of toxic compounds, their production, their activity, and their overexpression are written in this section.

Each chapter in the second part of the book deals with the degradation and/or removal of different toxic compounds. Examples of degradation of toxic xenobiotics are mentioned, which currently cause serious pollution problems, including emerging

pollutants. The degradation of lignin or hydrocarbons are not mentioned, because both topics are mentioned in all referenced books of fungal bioremediation.

The reader can get information about state-of-the-art advancements and knowledge in the field of remediation by fungi of chlorophenols, dyes, heavy metals, emerging pollutants, radioactive compounds, as well as pollutants present in the air. The chapters mention the most used fungi, the detoxification mechanisms of the compounds, and the most relevant research conducted till date.

In the third part of the book, tools and cutting-edge technologies used in the study of the removal and degradation of pollutants by fungi are presented. The first chapter of this part mentions how proteomics has helped to understand some of the degradation mechanisms done by fungi. The second one describes genomics as a very important tool in the understanding of the fungal metabolism, and technological advancements, such as the use of different bioreactors for the culture of fungi. The last chapter describes a tool which is not mentioned in other books about these topics, but which is just as important: microscopy. Topics ranging from the most basic techniques to the forefront of microscopy are discussed.

We want to thank all contributing authors that put in many hours to the creation of this book in an enthusiastic and professional manner. Thank you to all the authors that helped us achieve this project in the best way possible. Finally, we want to thank our families for their patience, support, and help, which were fundamental for the creation of this book.

Araceli Tomasini
H. Hugo León-Santiesteban
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CHAPTER-1

The Role of the Filamentous Fungi in Bioremediation

Araceli Tomasini[1,*] **and** *H. Hugo León-Santiesteban*[2]

1. Introduction

Environmental pollutants are a serious problem worldwide. They damage the ecosystems and all forms of life. Examples abound, such as climate change, diseases, and in some cases, mutagenic alterations that can lead to the death of organisms, just to name a few.

Pollution problems are increasing due to the rising human population and their anthropogenic activities. Compounds that cause pollution of the environment are increasing in quantity and diversity because of the lifestyle of this century that constantly demands new products and evolving technology. There are many toxic compounds used as pesticides, biocides, or as energy sources. Others are not toxic, but their accumulation in the environment is so high, that they can also cause pollution.

Given this problem, many studies have aimed at diminishing the toxic compounds present in the environment, either in the soil, water, or air. Physical, chemical, and biological processes have been proposed to remove the toxic compounds. All of them present advantages and disadvantages and their application depends on the type and concentration of pollutants and the site where they are present: soil, water, or air.

Biological processes involve organisms or biological activity to degrade or remove the pollutants; this is known as bioremediation. This chapter will describe the fundaments of bioremediation by fungi, deal with how they can remove toxic compounds, and provide some examples.

[1] Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, C.P. 09340, Ciudad de Mexico, Mexico.
[2] Departamento de Energía, Universidad Autónoma Metropolitana-Azcapotzalco, C.P. 02200, Ciudad de Mexico, Mexico.
* Corresponding author: atc@xanum.uam.mx

2. Bioremediation

Many definitions are given for bioremediation. One of the most accepted is: "bioremediation is a process to clean a polluted site using organisms as plants, algae, bacteria, and fungi" (Vidali 2001). Another definition used by many authors is the following: "bioremediation is a biological process to degrade or remove environmental contaminants from a polluted site, water, soil, and air, using organisms or biological activity as plants, algae, bacteria, fungi, and enzymes".

The principal advantage of bioremediation compared to physical and chemical processes is the cost. In general, it is lower than the physical and chemical processes, besides that it is a process that does not produce more contaminants. The disadvantages are that the bioremediation process takes more time than physical or chemical processes. The second one is that the site to be cleaned must have the right conditions for the growth and development of organisms that carry out the processes of removal or degradation of the contaminants.

Bioremediation includes two mechanisms to diminish the polluted compounds, through degradation involving the chemical modification of the molecules, and removal, which is a sorption process of the polluting molecules. Removal of toxic compounds is accomplished through a sorption process, the compounds are absorbed by a substrate; in this case, a biological substrate, biomass, or agriculture waste that can be used wet or dry. Biodegradation involves a chemical change of the molecule to produce other compounds, generally less toxic, and the live organisms or their enzymatic system are responsible for the compound's degradation.

Bioremediation involves different organisms. When plants are responsible for the remediation it is called phytoremediation. This process is one of the most economic ones, but it takes more time to be accomplished.

Bacteria, fungi, and algae can also be responsible for bioremediation; the efficiency depends on the type of compounds, the initial concentration of the toxic compounds, the site to be cleaned, and the microorganism to be used. In many cases, high efficiency is obtained using a microbial consortium containing a wide diversity of microorganisms, including different types of bacteria, fungi, and microalgae.

This chapter is focused on studying the role of filamentous fungi in bioremediation processes; the role of other organisms, such as plants, bacteria, and algae are not discussed, but it is important to know that bioremediation could also be using some of these organisms.

3. Filamentous Fungi

Fungi are eukaryotic and heterotrophic organisms that include unicellular and pluricellular fungi. The unicellular are yeasts, and the pluricellular are represented by all types of filamentous fungi, called as such due to their way of growth. Yeast are anaerobes and aerobes, and filamentous fungi are exclusively aerobes. This chapter will deal only with filamentous fungi.

They develop a tubular structure named hypha, the hyphae form the mycelium, fungi can reproduce by sexual or asexual spores, and by vegetative means from a mycelium segment. There are many types of filamentous fungi; they exist in terrestrial and aquatic habitats.

These fungi can grow as saprotrophs, which obtain their nutrients from dead organisms. They can grow also in symbiosis, which means a common life between two organisms including parasitism and mutualism. Parasitism is when only one of the organisms benefits and mutualism when both organisms are benefited; for example, mycorrhizae (mutualism with roots of plants) or lichens (mutualisms with algae) (Carlile et al. 2001).

They can grow in soil producing many hyphae that spread profusely all over the ground; despite fungi being microscopic, hyphae of *Armillaria ostoyae* can reach a spread rate of 1 m year^{-1} (Peet et al. 1996). *Armillaria bulbosa*, a filamentous fungus type, has been reported as one of the largest and oldest-living organisms (Smith et al. 1992).

Some fungi are pathogenic agents of plants and they are responsible for the loss of agricultural products, which are an important food source. However, many other filamentous fungi are used to produce secondary metabolites, such as antibiotics, immunosuppressors, anticholesterolemics, etc. (Barrios-Gonzalez et al. 2003). An important role to note is that fungi are the primary organisms responsible for degrading organic matter in nature.

Filamentous fungi grow in soil, and many of them degrade organic compounds present in dead organisms, both animal and vegetal. Organic matter is converted into small molecules that can be used by other organisms as a source of energy, carbon, and nitrogen. The ability of fungi to degrade organic compounds has been exceeded by the large number of organic compounds present in the soil due to the increase of human populations in our planet. This ability has also been affected by the presence of toxic compounds, such as xenobiotics. These last compounds are not present in nature, but they are produced by humans for specific uses, such as pesticides, biocides, or are the result of waste or byproducts from industrial processes.

Bioremediation by fungi is an alternative process to remove, degrade, or render harmless toxic compounds using natural biological activity. The fungi belonging to the basidiomycetes were the first assayed to degrade toxic compounds, as it was observed that this class of fungi grows on fragments of trees that are lying on the ground in forests, causing their rotting. Depending on the type of rotting that they cause on wood, fungi are called white-, brown-, or bland-rot fungi. These fungi have a common characteristic, the production of an enzymatic system able to degrade lignin. For this reason, they are also named ligninolytic fungi. *Phanerochatete chrysosporium* was one of the first white-rot fungi studied, showing its ability to degrade lignin, toxic compounds, and its relationship with the enzymatic system (Bumpus et al. 1985).

From the 1980s onwards, many fungi have been studied and found, which can be used in environmental bioremediation. It has been shown that ligninolytic fungi could degrade diverse types of toxic compounds, such as hydrocarbons, chlorophenols, polychlorinated biphenyls, etc., because of their enzymatic system.

Basidiomycetes, such as *Phanerochaete*, *Trametes*, *Pleurotus*, *Lentinus*, among others, can degrade toxic compounds (Table 1.1). Many of them have been isolated from natural sources, mainly from the soil or wastewater contaminated with toxic compounds (Singh 2006, Fackler et al. 2007, Harms et al. 2011, Seigle-Murandi et al. 1993, Lee et al. 2014, Mineki et al. 2015).

Table 1.1 Filamentous fungi used in bioremediation.

Class	Fungi	Use	References
Basidiomycete	*Phanerochaete chrysosporium*	Pentachlorophenol and anthracene degradation	Mileski et al. (1988), Mohammadi et al. (2010)
	P. chrysosporium	Dichlorophenol degradation	Huang et al. (2017)
	P. chrysosporium	Blue 4 dye sorption	Bayramoğlu et al. (2006)
	Phanerochaete velutina	Wood decomposing	Darrah and Fricker (2014)
	Phanerochaete sordida	Dioxin degradation	Sato et al. (2003)
	Bjerkandera adusta	Pentachlorophenol degradation	Rubilar et al. (2007)
	Trametes versicolor	Dye decolorization BTEX* oxidation	Libra et al. (2003), Aranda et al. (2010)
	Trametes modesta	Trinitrotoluene degradation	Nyanhongo et al. (2006)
	T. versicolor	Sulfonamide degradation	Rodríguez-Rodríguez et al. (2012)
	Pleurotus ostreatus	Olive cake degradation	Saavedra et al. (2006)
	Pleurotus pulmonarius	Aromatic hydrocarbons	D'Annibale et al. (2005)
	P. ostreatus	Ni, Cr(VI), Zn, Cd sorption	Javaid et al. (2011)
	P. ostreatus	Naphthalen sulphonic acid polymers degradation	Palli et al. (2016)
	B. adusta	Naphthalene sulphonic acid polymers degradation	Palli et al. (2016)
	Lentinula edodoes	Phenol degradation	Ranjini and Padmavathi (2012)
	Irpex lacteus	Soil bioremediation	Novotný et al. (2000)
	Panus tigrinus	Chlorophenol degradation	Leontievsky et al. (2002)
	Anthracophyllum discolor	Pentachlorophenol degradation	Rubilar et al. (2007), Cea et al. (2010)
	Geotrichum sp.	Azo dyes biotransformation	Máximo et al. (2003)
	Gloeophyllum striatum	Fluorophenol degradation	Kramer et al. (2004)
	Daldinia concentrica	Dibutylphthalate degradation	Lee et al. (2004)
	Agaricus augustus	Tribromophenol degradation	Donoso et al. (2008)
	Agaricus bisporus	PAH and Pb removal	García-Delgado et al. (2015)
	Funalia trogii	Dyes decolorization	Özsoy et al. (2005)
	Phlebia tremellosa	Lignin degradation	Fackler et al. (2007)
	Oxyporus latemarginatus	Lignin degradation	Fackler et al. (2007)

	Lentinus tigrinus	Phenol degradation	Kadimaliev et al. (2011)
	Eisenia fetida	Olive cake transformation	Saavedra et al. (2006)
	T. versiclor	Naproxen degradation	Marco-Urrea et al. (2010)
	Pycnoporus sanguineus	Metal sorption	Zulfadhly et al. (2001)
	P. sanguineus	Cu(II) sorption	Yahaya et al. (2009)
Hyphomycete aquatic	*Clavariopsis aquatica*	Nonylphenol degradation	Junghanns et al. (2005)
Ascomycete	*Aspergillus* spp.	Banana waste degradation	Shah et al. (2005)
	Asperigllus niger var tubingensis	Cr(VI) Sorption/reduction	Coreño-Alonso et al. (2009), (2014)
	Asperigllus niger	Pentachlorophenol sorption	Mathialagan and Viraraghavan (2009)
	Aspergillus awamori	Phenol, catechol, dichlorophenol, dimethoxyphenol degradation	Stoilova et al. (2006)
	Aspergillus fumigatus	Anthracene degradation	Ye et al. (2011)
	A. fumigatus	Cd, Cu, Ni, Pb and Zn accumulation	Dey et al. (2016)
	Aspergillus spp.	Cr and Cd bioaccumulation	Zafar et al. (2007)
	Aspergillus oruzae	Anthraquinone bisorption	Zhang et al. (2015)
	Phylosticta spp.	Banana waste degradation	Shah et al. (2005)
	Trichoderma virgatum	Pentachlorophenol degradation	Cserjesi and Johnson (1972)
	Gloeophylhum striatum	Dichlorophenol and pentachlorophenol degradation	Fahr et al. (1999)
	Trichoderma longibrachiatum	Pentachlorophenol degradation	Carvalho et al. (2009)
	Trichoderma spp.	Soil bioremediation	Harman et al. (2004), Ezzi and Lynch (2005)
	Trichoderma spp.	Cyanide degradation	Ezzi and Lynch (2005)
	Trichoderma harzianum	Soil bioremediation	Matsubara et al. (2006)

Table 1.1 contd. ...

...Table 1.1 contd.

Class	Fungi	Use	References
Ascomycete	*Fusarium* spp.	Cyanide degradation	Ezzi and Lynch (2005)
	Fusarium solani	DDT degradation	Mitra et al. (2001)
	Fusarium oxysporum	Phthalate degradation	Kim et al. (2003)
	Fusarium spp.	Zn sorption	Velmurugan et al. (2010)
	F. solani	Anthracene and benzl[a]anthracene degradation	Wu et al. (2010)
	Penicillium janczewskii	Pentachlorophenol degradation	Carvalho et al. (2009)
	Penicillium restictum	Black 5 dye sorption	Iscen et al. (2007)
	Cordyceps sinensis	Dioxane degradation	Nakamiya et al. (2005)
	Talaromyces helicus	Biphenyl degradation	Romero et al. (2005)
	Byssochlamys nivea	Pentachlorophenol degradation	Bosso et al. (2015)
	Scopulariopsis brumptii	Pentachlorophenol degradation	Bosso et al. (2015)
Zygomycete	*zygomycetes*	Vanillic acid transformation	Seigle-Murandi et al. (1992)
	Cunninghamella echinulata	Pentachlorophenol degradation	Seigle-Murandi et al. (1993)
	Rhizopus oryzae	Pentachlorophenol degradation	León-Santiesteban et al. (2014), (2016)
	Rhizopus spp.	Cr and Cd bio-accumulation	Zafar et al. (2007)
	Rhizopus nigricans	Cr(VI) sorption	Bai and Abraham (2003)
	Rhizopus sp.	Arsenic sorption	Jaiswal et al. (2018)
	Amylomyces rouxii	Pentachlorophenol degradation	Montiel et al. (2004), Marcial et al. (2006)
	Mucor rouxii	Pb, Ni, Cd and Zn sorption	Yan and Viraraghavan (2003)
	Mucor plumbeus	Pentachlorophenol degradation	Carvalho et al. (2011), (2013)
	Mucor ramosissimus	Pentachlorophenol degradation	Szewczyk et al. (2003), Szewczyk and Dlugoński (2009)
	Mucor hiemalis	Ni(II) sorption	Shroff and Vaidya (2011)
	Mucor hiemalis	Cr(VI)	Tewari et al. (2005)
	Mucor sp.	Cd and Pb sorption and accumulation	Deng et al. (2011)

*benzene, toluene, ethyl benzene, and xylene

4. Biosorption

Removal of toxic compounds through sorption by fungal biomass is one of the bioremediation processes. The compounds are not modified, they are only removed or eliminated from the polluted site and concentrated into a support, and this is called sorption. If the support is biological, the process is called biosorption.

Sorption is a physicochemical process where the molecules from a fluid phase, also called sorbate, are adhered to a surface of a solid, the sorbent. Adsorption processes are useful tools to remove chemicals from polluted sites, adsorption technologies can use inorganic and organic sorbents and they may be physical or chemical. In physical sorption, the involved forces are weak, such as van der Waals interactions, and electrostatic interactions formed by gradient-dipole or -quadruple interactions. Chemical sorption is due to the formation of a chemical bond between the sorbate and the sorbent. These interaction forces are stronger and more specific than the forces in physical sorption.

The main advantage of biosorption is that sorbents are cheap and biodegradable; they include agricultural wastes, such as wood chips, sugar cane bagasse, wheat shell, microbial biomass from bacteria, fungi, yeasts, and microalgae, etc. Fungal biomass has been studied to remove toxic compounds from polluted water. It was applied first to the removal of heavy metals, because fungal biomass is efficient to remove these ions. Biosorption occurs when the metal is fixed to the inactive or non-viable biomass, wet or dry.

Biosorption can occur in different parts of the cell, mainly in the cell wall by mechanisms, such as ionic exchange, chelation, or formation of a complex. Fungal biomass waste from the fermentation industries could be used as sorbent (Kuyucak and Volesky 1988, Volesky 1990). Biosorption of toxic compounds or heavy metals by fungal biomass is due mainly to the cell wall of fungi, which contains between 80 and 90% of polysaccharides, mainly chitin, glucan, mannan, galactosan, and chitosan, in some cases, cellulose. The rest, 10–20%, is constituted by proteins, lipids, polyphosphates, and inorganic ions that constitute the cement of the cell wall. The specific composition depends on the fungus and the identity and quantity of the different functional groups of the fungal biomass. Sorption ability of fungi is related to the functional groups present in the cell wall.

Metals can be removed also by living biomass involving metabolic processes; in this case the process is named bioaccumulation. Removal of heavy metals by bioaccumulation and biosorption by fungi has been studied for approximately 40 years. Filamentous fungi, such as *Aspergillus* sp., *Rhizopus* sp., *Mucor rouxii*, *Penicillium* sp., *Trametes* sp., among others, have been widely used to remove heavy metals, including As, Cr, Ni, Pb, Cd, Zn, Hg, Al, etc.

Mechanisms used by fungi to remove metals from the environment have been reported (Tsezos et al. 1997, Coreño-Alonso et al. 2014, Awasthi et al. 2017). Biosorption of metal ions is due to the physicochemical interactions between the functional groups of the fungal cell wall and the metal ions by mechanisms, such as physical sorption, ion exchange, and chemical sorption, such as chelation, complexation, and coordination. This sorption can be reversible, which means the metal can be desorbed from the biomass. Metal bioaccumulation by fungi involves

cell metabolism; the metal must pass through the cell membrane into the cytoplasm and this action has been related to different defense mechanisms of fungi against toxicity caused by metals. In some cases, it can be metal precipitation or extracellular bioaccumulation, involving both mentioned mechanisms (Sağ 2001, Dhankhar and Hooda 2011). The characteristics of fungal biomass have allowed adsorbing many types of toxic compounds besides metals.

The fungal biosorption approach is an alternative technique to remove different types of pollutants, such as phenolic compounds and dyes (Crini 2006, Kumar and Min 2011). The type of biomass and their previous treatment, type of pollutant, initial concentration of the compound, biomass concentration, pH, and temperature influence the process.

Fungal biomass is washed with water and it can be used either wet or dry. In some cases, the biomass is pre-treated to modify the functional groups of the fungal biomass, it can also be used immobilized, in both cases, the modification is made to increase the sorption ability. pH plays an important role because the solubility and ionization of toxic compounds depend largely on the pH of the phase where the toxicants are in the environment, mainly if the pollutants are in aqueous phase (Aksu 2005, León-Santiestebán et al. 2011, Kumar and Min 2011).

Fungal biosorption has been studied for many years; however, its application is limited mainly because more basic knowledge is needed on aspects such as physicochemical conditions, effect of parameters, and because the technology has not been fully developed yet.

5. Biodegradation

The degradation process is the chemical modification of the toxic compounds into less toxic or innocuous molecules. The mineralization process refers to the complete degradation of the toxic compound until CO_2 and water are obtained. However, mineralization is not always achieved, the most common is a partial degradation, in this case, some metabolites derived from the toxic compounds are produced. Generally, these metabolites are less toxic or completely innocuous; however, in a few cases the metabolites produced could be even more toxic than the original.

Fungi can degrade a vast variety of toxic compounds due to their enzymatic system. White rot-fungi degrade lignin, the most abundant resistant biopolymer in nature, have shown the ability to degrade toxic and xenobiotic compounds, using the same mechanisms that they use to degrade lignin. *Phanerochaete chrysosporiun* is one of the first and most studied fungus to degrade toxic compounds, its study began in the 1970s and, since then, many studies on the ability to degrade toxicants with this fungus have been reported (Mileski et al. 1988, Bogan and Lamar 1995, Laugero et al. 1997, Yadav et al. 1995). Table 1.1 shows some of the class and type of fungi used in bioremediation. These fungi have the characteristic to produce oxidase enzymes, which are involved in the degradation of the recalcitrant and xenobiotic toxicants, such as polycyclic aromatic compounds (PAHs), chlorophenols, polychlorinated biphenyls, synthetic polymers, dyes, pesticides, benzene, toluene, ethyl benzene, and xylene (BETX), chlorinated monoaromatics, halogenated organic compounds, and others. These compounds can be degraded by different fungi, mainly basidiomycetes,

such as the white-rot fungi species that include *Phanerochaete*, *Bjerkandera*, *Trametes*, *Pleurotus*, *Phlebia*, and *Ceriporipsis* (Cameron et al. 2000), and brown-rot fungi, for example *Lentinus*, *Lentinulla*, and *Gloeophyllum.* Fungi different from basidiomycetes have also been studied regarding their ability to degrade toxic compounds.

Degradation of polycyclic aromatic hydrocarbons (PAHs) by fungi has been reported by many authors. Most studies showed that fungi degrade this group of toxicants by co-metabolism, which means fungi cannot use the PHAs as the sole source of carbon, as accomplished by bacteria (Ghosal et al. 2016). Ligninolytic and non-ligninolytic fungi can degrade PHAs, however ligninolytic fungi have been studied more and present some advantages.

The metabolic pathway of degradation of PHAs by non-ligninolytic fungus is different from the degradation pathway used by ligninolytic fungi. Degradation of PHAs by non-ligninolytic fungi, involving cytochrome P450, can produce dihydrodiols and epoxides that are more toxic than the original PHAs. Ligninolytic fungi degrade PHAs by peroxidases and phenoloxidases producing quinones, i.e., less toxic molecules (Hammel 1995, Tortella et al. 2005, Cerniglia and Sutherland 2010, Treu and Falandysz 2017). Co-metabolic anthracene and pyrene degradation by *A. fumigatus* and *Pseudotrametes giboosas*, respectively, was reported by Ye et al. (2011) and Wen et al. (2011), the last authors indicated that the degradation rate is high in co-metabolic conditions.

Romero et al. (2005) reported biotransformation of biphenyls by an ascomycete of the genus *Talaromyces*, finding that its oxidation produces dehydroxylate derivatives and their glucosyl conjugates. Pentachlorophenol degradation by zygomycetes, such as *Mucor*, *Amylomyces*, and *Rhizopus* has been reported by Carvalho et al. (2011), Montiel et al. (2004), and León-Santiesteban et al. (2014). Table 1.1 depicts other examples of toxicants' degradation by different filamentous fungi.

There are diverse mechanisms of toxic compound degradation by fungi, for example, dehalogenation, which could be through oxidative or reductive reactions, methylation, hydroxylation, conjugation, breaking of the aromatic rings, and polymerization are the most common reactions. The mechanism to degrade a toxicant by fungi depends on the fungi, the characteristic of toxic compounds, the conditions of the reaction, the composition of the culture media. For example, if fungi are grown in nitrogen-deficient cultures, or with glucose or another carbon source, the mechanism to degrade a specific toxicant would be different. León-Santiesteban et al. (2014) reported that in PCP degradation by *R. oryzae* ENHE, nine metabolites were found when the fungus grew with glucose-ammonium sulfate as source of carbon and nitrogen. When the fungus grew in glutamic acid-sodium nitrate as source of carbon and nitrogen, only three metabolites from PCP degradation were found.

Szewczyl et al. (2018) propose a biodegradation pathway of the herbicide ametryn, a herbicide from the triazines group, by *Metarhizium brunneum*. This fungus is an entomopathogen fungus tested to infect insects. These authors showed the ability to degrade ametryn and reported the metabolites produced from the degradation process.

As mentioned before, a great diversity of toxicants has been degraded by fungi, and in some cases, the mechanisms of degradation have been elucidated. We mention

only some examples, there is much information about the degradation by fungi of toxic compounds including pesticides, PHAs, halogenated compounds, and emergent pollutants, such as pharmaceutical and personal care products (Table 1.1). Authors have focused the study on toxic degradation by fungi using different tools, such as fungi isolation, biochemistry, molecular biology, and analytical chemistry in order to understand and improve fungal bioremediation. Regardless to the focus of the study, the goal is to propose efficient processes of toxicants' degradation by fungi.

Currently, the enzymatic, redox reactions, and degradation pathways are known, as well as the metabolites produced from some toxic compounds degradation, such as PHAs, chlorophenols, azo dyes, by fungi. However, the degradation pathways by fungi of many recalcitrant compounds, such as emergent pollutants, are not yet known, just as the role of enzymes involved in degradation is not understood in many cases.

Molecular biology studies of fungi have been helping to understand and increase the ability of these microorganisms to degrade toxicants (Bogan et al. 1996, Huang et al. 2018, Szewczyk et al. 2018). Many studies about genetic improvement of fungal strains for degradation of toxic compounds have been reported (Mitra et al. 2001, Gao et al. 2010, Tripathi et al. 2013). For instance, Syed et al. (2010) reported the identification and functional characterization of P450 monooxygenase involved in the oxidation of PAHs by *P. chrysosporium* using a genome-to-function strategy. Molecular technologies have allowed better understanding of the role of *Ascomycota* fungi in the transformation of toxicants (Aranda 2016).

In addition, some other applications have been proposed to remove toxic compounds from polluted sites. Bioaugmentation or biostimulation is referred to increase the presence of fungi responsible for or involved in degradation processes of some compounds *in situ*. The above mentioned can be made first by isolating fungi from the site, then culturing them in the laboratory and, finally, inoculating the polluted soil with the cultured fungi. Lladó et al. (2013) reported the use of two fungi, *Trametes versocilor* and *Lentinus tigrinus*, for PAH removal from contaminated soil. The authors reported that PAH fractions remain in the soil contaminated by creosote after 180 days and that the biostimulation of the soil increases degradation rates. Degradation of anthracene by bioaugmentation of *T. versicolor* in a composting process was reported by Sayara et al. (2011).

While the use and the ability of fungi to degrade toxic compounds have been studied extensively, more studies are still needed for the scaling-up of the process. *In situ* bioremediation is still the objective to achieve.

6. Enzymes

The chemical modification of the toxicant is carried out by enzymatic reactions, involving oxidoreductase enzymes. This class of enzymes catalyzes redox reactions with transfer of hydrogen and oxygen atoms or electrons among molecules. Oxidoreductases include dehydrogenases, oxidases, peroxidases, and oxygenases. Table 1.2 shows the mechanisms of actions of these enzymes.

Peroxidases and phenoloxidases are the main enzymes produced by fungi. Peroxidases are hemoproteins and the hydrogen peroxide is necessary as an electron

acceptor. Phenoloxidases are enzymes containing copper that catalyze the oxidation of phenolic compounds without cofactor, using molecular oxygen for the reaction.

Table 1.2 Mechanisms of action of oxidoreductase enzymes.

Enzyme	**Mechanism of action**
Oxygenases	Transfer oxygen atoms from diatomic oxygen
Oxidases	Transfer electrons to diatomic oxygen
Peroxidases	Transfer electrons to hydrogen peroxide
Dehydrogenases	Transfer hydride ions

Some of these enzymes were found in the culture broth of *P. chrysosporium*, and it was shown that their activity is dependent on H_2O_2 (Forney et al. 1982, Paszczyński et al. 1985, Hatakka 1994), and they were named lignin peroxidases by Tien and Kirk (1988). It was shown that the enzymatic system produced by *P. chysosporium* and other basidiomycetes can degrade lignin; these enzymes are called ligninolytic. Lignin peroxidase and manganese peroxidase use hydrogen peroxide produced by glyoxal oxidase or aryl alcohol oxidase produced by the same fungi. Laccases use the four copper atoms to catalyze the electron reduction of molecular oxygen to water (Kudanga et al. 2011, Sinsabaugh 2010). The main feature of these enzymes is that they are non-specific; thus, they can degrade diverse substrates, including toxic compounds. Many authors have studied fungal ligninolytic enzymes and their activity and ability to degrade recalcitrant compounds, such as BTX, polyaromatic hydrocarbons (PHA), polychlorinated biphenyls, etc.

The most studied fungal enzymes to degrade toxic compounds are lignin and manganese peroxidases, as well as the phenoloxidases, laccase, and tyrosinase. These enzymes are mainly produced and excreted by basidiomycetes, such as *P. chrysosoporium*, *Trametes*, *Pleurotus*, *Pycnoporus*, etc. Other fungi, such as *Ascomycetes* that include *Aspergillus*, *Penicillium*, *Trichoderma*, and *Zygomycetes* such as *Mucor*, *Rhizopus*, as well as *Amylomyces*, also produce peroxidases and phenoloxidases (Durán and Esposito 2000, Montiel et al. 2004). Other enzymes, such as versatile peroxidases, reductive dehalogenases, cytocrome P450, monooxygenases, and peroxygenases, produced by fungi are involved in the degradation process of toxicants (Hofrichter et al. 2010, Subramanian and Yadav 2009, Reddy and Gold 1999, McErlean et al. 2006, Syed et al. 2010). *P. chrysosporium*, *P. ostreatus*, and *B. adusta* showed ability to degrade PHAs and the enzymes produced by these fungi involved in the degradation pathways have been reported (Kadri et al. 2017).

Molecular studies aimed at increasing the enzymatic activity produced by fungi have also been published. There are works reporting the regulation and expression of peroxidases and phenoloxidases, heterologous expression, transcriptome and proteomic studies of fungi producing these enzymes (Kellner and Vandenbol 2010, Piscitelli et al. 2010, Manavalan et al. 2011, Huang et al. 2011, Huang et al. 2017, Carvalho et al. 2013, Janusz et al. 2013, Montiel-González et al. 2009).

Immobilization of enzymes or of the enzyme producing microorganism used in biodegradation processes has been proposed with the advantage that it could be used several times and the degradation process would be environmentally friendly (León-

Santiestebán et al. 2011, Voběrková et al. 2018). Immobilization of enzyme could be accomplished on inert supports such as nylon fibers or on organic ones such as sugar cane bagasse (León-Santiestebán et al. 2011, Mohammadi et al. 2010). Yang et al. (2017) reported the degradation of antibiotics by immobilized laccase at pH 7.0 in 48 hours, removing approximately 60% of tetracycline and oxytetracycline.

Enzymes play a key role in the degradation processes and have been widely studied; the next two chapters deal with these fungal enzymes.

Conclusions

The role of fungi as natural degraders of organic material is very important in the environment, although their capacity has been exceeded by the amount and the diversity of compounds. The interest of using fungi to degrade toxicants has increased, due to the current pollution problem and to the low cost that their use may imply.

To understand how fungi can degrade toxicants and increase their ability, physiological, biochemical, and molecular studies, including genomics, transcriptomics, and proteomics, have been made. All this effort has generated a very broad knowledge of fungi, revealing their potential in bioremediation processes to eliminate hazardous compounds.

However, for a practical application, bioremediation *in situ*, more studies are needed to know the technology that could be used. Bioaugmentation is not an efficient technology, so far, because of the competition among native microorganisms. Another drawback is the control of the environmental conditions required for fungi growth. The addition of enzymes to the site is complicated, since the enzymatic activity depends on parameters, such as pH, humidity, and temperature, which are not possible to control *in situ*. There are some studies reporting the scaling-up from laboratory to field applications; however, it is necessary to study this subject more to propose potential soil bioremediation processes using fungi.

Studies and financial investment should be directed to implement technologies applied *in situ*, which will help and increase the ability of fungi to degrade hazardous compounds, for the bioremediation of polluted environments. It should be emphasized that although bioremediation is a very slow process compared to chemical processes, it is not expensive and is friendly for the environment.

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CHAPTER-2

Fungal Peroxidases Mediated Bioremediation of Industrial Pollutants

*Misha Ali, Qayyum Husain** **and** *Hassan Mubarak Ishqi*

1. Introduction

The use of chemicals in many aspects of our daily lives, industrial processes, and agricultural practices results in the deliberate or accidental spillage of potentially toxic chemicals into the environment. A major portion of the pollutants is constituted by aromatic compounds released by various industrial processes including petroleum refining, dye manufacturing, textile, leather, plastic and resin synthesis, coking and coal conversion, chemical and pharmaceutical industries, foundries, and pulp and paper plants (Husain and Ulber 2011). There are several classes of aromatic compounds that have been selected as priority pollutants by the United States Environmental Protection Agency due to their negative impact on the environment and aquatic and human life. Some of these classes include polycyclic aromatic hydrocarbons (PAHs), pentachlorophenol (PCP), polychlorinated biphenyls (PCBs), phenolic compounds, dyes, pesticides, explosives, and pharmaceuticals, etc. These aromatic pollutants are persistent/recalcitrant in the environment, and some of them are known carcinogenic and/or mutagenic agents (Husain et al. 2009, Yu et al. 2016). Therefore, the removal of such compounds from the environment is necessary. Many conventional methods which have been employed for the treatment of environmental pollutants include chemical, physical and biological methods, or a combination of these methods (Silva et al. 2013).

Conventional chemical and physical procedures which have been employed for the removal of aromatic pollutants include electrochemical oxidation, membrane

Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh-202002, India.
* Corresponding author: qayyumbiochem@gmail.com

filtration, coagulation, sorption, ion exchange, flocculation, electrolysis, adsorption, advanced oxidation processes, bleaching, chlorination, ozonation, Fenton oxidation, chemical reduction, and photocatalytic oxidation (Hao et al. 2000, Husain 2006). Although these methods are effective in the clearance of toxic compounds from the environment, most of them suffer from some drawbacks, such as partial removal, applicability to a limited concentration range, high cost, and the formation of toxic by-products (Ashraf and Husain 2010). These methods of remediation and degradation of organic compounds are actually outdated due to some unresolved problems. Recently, the biological techniques based on microbial degradation of organopollutants have attracted much attention of environmentalists for the treatment of wastewater. Several researchers have shown partial or complete biodegradation of such chemicals by pure and mixed cultures of bacteria, fungi, and algae (Husain et al. 2009). The treatment of aromatic pollutants using microbiological tools has some of its own demerits, such as limited mobility and survival of cells in the soil, high cost of microbial culture production, alternative carbon sources, completeness of the indigenous populations, and metabolic inhibition (Steevensz et al. 2014). Numerous kinds of organisms have been used for the complete degradation of toxic compounds, but much success has not yet been achieved. Aromatic compounds are degraded by secondary metabolic pathways of bacteria, fungi, and algae, which require appropriate growth conditions aided by additional loads of chemicals (Robinson et al. 2001, Rabinovich et al. 2004). Moreover, the degradation of aromatic compounds is not constant with time, as the expression of enzymes involved in degradation depends on the growth phase of the organisms which is in turn influenced by inhibitors present in wastewater (Wesenberg et al. 2003, Kurnik et al. 2015).

In an attempt to overcome the problems associated with the traditional biological and chemical waste treatment procedures, recent researches have been focused on the applications of enzymes in such fields (Husain and Jan 2000, Rao et al. 2014). Due to a variety of chemical transformations catalyzed by enzymes, the enzyme based technology brought the focus of biotechnologists towards this novel approach. Cheap and more readily available enzymes are produced through better isolation and purification procedures due to recent advances in enzyme based technology. These can be applied in many remediation processes to target specific recalcitrant pollutants present in wastewaters (Xu and Salmon 2008). Enzymes isolated from microorganisms have been often preferred over intact organisms due to easy handling and storage, greater specificity, higher stability, and better optimization (Satar et al. 2012, Husain and Husain 2012). Moreover, unlike chemical catalysts, the enzymatic systems have the potential of carrying out complex chemical transformation under mild environmental conditions with high reaction velocity and efficiency (Husain and Husain 2007, Michniewicz et al. 2008). Due to high specificity for individual compounds, enzymatic processes have been developed specifically to target selected aromatic pollutants, which cannot be treated effectively using traditional methods (Alarcón-Payán et al. 2017).

The enzymatic treatment has an edge over the conventional methods of pollutants treatment because this technique can be applied—at very low to high concentrations of contaminants over the broad range of pH, temperature, and ionic strength, to persistent and recalcitrant materials, and in the absence of shock loading

effects. It reduces sludge volume and delays associated with the acclimatization of biomass (Husain and Husain 2012, Goncalves et al. 2015). Among various enzymes being actively pursued for environmental purposes, oxidoreductases have attracted more attention. These are a large group of enzymes catalyzing oxidation/reduction reactions. Oxidoreductases catalyze the electron transfer from one substrate to another, where the oxidized substrate is referred to as electron donor in contrast to the reduced substrate which is electron acceptor (Husain 2017a,b). The potential application of oxidoreductases from bacteria, fungi, and plants is increasingly being demonstrated, and among these enzymes are peroxidases, laccases, and polyphenol oxidases (Durán et al. 2002).

Peroxidases are suitable catalysts for the bioremediation, as they exhibit broad substrate specificity and high redox potential (up to 1.3 V). The bioremediation mediated by these enzymes relies on the oxidation of toxic aromatic compounds into less toxic derivatives and also removes them from the polluted water, e.g., phenols are being oxidized and polymerized by such enzymes into insoluble products which can be easily removed from the wastewater (Ayala and Torres 2016). Peroxidases [donor: H_2O_2 oxidoreductases] are predominantly heme proteins which utilize H_2O_2 or organic hydroperoxides as co-substrate to oxidize a variety of organic and inorganic substrates (Fatima and Husain 2008). They are found in all five kingdoms of life. The "Non-animal heme-peroxidases" include intracellular peroxidases of bacterial and eukaryotic origin (Class I, e.g., yeast cytochrome c peroxidase), secreted fungal peroxidases (Class II, e.g., lignin, manganese, and versatile peroxidases), and classic secretory plant peroxidases (Class III, e.g., horseradish, turnip, and bitter gourd peroxidases) (Battistuzzi et al. 2010, Hofrichter et al. 2010).

2. Fungal Peroxidases

2.1 Ligninolytic peroxidases

Lignocellulose is the most abundant recalcitrant renewable material available in nature. Lignin is an amorphous, complex and three-dimensional aromatic polymer. These polymers have compact structures that are insoluble in water, as well as difficult to penetrate by microbes or enzymes (Sánchez 2009). The rigidity of lignin can be accounted to the intermonomeric linkages that comprise of many kinds of C–C and C–O bonds, with the β-aryl ether linkage being the most significant and these linkages are not hydrolysable. A conclusion from the above items is summarized as follows: (i) polymeric lignin degradation requires extracellular enzymes and/or small molecular weight mediators or factors, such as radicals, (ii) the lignin degrading system must be unspecific, and (iii) the enzymes must be oxidative, not hydrolytic (Hatakka and Hammel 2011, Satar et al. 2012). Fungi are the only organisms known that are able to completely mineralize lignocellulose by using their ligninolytic enzymes system. One of the major components of the ligninolytic system is the peroxidase enzyme family, which is one of the major components of the ligninolytic system and is comprised of several members. Peroxidases are oxidoreductases that utilize H_2O_2 to catalyze oxidative reactions of diverse substrates. The enzymatically generated H_2O_2 oxidizes lignin polymer in a reaction catalyzed by ligninolytic peroxidases, and this

process is described as a key reaction in the process of enzymatic combustion (Ruiz-Dueñas and Martínez 2009).

The main lignin modifying enzymes which play a central role in lignin modification are lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP). These peroxidases oxidize specific components of the lignin structure and may act in synergy if they are produced by the same organism. While VP has the capability of oxidizing both phenolic and non-phenolic structures, MnP oxidizes only phenolic structures of lignin, and LiP targets the non-phenolic components (Falade et al. 2017). These extracellular lignin degrading enzymes belong to Class II heme-peroxidases. Ligninolytic enzymes have very high reducing specificity for the substrates, because ligninolytic fungi grow on a variety of lignocellulosic substrates, and produce ligninases, which have the ability to mineralize distinct structures of lignin (Knop et al. 2015). The low specificity of many fungal enzymes enables the organisms producing them to co-metabolize structurally distinct compounds belonging to different pollutant classes, e.g., benzene, toluene, ethylbenzene and xylenes (BTEX) compounds, organochlorines (chloroaliphatics, chlorolignols, chlorophenols, polychlorinated biphenyls, and polychlorinated dibenzo-p-dioxins (PCDD)), 2, 4, 6-trinitrotoluene (TNT), PAHs, pesticides, synthetic dyes, and synthetic polymers (Harms et al. 2011). Interestingly, the structurally diverging representatives of a particular pollutant class (such as various low- and high-molecular-mass PAHs and different PCDD congeners) can be degraded by the same fungal organism, even in the mixture (Nakamiya et al. 2005, Cerniglia and Sutherland 2010).

2.2 Non-ligninolytic peroxidases

Other fungal peroxidases which belong to Class II heme-peroxidases are non-ligninolytic *Arthromyces ramosus* peroxidase (ARP) and *Coprinus cinereus* peroxidase (CIP). Because of the catalytic similarity of these enzymes to horse radish peroxidase (HRP), their application in the removal of phenolic compounds from wastewater has also drawn great attention. It was exhibited that various phenolic compounds could be removed from their aqueous solutions using ARP or CIP (Ikehata et al. 2004).

The other peroxidases that are less studied, but have shown their potential in the degradation of organopollutants, are heme-thiolate peroxidases (HTPs) and dye decolorization peroxidases (DyPs). They have considerably divergent protein sequences and unique catalytic properties, which does not allow their classification into the class II peroxidase family, but justifies their affiliation to separate (super) families of heme peroxidases (Sugano 2009, Lundell et al. 2010). Two main enzymes from the HTPs family are *Caldariomyces fumago* chloroperoxidase (CfuCPO) and aromatic peroxygenases (APOs). It is evident that the main activity of CPO is the oxidation of chloride (Cl^-) into hypochlorous acid at pH < 5, which in turn acts as a strong oxidant and can chlorinate organic compounds. However, it is still unclear to which end, this reaction is catalyzed. Initially, it had been thought that CfuCPO was involved in the synthesis of chlorinated metabolites, such as caldariomycin (Hofrichter et al. 2010), but later this assumption was questioned (van Pée 2001).

Recently, CfuCPO and also non-heme chloroperoxidases are rather believed to be involved in antimicrobial antagonism, since the hypochlorite formed has strong biocidal activities (Bengtson et al. 2009). The natural functions of APOs are still not known. Due to their versatility, they could be involved in the non-specific oxidation and detoxification of microbial metabolites or plant ingredients (e.g., methoxylated phytoalexins) and also in the conversion of lignin-derived compounds, *o*-demethylation of mono- and oligomeric lignin fragments (Hofrichter et al. 2010).

HTPs catalyze a wide range of reactions, including oxidations of various aliphatic and aromatic compounds (Ullrich and Hofrichter 2005, Gutiérrez et al. 2011). DyP-type peroxidases are catalytically highly stable and versatile (Pühse et al. 2009). The physiological role of DyPs is also still not clear, but it has been suggested to be a part of the catalytic toxic aromatic products, or as a defense mechanism against oxidative stress. They possess a high redox potential (1.1–1.2 V) so that numerous substrates can serve as electron donors (Liers et al. 2013, Liers et al. 2014). DyP-type peroxidases are therefore able to oxidize a wide spectrum of complex dyes, in particular, xenobiotic anthraquinone derivatives, typical peroxidase substrates, such as ABTS (2,2′-azino-bis(3-ethylthiazoline-6-sulfonate)) and phenols (Sugano 2009). Figure 2.1 illustrates all the fungal peroxidases involved in bioremediation of different kinds of pollutants.

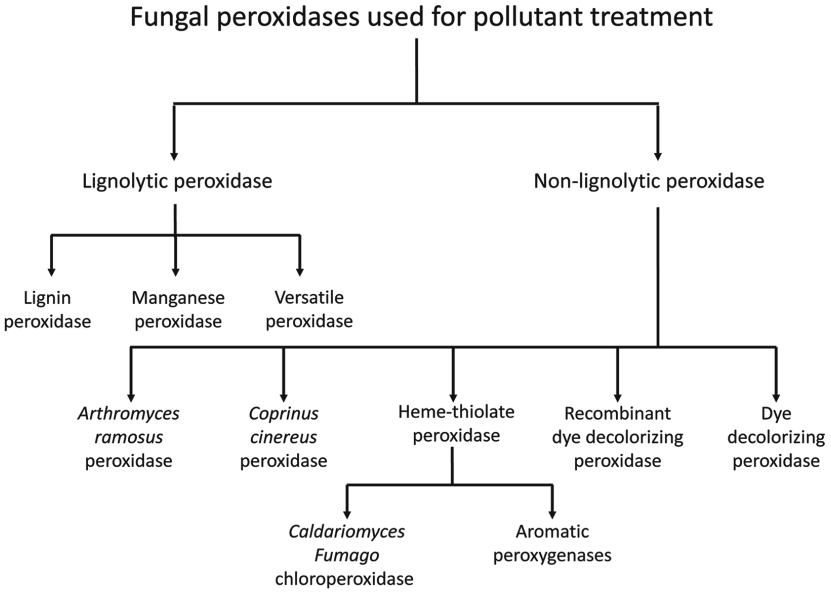

Figure 2.1 Demonstrates fungal peroxidases employed for bioremediation.

3. Main Fungi Producing Peroxidases

The organisms predominantly responsible for lignocellulose degradation are fungi, which are classified by the type of degradation. They are, namely, white-rot fungi (WRF), brown-rot fungi (BRF), and soft-rot fungi (SRF). WRF break down lignin in wood, leaving lighter colored (white) cellulose behind. Some of these fungi break down both lignin and cellulose by producing powerful extracellular oxidative and hydrolytic enzymes (Manavalan et al. 2015). WRF is a heterogeneous group of fungi that usually belong to basidiomycetes, although there are ascomycetous fungi that cause pseudo-white-rot, also designated as soft-rot type II, such as fungi belong to the family Xylariaceae (Liers et al. 2006). Basidiomycetous WRF and some related litter-decomposing fungi are the only organisms known which are capable of efficiently mineralizing lignin. BRF also belong to basidiomycetes that degrade wood in a manner which yields brown, shrunken specimens that typically exhibit a pattern of cubical cracks and easily disintegrate upon handling. They degrade wood polysaccharides, cellulose, and hemicellulose, by producing hydrolytic enzymes cellulases and hemicellulases, while they catalyze partial modification of lignin. SRF from ascomycetes secretes cellulase that breaks down cellulose from their hyphae, leading to the formation of microscopic cavities inside the wood and sometimes to a discoloration and cracking pattern resembling that of BRF (Manavalan et al. 2015). Xylariaceous ascomycetes from genera, such as *Daldinia*, *Hypoxylon*, and *Xylaria* are grouped with the SRFs, they cause a typical erosive soft rot. Compared to basidiomycetous fungi, the knowledge about lignocellulose degradation by ascomycetes is limited, and very little is known about its mechanism of lignin degradation (Hatakka and Hammel 2011). Hence, it can be concluded that the main fungi responsible for lignin degradation is WRF.

LiP, MnP, VP, and laccase are the major lignin degrading enzymes from WRF. LiP and MnP were discovered in the mid-1980s and were purified from extracellular culture medium of a basidiomycete *Phanerochaete chrysosporium*. *P. chrysosporium* is certainly the most studied fungus for ligninolytic peroxidase production. Soon after the discovery of *P. chrysosporium* LiP and MnP, the production of these enzymes by many different WRF were reported, such as *Trametes* (*Coriolus* or *Polyporus*) *versicolor*, *Chrysonila sitophila*, and *Phlebia radiata* among many others. Besides *P. chrysosporium*, LiP activity was found in the culture filtrates of *Chrysosporium pruinosum* and *Coriolus* (*Trametes*) *versicolor* (Lundell et al. 2010, Liu et al. 2012, Pinto et al. 2012).

Pleurotus species is another class of extensively studied WRF and these fungi have also been exploited for the production of ligninolytic peroxidases. The ligninolytic peroxidases produced by one of *Pleurotus* spp., *P. eryngii* had remarkably high catalytic activities as compared to the conventionally produced LiP and MnP by *P. chrysosporium* (Gómez-Toribio et al. 2001). Papinutti et al. (2003) discussed the production of MnP and laccase by a WRF *Fomes sclerodermeus BAFC 2752*. Some other distinguished fungi discovered to produce either LiP or MnP are marine

ascomycetes *Sordaria fimicola* and *Halosarpheia ratnagiriensis*; MnP and laccase, a marine basidiomycete *Flavodon flavus*; LiP, MnP, and laccase (Raghukumar 2000), litter-decomposing basidiomycetes *Agrocybe praecox*, *Collybia dryophila*, and *Stropharia coronilla*, MnP (Steffen et al. 2002), and yeasts *Candida tropicalis* and *Debaryomyces polymorphus*, MnP (Yang et al. 2003).

DyP was isolated for the first time from WRF, *Bjerkandera adusta* (Kim et al. 1995). It was also reported that DyP was produced by jelly fungus *Auricularia auricula-judae*, *Exidia glandulosa*, *and Mycena epipterygia* (Liers et al. 2010, Liers et al. 2013, Linde et al. 2014). DyPs were also reported from *Irpex lacteus*, a white rot basidiomycete and WRF *Pleurotus sapidus* (Salvachúa et al. 2013, Lauber et al. 2017). One of the HTP, i.e., chloroperoxidase, was isolated from the ascomycete *Leptoxyphium fumago*. A second HTP described as an aromatic peroxygenase (APO) has been produced from agaric basidiomycetes, *Agrocybe aegerita* (Ullrich et al. 2004, Gutiérrez et al. 2011). APO of somewhat different catalytic properties has been isolated from ink cap mushroom *Coprinus radians* (Anh et al. 2007). Gröbe et al. (2011) described isolation of APO from agaric fungus *Marasmius rotula*. ARP and CIP were mainly produced by the imperfect fungus (deuteromycete) *Arthromyces ramosus* obtained from soil, and from an inky cap basidiomycete *Coprinus cinereus*, respectively.

4. Characteristics of Fungal Peroxidases

4.1 Lignin peroxidase (LiP)

LiP is a heme-containing glycoprotein oxidoreductase which was first found and reported 25 years back in white-rot basidiomycete *P. chrysosporium* as lignin degrading fungi. However, many other fungi have also been known to produce LiP (Miki et al. 2010). Most of the LiPs used these days in different bioremediation processes and lignin degradation has been produced by fungus *P. chrysosporium* where this enzyme is secreted during secondary metabolism as a result of nitrogen limitation (Martínez et al. 2009, Hofrichter et al. 2010). The catalytic cycle involves initial oxidation of the enzyme by H_2O_2 to form a two-electron deficient intermediate named as compound I. The compound I consists of a Fe(IV) oxoferryl centre and a porphyrin-based cation radical. It further oxidizes substrates by one electron and forms compound II, which is a more reduced Fe(IV) oxoferryl intermediate (Breen and Singleton 1999).

LiP is catalytically the most prominent fungal peroxidase in class II. It has the ability to oxidize directly several phenolic and non-phenolic substrates with calculated ionization potential up to 9.0 eV (ten Have and Teunissen 2001). It completely oxidizes methylated lignin and lignin model compounds in addition to several polyaromatic hydrocarbons, PAHs. Among the different oxidation reactions catalyzed by LiP are the cleavage of Cα–Cβ and aryl Cα bond, demethylation, and aromatic ring opening. Veratryl alcohol (VA), a secondary metabolite, has been the focus of many studies. VA is a rich substrate for LiP and increases the oxidation of otherwise weak or terminal LiP substrates (Ollikka et al. 1993). VA is playing three important roles. Firstly, VA behaves as a mediator in electron-transfer reactions.

Secondly, it is a good substrate for compound II and for that reason; it is required for completing the catalytic cycle of LiP via oxidation of terminal substrates. Thirdly, H_2O_2-dependent inactivation of LiP by reducing compound II back to native LiP is prevented by VA. Moreover, if the inactive LiP compound III is established, the intermediate VA+ is able to reduce LiP compound III back to its original form (ten Have and Teunissen 2001). The oxidation site in LiP is confined to catalytically active tryptophan (Trp171 in *P. chrysosporium*) on the enzyme surface for the high-redox potential compound like VA. This site is very important as it has been demonstrated by the mutagenesis studies. It is a substrate-intermediate-protein radical center upon LiP catalysis and initiation of the long-range electron transfer pathway leading to the heme (Smith et al. 2009).

4.2 Manganese peroxidase (MnP)

Another heme-containing glycoprotein oxidoreductase which preferentially uses Mn^{2+} as the electron donor is MnP. This enzyme has been produced by only a few basidiomycetous fungi and there is no record of its isolation from bacterium, yeast, mold, or mycorrhiza-forming basidiomycete (Hofrichter 2002). It was first identified in WRF *P. chrysosporium* along with LiP. The first phase of the catalytic cycle involving 2 electron oxidation of enzyme by H_2O_2 to compound I and subsequent reduction to compound II by oxidation of the suitable substrate (numerous monomeric and dimeric phenols which also includes phenolic lignin model compounds) or Mn^{2+}, is analogous to that of other peroxidases. However, unlike other peroxidases, compound II strictly requires Mn^{2+} as an electron donor (Kuan et al. 1993).

MnP is one of the most dominating enzymes of the class II peroxidases. Thus, the MnP encoding genes are much more widely prevalent compared to LiP in agaric (cap forming) and polyploid as well as corticoid fungi, all of which belong to Agaricomycotina subphylum (Morgenstern et al. 2008). Recent phylogenetic analysis has revealed that there were at least two pre-MnP lineages that separated early in the lignin-degrading fungi. This led to the formation of two groups of MnPs, namely, the short hybrid MnP variants or group A, and the characteristic long MnP enzymes or group B. The former are evolutionarily more relevant to VPs and LiP than to classical MnPs (Lundell et al. 2010). The MnP is around 20 to 30 amino acid long protein (longer than LiP or VP) having a 5th cysteine disulfide bond with a Mn^{2+} binding site near the C-terminus (Sundaramoorthy et al. 2005). MnP is exceedingly particular for oxidation of Mn^{2+} ions in an acidic environment (Gold et al. 2000). This is due to a solvent exposed and conserved Mn^{2+} binding site neighborhood with either of the 2 heme propionates. It was first observed in the crystal structure of *P. chrysosporium* MnP1, consisting of three acidic amino-acid residues (two Glu, E35 and E39; and one Asp, D179). These amino acids are essential for the Mn^{2+} ion hexa-coordination, thus enabling the rapid electron transfer to the ferryl iron and heme (Sundaramoorthy et al. 2005).

The catalytic cycle of MnP completes when two Mn^{2+} ions are oxidized and converted into two Mn^{3+} ions. These ions form chelate complexes with anions of dicarboxylic acids such as oxalate or malonate (produced by the WRF in copious amount) and are dispersed out of the binding site (Gold et al. 2000). The scope of the

oxidation capability of MnP is clear in its products, the chelated Mn^{3+} ions, which act as diffusible mediators for charge transfer and then attack phenolic compounds containing bigger biopolymers, such as natural and synthetic lignin, milled wood, humic substances, and various xenobiotic compounds (Hofrichter et al. 2001, Husain et al. 2009). The cloned and enzymatically described long, classical, and highly conserved MnPs have only been described for the efficiently wood-lignin-degrading fungi of the basidiomycetous families *Corticiaceae* or *Polyporaceae*, such as *Ceriporiopsis subvermispora*, *Dichomitus squalens*, *P. chrysosporium*, *Phanerochaete sordida*, *Phlebia radiata*, and *Phlebia* spp. (isolates Nf-b19 and MG-60), thus indicating the importance of this particular peroxidase for the development of typical white-rot decay of wood (Lundell et al. 2010).

4.3 Versatile peroxidase (VP)

VP is the third largest lignin-degrading and high redox potential peroxidase of class II fungal peroxidases. It was first described in the plant roots- and wood colonizing white-rot basidiomycete *Pleurotus eryngii* (Pérez-Boada et al. 2005) and is also found in *Bjerkandera* spp. (Moreira et al. 2005). It is a hybrid-type heme containing enzyme that combines the catalytic functions of both MnP and LiP. They can oxidize Mn^{2+} as well as high redox-potential compounds, such as Reactive Black 5. Physical and catalytic properties have shown that the multiple catalytic properties are due to co-existence of different catalytic sites on the same enzyme. In VP, the three specific MnP amino acid residues involved in the Mn^{2+} binding and specific LiP amino acid exposed tryptophan residue (Trp-164) is found (Ruiz-Dueñas et al. 2009).

The catalytic cycle of the enzyme combines activities resonant of both MnP and LiP. At the lignin-peroxidase site, the enzyme is capable of Mn^{2+}-independent oxidation of various organic compounds, including high redox potential non-phenolic aromatics and dyes following the catalytic cycle in a similar fashion as that of LiP, as described earlier. Due to steric hindrance, long-range electron transfer from a protein radical at the surface of the enzyme (which would act as the substrate oxidizer) to the heme cofactor, has been suggested to explain oxidation of large compounds, such as the large aromatic substrates, lignin polymer, or complex redox mediators (Ruiz-Dueñas et al. 2009). At the manganese oxidation site, Mn^{2+} is oxidized to Mn^{3+} employing the mechanism identical to the MnP reaction mechanism. The generated Mn^{3+} acts as a diffusible oxidizer for free phenols and phenolic lignin and has also been reported to initiate lipid peroxidation reactions, which may be implicated in the biodegradation of recalcitrant materials (Kapich et al. 2005). The *Bjerkandera* spp. peroxidase is apparently another VP with the conserved Trp and Mn^{2+}-binding site residues found in the primary structure (Moreira et al. 2005). Moreover, in *P. radiata*, two divergent MnPs have been described, the first of the classical-long MnP type and the second of a short-VP-type comprising the conserved Mn^{2+}-binding site but lacking the exposed tryptophan (Hildén et al. 2005). Due to this, the latter MnP is unable to oxidize veratryl alcohol but shares with VP the Mn^{2+}-independent ability to oxidize small dye phenolic and amine compounds.

4.4 Arthromyces ramosus peroxidase (ARP) and coprinus cinereus peroxidase (CIP)

Arthromyces ramosus peroxidase (ARP) and *Coprinus cinereus* peroxidase (CIP) of class II fungal peroxidases with no ligninolytic activities were discovered in the mid-1980s. ARP and CIP were found in an imperfect fungus (Deuteromycete) *Arthromyces ramosus* and inky cap basidiomycete *Coprinus cinereus*, respectively (Morita et al. 1988). *Coprinus macrorhizus* peroxidase (CMP) is another group of fungal peroxidase obtained from *Coprinus macrorhizus*. However, *C. macrorhizus* is recognized as the same species as *C. cinereus*, and these two *Coprinus* peroxidases were suggested to be identical. Certainly, the analyses of three-dimensional molecular structures, amino acid sequences, and catalytic activities of these fungal peroxidases; CIP, CMP, and ARP have illustrated that the enzymes are identical only with a minor change in glycosylation, which did not influence the reaction rate of the enzyme (Sawai-Hatanaka et al. 1995).

These three peroxidases readily oxidize phenols and smaller dye molecules similar to the class III horse radish peroxidase (HRP). However, the 3-D structure and heme packing with conserved helices, two domains with bound Ca^{2+} ions, N-glycosylation sites, and the conserved proximal and distal residues near the heme resembled other class II peroxidases (Houborg et al. 2003). The distal side substrate channel of CIP/ARP is more open and accessible to phenolic and aromatic reducing compounds (Kunishima et al. 1994, Petersen et al. 1994). There is a 40–50% amino-acid identity of CIP with LiPs, VPs, and MnPs, and the size (340 amino acids in the mature protein) is almost the same. Instead of very similar catalytic activities of ARP and CIP to a classical plant peroxidase, HRP, the similarity in overall amino acid sequences among the genes encoding these plant and fungal enzymes is less. Although ARP and CIP amino acid sequences have shown better agreement with those of LiP and MnP compared to those of HRP, but no ligninolytic activity is exhibited by these enzymes. Some significant structural differences revealed by X-ray crystallography at the active sites of the two fungal peroxidases have demonstrated the reason for the differences in substrate specificity (Kunishima et al. 1994, Petersen et al. 1994).

4.5 Dye decolorization peroxidases (DyP)

DyPs are a new superfamily of heme-containing glycoproteins that oxidize typical peroxidase substrates and also additionally oxidize anthraquinone-type dyes and their derivatives (Sugano et al. 2009, Sugano 2009). Furthermore, some DyPs are able to oxidize aromatic sulphides (van Bloois et al. 2010), non-phenolic β-O-4 lignin model compound (Liers et al. 2010), as well as cleave β-carotene and other carotenoids (Zelena et al. 2009). The enzyme was first identified in the fungus *Geotrichum candidum* Dec 1, which was later assigned to the phytopathogenic basidiomycete *Thanatephorus cucumeris* that was a misidentified strain (Strittmatter et al. 2013). The present correctly identified strain for DyP secretion is *Bjerkandera adusta* (Ruiz-Dueñas et al. 2011). Afterwards, it was also isolated from bacteria (Hofrichter et al. 2010).

DyP is a glycosylated heme protein (17% sugars) with a Soret band at 406 nm, a pI of 3.8, and a molecular mass of 60 kDa. The sequence alignments and structural comparison of DyPs with representative members of all classes of the plant, bacterial, and fungal peroxidases demonstrated that DyPs do not belong to either of these families (Sugano et al. 2007, Zubieta et al. 2007). These are structurally different from all other peroxidases, however, these enzymes showed little sequence similarity (0.5–5%) to classic fungal peroxidases and lack the typical heme-binding region, which was conserved in plant peroxidases (Faraco et al. 2007, Sugano 2009). A characteristic feature of all DyPs investigated so far is their ability to oxidize synthetic high-redox potential anthraquinone dyes, which were recalcitrant to oxidize by other peroxidases. This catalytic feature made DyPs a potential tool to target pollutants (Syoda et al. 2006). However, the mechanism of the catalytic cycle of DyP and the cleavage of dyes are not fully understood yet, but there are interesting indications that includes, besides typical peroxidative reactions, the hydrolytic fission of the anthraquinone backbone (Sugano et al. 2009). The latter notion is supported by the identification of phthalic acid as a cleavage product of the recalcitrant dye Reactive Blue 5. The spectroscopic characteristics of DyP in the presence of an equivalent of peroxide showed a similarity to those of a typical peroxidase compound I but the formation of compound II has not been noticed so far (Sugano et al. 2007). DyPs differ from other heme peroxidases in the process of compound I formation, since an aspartate replaces the distal histidine that is usually responsible for H_2O_2 binding. In this respect, DyPs resemble heme-thiolate peroxidases, HTPs (CfuCPO, APOs), which contain identical amino acid glutamate at the respective position (Pecyna et al. 2009).

The high stability of DyPs is another significant property that attracted it for applications under rather harsh environmental conditions. Pühse et al. (2009) reported that purified dimeric MsP1 (DyP-type peroxidase of *M. scorodonius*) was markedly more stable at high temperatures (up to 70°C) and pressures (up to 2,500 bar). DyPs also appeared quite stable under acidic conditions. DyP isoform of *A. auricula-judae* exhibited very high stability in a buffer of pH 2.5 even after 2 hours of incubation. The secretion of this DyP by *A. auricula-judae* under conditions close to nature, i.e., in wood cultures and their ability to oxidize non-phenolic lignin model compounds (indicative for a ligninolytic activity) may throw some light on the possible physiological function of these enzymes (Liers et al. 2010). According to a method of Ayala et al. (2007), which is based on the oxidation of different *p*-substituted phenols, there are indications that the redox potential of DyPs range between those of high-redox potential peroxidases (i.e., ~ 1.5 V for LiP and VP) as well as low-redox potential peroxidases, i.e., ~ 0.8–1.0 V for plant peroxidases.

4.6 Heme-thiolate peroxidases (HTPs)

The most interesting catalytic activity of secreted HTPs is the transfer of peroxide-oxygen (from H_2O_2 or R-OOH) to substrate molecules, and has been described as a peroxygenase activity. The most relevant enzymes in this category include CfuCPO (EC 1.11.1.10) and APOs (EC 1.11.2.1).

4.6.1 Caldariomyces fumago chloroperoxidase (CfuCPO)

CfuCPO is a halogenating enzyme and it does not fit into the class II of heme peroxidases, due to the difference in amino-acid sequences and type of reactions they catalyze (Passardi et al. 2007). This enzyme also illustrates remarkable structural differences compared to all other non-animal peroxidases, since it bears a cysteine-ligated heme, while the other peroxidases have a histidine as axial heme ligand, heme-imidazole type (Liu and Wang 2007). It has been the only HTP characterized on the protein level for almost 50 years, until similar enzymes were discovered in several agaric basidiomycetes (Omura 2005, Hofrichter and Ullrich 2006, 2011).

The main activity of CPO is to oxidize chloride (Cl^-) into hypochlorous acid at low pH (< 5). Thus, it appears as a strong oxidant that catalyzes chlorination of organic compounds. The first part of the cycle including peroxide binding and formation of compound 0, heterolytic O–O fission, and compound I formation is similar to other heme peroxidases (even if a Glu residue is involved in H_2O_2 binding instead of a His). However, the subsequent oxidation of halide by compound I via two electron oxidation to form compound X, a hypothetic Fe(III) hypohalite intermediate, differs from the typical cycle of heme peroxidases (Kühnel et al. 2006). This unstable compound X is proposed to rapidly decay into the native ferric enzyme and hypohalous acid that oxidizes electrophilic substrates via halogenation. Compound II is not formed in this cycle; however, CfuCPO can act as a "normal" peroxidase in the absence of halides (Hofrichter et al. 2010).

It catalyzes the oxidation of phenols and anilines via a peroxidative mechanism in the absence of halides. These reactions include also the oxidation of highly chlorinated derivatives; e.g., PCP, which were earlier reported to be partially dehalogenated while being polymerized into insoluble polymeric materials. Contrasting to simple phenol oxidation, the conversion of PCP by CfuCPO was one to two magnitudes faster than the reactions of HRP, LiP, or VP (Longoria et al. 2008). These one-electron oxidations/H abstractions follow the typical cycle of heme peroxidases involving compound I and II formation, and may occur in distance to the active site heme at the protein surface. CfuCPO is also able to catalyze oxygen transfers like it epoxidizes double bonds of alkenes and cycloalkenes as well as hydroxylates benzylic carbon via a peroxygenase mechanism (oxygenation mechanism/two-electron oxidation) that resembles the peroxide "shunt" pathway of P450s (Osborne et al. 2007, Manoj and Hager 2008). However, oxygen transfers to less activated molecules, such as aromatic rings or n-alkanes cannot be catalyzed by CfuCPO (Ullrich and Hofrichter 2007).

The reasons for describing CfuCPO as a "heme peroxidase cytochrome P450 functional hybrid" or a "Janus enzyme" were the different catalytic properties of CfuCPO, i.e., one electron oxidations as well as two-electron oxidations coupled with halide oxidation or oxygen transfer (Manoj and Hager 2008). It is important to remember that CfuCPO does not share any sequence homology with P450s. In contrast, molecular architecture (arrangement of α-helices in the tertiary structure) of both enzyme types was reported to show some similarity. A problem that limited the practical utility of CfuCPO is its inherent activity to dismutate parts of the