Fungal Biology

Ram Prasad *Editor*

Mycoremediation and Environmental Sustainability

Volume 2

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Fungal Biology

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About the Series

Fungal biology has an integral role to play in the development of the biotechnology and biomedical sectors. It has become a subject of increasing importance as new fungi and their associated biomolecules are identified. The interaction between fungi and their environment is central to many natural processes that occur in the biosphere. The hosts and habitats of these eukaryotic microorganisms are very diverse; fungi are present in every ecosystem on Earth. The fungal kingdom is equally diverse, consisting of seven different known phyla. Yet detailed knowledge is limited to relatively few species. The relationship between fungi and humans has been characterized by the juxtaposed viewpoints of fungi as infectious agents of much dread and their exploitation as highly versatile systems for a range of economically important biotechnological applications. Understanding the biology of different fungi in diverse ecosystems as well as their interactions with living and non-living is essential to underpin effective and innovative technological developments. This series will provide a detailed compendium of methods and information used to investigate different aspects of mycology, including fungal biology and biochemistry, genetics, phylogenetics, genomics, proteomics, molecular enzymology, and biotechnological applications in a manner that reflects the many recent developments of relevance to researchers and scientists investigating the Kingdom Fungi. Rapid screening techniques based on screening specific regions in the DNA of fungi have been used in species comparison and identification, and are now being extended across fungal phyla. The majorities of fungi are multicellular eukaryotic systems and therefore may be excellent model systems by which to answer fundamental biological questions. A greater understanding of the cell biology of these versatile eukaryotes will underpin efforts to engineer certain fungal species to provide novel cell factories for production of proteins for pharmaceutical applications. Renewed interest in all aspects of the biology and biotechnology of fungi may also enable the development of "one pot" microbial cell factories to meet consumer energy needs in the 21st century. To realize this potential and to truly understand the diversity and biology of these eukaryotes, continued development of scientific tools and techniques is essential. As a professional reference, this series will be very helpful to all people who work with fungi and should be useful both to academic institutions and research teams, as well as to teachers, and graduate and postgraduate students with its information on the continuous developments in fungal biology with the publication of each volume.

More information about this series at http://www.springer.com/series/11224

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Foreword


Fig. 1 (**a**) George Robinson, the inventor of bioremediation; (**b**) Mycoremediation– the remediation of waste land with fungi

Bioremediation refers to cost-effective and eco-friendly method for converting the toxic, recalcitrant pollutants into environmentally benign products through the action of various biological treatments (White et al. 1998; Lovley and Lloyd 2000; Harms et al. 2011). The process of bioremediation was reportedly devised by George M Robinson (1960s) during experimentation with dirty jars (Fig. 1a). It is estimated that severe degradation of 10–20% of global drylands could affect up to 250 million people, mostly in the developing world. That would have a detrimental impact on the United Nations' Sustainable Development Goals, particularly the eradication of poverty and ensuring environmental sustainability (Singh and Baquerizo 2016). The global market for bioremediation technology and services market was valued at USD 32.2 billion in 2016 and is estimated to reach USD 65.7 billion by 2025 at a CAGR of 8.3% from 2017 to 2025 (http://wardsauto.com/%5Bprimary-term%5D/bioremediation-technology-services-market-reach-us657-bn-2025-globally-transparency-m).

Fungi play a key role in bioremediation owing to their robust morphology and diverse ecosystem amenities (Deshmukh et al. 2016). However, many of the environments where fungi provide these products are under extreme stress. For example, to produce ethanol, the yeast *Saccharomyces cerevisiae* needs to cope with high

ethanol concentrations, oxidative and osmotic stress, as well as high temperatures generated by fermentation (Nguyen et al. 2017). Soil-dwelling fungi may enhance plant health and crop production in arid environments and also help degrade and valorize organic waste materials (Fig. 1b) (Pennisi 2004). Mycoremediation has a major section of technology in the bioremediation and services market, due to increase in use of fungal mycelium to disintegrate contaminants from waterways, soil, or even radioactive contaminated areas. This will increase the usage of fungus for treatment procedures of soil which are being polluted by mercury and other heavy metals. Mycoremediation has ability to transform contaminated wasteland into a diversified ecosystem. However, application of this technology is difficult; skilled and trained personnel with proper knowledge of the subject are required to do the work of fungal remedy. Mycoremediation is one of the green chemistry strategies which are considered as the safest, least disruptive, and most cost-effective treatment in comparison with traditional physicochemical treatments for decolorization processes. Three essential mechanisms (biosorption, biodegradation, and bioaccumulation) are utilized by fungal strains for the removal of recalcitrant dyes from environment (Harms et al. 2011; Deshmukh et al. 2016; Prasad 2017).

The first chapter by Saglam et al. reviews fungal bioremediation with special reference to industrial wastewaters such as olive oil mill wastewater and alcohol factory wastewater (vinasse). Chap. 2 by Chaudhary et al. highlights the roles of phytochelatins (PC), metallothioneins (MT), and heavy metal ATPase (HMA) genes play a crucial role in signaling, uptake, detoxification, and accumulation of metal. In Chap. 3, Laura Bardi described metabolic engineering strategies aimed to improve quantity and quality of single cell oils production, in particular in the fields of nutrition-nutraceuticals and of biofuels. Extensive studies desired for exploration of fungi as a potential mycoremediator in order to attain agricultural sustainability are discussed by Purohit et al. in Chap. 4. In Chap. 5, Yeislada et al. highlighted on the textile dye bioremediation, decolorization, and detoxification abilities of white rot fungi and wide application of laccases. In Chap. 6, Pandey et al. highlighted on mycoremediation of common agriculture pesticides (Fig. 2). In Chap. 7, Mohapatra

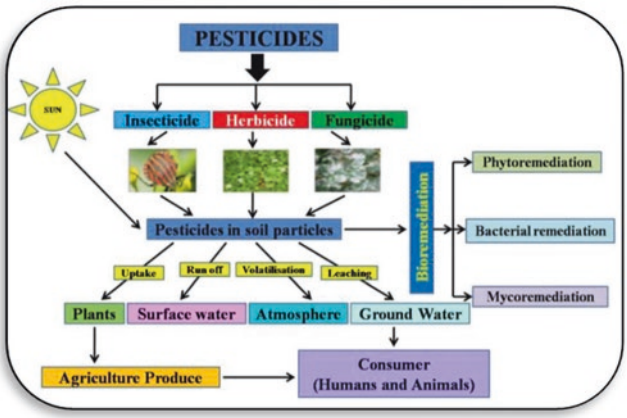

Fig. 2 Fate of pesticides in environment and bioremediation

et al. describe that the lignolytic, white rot fungi produces extracellular enzymes which are suitable for degradation of many different compounds notably organopollutants. In Chap. 8, Reina et al. highlighted on usage of white rot fungi in the transformation of dry mill olive residue (agricultural by-product) for an environmentally sustainable scenario. In Chap. 9, Dias et al. highlighted on fungal biodegradation of winery wastes as well as extraction of bioactive compounds. Meena and Busi detail that biosorption method is a cost-effective, non-toxic, green approach for the removal and recovery of dyes and heavy metals from industrial effluents in Chap. 10. In Chap. 11, Deka et al. give an overview of production of nanoparticles using different fungal species and its potential applications in agriculture for enhancing crop production by improving growth and protection against plant diseases. The prospects of obligate marine fungi in bioremediation are discussed in Chap. 12 by Sarma. The bioremediation potential of fungal-derived chitosan and chitosan nanocomposites is discussed by Pattnaik and Busi in Chap. 13. Finally, strategies and applicability of mycoremediation mechanisms for heavy metal resistance/tolerance in plants are presented by Singh et al. in Chap. 14.

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Eric Lichtfouse, is a biogeochemist at the University of Aix-Marseille, CEREGE, Aix-en-Provence, France. He obtained his PhD in Organic Geochemistry from the University of Strasbourg in 1989 for the discovery of new fossil steroids in sediments and petroleum. He has invented the ^{13}C-dating method allowing to measure the dynamics of soil organic molecules. He is Chief Editor of the journal *Environmental Chemistry Letters* and former Chief Editor of the journal *Agronomy for Sustainable Development*. He has published the book *Scientific Writing for Impact Factor Journal*, describing the micro-article, a new tool to identify the novelty of experimental results. He has published 85 research papers, with an h index of 23, and edited more than 50 books. He got the Analytical Chemistry Prize from the French Chemical Society, the Grand University Prize from Nancy University, and a Journal Citation Award by the Essential Science Indicators.

Preface

Mycoremediation is a procedure of bioremediation in which fungal-based technology is used to decontaminate the environment. Fungi have been demonstrated to be a very cost-effective and environmentally sound way for helping to remove an extensive array of toxins from damaged environments or wastewater. The toxins include heavy metals, persistent organic pollutants [polycyclic aromatic hydrocarbons (PAHs), pesticides, and herbicide], textile dyes, leather tanning industry chemicals and wastewater, petroleum fuels, pharmaceuticals, and personal care products. The by-products of the remediation can be appreciated constituents themselves, such as enzymes (like laccase), edible or medicinal mushrooms, making the remediation process even lucrative. Mycoremediation practices involve placing of mycelium into contaminated soil and placing mycelial mats over toxic sites, or a combination of these techniques, in one or more treatments. Toxins in our food chain (including heavy metals, PCB's, and dioxins) become more concentrated at each step, with those at the top being contaminated by ingesting toxins consumed by those lower on the food chain. Fungal mycelia can destroy these toxins in the soil before they enter our food supply.

Fungi are among the primary saprotrophic organisms in an ecosystem, as they are efficient in the decomposition of material. Wood-decay fungi, especially white rot, secrete extracellular enzymes and acids that break down lignin and cellulose. Fungi feature among nature's most vital agents for the decomposition of waste matter and are crucial components of the soil food web, providing nourishment for the supplementary biota that live in the soil environment. The degree of sustainability of the physical environment is an index of the survival and well-being of the all-inclusive components in it. Additionally, it is not sufficient to try disposing toxic/deleterious substances with any known method. The best method of sustaining the environment is to return back all the components (wastes) in a recyclable way so that the waste becomes useful and helps the biotic and abiotic relationship to maintain an aesthetic and healthy equilibrium that characterizes an ideal environment.

This book should be enormously advantageous for researchers, technocrats, policy maker, and scientists of fungal biology and those who are interested in environmental suitability. I am honored that the leading scientists who have extensive, in-depth understanding and expertise in fungal biology and environmental concern took the time and effort to develop these outstanding chapters. Each chapter is written by internationally recognized academicians so the reader is given an up-to-date and detailed account of our knowledge of the fungal system and numerous applications of fungi.

We are indebted to the many people who helped to bring this book to light. I wish to thank series editors Dr. Vijai Kumar Gupta and Dr. Maria G. Tuohy and Dr. Eric Stannard, Senior Editor, Botany, Springer, for their generous assistance, constant support, and patience in initializing the volume. Editor in particular is very thankful to Springer group Tanya Chacko, Anthony Dunlap (Project Coordinator), and P. Abitha (Project Manager) for the kind care and constant encouragement received. Special thanks go to my exquisite wife Dr. Avita Maurya for her continuous support and inspirations in putting everything together. Dr. Prasad is very thankful to Professor Ajit Varma at Amity University and Dr. Wang Shanquan at SESE, Sun Yat-Sen University, for the kind support and constant encouragement. Special thanks are due to my well-wisher father Mr. Shyam Sundar Prasad and mother Mrs. Shyama Devi, and other family members and friends.

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Chapter 1
Bioremediation Applications with Fungi

Necdet Saglam, Ozfer Yesilada, Semran Saglam, Elif Apohan, Mesut Sam, Sedef Ilk, Ezgi Emul, and Ekrem Gurel

1.1 Introduction: Role of Fungal Enzymes in Bioremediation

Scientists began to study for cleaning up industrial wastes and pollutants using microorganisms in 1988. Thanks to advances in these studies, some bacteria can turn industrial wastes into more beneficial sources or enzymes. This kind of eco-friendly utilization of the microorganisms was known as bioremediation (Tortora et al. 1995).

Bioremediation, the use of organisms for the treatment of environmental pollution techniques, have received considerable interest in recent years because of potential cost savings compared to conventional nonbiological techniques. Bioremediation is composed of biotechnological applications that are used for the destruction process of wastes (Leung 2004; Prasad 2017). Much of the bioremediation is comprised of physicochemical or biological processes. Enzymatic bioremediation is among the

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two traditional categories involving biological catalysts and chemical processes (Paul et al. 2005). Biocatalysis process provides to improve the development of products and processes for the reduction of industrial costs and the generation of toxic products (Alcalde et al. 2006; Sutherland et al. 2004).

In Turkey and other Mediterranean countries, the extraction process of olive oil causes a serious polluting problem known as olive mill wastewater (OMWW). High amounts of toxic phenolic and nitrogenous compounds, volatile acids, and polyalcohols including OMWW spread widely in lands and are a threat to every kind of living organisms. Because of technical and economical limitations for OMWW treatments, fungal remediation techniques and the ability of white rot fungi in degradation have been widely studied (Aytar et al. 2011). On the other hand, there is a wide usage of fungal microorganisms in heavy metal removal. The potential use of fungi in heavy metal-contaminated waters and metal recovering methods in mining wastes has gained special importance. Some heavy metals such as cadmium, mercury, lead, iron, and copper have been removed by different species of fungi like *Pleurotus sajor-caju* and *Phanerochaete chrysosporium* (Saglam et al. 1999; Cihangir and Saglam 1999).

Enzymes are the active agents for biocatalysis process which take place through bioremediation. The biochemical transformation process occurs when the enzyme encounters its substrate which is the target pollutant for the bioremediation and removes part of the molecule. The use of enzymes in textile, leather, and detergent industries and especially in the bioremediation process has gained more attention owing to enzymatic processes that lead to less environmental pollution than chemical processes by means of moderate conditions and economic reasons (Sheldon and van Rantwijk 2004). Enzymes are used in many fields such as organic synthesis, clinical analysis, pharmaceutical production, detergent food production, and fermentation because they can be biodegradable and work under stable, efficient, and selective conditions (Alcalde 2006). White rot fungus can oxidize many of the destruction processes that causes pollution of powerful organic compounds by extracellular lignolytic enzyme systems (Baldrian 2006). The lignolytic enzymes of white rot fungus are used in environmental biotechnology for the removal of phenolic compounds, chlorinated phenolic compounds, polyaromatic hydrocarbons, starchy wastes, protein wastes, high-fat waste, pesticides, chlorine, paper waste, heavy metal pollution, surface contaminants, aromatic amines, soil decontamination, and in coloring agents (Claus and Filip 1988).

The enzyme laccase secreted by fungus is generally used for bioremediation process in industry. Laccases of fungi, copper-containing oxidases, can have the ability for the transformation of various phenolic compounds such as the humic and polymeric lignin substrate. The degradation of lignin and phenolic compounds by laccases has been evaluated in several biotechnological applications such as bioremediation of some toxic chemical wastes (e.g., polycyclic aromatic hydrocarbons (PAH), chlorinated aromatic compounds, nitroaromatics, and pesticides) and dye degradation (Samanta et al. 2002; Whiteley and Lee 2006). In recent years, the white rot fungi have been studied to produce laccase. White rot fungi provide powerful oxidative laccase enzyme systems that could degrade lignin to carbon dioxide (Ten Have and Teunissen 2001). The recent treatments of the use of laccase

have enabled the use of mediators, which promote or facilitate enzyme action. Laccases role by white rot fungi in bioremediation could be summarized by means of degradation of xenobiotics including chlorinated phenolics, pesticides, and polycyclic aromatic hydrocarbons (Torres et al. 2003; Pointing 2001); decolorization of dyes (Banat et al. 1996); pulp bleaching (Soares 2001); effluent treatment (Taşpınar and Kolonkaya 1998); and biosensor technology for the detection of a broad range of phenolics (Palmore and Kim 1999). Especially for the purpose of lignin and color removal, the most commonly used white rot fungus species are *Phanerochaete chrysosporium* and *Trametes* (*Coriolus*) *versicolor* (Rogalski 1991), while *Funalia trogii*, *Lentinula edodes*, *Pycnoporus coccineus*, *Coriolopsis polyzona*, *Pleurotus ostreatus*, species of *Cerrena*, *Byssochlamys*, *Lasiodiplodia*, and *Bionectria* have been used for remediation of OMWW (Aytar et al. 2011).

1.2 Detection of Fungus in Purification Technology

Fungi fulfill an important function, such as the disintegration of organic substances, but unfortunately, we do not have adequate knowledge about them. Enzymes and applications of fungi have an active role in bioremediation technology, but the fungus did not gain any interest which it deserved to be due to the lack of sufficient information.

The most important step in a process in which microorganisms are used is the selection of microorganisms. The first step is the selection of isolation and pure culture defined to the organism because right pure culture is important to increase product efficiency. The third application is to ensure the continuity of pure cultures. The forth process is to keep product efficiency at the highest level for strain. Mutant strains are produced for this purpose. When the products of microorganisms are examined, it is observed that they synthesize different products called primary and secondary metabolites. The primary metabolites occur at the highest level of the reproduction and at the end of this phase but secondary metabolites occur in the so-called stagnant phase (Anastasi et al. 2013; Hamman 2004). Examples of primary metabolites include organic acids such as alcohol, acetone, amino acids and vitamins, or organic solvents and microbial enzymes. Examples of secondary metabolites are antibiotics and hormones.

Currently, two basic methods are used to detect potential fungi that can be used in bioremediation studies and immunological and molecular assays.

1.2.1 Immunological Assays

Immunological method was first used with the detection of fungi in the last quarter of the twentieth century. Monoclonal and polyclonal antibodies, that have potential of the binding to the enzymes that are used in bioremediation such as manganese peroxidase, laccase, and lipase, are used for creating new systems that enable to

detect fungi. In this way, these fungi, which can synthesize important enzymes valuable for the industry, can be easily distinguished from others. Between the years 1980 and 2000, researchers have developed many methods based on the immunological probes, immunofluorescence, ELISA (immunization), and immunoblotting.

In particular, polyclonal antibodies have been successful in detecting brown rot fungi, while immunofluorescence stains (immunofluorescence dyes) have been used in the identification of *Basidiomycetes* fungi, which contain many microorganisms used in the purification technologies (Peruski and Peruski 2003).

1.2.2 Molecular Assays

The rapid development of molecular biology from the 1980s led to the use of new molecular biology-based methods instead of morphological methods, which are known to be unstable in characterization of fungi and that may vary with environmental conditions. With these techniques based on molecular biology, microorganisms with the ability to synthesize enzymes at the high rates can be used in bioremediation studies, and it could be detected more quickly and precisely than other methods (Jafari et al. 2013).

The techniques of molecular assays are divided into two methods depending on whether they eventually require or not require a gene sequence analysis.

The method called ITS (internal transcribed spacers) is widely used in taxonomic studies in species detection and interspecific relationships. The ITS regions are a repeating region within the ribosomal RNA. The regions of the ITS have become an official molecular bar code accepted by the fungus taxonomy. With this barcode, it is possible to determine if the microorganisms have the desired feature.

The PCR method is also a common technique used for the same purpose. PCR is an enzymatic reaction that is widely used in molecular biology and aims to replicate DNA strands in organisms. In this method, the DNA in the organisms is first isolated, and then the isolated DNA is amplified with various enzymes and reaction components. When replication is complete, DNA is kept in an agarose gel container. After these steps, it is possible to detect the fungus carrying the desired characteristics by setting the target gene sequence or considering the bands according to the DNA size.

1.3 Fungal Polymers

Nations are supposed to produce cheaper but better quality products in order to increase social and prosperity levels and not to lose market competition of the industrial organizations they own. Especially for developing countries like Turkey, it is imperative that they develop their own technologies and apply them to their industry so that they can compete with the developed countries dominating the world market. However, while developing new technologies, cleaner technology for a cleaner environment is one of the necessary criteria to consider.

Biopolymers, which are the most common application areas within biomaterials, are widely used in medicine and biotechnology as well as in the food and cosmetic industries. Biopolymer applications include enzyme and cell support materials for immobilization, surgical instruments, implant and support materials (e.g., artificial organs and prostheses), drug delivery systems, biosensors, and components of diagnostic testing.

There are many practical applications in the fields of textile, food, cosmetic, chemical, and pharmaceutical industries for microbial-based biopolymers and derivatives. Due to the high application potential and broad structural, functional, and physicochemical properties of these natural polymers, a brand new product is added to the research in this field almost every single day.

It is becoming increasingly common for these polymers to gain new properties and to increase their application area by making various modifications (such as methylation, which increases binding and stabilization properties) on natural polymers of cellulose and other polysaccharide structures of algae and bacterial origin (Gersh et al. 2000).

Depending on developments in immobilized enzyme systems, the development of cheap and durable biodegradable support materials is an important research area. As is known, synthetic polymers are highly resistant to microbial degradation, and waste constitutes an important contaminant group for the environment. For this reason, efforts to develop biodegradable alternatives of synthetic polymers that are currently in widespread use are at the forefront of biotechnological research. The first natural polymer discovered for this purpose is poly-P-hydroxybutyrate, which has many uses as bacterial plastic (Angelova and Hunkeler 1999). Biologically biodegradable biopolymers, such as this mentioned polymer, which can be naturally produced and destroyed by biological means, are used for various purposes (Dlamini and Peiris 1997; Skvortsov and Ignatov 1998).

During our laboratory studies, we have found that *T. versicolor*, a white rot fungus, produces an extracellular biopolymer in a special feed developed by us. It has not been reported in the literature that an extracellular biopolymer is produced by this microorganism, but it has been reported that *Schizophyllum commune* ATCC 38548, which is also a white rot fungus, produces an extracellular polysaccharide called "schizophyllan" (Münzberg et al. 1995). The polysaccharide structure, schizophyllan, is formed in the glucose residue bound to the parent chain p-1,6-glycosidic bond, which is formed by glucose monomers polymerized by the p-1,3-glucosidic linkage and used as antitumor, anti-hepatitis, anti-HIV, and antiviral agents. It has also been reported that there may be a new support material for enzyme immobilization and tissue culture (Münzberg et al. 1995).

1.4 Fungal Enzymes and Application Areas

Efforts to increase the efficiency of these types of enzymes are rapidly continuing in biological processes that are still widely used in waste treatment and developed as an alternative to uneconomic chemical processes due to high energy and chemical

consumption. These studies have focused on developing potent commercial offenses, increasing enzyme stabilization, and producing low-cost enzymes. In addition, enzymes in both groups were separately arrested and used as biological catalysts in waste purification, with different support materials (alginate, carrageenan, magnetite, chitin, chitosan, polyurethane foam) (Kirkpatrick et al. 1990; Sun et al. 1992; Sun and Payne 1996; Wada et al. 1992, 1993).

Aromatic compounds containing phenols and aromatic amines form an important group of pollutants, and environmental pollution caused by these chemicals is strictly controlled in many countries. Phenolic pollutants are found in wastewater from a wide variety of industries such as coal, oil, wood, metal coating, resin and plastic, paint, textile, mine, paper, and paper clay (Klibanov et al. 1980, 1983, 2000). In fact, almost all phenolic compounds and their derivatives are toxic compounds and are hazardous to the environment. For this reason, it is necessary to be removed from the wastewater before discharge to the environment.

Conventional methods for removing phenols from industrial wastewaters include solvent extraction, microbial degradation, adsorption on activated carbon, and chemical oxidation (Atlow et al. 1984). Although these conventional methods are useful and effective, they also have significant disadvantages, such as high cost, inadequate elimination, the formation of harmful by-products, and the application to only a limited concentration of phenol. For this reason, the development of alternative technologies is very important.

1.4.1 Phenol Oxidase Group Enzymes

Phenol oxidases are part of the enzymes of the oxidoreductase class, which catalyze the oxidation reactions of phenolic compounds. This enzyme group is divided into two main subgroups, namely, tyrosinase- and laccase-type phenol oxidases (Karam and Niceli 1997). Both enzyme groups require the presence of molecular oxygen to be active. In addition, both enzymes have no coenzymes.

Tyrosinase enzyme (EC 1.14.18.1) (Fig. 1.1), commonly referred to as polyphenol oxidase, catecholase and phenolase. This enzyme catalyzes two sequential reactions (Atlow et al. 1984); (1) the hydroxylation of ortho-phenols in the presence of molecular oxygen in the presence of monophenols (cresolase activity), (2) is formed by dehydrogenation (catecholase activity) to form o-kines. Quinones, the end product of sequential reactions, turn into water-insoluble polymeric structures that can be easily removed from wastewaters by simple filtration methods. Thus, phenolic compounds, which are among the most important environmental pollutants, become easily removable from wastewater (Atlow et al. 1984; Sun et al. 1992; Wada et al. 1995).

Tyrosinase is typically active on tyrosine, 3,4-dihydroxyphenylalnine occuring at the end of this reaction. The activity of the result 3,4-dihydroxyphenylalanine enzyme is oxidized to quinones. The heterocyclic red compounds formed from the quinones then become melanin polymerized. Atlow et al. (1984) reported that they successfully pressurize phenols with the tyrosinase enzyme and remove them from

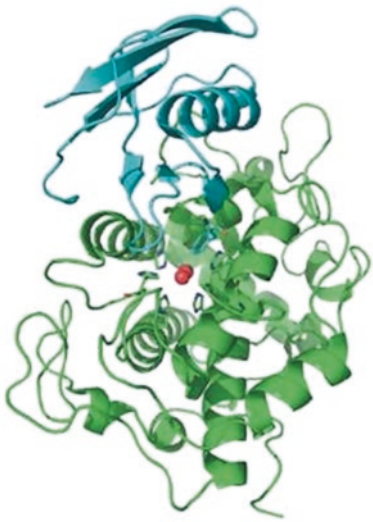

Fig. 1.1 Structural features of tyrosinase

the wastewater (Atlow et al. 1984). In contrast, Wada et al. (1993) reported that the results obtained in their studies contradicted Atlow et al.'s studies and that in their studies, precipitation did not occur as a result of polymerization of thiolsinase and phenols, but that the colorless solution turned into a dark brown color (Wada et al. 1993). Since these investigators observed precipitate formation, they used chitin and chitosan to adsorb the reaction products. According to Sun and Payne (1996), absorption of quinones and other intermediates formed by oxidation of phenols in tyrosinase on chitosan was successfully accomplished. The main advantage of using chitin and derivatized chitosan in order to adsorb the oxidation-inhibiting compounds of phenols is to convert a seashell-derived waste into a useful product (Wada et al. 1993). By using chitosan in combination with immobilized tyrosinase to purify the phenols, it provided a 100% phenol removal within 2 h. In contrast, some investigators used tyrosinase inhibition as chitin and chitosan support material (Wada et al. 1993). Immobilization of tyrosinase has the advantages of allowing the enzyme to remain in the reactor and preventing inactivation of the reaction terminator with quinones that are released. For this reason, immobilization of tyrosinase is an effective tool for eliminating toxic phenols.

As already mentioned, because of the use of oxygen as an oxidizing agent, the tyrosinase enzyme reduces the phenol digestion compared to other technologies, but this enzyme is already produced from the capped fungi via costly chemical processes, which again creates a negative position for the cost of the treatment. In fermentative conditions, it is a fact that the production and use of antibiotics in filamentous fungi is more efficient and cheaper (Tsuruta and Kawai 1983).

Laccase (EC 1.10.3.2) is a copper blue oxidase and laccase capable of oxidizing of ortho- and para-phenols (Arcand and Archibald 1991) (Fig. 1.2). Separation of a proton and electrons from the hydroxyl groups of aromatic compounds forms free

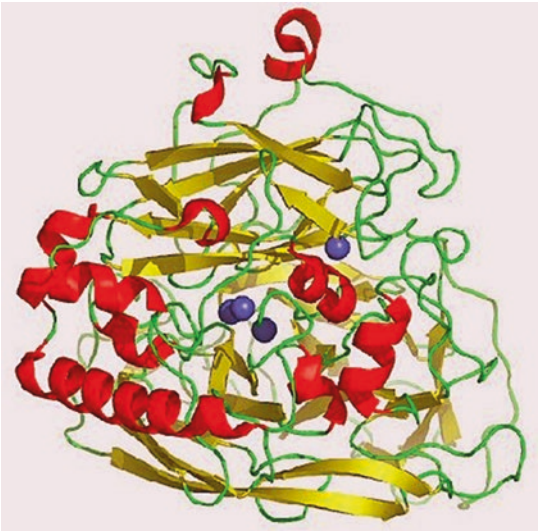

Fig. 1.2 Structure of fungal laccase

radical forms (Mason 1955). Laccases are widely distributed in nature and found in many plant and fungus species. The literature on the application areas and use of laccase enzyme is quite extensive.

Comparative studies with extracellular fungal laccases were performed by several investigators. Various basidiomycetes, ascomycetes, and deuteromycetes group microorganisms have good reproduction in rich sweet liquids. This group of microorganisms is capable of producing a large amount of laccase enzyme. 2,5-Xylidine has a fascinating effect on laccase production (Zawistowski et al. 1991). Studies on laccase immobilization are quite extensive. Alginate was used as support material for the immobilization of the enzyme. Free and immobilized enzymes are used as catalysts for the transformation of phenol compounds. Another scientific study in the literature is the detoxification of phenolic compounds via laccase enzyme (Niceli et al. 1993). Studies have been carried out by various investigators to improve the stability of immobilized fungal laccases. It has been shown that the activity of the enzyme laccase is able to remain unchanged for a long period of time, with a high level of stability. In this regard, especially the free and immobilized enzyme forms were compared, investigating the effects of pH, temperature, and different substrates on free or immobilized enzyme activity. Glass beads have been used as a method for the immobilization of laccase from *Trametes versicolor*, and this method has been shown to be more active and more tolerant at elevated temperatures. In the future, it could be expected that immobilized saccharides and various agricultural and industrial phenolic compounds may be detoxified. In addition, according to some literature, direct chlorination of chlorophenolic compounds with laccase enzyme obtained from *Trametes versicolor* was possible. It has been used for dechlorination of various chlorinated aromatics such as laccase enzyme tetraguaia-

col obtained from *Trametes versicolor* which is from white rot fungi in Basidiomycetes group. A downfall of approximately 85% was observed in the chlorinated organic matter. Purification studies of extracellular enzymes from *Trametes versicolor* have also been carried out. Techniques such as affinity chromatography and polyacrylamide gel electrophoresis have been used in purification processes.

1.4.2 Peroxidase Group Enzymes

Peroxidases are oxidoreductases that are produced by a group of microorganisms and plants. This group of enzymes requires H_2 for their catalytic activities. The best known of these group of enzymes are manganese peroxidase (MnP) (Fig. 1.3) and lignin peroxidase (LiP). Manganese peroxidase enzyme (EC unknown) was first detected in 1983 in the extracellular culture fluid of *P. chrysosporium*, a white rot fungus (Kuwahara et al. 1983). The MnP enzyme is a molecule in the glycoprotein, carrying a prothrombin-DC prosthetic group with a molecular weight of 46,000 structures (Perez and Jeffries 1993; Wariishi et al. 1989).

The enzyme acts by oxidizing Mn^{+2} to Mn^{+3}. The peroxidative effect of the enzyme is dependent on H_2O_2 and $Mn^{+}2$. However, in the absence of H_2O_2, the enzyme produces H_2O_2 by oxidizing NADPH, GSH, dithiothreitol (DTT), and dihydroxymaleic acid (using oxygen as oxidant), and this activity is also dependent on Mn^{+2} (Paszczynski et al. 1985). Enzyme activity can be stimulated by α-hydroxy acids (lactate, malate, tartrate, and citrate) and proteins (Glenn et al. 1986). In addition, kinetic and spectroscopic studies have shown that organic acids such as

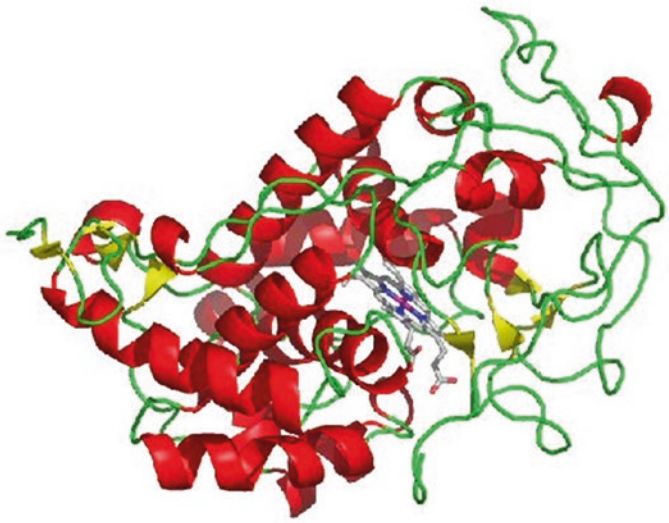

Fig. 1.3 Structure of manganese peroxidase obtained from *P. chrysosporium*

lactate facilitate the dissociation of Mn^{+3} from the enzyme-manganese complex by chelating Mn^{+3} and the dissolution of lignin as a function of the lignin polymer which is very difficult to dissolve in this way. In addition, organic acids also provide stabilization of Mn^{+3}, which has a very high redox potential (0.9–1.2 V) (Wariishi et al. 1989).

MnP is synthesized during the secondary metabolism, as part of the lignin-degrading enzyme system, as observed in many white rot fungi. MnP is completely dependent on Mn^{+2} in MnP of the organic substrates. No other metal ion can substitute for Mn^{+2} (Gold and Glenn, 1988). MnP, Mn^{+2} is oxidized to Mn^{+3}, and this Mn^{+3} is also a nonspecific oxidant that oxidizes various organic compounds. Previously made transient-position kinetic assays revealed that Mn^{+2}/Mn^{+3} acted as a redox couple rather than an enzyme-binding activator.

Simple phenols, amines, and various dyes have been used for testing substrate spectroscopy of enzyme systems. The activity of the enzyme manganese peroxidase is limited by the conditions of production and especially the presence and amount of trace elements in the production environment. The relative amount of Mn^{+2}/NlV ions from these trace elements is the most important criterion not only for the MnP enzyme but also for the synthesis of other lignolytic enzymes. While the NHt + ion is limiting for the synthesis of the MnP enzyme, the Mn^{+2} ion has a stimulating effect on the enzyme synthesis. Two investigators, Bonnarme and Jeffries (1989), reported that, in the culture location without Mn^{+2} ion, lignin peroxidase activity measured both activity and activity 2.5 times higher than those detected in the early days of incubation, but MnP production remained very low. On the other hand, MnP activity was measured 24 times more in the culture medium with 11–15 ppm (amount found in the base medium) Mn^{+2} ion (Bonnarme and Jeffries 1989).

Addition of veratryl alcohol to the incubation environment and *Chrysosporium purinosum*, which stimulates the synthesis of manganese peroxidase as well as ligninase, have been shown in studies conducted with *C. versicolor* ve *P. ostreatus*.

The effect of oxygen on the lignolytic systems of white saprophytes has been extensively studied. In this regard, some researchers suggested that the stimulating effect of oxygen on the lignolytic system is caused primarily by the increased oxygen synthesis of veratryl alcohol. In the static and submerged culture of *P. chrysosporium* white rotifer acculturation, periodic or continuous administration of oxygen bubbles shows stimulating effect of MnP and ligninase synthesis.

It has also been understood that the buffers used in the preparation of fattening sites have an effect on lignolytic enzyme systems. For example, when cultivation of *P. chrysosporium* fungus was carried out in environment where tartrate or oxalate buffers were used, it was reported that MnP and LIP activity could not be detected in these environments. In addition, the use of acetate buffer in place of the dimethyl succinate buffer has also been shown to produce quantitative and qualitative changes in extracellular MnP and LiP production.

The pH of the incubation environment has an important role in the synthesis of MnP as well as in other lignolytic enzymes. The pH changes slightly compared to the white rot fungus cultured. It has been determined that the mentioned pH limit

is between 4.5 and 5.5 in experiments where many white rot fungi are used (Elisashvili 1993).

Lignin peroxidase or diarylpropane oxygenase (ligninase; EC not known) was first discovered by two groups of investigators in the culture fluid of *Phanerochaete chrysosporium* (Karam and Niceli 1997). The enzyme is a hemoprotein with a molecular weight of 42 kDa. Ligninase (LiP) is synthesized generally from the white rot fungi during the secondary metabolites of microorganisms as extracellular lignin catalyzes reactions such as peroxidase, oxidation, hydroxylation, and breakage of aromatic bonds. The enzyme does not only succeed to the depolymerization of natural and synthetic lignin (Elisashvili 1993) but also mineralize many polycyclic aromatic and phenolic compounds with various recalcitrant aromatic compounds. All these reactions occur by the oxidation of the lignin component by the enzyme with the direction of the single electron oxidation via H_2O_2 (Karam and Niceli 1997). In the ligninase activity, veratryl alcohol is used as the model compound. Veratryl alcohol is a secondary metabolite and converted to aldehyde by ligninase (Leisola et al. 1987).

In studies conducted with *P. chrysosporium* white rot fungus, nutritional factors in the reproductive environment were found to be very important for observing lignolytic activity. In studies using methods based on separation of ^{14}C-labeled CO_2, investigators were able to detect the activities of *P. chrysosporium* lignolytic enzymes in high quantities in environments where the amount of nitrogen was very low. This result once again showed the presence of the suppressive and regulatory effect of nitrogen on the lignolytic activity. It has also been found in studies with different microorganism, in which the amount of carbon source and the variety play an important role in the production of lignolytic enzymes. In a series of experiments conducted by Jager et al. (1985), they found that lignolytic activity was increased in cultures where confounding and various detergents (such as Tween 80) were added (Higuchi 1987). Leisola and Fiechter (1985) have shown that lignolytic activity is induced by the addition of veratryl alcohol to the incubation environment. Also three more important functions were found besides induction of veratryl alcohol (Higuchi 1987): stabilization of ligninase enzyme, activation of electron transfer processes, and occurrence of activated oxygen species.

In addition to these factors in LiP production, it has been supported by many studies repeated that the effects of trace elements are not to be underestimated. Initial studies of optimal mineral nutrient balance have shown that ligninase activity is increased by a decrease of the Mn^{+2} ion concentration. So according to the Bonnarme and Jeffries (1989), the LIP activity in the environment without Mn^{+2} ions contain 2.5 times more than the activity measured in environment containing Mn^{+2} at 12 ppm. The results show that the Mn^{+2} ion concentration is important; the source of the Mn^{+2} ion is not important according to this study (Bonnarme and Jeffries 1989).

The regulatory effect of the presence of oxygen on lignolytic activity has been examined by many investigators. According to some investigators, the stimulating effect of oxygen on lignolytic activity is due to the effect of enhancing the synthesis of veratryl alcohol. The studies show that periodically or continuously supplied

oxygen bubbles has stimulating effects on *P. chrysosporium* static, submerged culture environment. In contrast to the increase in lignin synthesis in *P. radiata* cultures containing 100% oxygen, the production of laccase was suppressed. In experiments conducted by Desorets et al. (1990), they showed that LiP was not produced in submerged cultures of *P. chrysosporium* aerated with air. The production of fungi in these conditions was achieved by producing significant amounts of free or semi-polysaccharides and by increasing the environment viscosity (in this case, the passage of oxygen and nutritional compounds to pellets or cells). LiP activity increases when oxygen bubbles that is transferred to periodic or continuous culture media at day three. In addition, continuous oxygen supplementation to cultures increases the rate of glucose absorption and leads to the formation of fasting-inducing proteases, which are secondary metabolites, leading to a rapid decrease in LiP and MnP activity. At the end of these studies, it has been understood that the level of oxygen in culture environment plays a regulatory role both in the formation of lignin-depleting enzymes and in their degradation mechanisms, as well as in the production of specific proteases and polysaccharides (Elisashvili 1993).

The application areas of these two industrial enzymes originating from microorganisms can be classified under three groups:

1. Increased biodegradation of lignocellulosic materials to intramolecular digestibility
2. The production of cellulose pulp (biopulp), which is biologically required by the paper industry
3. Color removal of high-content dye-containing waters, especially of cellulose, paint, and textile industries

1.5 Bioremediation of Olive Oil Mill Wastewater and Molasses Wastewaters by White Rot Fungi

1.5.1 Composition of Olive Oil Mill Wastewater

Olive oil extraction produces an oily phase (20%), a solid residue (30%), and a wastewater (50%) called olive oil mill wastewater (OOMW) (Borja et al. 1995). The annual OOMW production in the Mediterranean area is more than 3×10^7 m^3 (Yesilada et al. 1995). Its composition depends on maturity and variety of olive fruit and mainly on the oil extraction technology used (Tsioulpas et al. 2002) (Table 1.1). Due to its organic load, it has high pollution potential. Its biochemical oxygen demand (BOD) and chemical oxygen demand (COD) may be as high as 100 and 200 g/L, respectively (Sayadi and Ellouz 1992). Its organic compounds include sugar, tannins, polyphenols, polyalcohols, pectins, and lipids (Hamdi and Garcia 1993; Yesilada et al. 1995). Phenolic compounds give its phytotoxic and antimicrobial effects and also dark color. This dark color is mostly due to polyphenols (Yesilada and Sam 1998). OOMW contains several types of phenolics (Sanjust et al. 1991).

Table 1.1 Main physicochemical characteristics of OOMWs

Characteristics	Yesilada and Sam (1998)	Dias et al. (2004)	Fountoulakis et al. (2002)	Dhouib et al. (2005)	D'Annibale et al. (2006)	Fenice et al. (2003)
Color	108 A_{395}	21 A_{465}	–	82 A_{395}	2500 CU	32000 CU
COD	112 g/L	130.5 g/L	105.4 g/L	117 g/L	43 g/L	85 g/L
Total phenols	10 g/L	4 g/L	10.2 mg/L	–	2.9 g/L	5.5 g/L
Total sugars	–	2.1 g/L	–	–	7.5 g/L	–
Total solids	85 g/L	49.7 g/L	–	11.40%	–	45 g/L (suspended solids)
Total volatiles	53 g/L	–	–	9.3%	–	–
pH	5.3 g/L	4.7	5.4	5.4	5.4	5.3

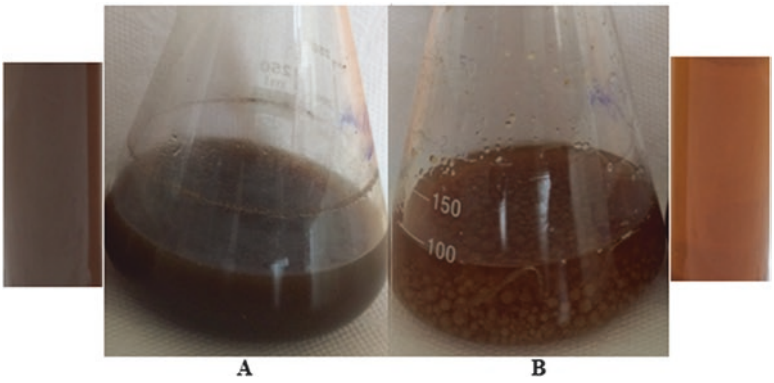

Fig. 1.4 Color of OOMW before (**a**) and after 24 h of incubation (**b**) by white rot fungal pellets

Direct disposal of OOMW to water bodies or soils causes serious environmental problems. Therefore, this wastewater should be valorized or remediated before discharged into environment. Several methods used to valorize or treat industrial wastewaters have some disadvantages. Due to its recalcitrant nature, specific biological system must be used to solve this problem.

1.5.2 Bioremediation of Olive Oil Mill Wastewater by White Rot Fungi

White rot fungi can mineralize lignin and phenolic compounds. Since the structure of phenolic compounds in OOMW is similar to lignin, white rot fungi have been tested for valorization or bioremediation of OOMW (Fig. 1.4). Their nonspecific lignolytic enzymes can degrade or transform various pollutants such as phenolic compounds. Laccases are the main enzymes responsible for phenolic compound

degradation. OOMW contains some essential elements for microbial growth. It also induces production of biotechnologically and industrially important lignolytic enzymes of white rot fungi. Because of high organic contents of OOMW, use of this wastewater as a growth medium to produce enzymes such as laccase may be the correct approach. Therefore, valorization and/or treatment of OOMW by white rot fungi or by their enzymes may be good solution. Because OOMW contains several growth inhibitors such as organic acids and phenolic compounds, biological efficiency depends on various factors such as composition and dilution of OOMW, type of bioreactors, and strains used.

Pleurotus genus includes various species with biotechnological and medical importance. Several *Pleurotus* strains such as *Pleurotus ostreatus*, *P. florida*, *P. sajor-caju*, and *P. eryngii* were grown in Erlenmeyer flasks containing olive oil mill wastewater (OOMW) under stationary conditions. All fungi reduced at least 90% phenolics within 25 days. Moreover, toxicity of OOMW on HEp2 cells significantly decreased. On the other hand, these fungi were not efficient to decolorize OOMW. However, addition of sucrose (0.5% w/v) or lactose (0.5% w/v) induced the decolorization activity, and the best results were obtained with *P. florida* as 40% and 60% in the presence of sucrose and lactose, respectively. The best result with *P. eryngii* was 40% (Sanjust et al. 1991). In another study, *P. ostreatus* was used to reduce the color, phenol content, and toxicity of 10% OOMW in liquid media. After 15 days, the decrease in the absorbance at 600 nm was 71%. Pre-grown mycelia (1 g wet weight/250 ml OMMW) reduced the phenol content (90%). Toxicity on *Bacillus cereus* also decreased sevenfold. Fungal biomass could be reused for three cycles. Therefore, this fungus was able to remove phenols and detoxify OOMW. Purified phenol oxidase enzyme could also remove up to 90% phenols extracted from OOMW but without any toxicity reduction. On the other hand, spectrophotometric and chromatographic analyses of raw OOMW incubated with phenol oxidase showed no detectable modification. It was suggested that transformation of toxic compounds follows a pathway in which phenol oxidases could play an important, but not exclusive, role (Martirani et al. 1996). Agitated cultures of *Pleurotus* spp. strains were able to grow in OOMW without addition of nutrients and pretreatment. OOMW induced the laccase synthesis of *Pleurotus* spp., and thus high laccase activity was measured in the growth medium. OOMW proportion was detected as an important parameter for laccase production. Phenolic compound removal capability of the strains was not closely associated to the laccase activity in the growth medium. Maximum phenolic removal ranged between 69% and 76% of the initial phenolic content. A decrease of phytotoxicity occurred in the treated OMW with some *Pleurotus* spp. strains. However, this decrease was not proportional to the phenolic removal (Tsioulpas et al. 2002). The bioremediation activity of *P. ostreatus* LGAM P69 and effect of this pretreatment on the subsequent anaerobic digestion of 50% OOMW were reported (Fountoulakis et al. 2002). The phenol concentration was reduced up to 78% after 21 days of growth. However, COD was not reduced as much as phenol concentration. Anaerobic digestion of OOMW was enhanced by the pretreatment of this fungus. In another study, *P. ostreatus* strains LGAM P113 and P115 grown in bioreactor batch cultures in a diluted and sterilized OOMW reduced

the phenolic content. The treatment reduced the toxicity of OOMW against the seeds of *Lepidium sativum* and *Artemia* sp. However, the toxicity against *Daphnia magna* and *Heterocypris incongruens* was unaffected by the treatment. Laccase was the sole lignolytic enzyme detected in OOMW-based media (Aggelis et al. 2003). It was reported that *P. ostreatus* could reduce the organic load, but OOMW phytotoxicity reduction was limited. It was stated in this study that the phenol reduction could not be used as sole indicator of the bioremediation of OOMW, and use of phytotoxicity and microbial toxicity assays together may provide better evaluation of OMMW toxicity (Aviani et al. 2009). In a study by Olivieri et al. (2012), dephenolization activity of the same fungus in an airlift bioreactor and in aerated flasks and under batch, continuous, and biomass recycling modes was investigated. *P. ostreatus* ATCC MYA-2306 reduced 70% phenol of raw OOMW under single batch, and high dephenolization values were obtained after recycled up to six times. Process with biomass recycling and added nutrients was detected as the most effective way for polyphenol reduction. Static batch cultures of *P. ostreatus* LGMACC 22, *P. eryngii* LGAM P66, *Ganoderma australe* IK-1600, and *Ganoderma carnosum* IK-1642 effectively reduced (71–77%) the phenolic content of 25% OOMW. While the former two decolorized OOMW by 41–44% and 60–65% after 20 days and 30 days incubation periods, respectively, the last two decolorized 40–32% after 20 days. While *G. carnosum* reduced COD by 29%, the others showed 12–19% reduction. *Pleurotus* strains were efficient in reducing phytotoxicity. However, no positive effect on phytotoxicity was detected with *Ganoderma* strains. On the other hand, bioluminescence assay showed that all fungi could reduce OOMW toxicity by approximately 5–15 compared to the control. The results indicate the suitability of such fungi in the solution of OOMW pollution problem. These results pointed out that OMW decolorization by *Ganoderma* strains was correlated to the reduction of phenols, whereas laccase activity of *P. eryngii* was correlated with the decolorization (Ntougias et al. 2012). Total phenol reduction and decolorization values obtained with *Abortiporus biennis* and *Irpex lacteus*, *G. carnosum*, and *Pleurotus* spp. were correlated with laccase and/or peroxidases activities (Koutrotsios and Zervakis 2014). *Ganoderma applanatum* Ga-20 grown in 20% OOMW supplemented with various sources such as carbon/energy and minerals at 28°C and 100/140 rpm for 10 days exhibited strong laccase (the only enzyme detected) induction and significant reduction in phenols, color, and COD amounts. Strong induction of laccase production by OOMW shows its role in bioremediation. Strong depletion of aromatic compounds with high and low apparent molecular mass fractions was also determined (Matos et al. 2007).

Bioremediation activity of the white rot fungus, *P. sajor-caju*, was also investigated. Several studies demonstrated its effective role in bioremediation process. Bioremediated OOMW by *P. sajor-caju* was reported to be a promising substrate for bioethanol production process. Erlenmeyer flask cultures were able to grow and remove phenolics in 50% OOMW without addition of nutrients. There was a notable correlation between the removal of phenolic compounds and decolorization. Ethanol production can be enhanced by the pretreatment of *P. sajor-caju* (Massadeh and Modallal 2008). In a study by Justino et al. (2009), pellets of fungi incubated at

120 rpm in a batch bioreactor, and fungi especially *Pleurotus sajor-caju*, promoted a reduction in acute toxicity of diluted OOMW on *Daphnia longispina*. It was also stated in this study that photo-Fenton oxidation might be an interesting solution, especially for color removal, before and after tertiary biological treatment. On the other hand, works of this study showed that more attention should be given to the toxicity of treated effluents. This fungus could also degrade phenolic compounds and reduce the color (60–70%) and COD and BOD values of 50% OOMW in a stirred bioreactor (Fraij and Massadeh 2015). Nogueira et al. (2015) investigated the treatability of OMW by the combination of photocatalytic oxidation with biological degradation by the pre-grown pellets of *Pleurotus sajor-caju* and *Phanerochaete chrysosporium* (Burdsall 38388). They reported that combination with a fungal treatment increased the reduction of COD and total phenolic content as well as a reduction in toxicity. The results showed that photocatalytic oxidation combined with biological treatment with fungi is appropriate for reducing COD, total phenolic content, and ecotoxicity.

OOMW contains polyphenols with different molecular mass. These compounds are responsible for the dark color, phytotoxic, and antibacterial effects (Sayadi et al. 2000). Therefore, these authors used three polyphenolic fractions (low, medium, and high) of crude OOMW and cultivated *P. chrysosporium* and also bacteria in the presence of these fractions under static and agitated conditions, respectively. The decolorization/depolymerization activity of *P. chrysosporium* ATCC 24725 was high. However, cultivation in the presence of high molecular mass polyphenols inhibited lignin peroxidase, which led to decrease in color and COD removal efficiencies. High molecular mass polyphenols were also resistant to bacterial degradation. Medium and particularly high molecular mass fractions were slightly degraded with bacteria, *P. putida* or *A. eutrophus*. COD removal with activated sludge cultures was also low. The authors concluded that high molecular mass compounds should be removed from OOMW prior to conventional treatment (Sayadi et al. 2000). *P. chrysosporium* Burdsall MI (DSM 13583) was able to grow using diluted OOMW without any supplements and to reduce color, phenol content, and COD. They hypothesized that lignin peroxidase plays a key role in biodegradation. Fungal treatment caused complete loss of toxicity on *B. cereus* (Kissi et al. 2001; Sayadi and Ellouz 1992). Sayadi and Ellouz (1992) tested the OOMW decolorization activity of static cultures of *P. chrysosporium* ATCC 24725 and proposed that lignolytic system of this fungus, especially lignin peroxidase enzyme, has a role in bioremediation of OOMW. This fungus significantly decolorized the effluent and also degraded high and low molecular mass aromatics under the conditions used. The periods of decolorization and COD removal were similar, suggesting that much COD originates from colored substances. Similarly, Sayadi and Ellouz (1995) reported the role of lignin peroxidase of *P. chrysosporium* ATCC 24725 in OOMW decolorization and high molecular weight aromatics depolymerization. Ayed et al. (2005) suggested that lignin peroxidase of *Geotrichum candidum* may be the major system responsible for the decolorization of OOMW. In another study, Sayadi et al. (1996) tested the decolorization activity of free and immobilize *P. chrysosporium* ATCC 24725 under agitated conditions and reported that no decolorization of crude

OOMW was observed when *P. chrysosporium* was cultivated as free pellets. It was reported that agitation and high molecular polyphenolics have negative effects on OOMW decolorization as well as lignin peroxidase activity. However, the use of immobilized fungus on polyurethane foam showed high decolorization activity. Therefore, immobilized organisms may be an attractive alternative for OOMW decolorization. Dhouib et al. (2005) used OOMW as a sole carbon and energy source for testing the bioremediation activity of *P. chrysosporium* DSM 6909 in a 120 L stainless steel airlift reactor composed of bubble column reactor in a semi-continuous mode. This fungus needed high oxygen for biological reactions. 0.25 vvm aeration was not sufficient for bioremediation, and only 26% toxicity removal was achieved. However, higher aeration rates gave better results, and the toxicity on *V. fischeri* decreased from 100% to 62% and 49% for the aeration rate of 1 and 1.5 vvm, respectively. On the other hand, treatment of OOMW with *P. chrysosporium* resulted in decreasing the toxic effect on anaerobic digestion. Salman et al. (2014) firstly incubated *P. chrysosporium* on PDA media containing OOMW and then investigated its crude OOMW bioremediation activity under agitated liquid culture conditions. This fungus was able to grow in OOMW without enrichment. Total phenols and phytotoxicity of OOMW were reduced by 60% and 82% after 2 weeks of treatment under agitated conditions, respectively.

It was reported that while no laccase activity was detected in the control cultures of *Phanerochaete flavido-alb*a FPL 106507, it could be detected in OOMW-containing cultures. Mn-peroxidase was the predominant enzyme in OOMW decolorized by *P. flavido-alb*a, and laccase was strongly induced in OOMW. Therefore, this wastewater influences the production of lignolytic enzymes, and laccase and Mn-peroxidase have an important role in this bioremediation (Perez et al. 1998). Similarly, Ruiz et al. (2002) stated that laccase was the main enzyme responsible for the degradation of OOMW phenolics by *P. flavido-alba. P. flavido-alba* laccase plays an important role in monomeric aromatic degradation during decolorization, dephenolization, and detoxification of OOMW. Mn-peroxidase is involved in polymeric pigment degradation along with laccase (Ruiz et al. 2002). A laccase-producing strain, basidiomycete Euc-1, removed 90% of phenols, 73% of color, and 45% of COD in agitated batch cultures containing OOMW. Laccase activity had increased sixfold at the end of the incubation period in the 20% OMW medium. There is a significant correlation between laccase production and decolorization. Therefore, the color removal was a laccase-dependent extracellular process (Dias et al. 2004). It was reported that *P. flavido-alba* FPL 106507 could decolorize OMW in submerged cultures and reduced OMW toxicity at room temperature (25 °C) in a laboratory-scale bioreactor. Color, aromatic compound, and toxicity reductions were 70%, 51%, and 70%, respectively. Mn-peroxidase and laccase enzymes were detected; however, lignin peroxidase has not been detected. It was stated that lignolytic enzymes produced by *P. flavido-alba* are capable of decolorizing OMW, reducing aromatic compounds and toxicity, simultaneously (Blanquez et al. 2002).

Panus tigrinus CBS 577.79 was tested for its bioremediation activity on filtered and centrifuged OOMWs differing mainly in their COD and total phenol contents (low or high strength wastewaters). Experiments were carried out in Erlenmeyer

flasks containing 50 mL OOMW either in the absence or in the presence of 5% sucrose and 0.1% yeast extract. Treatment was more effective on low strength OOMW. This fungus highly removed total phenols from low strength OOMW, and color removal from low strength OOMW was more extensive. The initial organic load affected the levels and time courses of laccase and Mn-peroxidase (D'Annibale et al. 2004b). The same fungus was also used to bioremediate OOMW in mechanical (stirred tank bioreactor) and pneumatically (bubble column bioreactor) agitated bioreactors. OOMW depollution in bubble column bioreactor was generally higher and faster than in stirred tank bioreactor. Agitation and aeration were important for treatment efficiency. COD reduction, phenol reduction, and decolorization were 61%, 97%, and 75%, respectively, in bubble column bioreactor. Mn-peroxidase and laccase peaks in bubble column bioreactor were achieved earlier than stirred tank bioreactor. Low aeration for good mixing and mass transfer and also less shear stress in bubble column reactor makes it as a preferred bioreactor for OOMW depollution (D'Annibale et al. 2006). OOMW (50%) supplemented with 0.5% sucrose and 0.1% yeast extract was tested as a growth medium for production of laccase and Mn-peroxidase by *Panus tigrinus* CBS 577.79 in various bioreactors such as stirred tank bioreactor, airlift bioreactor, and rotary drum bioreactor. Volumetric productivity of enzymes in airlift bioreactor was about twice that in stirred tank bioreactor. Laccase production was significantly induced in OOMW-moistened solid substrate during solid-state fermentation in rotary drum bioreactor. Liquid-state fermentation was detected more effectively than solid-state fermentation due to the unitary volumetric activity. Both airlift and stirred tank bioreactors had better volumetric productivity and adequacy for effluent utilization than solid-state fermentation (Fenice et al. 2003).

Lentinus edodes (synonym, *Lentinula edodes* SC-495), which was grown in 20% OOMW at 30 °C and 140 rpm for 12 days, was able to decolorize and reduce the organic matter and phenolic compound content of this wastewater. The maximum color and total organic carbon reduction amounts were 72% and 85% after 12 days, respectively. However, this fungus caused 45% decolorization and 70% total organic carbon reduction only after 4 days of growth. OOMW addition greatly induced the phenol oxidase and Mn-peroxidase production of this fungus. Therefore, this wastewater could also be used to improve the production of such biotechnologically important enzymes (Vinciguerra et al. 1995). Same strain immobilized on polyurethane cubes of sponge was used to biodegraded undiluted OOMW supplemented with sucrose, yeast extract, and tween 80. It was cultivated in 300 mL with the treatment time of 8 days for each batch. The cultures were purged daily with filter-sterilized oxygen. The maximum amount of color removal was detected at end of first and second cycle as 75% and 72%, respectively. Depolymerization of the high molecular weight fraction was detected at the end of the second and third cycles. There is no correlation between OOMW decolorization and laccase and Mn-peroxidase production. Total organic carbon (TOC), total phenols, and ortho-diphenol were reduced. TOC removal was 73% in the first batch. In the second and third cycles, the removal values were 88% and 75%, respectively. The maximum phenol reduction amounts were 84%, 89%, and 78%, respectively, in three batches.

Therefore, it was possible to remove phenolics, aromatic components and color, and depolymerize high molecular weight fraction by this system (D'Annibale et al. 1998). OOMW-preadapted *L. edodes* SC-495 culture was also able to decolorize undiluted OOMW without any supplements, during incubation at 28 °C and 80 rpm for 10 days. This fungus reduced the total phenols by about 88% in 240 h. Its maximum color and COD removal values were 70% and 67%. Significant levels of laccase activity (2.8 IU/mL) were produced under this condition. However, no Mn-peroxidase activity was detected. OOMW phytotoxicity was also significantly reduced by these cultures (D'Annibale et al. 2004a). Several *L. edodes* strains were tested for their OOMW (10%) bioremediation activities, and *L. edodes* Le119 was reported as the most effective one. This fungus showed 65% and 75% color and total phenol reductions. Total phenol degradation was correlated with decolorization. Laccase was the main enzyme detected under this condition (Lakhtar et al. 2010).

White rot fungi *Trametes trogii* ATCC 200800 and *Trametes versicolor* ATCC 200801 are able to bioremediate various wastewaters. These fungi can produce high amounts of laccase. Yesilada et al. (1995) tested the OOMW (30% without any supplement) bioremediation capacity of *T. trogii* ATCC 200800. This fungus showed 31% and 38% color removal and 77% and 72% phenol removal under the static and agitated condition, respectively. In a study by Yesilada et al. (1998), initial COD concentration, agitation, and amount of inoculum were detected as the important parameters for OOMW (without any supplement) biodegradation activity of *T. trogii* ATCC 200800 and *T. versicolor* ATCC 200801. While *T. versicolor* ATCC 200801 removed 63% COD, 90% phenol, and 65% color within 6 days, *T. trogii* ATCC 200800 removed 70%, 93%, and 81%, respectively. High biodegradation yields were also obtained with the alginate-immobilized forms of these fungi. These two fungi produced high amounts of laccase enzyme during bioremediation studies. *T. trogii* ATCC 200800 and *T. versicolor* ATCC 200801 were efficient in reducing toxicity on *P. aeruginosa* and tadpoles, respectively (Yesilada and Sam 1998; Yesilada et al. 1999). Dhouib et al. (2006) tested the OOMW detoxification activity of white rot fungi in the presence of a complex activated sludge in a 25-L airlift reactor during batch incubation. COD removal efficiency (65%) of the fungi+activated sludge was better than the COD removal efficiency (34%) of activated sludge alone. Activated sludge + *T. versicolor* highly reduced the major OMW phenolic monomers such as tyrosol and hydroxytyrosol. This reduction was less important in OMW pretreated with activated sludge alone. Moreover, anaerobic digestion of the OMW pretreated with activated sludge+white rot fungi showed higher biomethanization yields than that pretreated with activated sludge only. Ergul et al. (2009) reported that agitated cultures and static cultures of adapted *T. versicolor* FPRL 28A INI could reduce 78% and 39% total phenolics, respectively, in undiluted OOMW without addition of supplements. Laccase and Mn-peroxidase activities were observed in the culture media but no lignin peroxidase. This value was 70% in continuously stirred tank reactor. However, the decolorization was low in both shake flasks and continuously stirred tank reactor. Adapted *T. versicolor* could dephenolize OMW efficiently without any dilution, pretreatment, or without any addition of nutrients.

Coriolopsis polyzona MUCL 38443 reported as good choice for OOMW decolorization. Lignin peroxidase enzyme of *Coriolopsis polyzona* had a predominant role in OOMW decolorization. Lignin peroxidase induction condition gave a better decolorization than when lignin peroxidase was repressed. Laccase was rapidly produced. Therefore, this enzyme could play a predominant role during the initial stage of decolorization, while lignin peroxidase could be involved in the following stages (Jaouani et al. 2006). Neifar et al. (2012) compared the OOMW bioremediation activity of the *Pycnoporus coccineus* MUCL 38527 and *Coriolopsis polyzona* MUCL 38443 immobilized on polyurethane foam with the activity of free cells. COD and color removal activity immobilized cells were comparatively higher than that by free cells. Immobilize cells showed better COD decreases during three consecutive batches. The reusability of immobilized cells of *P. coccineus* was higher than *C. polyzona*. Results suggested that immobilized *C. polyzona* and especially immobilized *P. coccineus* might be applicable to a large scale for the removal of color and COD of OOMW.

White rot fungus *Cerrena* sp. (identified by Mann et al. (2015) as *C. consors*) was able to reduce phytotoxicity on *Lepidium sativum* and phenols in 50% OOMW (Mann et al. 2010). This fungus could also produce laccase in flasks containing the 50% OOMW. Total phenol reduction activity of crude laccase from *C. consors* in a solution of phenolic acids was enhanced when 1% OMWW was added. OMWW, a good substrate for laccase production, is reported as an apparent source of laccase mediators (Mann et al. 2015).

Hericium erinaceus HE 4514 had high color and phenolic compound toxicity reduction. Its decolorization activity was correlated with phenol reduction ability. Moreover, dephenolization was also correlated with laccase activity and biomass production. High phytotoxicity reduction values such as 98% and 52% for OOMW 12.5% and OMW 25%, respectively, were obtained in large dilutions. In the case of OMW 50%, the detoxification value was only 7% (Koutrotsios et al. 2016).

1.5.3 Bioremediation of Olive Oil Mill Wastewater by Laccase

The advantage of the enzymatic treatment compared with the use of fungal cultures is principally related to a shorter effluent treatment period. It was concluded that the fungal laccases are the most easily produced lignin-modifying enzymes or the ones with the highest stability in OOMW, and they therefore may participate in OOMW bioremediation. Growth inhibition zone of *B. megaterium* showed poor correlation with the amount of total phenols, laccase activity, and fungal growth (de la Rubia et al. 2008). On the other hand, it was also concluded that, at least in part, the detoxification of OOMW could be nonenzymatic. Laccases are extracellular enzymes being able to oxidize different phenolic compounds in OOMW. This enzyme was purified from *Pycnoporus coccineus* MUCL 38527 and used to degrade OOMW fractions. The results of OOMW treated with purified laccase indicated that laccase plays an important role in the bioconversion of this wastewater by *P. coccineus*

(Jaouani et al. 2005). OOMW treatment with laccase enzyme purified from solid-state culture of *L. edodes* also resulted in 65% total phenols and 86% ortho-diphenol reductions due to their polymerization. When OOMW at a 1:8 dilution and also at a 1:2 dilution was treated with laccase, phytotoxicity was also reduced and germinability was increased by 57% and 94%, respectively. Reduction in germinability inhibition by laccase suggested that total phenols are the main determinants responsible from OOMW phytotoxicity. However, undiluted OOMW treated with laccase suppressed seed germination (Casa et al. 2003). Immobilize laccase have many advantages as they can be repeatedly used. Therefore, immobilized laccase may offer an alternative approach for bioremediation. Purified laccase from solid-state cultures of *L. edodes* SC-495 was immobilized on chitosan, and then, it was used to bioremediate OOMW under batch or batch with recirculation mode in a column operated as a fluidized bed reactor. It reduced total phenols and ortho-diphenols by 67% and 72% after 24 h incubation under batch condition. The treatment in column system performed on six consecutive batches resulted in significant total phenols, ortho-diphenols, and color reductions especially in the first four batches. This proves the responsibility of laccase for bioremediation. The growth suppressive effect of eight-fold diluted OOMW appeared to be completely removed when previously treated with this immobilized laccase for 24 h (D'Annibale et al. 1999). The same group also tested the phenolic removal efficiency of oxirane-immobilized *L. edodes*-purified laccase. Immobilized laccase showed significant total phenols and ortho-diphenols reduction at the end of eight consecutive batches; each batch operation time was 2 h, in a fluidized bed operated in a batch with recirculation mode. This immobilized laccase was stable in removing the phenolics (D'Annibale et al. 2000). In another study, purified laccase from *Pycnoporus coccineus* MUCL 38527 immobilized into Ca-alginate beads and reused for five consecutive batches showed each time a total phenol reduction of about 45% (Alaoui et al. 2013).

1.5.4 Valorization of Olive Oil Mill Wastewater (OOMW) by White Rot Fungi

Olive oil mill wastewater contains high amounts of organic and inorganic compounds. Therefore, biological valorization of OOMW as a substrate may be another treatment method. Kahraman and Yesilada (2001) reported that these fungi could produce laccase in diluted OOMW without any supplement and addition of cotton stalks enhances the laccase production. Therefore, OOMW could be used as a substrate for enzyme production. Liquid fermentation by pre-grown pellets and solid-state fermentation may be other valorization methods. Laccase production activity of the pellets of *T. trogii* ATCC 200800 and *T. versicolor* ATCC 200801 in OOMW was investigated under repeated batch process. It was reported that this wastewater provides necessary nutrients, and pre-grown pellets could be used repeatedly to produce laccase in OOMW media during repeated-batch process under optimized conditions. *T. trogii* ATCC 200800 and *T. versicolor* ATCC 200801 pellets were also

able to remove 33% and 28% COD during this valorization process. Given their reusability and maintenance of high- and long-term enzyme production abilities, pre-grown pellets have great benefits (Apohan and Yesilada 2011). Laccase production activity of these two fungi was also investigated on wheat bran moistened with OOMW under solid-state fermentation conditions. *T. versicolor* ATCC 200801 culture reported as the more effective laccase producer than *T. trogii* ATCC 200800 without inducer. It was concluded that lignocellulosic compounds moistened with OOMW could be used as a solid substrate for fungal growth and laccase enzyme production, and this method could also be an alternative method for valorization and bioremediation of wastewaters (Boran and Yesilada 2011). Yurekli et al. (1999) used 20% OOMW as a growth media for plant growth hormone (gibberellic acid, abscisic acid, and indole-3 acetic acid) production by *T. trogii* ATCC 200800 and *T. versicolor* ATCC 200801. These fungi were able to produce high amounts of plant growth hormones and to bioremediate OOMW under agitated condition. Valorization of OOMW as a low-cost growth medium for laccase production by white rot fungi is an environment-friendly solution. *T. trogii* could produce laccase in OOMW-based media, and the growth rate of this fungus on OOMW was depending on phenolic compounds concentration. Because high amount of phenolic compounds in undiluted OOMW inhibits the growth and OMWW is also insufficient to support growth, a light dilution of OOMW and correction of the C:N ratio by urea addition transformed this wastewater to a suitable culture medium. *T. trogii* was able to overcome this toxicity, and its laccase production reached 25,120 U/L in a OMW-based medium (80%) containing 2 g/L of urea corresponding to initial BOD_5 and COD values of 18.4 and 46 g/L, respectively (Chakroun et al. 2009).

1.6 Composition of Molasses Wastewaters

Sugar industry by-product molasses contain about 52% sugars and other various compounds and minerals. It can be used as a substrate for various fermentation processes, such as alcohol, baker's yeast, and amino acid fermentation (Yesilada and Fiskin 1995a). These fermentation processes generate wastewater with a high organic load and dark color (Kahraman and Yesilada 2003) (Table 1.2). A traditional ethanol plant generates between 9 and 15 l of vinasse per liter of ethanol (Espana-Gamboa et al. 2015). Vinasse (molasses distillery wastewater) is a dark brown liquid with high organic content and acidic pH (Zayas et al. 2007). Its color is from brown polymer melanoidins that are formed in Maillard reactions between amino acids and carbohydrates. Melanoidin pigments are one of the major pollutants (Raghukumar et al. 2004). Sugarcane vinasses are complex wastewaters because of the presence of recalcitrant compounds such as melanoidins and phenolics (Tapie et al. 2016). The COD and BOD values of these wastewaters may be as high as 100 and 80 g/L, respectively. When discharged without treatment, these types of colored wastewaters having high organic load negatively affect the photosynthetic activity and dissolved oxygen concentration. Because of high organic content of vinasse, it

Table 1.2 Main physicochemical characteristics of vinasse

Characteristics (g/L)	Yesilada and Fiskin (1995a)	Espana-Gamboa et al. (2015)	Gonzalez et al. (2000)	Potentini and Rodriguez-Malaver (2006)	Aguiar et al. (2010)	Sun et al. (2013)
COD	73	110	55.5	40	42	171
Total phenols	–	10.9	–	–	–	–
Total sugar	21	–	–	25	–	133
Reducing sugar	–	–	–	–	0.1	16
pH	4.40	4.39	4.1	4.4	3.95	4.16

may cause eutrophication and thus hyper-eutrophication in aquatic systems. Vinasse in large quantities can saturate soil and contaminate nearby water bodies. It also acidifies soil and affects agricultural crops. Therefore, it has a high pollution potential. Thus, it must be valorized or treated before discharged into the environment. Various processes such as physical, chemical, or biological treatment (aerobic and anaerobic), either alone or in combination, have been tested to solve the pollution problem of vinasse. Conventional treatments such as activated sludge treatments are inefficient to decolorize this type of melanoidin-containing wastewaters (Miyata et al. 2000; Dahiya et al. 2001). Phenols (8000–10,000 mg/L) and melanoidins may also show toxic or inhibitory activity on anaerobic microorganisms in an anaerobic process (Espana-Gamboa et al. 2017).

1.6.1 Biodegradation of Molasses Wastewaters and Melanoidins by White Rot Fungi

There is a need to develop alternative biotechnological processes to bioremediate molasses wastewaters. Because white rot fungi produce various extracellular enzymes such as laccase, lignin peroxidase, and manganese peroxidase that are capable of degrading various environmentally persistent xenobiotics and organopollutants, use of these fungi for elimination of phenolic compounds and melanoidins may be the best option (Espana-Gamboa et al. 2015; Tapia-Tussell et al. 2015). Molasses wastewater bioremediation activity of white rot fungi has been studied for more than 40 years. These fungi have also been studied in terms of their bioremediation and enzyme production abilities (Apohan and Yesilada 2017; Espana-Gamboa et al. 2017; Aguiar et al. 2010).

Trametes strains have high bioremediation activity. Watanabe et al. (1982) found that *Coriolus* (*Trametes*) sp. No. 20 could decolorize (80%) distillery waste liquid under agitated conditions. Intracellular sorbose oxidase was reported as a responsible enzyme for melanoidin-decolorizing activity. It was considered as having the most important role in melanoidin decolorization. In another study, various *Basidiomycetes* were tested for their molasses pigment (wastewater from baker's yeast factory, treated with activated sludge) decolorizing activity, and *C. versicolor*

Ps4a was determined with high decolorization activity under agitated conditions. Addition of nitrogen and carbon sources showed that peptone (nitrogen source) and glucose, maltose, and sucrose (carbon sources) were the most efficient for decolorization. Decolorization activity detected was due to intracellular enzymes (Aoshima et al. 1985). Ohmomo et al. (1985) investigated molasses wastewater (from baker's yeast factory, treated by means of methane fermentation and with activated sludge) decolorization activity of the mycelia of the same strain during continuous decolorization process. Added glucose and peptone were necessary to maintain the decolorization activity. Continuous decolorization in a bubbling column reactor with pellet-type mycelia showed a decolorization yield of approximately 75% in only 20 h under the optimum conditions. Immobilized mycelia into alginate beads showed an almost constant decolorization yield (66%) during continuous decolorization for 16 days. On the other hand, it was reported that crude culture filtrate of *T. versicolor* DSM 3086 with melanoidin decolorization activity is a Mn-dependent enzymatic activity (Dehorter and Blondeau, 1993). Liquid cultures of *T. versicolor* ATCC 200801 and *T. trogii* ATCC 200800 could efficiently decolorize vinasse. They showed 75% and 62% decolorization values, respectively, under optimum conditions (Yesilada and Fiskin 1995a). These two fungi were also able to remove 43% and 32% COD and showed high laccase and peroxidase production abilities (Yesilada and Fiskin 1995b). Bioremediation activity of *T. versicolor* pre-grown pellets on wastewater from alcoholic fermentation was tested in batch mode at 30 °C and 200 rpm. 82% and 77% decolorization and COD removal values were obtained under the best conditions (sucrose 3 g/l, $KHPO_4$ 1 g/l, and initial pH of 5) (Benito et al. 1997). Kumar et al. (1998) reported that carbon source was necessary for growth of melanoidin decolorization of liquid cultures of *C. versicolor* and *P. chrysosporium* ATCC 24725. However, lack of additional nitrogen source had no dramatic effect on growth or decolorization. Increased wastewater concentrations generally reduced color and COD removal. Compounds such as gallic and vanillic acid may be responsible for this inhibitory effect. It was concluded that this problem might be solved by combining this wastewater with different food processing or agricultural effluents as an additional source. *C. versicolor* was more efficient in both color and COD reduction. Maximum color and COD reduction values by this fungus were 71% and 90%, respectively, in 6.25% wastewater. However, these values decreased at higher concentrations (12.5% and 25%). Miyata et al. (2000) proposed the participation of *Coriolus hirsutus* IFO 4917 extracellular Mn-independent peroxidase and Mn-peroxidase as the key enzymes in melanoidin decolorization. Gonzalez et al. (2000) reported that *Trametes* sp. I-62 was able to remediate 20% molasses wastewater and produce laccase alone. Color and COD removal values after 7 days were 73% and 62%, respectively. Under this condition, 35-fold increase in laccase production was obtained. It was postulated that the results strongly suggest the role of extracellular laccase in decolorization of melanoidins from distillery vinasse. Another study of Gonzalez et al. (2008) showed that molasses wastewaters and molasses melanoidins induce gene expression of laccases from *Trametes* sp. I-62. Flask cultures of *Trametes* sp. I-62 were grown in 20% molasses wastewater at 28 °C and 100 rpm. Neither LiP nor MnP activities could be detected in the cultures

with effluent or in the controls during the 16 days of the experiment, under the assayed conditions. Laccase was the only detectable lignolytic activity produced under these conditions. Levels of this enzyme in the medium supplemented with effluent were always significantly higher than those of the controls. Decolorization of 77% and COD reduction of 71% with respect to the initial values were achieved at the end of the experiment. Both the complete effluent and isolated molasses melanoidins caused an increase in extracellular laccase activity. Results strengthened the role of laccases in MWW color reduction. Complete molasses effluents and melanoidins selectively induced lcc1 and lcc2 laccase gene transcription. Results indicated a close relationship between decolorization of MWW and selective induction of laccase activity in *Trametes* sp. I-62. Flask cultures of *T. pubescens* MB 89 were able to remove 79% COD, 80% total phenols, and 71% color of the distillery wastewater. Laccase enzyme was also produced in this media. The highest laccase activity in flask cultures was 4.6 units/L, while the activity in a 50 L bubble lift reactor peaked was 13 U/L (Strong and Burgess 2007). Color of wine distillery wastewater is generally due to phenolic compounds, although in some distillery wastewaters, such as those derived from molasses, brown color is from melanoidins. Therefore, flask cultures of *Trametes pubescens* MB 89 were also used to bioremediate various wine-related wastewaters at 28 °C and 150 rpm. Fungal treatment improved the quality of the wastewaters and resulted in 83%, 87%, and 88% COD, phenolic compounds, and color removals, respectively. Laccase activity greater than 1500 U/l was obtained in all wastewaters, with a maximum of 8997 U/L. Crudely purified laccase reduced the total phenolic compounds (61%) but did little to improve the color of the wastewaters. The complete fungal system was found to be superior to enzymatic treatment alone (Strong and Burgess 2008). Tapia-Tussell et al. (2015) investigated the bioremediation activity of *T. hirsuta* Bm-2 and reported the highest laccase production corresponding to a high vinasse discoloration value. This suggested that *T. hirsuta* Bm-2 laccase overproduction might play an important role in vinasse discoloration. Espana-Gamboa et al. (2015) tested the sterilized hydrous ethanol vinasse (10%) bioremediation activity of *T. versicolor* ATCC 42530 pellets in an air-pulsed bioreactor and found that continuous operation removed 80% phenol and 60% COD. It was reported that pellets of this fungus successfully removed phenolic compounds and COD of unsterilized hydrous ethanol vinasse during continuous operation in a fluidized bed bioreactor. Coupling of fluidized bed bioreactor with upflow anaerobic sludge blanket gave a better quality of effluent and higher methane content in biogas. No correlation was detected between laccase activity and phenol degradation, suggesting the possibility of other enzymes in phenol degradation activity of this fungus (Espana-Gamboa et al. 2017).

Strains of *Phanerochaete* were also investigated for their bioremediation abilities. *P. chrysosporium* ATCC 24725 also decolorized molasses spent wash (MSW) (6.25% v/v) effectively in the absence of peptone and in the presence of glucose (25 g/L) by 85% after 10 days at 150 rpm. Decolorization was 49% in the absence of either glucose or peptone. Increase in the wastewater concentration greater than 6.25% decreased the decolorization ability. Immobilized cells into alginate beads resulted in a much more rapid decolorization of the wastewater than with free cells

(Fahy et al. 1997). FitzGibbon et al. (1998) reported that growth rate of *G. candidum* and *P. chrysosporium* ATCC 24725 increased with increasing concentrations of molasses spent wash up to a maximum at 50%. However, growth rate of *C. versicolor* was inhibited above 5%. In another study, *Phanerochaete chrysosporium* JAG-40 was able to decolorize synthetic and spent wash melanoidins up to 80% in 6 days at 30 °C and 150 rpm. Larger molecular weight fractions of melanoidin were decolorized more rapidly than small molecular weight fractions. Inoculum size was important for decolorization, and 5% w/v (dry weight) mycelial suspension was found optimum for maximum decolorization in melanoidin medium supplemented with glucose and peptone. However, higher concentrations producing more fungal biomass did not improve the decolorization. Best sugars for decolorization were glucose and glycerol. Organic nitrogen sources were important for decolorization. Peptone was the best for decolorization. Culture filtrate of this fungus showed 40–50% decolorization of synthetic melanoidin (Dahiya et al. 2001). Temperature is an important parameter for bioremediation activity of *P. chrysosporium.* Bioremediation activity of *P. chrysosporium* in ethanol production wastewater (vinasse) at 39 °C was compared with its activity at room temperature. The activity was better at 39 °C. While the COD, total phenol, and color removals were 48%, 55%, and 45% at 25 °C, these values were 54%, 59%, and 57%, respectively, at 39 °C (Potentini and Rodriguez-Malaver 2006). It was concluded that enzymes (laccase, lignin peroxidase, and Mn-peroxidase) of *P. chrysosporium* BW808 (MTCC 787) have a key role in the decolorization of melanoidins, and this decolorization was closely related to the composition and pH of the medium (Saoji and Khan 2015).

White rot fungus, *Flavodon flavus* NRRL 30302 immobilized on polyurethane foam removed 60% and 73% of the color from 10% diluted wastewaters of molasses-based alcohol distilleries by 60% within 5 and 7 days, respectively. Similar percentage of decolorization was achieved within days 3–5 by using free or immobilized fungus. Immobilized fungus could be effectively used for a minimum of three cycles repeatedly. Reduction in color of high molecular weight fractions after 5 days was proportionately higher than the low molecular weight fractions. Furthermore, fungus totally removed the toxicity of this wastewater on fish *Oreochromis mossambicus*. Polycyclic aromatic hydrocarbon (benzopyrene) was detected in this wastewater, and this fungus reduced benzopyrene by 68% by day 5. Immobilized fungus remained viable up to 75 days. The author proposed that hydrogen peroxide produced as a result of glucose oxidase activity might act as a bleaching agent on this wastewater (Raghukumar et al. 2004).

Chairattanamanokorn et al. (2005) compared the alcohol distillery wastewater decolorization activity of free and immobilized *Pycnoporus coccineus* at 43 °C and 100 rpm. Immobilized fungi on polyurethane foam removed 3.2 and 1.5 times as much total phenol and color than the free mycelia. COD removal was about 17% and 19% for free and immobilized mycelia. Moreover, immobilized mycelia removed nearly 50% more color than did free mycelia. Activities of enzymes secreted by immobilized mycelia were 3.2, 2.5, and 18.9 times as high as by free mycelia for MnP, MIP, and laccase, respectively. Increase in the removal of phenol

and color with increasing MnP activities suggested the major role of Mn-peroxidase in this bioremediation activity.

P. ostreatus was also reduced 83%, 87%, 92%, 83%, and 72%, color, COD, BOD, total suspended solids, and volatile suspended solids of sugarcane vinasse, respectively, in a fixed-bed bioreactor (Tapie et al. 2016).

1.6.2 Valorization of Molasses Wastewaters

Laccase is biotechnologically and industrially important enzyme. Therefore, its production using cheap and natural wastes is important. Vinasse could be used as a safe and cheap substrate for laccase production by *C. versicolor* (Yesilada et al. 1991). Kahraman and Yesilada (2001) found that vinasse concentration was important for laccase production by these two fungi and the increase in wastewater concentration significantly enhanced the laccase activity. High amounts of laccase enzyme could be obtained by the use of vinasse as moistening agent during solid-state fermentation. Copper was reported as a more effective laccase inducer than 2,5-xylidine in a solid-state fermentation (Boran and Yesilada 2011). Immobilized fungi (*T. trogii* and *T. versicolor*) either into alginate beads or on pinecone produced this enzyme, repeatedly and successfully, in vinasse media (Apohan and Yesilada 2017). Various factors can induce the laccase production of white rot fungi. Strong (2011) tested the effect of several factors on laccase production of *Trametes pubescens* MB 89 at 150 rpm and 28 °C and reported that pH 5; various carbon sources such as fructose, glucose, sucrose, and cellobiose; and peptone all improved laccase production. The highest laccase activities were detected when the combination of copper, 2,5-xylidine, and glucose was added in wastewaters. Moreover, multiple doses of 2,5-xylidine significantly induced laccase synthesis. *Coriolus hirsutus* was able to produce laccase enzyme in media containing 47% molasses distillery wastewater and 0.5% urea. Laccase production was 2198 U/mL under optimized conditions. Furthermore, the removal of COD and color were 63% and 42% after fermentation. This simultaneous treatment of wastewater and production of valuable products is an environmentally friendly and economical approach (Sun et al. 2013).

Yesilada et al. (1990) reported the abscisic acid production ability of various white rot fungi (*Polyporus versicolor*, *Pleurotus florida*, and *Pleurotus ostreatus*) in vinasse media. Enhanced levels of gibberellic acid, abscisic acid, and indole acetic acid production with white rot fungi were also reported by Yurekli et al. (1999).

It was reported that vinasse, in combination with bagasse, could be reused as a supplement to the production for these enzymes as well as the production of fungal biomass (Aguiar et al. 2010). According to Ferreira et al. (2010), *P. sajor-caju* CCB 020/vinasse can be utilized as a bioprocess for color removal and degradation of complex vinasse compounds. Treated vinasse could be utilized as reused water, laccase, and Mn-peroxidase production and for fungal biomass production with a high nutritional value. In a study by Ferreira et al. (2011), *P. sajor-caju* CCB 020 removed

99%, 83%, and 75% of the color, COD, and BOD of undiluted vinasse under submerged fermentation at 28 °C for 15 days. There was also reduction in other analyzed parameters, such as phenols (98%), total suspended solids (98%), phosphate (86%), calcium (70%), and reducing sugars (34%). Toxicity of vinasse was also reduced after fungal treatment. The same group concluded that systemic use of vinasse and *P. sajor-caju* CCB 020 could be applied in the process of color removal and degradation of the complex compounds found in vinasse promoting detoxification and improving its quality for a potential use as recycled water. In another study, *P. sajor-caju* CCB 020, *P. albidus* CCB 068, *P. ostreatus*, and *P. flabellatus* CCB 396 showed satisfactory mycelia growth in vinasse (alcohol fermentation wastewater) without chemical changes at 28 °C, 150 rpm for 5 days. The lyophilized mycelia were used as a complementary diet for *Danio rerio* fish. The results showed that these mycelia can be used as a dietary supplement for fishes (Sartori et al. 2015).

1.7 Nanotechnological Approaches to Bioremediation

The word "nano" comes from the Greek word root and is used to mean dwarf. A nanometer (nm) has a length of about one billionth of a millimeter, and theoretically the millimeter is about a millionth of a length. When we make a comparison with human hair, we have approximately one-tenth of the hair's weight. If we compare the world with a soccer ball, there will be no wrong comparison without expressing nano size. The term nanotechnology is used to express the smallest units of atoms and molecules and to control the substance by its atomic size. It involves the work of creating new substances by placing atoms on the atom and changing the molecular structure of the existing substances. Nanotechnology has also given a new perspective to the treatment technology. Especially in the field of water treatment, it is possible to collect these environmentally friendly techniques under the titles of photocatalysis and nanofiltration (Prasad et al. 2014, 2017).

Photocatalysis is the substance that causes some reactions with light, just like chlorophyll in photosynthesis. When exposed to light, it is active throughout the day as it is in the photosynthesis process. The most powerful and inexpensive photocatalyst is titanium dioxide. It is this function that titanium dioxide is a semiconductor metal. Titanium dioxide is found in a different structure in almost all white colored objects that we see in our environment. It is neither chemically nor biologically active. It prefers not to enter the reaction causing it to remain in the continuous environment and perform the cleaning process. Even though it is very active with light, the light can't shred it. Photocatalytic reaction is the electrochemical energy transfer of the light energy of the photons through a catalyst. In short, the photocatalyst is a semiconductor where strong oxidation occurs on the surface through the light energy. The basic function of photocatalysis is to increase the rate of reaction by reducing the activation energy.

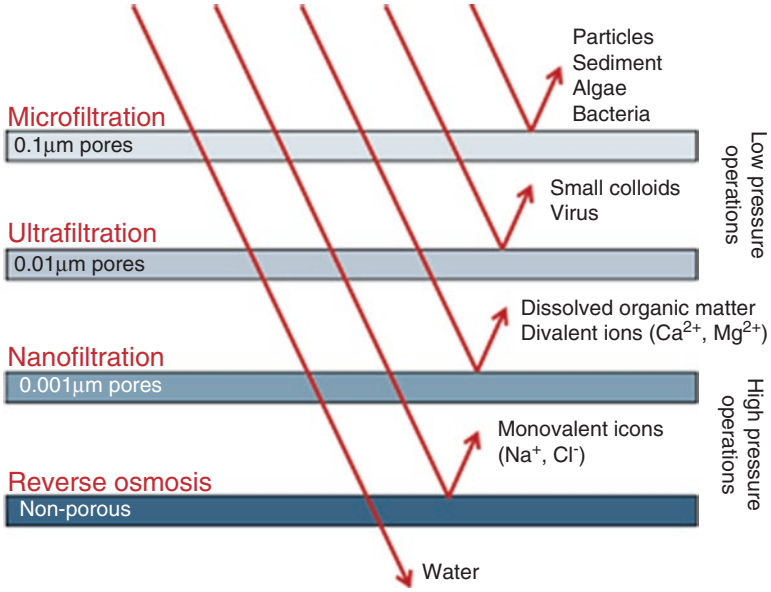

Fig. 1.5 Water filtration system

In recent years, it has been determined that the most effective alternative method is photocatalysis. This method involves the conversion of harmful substances into a non-harmless material (water, carbon dioxide, mineral salts) by splitting the harmful substances in the wastewater with a semiconductor activated by UV light.

The industrial pollution and the atmospheric layer cause more and more damage to the environment every day, and it has become a necessity to continuously clean up this pollution by developing new technologies. This cleanliness should not cause any harm to the environment; it should be a completely clean chemical technology. Bad odors, pesticides in the water, bacteria, nicotine, toxins, isotonic nitrogenous compounds, and even viruses can be cleared by this mechanism. Only organic impurities are cleaned by this mechanism. The necessity of withdrawing harmful compounds in the air to very low limits has led to the idea that even the air in the streets, bus stops, and tunnels can be cleaned with this mechanism.

Nanofiltration systems are members of the membrane technology class (Fig. 1.5). Membranes with similar physical structures, such as reverse osmosis membranes, are found but differ in terms of function and operating principle from reverse osmosis. Nanofiltration membrane allows only monovalent ions such as sodium and chloride to pass, but it removes divalent and larger ions from the water.

Concentrated lactose can be used in dairy processes or cooling towers, in hardness removal in industrial treatment systems, or in caustic recovery processes.

Applications of nanofiltration membranes are color removal, carbon removal, water-softening systems, and metal removal.

1.8 Conclusion

Fungi can produce unusual beneficial enzymes that have abilities in performing complex chemical and biological reactions. These phenol oxidase and peroxidase group enzymes such as laccases are gaining interest owing to their high capability in detoxification, high selectivity, and efficiency. The use of these kinds of enzymes has become more attractive because of their ability in converting pollutants into healthy compounds especially in bioremediation. Not only the fungal enzymes but also fungal polymers have many applications in textile, pharmacy, medicine, cosmetic, nanotechnology, etc. The biopolymer industry has been improving and getting more attraction in these days due to their antitumor, anti-hepatitis, anti-HIV, and antiviral properties.

Bioremediation uses nontoxic chemicals and organisms for removing and neutralizing contamination from industrial waste and other harmful sources like olive oil mill wastewater just as our chapter focuses. Effective and harmless bioremediation in reduction of toxic sources and heavy metals allows preferable and ecofriendly disposal of OMWW. The gaining interest in using fungal enzymes and polymers for many technological applications includes bioremediation and filtration, and catalysis systems will provide a well-deserved popularity to fungal world.

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Chapter 2
Role of Phytochelatins (PCs), Metallothioneins (MTs), and Heavy Metal ATPase (*HMA*) Genes in Heavy Metal Tolerance

Khushboo Chaudhary, Swati Agarwal, and Suphiya Khan

2.1 Introduction

Heavy metal pollution is a worldwide problem. The chemicals released into the soil are in the form of cadmium (Cd), cobalt (Co), copper (Cu), lead (Pb), zinc (Zn), mercury (Hg), chromium (Cr), antimony (Sb), and nickel (Ni) elements. These elements are essential for many physiological functions in living beings, while others have no such biological function in required level (Fassler et al. 2010; Dubey et al. 2014). These elements are commonly found in fungicides, animal waste, fertilizers, and sewage sludge in soil. Deposition of these industrial wastes can increase the concentration of the elements at toxic level (Fassler et al. 2010). The use of a lot of fertilizers results in the release of heavy metals into the soil which dramatically affects the agriculture (Saba et al. 2015). Lead (Pb) is the most toxic heavy metal found in several fertilizers, which is further translocated into plants and easily enters into the food chain. The movement of heavy metal contaminants from soil to water (ground level) is very slow because of low mobility, and it is the major cause of their absorption by plants (Rodriguez et al. 2011; Lori et al. 2015).

Plants under heavy metal stress produce reactive oxygen species (ROS). ROS include hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-), hydroxyl radicals (OH), and catalase which result in damages to plant cell or tissue (Migocka et al. 2014; Wang et al. 2015). ROS are produced continuously in different compartments of the plant with a number of antioxidant molecules as by-products (Kwankua et al. 2012; Palma et al. 2013; Dubey et al. 2010). However, the critical imbalance and an excess amount of production of ROS and antioxidant molecules in plant creates disorder and affects the plant's enzymatic activity (Gratao et al. 2005; Qiao et al. 2015). Several remediation technologies are used such as landfill, excavation, thermal treatment, recovery by means of electricity, and leaching of acids, but these are

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not suitable due to their high cost and very low success and less consistency on the specific metal contamination on the site and its properties. Therefore, it is important to develop an economically practical and more effective method to decontaminate soils from heavy metal contamination.

Phytoremediation is a low-cost technology (Abdul and Schroder 2009). It is about 1000-fold cheaper compared to other conventional methods such as (flotation-filtration, evaporation, ion exchange, electrodialysis, and ultrafiltration). For example, to clean about 1 acre of sandy soil (55 cm depth) will cost around $60,000–$100,000 compared to $400,000 for conventional methods (Ali et al. 2013). Phytoremediation technique involves several techniques such as phytofiltration, rhizofiltration, phytoextraction, phytostabilization, phytoimmobilization and phytodegradation, and rhizodegradation (Fig. 2.1) (Abdul and Schroder 2009; Ali et al. 2013). Phytoextraction is the most efficient and useful technique among them, but it is much more difficult than others. This technology involves the growth of tolerant plants in the contaminated soil which uptake large amounts of heavy metals from the soil and translocating into the aerial parts of the plant. Hyperaccumulator plants are those which uptake and tolerate about a hundred times greater contaminants than normal plants without any visible symptoms (Memon and Schroder 2009). Some different 450–500 plants have been identified as hyperaccumulator include *Thlaspi caerulescens* that accumulate Pb, Ni, Cd, and Zn; *Arabidopsis halleri* that can accumulate high levels of heavy metals Cd and Zn, but not Pb; and *Alyssum bertolonii* that can uptake Ni and Co (Padmavathiamma and Li 2007; Maestri et al. 2010).

Phytochelatins (PCs) are the most important class of metal chelators; it is used to chelate a variety of toxic metals. PCs are produced in a cell under stress condition

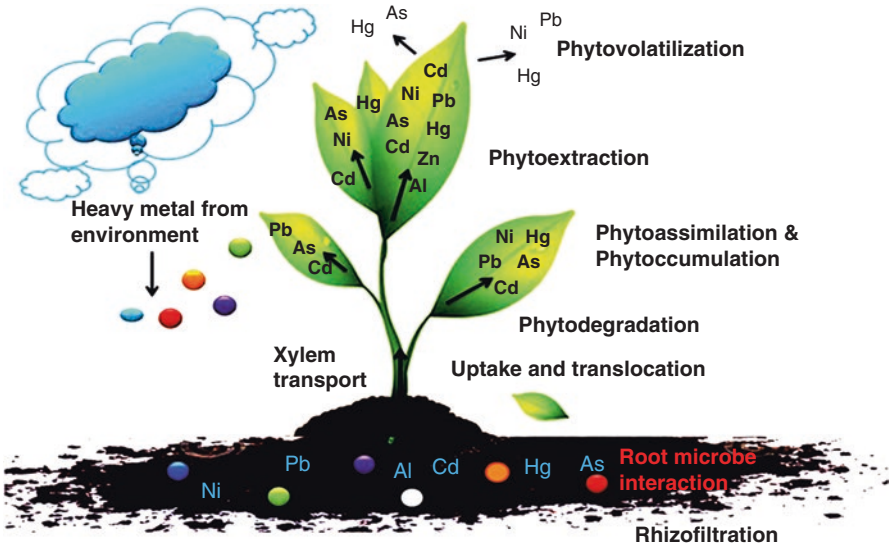

Fig. 2.1 Diagrammatic representation of phytoremediation technique

and in reaction to the high concentration of heavy metals. Some important PC-metal complexes have been a derivative from different microorganisms, fungi, and plants. The work of PC-metal complexes is to lower down the binding capacity of heavy metals to the cell wall and, at the same time, detoxify the cell compartments. It can resist very high concentration of heavy metals without causing toxicity (Sunitha et al. 2013; Sharma et al. 2016). In assessment to free metal ions, the PC-metal complexes are in complex form and much more stable.

Metallothioneins (MTs) are also a group of phytochelatins which binds heavy metals through a thiol group of cysteine and also plays an important role in detoxification of heavy metals. These MTs have a different mechanism to protect the plant from heavy metals by scavenging of the ROS and sequestration (Huang and Wang 2010). It regulates the action of metallodrugs, their transcriptional genes activation, and the activity of metalloenzymes under any stress condition (Bractic et al. 2009; Gautam et al. 2012). The regulation of MT genes was dependent on the type of plant tissue (Yuan et al. 2008). The genes are activated when a plant is under abiotic stress such as cold, heat, salt, drought, heavy metal, and oxidative stress (Usha et al. 2007, 2009; Singh et al. 2011; Gautam et al. 2012). MT genes helped to keep the plant from metal toxicity by their hyperaccumulation, and they are expressed in high concentration in hyperaccumulator plants as compared to non-hyperaccumulator plants.

Significantly, progress has been made in their recognition and their role in phytoremediation. Some of the other genes like heavy metal ATPases are also used for phytoremediation; these are *HMA2*, *HMA3*, and *HMA4* (Chaudhary et al. 2016). The expression of these genes is responsible for heavy metal uptake, translocation, and sequestration, allowing the yield of plants that can be effectively exploited in phytoremediation. The goal of this chapter is to discuss the new approaches of phytoremediation together with the important genes that play a crucial role in phytoremediation technology.

2.2 Phytoremediation Techniques: An Overview

Phytoremediation is a technology in which plants are used to remediate contaminated soil and water; it is a low-cost successful technique (Yao et al. 2012; Dixit et al. 2015). Based on remediation, technology can be divided into two main types (ex situ and in situ) method. Ex situ method is those which need physical removal (Dixit et al. 2015). In situ methods have need of biological mechanisms at the contaminated site and do not involve soil leaving its innovative site. However, there are more promising in situ methods to remove toxic metals and chemicals from the soil than ex situ methods because of its low cost, ease of use, and environmentally friendly nature (Salla et al. 2011).

Phytoremediation involves a substantial amount of biological mechanisms, including straight uptake, the discharge of exudates into the rhizosphere (to enhance bacterial and fungal processes), and metabolic processes within the root and shoot

cells (Singh et al. 2003). Selected plants are grown on the contaminated site, where they absorb pollutants and concentrate them within various tissues. The plants are then harvested and may be further treated by burning in a controlled system. The residue of the plants would then be very rich in metals and could be recycled or placed in landfills (Ma et al. 2011). Designing a phytoremediation system varies according to a wide range of factors counting the contaminant conditions at the site, the level of cleanup required, and the plant species to be used. All these factors need to be considered for a successful phytoremediation technique.

Phytoremediation is a broad term which involves more than a few different techniques such as, phytofiltration, phytovolatilization, phytodegradation, phytostabilization, and phytoextraction (Fig. 2.1) (Ali et al. 2013). Phytofiltration is used as a primary term for phytoremediation technique. It is mainly based on the adsorption of heavy metal contaminants from soil or water with the help of plant organs such as roots, shoots, and leaves (Mukhopadhyay and Maiti 2010). The second form of phytoremediation involves phytovolatilization; in this process, there is the conversion of the toxic pollutant compounds into a volatile form which makes possible their direct release from the soil into the atmosphere (Prasad and Freitas 2003). Next one is phytodegradation; in this process, the toxic metal pollutant is degraded into small particles for easy uptake by the roots (Dixit et al. 2015). Phytostabilization is used when metal pollutants are restricted to the particular soil zone mainly near the roots (Fig. 2.1) (Salt et al. 1998; Yao et al. 2012). One other technique is phytoextraction; it is a very useful process than the others. In this technique, contaminant in soils are taken up in large amounts by the roots and translocated into the aerial parts of the plant. It is natural that not all plants accumulate contaminants in equal proportion. Some plants accumulate in large quantities, and such hyperaccumulator plants are the base of phytoremediation technology.

2.3 Hyperaccumulator Plants: A Base for Phytoremediation Technology

Hyperaccumulator plants are those which uptake and tolerate more metal ion contaminants without any visible symptoms (Memon and Schroder 2009). Some 450–500 different plants have been identified as hyperaccumulator include *Thlaspi caerulescens* that accumulate Pb, Ni, Cd, and Zn; *Arabidopsis halleri* accumulate Cd and Zn, but not Pb; *Alyssum bertolonii* can uptake Ni and Co; and some other plants that belong to a different family, such as Caryophyllaceae, Fabaceae, Poaceae, Lamiaceae, Asteraceae, Cunoniaceae, and Cyperaceae, and many others plants can also participate to accumulate heavy metals (Maestri et al. 2010).

Plants have specific properties that give us some specific advantages to remediate the environment (Meagher 2000; Meagher et al. 2000). Plants absorb metal particles by the roots and root hairs that generate forces (cohesive and adhesive) through which pollutants can be absorbed from contaminated soil and water. Plants are

autotrophs; they take up nutrients directly from the environment in gaseous form with the help of the process of photosynthesis. Different crop plants like *Ipomoea alpine*, *Centella asiatica*, *Eichhornia crassipes*, *Euphorbia macroclada*, *Berkheya coddii*, *Alyssum* and *Thlaspi*, *Zea mays*, *Pteris vittata*, *Astragalus bisulcatus*, *Sesbania drummondii*, *Sedum alfredii*, *Euphorbia macroclada*, *Phragmites australis*, *Phytolacca americana*, *Astragalus bisulcatus*, *Cardamine hupingshanensis* and *Iberis intermedia* are reported as hyperaccumulators for phytoextraction of particularly Cu, Ni, Cd, Zn, Cr, As, Mg, Se, and Ti in their shoots (Sharma et al. 2003; Meers et al. 2005; Dong 2005; Israr and Sahi 2006; Jin et al. 2008; Pollard et al. 2009; Bani et al. 2010; Mehdawi et al. 2011; Mokhtar et al. 2011; Nematian and Kazemeini 2013; Tong et al. 2014).

The five willow tree species *Salix schwerinii* (Christina), *Salix fragilis* (Belgisch Rood), *Salix triandra* (Noir de villaines), *Salix purpurea* × *Salix daphnoides* (Bleu), and *Salix dasyclados* (Loden) accumulate Zn and Cd at a higher concentration in their shoots (Meers et al. 2007). Recently, a plant named *Lolium multiflorum* is also found and is used for phytoremediation of Mn, Cu, Pb, and Zn (Mugica-Alvarez et al. 2015). The aquatic plant *Hydrilla verticillata* also has a great capacity for heavy metal uptake and is considered a good accumulator of Cr and Cd from ponds (Phukan et al. 2015). *Sauropus androgynus* is a perennial plant that has the ability to phytoremediate soil, and this plant can be used commercially due to its high capacity of toxic metal accumulation (Xia et al. 2013). One other plant named *Pannicum virgatum* (switchgrass) is reported as a heavy metal accumulator of Zn, Cr, and Cd (Chen et al. 2012).

2.4 Heavy Metal Toxicity Mechanism

Some heavy metals such as Cu, Zn, Al, Cd, Ni, As, and Hg have negative effects on macronutrient and micronutrients (Mariano and Keltjens 2005; Baligar et al. 1993). Calcium (Ca) ions work as a secondary messenger in plant signaling and uptake by the roots and translocate into meristematic zones of young tissues. Ca is unable to recycle after deposition in the leaves (Hanger 1979). Heavy metals bind to all Ca-binding sites on the surface of the cell, and at low pH (4.5), it works with Ca absorption and uptake by the roots (Roy et al. 1988; Hossain et al. 2014). The amount of heavy metals exposes the inhibition (Ribeiro et al. 2013) of Ca ions (100 mM (Al) (Nichol et al. 1993). Al ions interfere with the action of guanosine 5′ triphosphate (GTP) binding protein as well as inhibit Ca ion uptake by binding the specific channels (verapamils) (Rengel and Elliott 1992). In beech plants, there is a decrease in Ca ions uptake due to the combination of high concentration of nitrogen and aluminum (Bengtsson et al. 1994).

Potassium (K) channel influx inhibits due to the toxicity of heavy metals like Al. Active pathway involvement of uptake of K is also inhibited by high concentration of Al (Hossain et al. 2014). At the low pH (4.5), concentration of K decreases when they are treated with heavy metals (Moustakas et al. 1995). The Al toxicity is caused

by decrease in K ions in the roots as well as guard cells, which is caused by the blocking of the pathway channels at cytoplasmic sites of the plasma membrane. The increase in K ion concentration in barley roots under stress of Al ions is somewhat significant (Kasai et al. 1992). The concentration of K ions increased in roots and shoots of pine trees with an increase in Al concentration (Huang and Bachelard 1993). Durum wheat is also very resistant to Al toxicity, and it shows a decrease in K ion concentration with winter wheat (Zsoldos et al. 2000).

Magnesium (Mg) ions are also affected by heavy metal stress more than other nutrients present in the plant (Wheeler and Follett 1991). Plant treated with different concentrations of heavy metals in acidic medium (pH 4.5) can be checked for a decreased level of Mg ions (Huang and Bachelard 1993). The uptake of Mg ions by the roots is much stronger than Ca ion uptake (Bose et al. 2011, 2013). Iron (Fe) concentration decreased to an acidic pH of 4.5 when the plants were treated with different concentrations of heavy metals, and root growth was also significantly affected (Moustakas et al. 1995). Some researchers focus on nutrition level value which is really affected by heavy metals. The accumulated nitrogen concentration in roots decreased as a result of high exposure to Al ions and translocated to the aerial parts of plants (Gomes et al. 1985; Purcino et al. 2003).

Under the heavy stress of toxic metal, there is a decrease in concentration of lipoxygenase enzyme, antioxidant enzyme, and ROS (Wang and Yang 2005). Soil contains large amount of heavy metals with less phosphorus and aluminum (Liao et al. 2006).When the concentration of metals in plants is low, there is an increase in micronutrients and also macronutrients, but metal concentration higher than the acceptable level results in toxicity and also decreases the level of phosphorus (Cumming et al. 1986; Nichol et al. 1993). Hydrogen Peroxide level increases the plant tolerance to the heavy metals stress (Hameed et al. 2016). Nevertheless, with the treatment given at 100 mM H_2O_2 for 1 day, it lowers the Cd stress by inducing some antioxidant enzymes such as CAT, SOD, GST, APX, and GPX and increases the concentration of GSH and AsA. The activity of POD, SOD, GSH, GPX, APX, AsA, MDHAR, and DHAR content and their redox state increases in response to the Al stress. Therefore, H_2O_2 pretreatment in Cd and Al stress makes the plant more tolerant, inducing "GSH, AsA, NPT, and PC" contents as well as the GST activity in root tissues (Fig. 2.2) (Cho and Seo 2005; Bai et al. 2011; Kumar and Trivedi 2016; Hameed et al. 2016). Al toxicity is also disturbed by the cellular redox homeostasis and causes the oxidative burst of mitochondria and programmed cell death (PCD) in plants (Li and Xing 2010).

2.5 Heavy Metal Transportation Pathway

Plants have the ability to store metals in cells and organs and tolerate high concentrations of toxic heavy metals. The uptake of heavy metals from the soil into roots from where they are translocated through different transporters to aerial parts of the

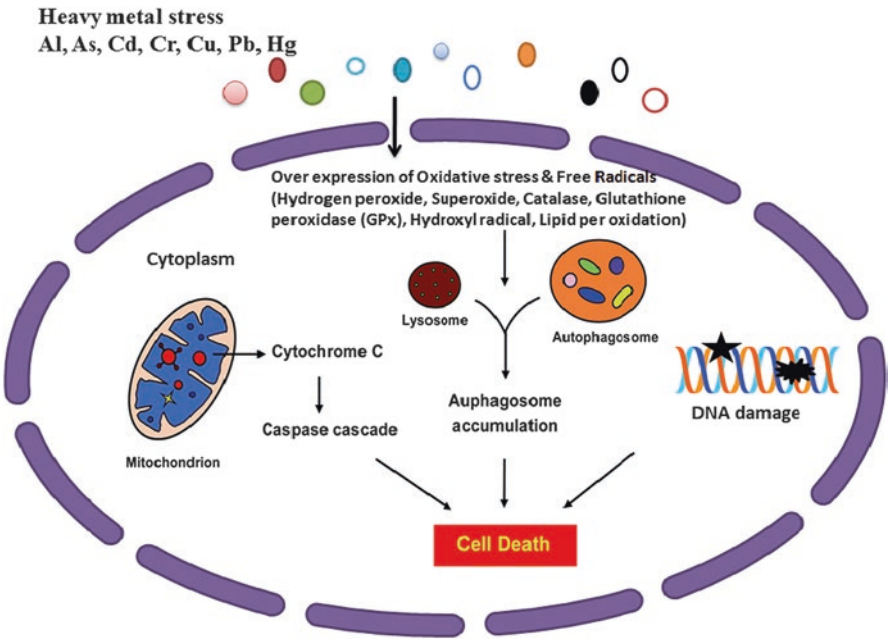

Fig. 2.2 Reactive oxygen species (ROS) signaling activates under heavy metal stress

plant is called phytoremediation (Fig. 2.1) (Mahmood 2010). This process involves several steps such as uptake of heavy metals from the soil by roots, translocation of heavy metals from roots to shoots throughout the xylem channels which is known as xylem loading, and the sequestration of heavy metals in leaves predominantly in vacuoles (Bhargava et al. 2012). Different plants have different mechanisms for the uptake of different heavy metals, and variations in the transport pathways are observed.

There are two pathways by which heavy metals can enter the roots. First is an apoplastic pathway and the second one is the symplastic pathway. The apoplastic pathway allows the soluble metals to travel without entering the cells and also through intracellular spaces. On the other hand, symplast pathway allows the movement of nonessential metals like Ni, Cd, and Pb through the cytoplasm by consuming energy (Lombi et al. 2002; Lu et al. 2009). Heavy metals transfer from soil to root and root to shoot through xylem tissues and need to be transferred from roots to leaves and vice versa. It is transferred through symplast pathway where they are an energy-consuming process that allows all nonessential heavy metals. This is due to normal intracellular transport movement pathway blocked by Casparian strip in the endodermis. Hence, the movement of heavy metals through apoplastic pathway gets blocked which means that the only way to enter xylem vessels is by taking the symplast pathway (Mahmood 2010).

2.6 Types of Genes Used in Phytoremediation

In this section, we are going to discuss three major genes which are most commonly expressed in plants under stress condition.

2.6.1 Phytochelatins (PCs)

Phytochelatins (PCs) are generally cysteine-rich small polypeptides with a specific structural formula (g-Glu-Cys)n-Gly (Fig. 2.3). They have low molecular weight and get synthesized under toxic metal stress condition. Generally, PCs are found in plants but their presence is also reported in some fungi and bacteria (Mirza et al. 2014). PCs are the most common and important group of heavy metal chelators, and are is mainly synthesized in the cell cytoplasm. The complex of PCs such as *PC*-metal and *PC*-metalloid are the most stable structures in nature which is found in sequestration of vacuolar compartments where heavy metal toxicity is less toxic in comparison to other cell parts (Shen et al. 2010; Dago et al. 2014).

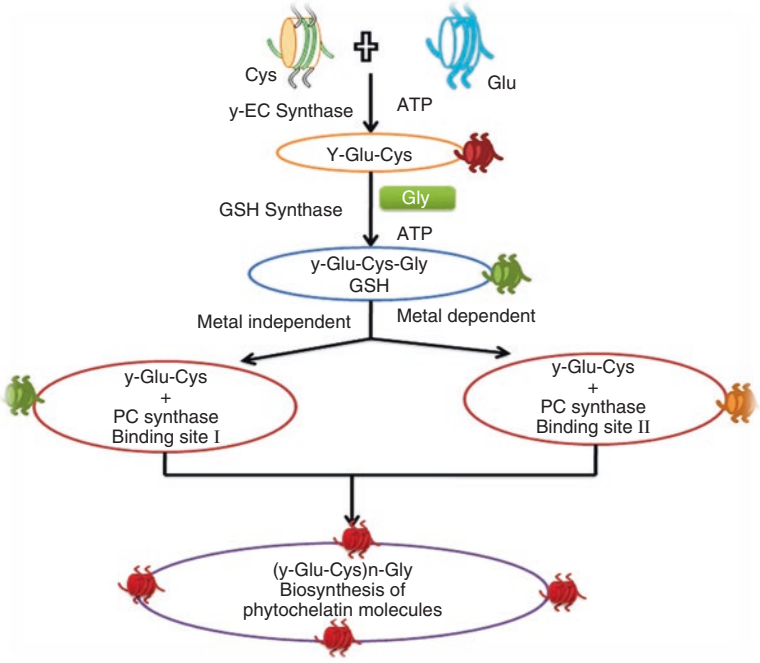

Fig. 2.3 Biosynthesis pathway of phytochelatins (PCs)

2.6.1.1 Biosynthesis of Phytochelatins (PCs)

Biosynthesis reaction of PCs is catalyzed by one of the important enzymes called phytochelatin synthase (Kuhnlenz et al. 2014). PCs belong to a peptide family which was first found in *Schizosaccharomyces pombe* as cadmium (Cd)-binding complex under Cd stress condition (Inhouhe 2005). L-glutamate (Glu), glycine (Gly), and L-cysteine (Cys) are the key amino acids which take part in the biosynthesis of PCs. Glutathiones (GSH) are used in the biosynthesis of PCs; therefore, the GSH biosynthesis pathway overlaps with PC biosynthesis. The general structure of *PC* oligomer is (g-Glu-Cys)n-Gly where n usually ranges from 2 to 5 but has been reported as high as 11 in some species (Cobbett 2000; Sharma et al. 2014).

PC biosynthesis starts with the formation of g-glutamylcysteine from L-glutamate and L-cysteine which is catalyzed by a specific enzyme named g-glutamylcysteine synthase. Further glycine moieties are added to g-glutamylcysteine, and GSH formation occurs. This reaction is catalyzed by glutathione synthase enzymes in the presence of adenosine triphosphate (ATP) (Meister 1988; Sharma et al. 2014). After GSH formation, the last step of *PC* biosynthesis is the formation of PCs in the presence of *PC* synthase enzyme (Fig. 2.4). Due to transpeptidation of a g-Glu-Cys moiety of GSH, this enzyme is named g-Glu-Cys dipeptidyl transpeptidase (Cobbett 2000; Sharma et al. 2014). However, before transpeptidation reaction, glycine moieties are cleaved from GSH which further in the next step (transpeptidation) forms a peptide bond with either *PC2* or GSH to produce np1 oligomer (Clemens 2006; Sharma et al. 2016).

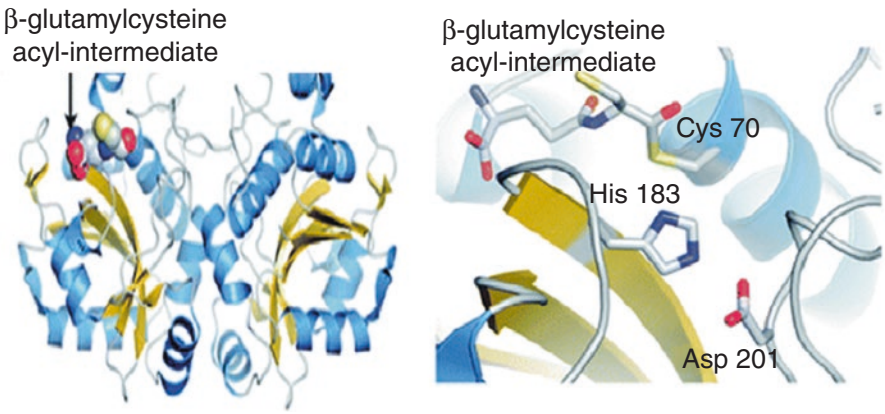

Fig. 2.4 Cysteine-rich small polypeptide phytochelatins structure

2.6.1.2 Phytochelatins Action Mechanism

The PCs are also bound to metalloids with heavy metals. *PC*-metal complexes are very stable and having less toxicity in comparison to free metal ions present in the cells. Under natural conditions, PCs are actively involved in degradation of various GSH conjugates in comparison to other metalloids (Franchia et al. 2015; Sharma et al. 2016). In *Arabidopsis thaliana*, PCs form a complex with Zn metal and compartmentalized it to overcome the toxic effect of Zn metal (Tennstedt et al. 2009).

Recently, it has been found that PCs are induced in various plants (non-hyperaccumulator) even at a low concentration of metalloids and heavy metals (Table 2.1). Large numbers of studies take place which suggest that lack of *PC* mutant expression results in an increase of Cd sensitivity (Andresen et al. 2013). *PC*-metal complexes are found in a plant named *Nicotiana caerulescens* which is a good example of copper hyperaccumulator plant. *PC*-metal complexes are formed in the presence of *PC* genes. When heavy metals ligate with GSH molecule, it forms thiolate which further leads to the formation of the *PC*-metal complex (Sharma et al. 2014). These *PC*-metal complexes are then transported to vacuolar compartments where they are called Mr-(*PC*-metal) complexes. These are less toxic and more stable form of heavy metal ions (Yadav 2010). The *PC*-metal complex formation has been reported in a plant named *Ceratophyllum demersum* L. under lead (Pb) toxicity and some other heavy metal toxicity.

Table 2.1 Phytochelatin (PC) genes expressed in various plants under heavy metal stress

S. No	PCs genes	Heavy metals and their effects	Plant	References
1.	AtBCC3	Cd tolerance	*Arabidopsis thaliana*	Brunetti et al. (2015)
2.	PCS1	Cd tolerance	*Schizosaccharomyces pombe*	Shine et al. (2015)
3.	ACR2, ACR3	As hypersensitivity Zn hyperaccumulation	*Pteris vittata, Arabidopsis halleri*	Indriolo et al. (2010)
4.	SpHMT1	Cd, Zn, as tolerance	*Schizosaccharomyces pombe*	Lee (2014)
5.	HMA4, PCS1	Zn homeostasis	*Liriodendron tulipifera*	Adams et al. (2011)
6.	CdPCS1	As and Cd assimilation	*Ceratophyllum demersum*	Shri et al. (2014)
8.	AtPCS2	PC synthesis	*Arabidopsis thaliana*	Kuhnlenz et al. (2014)
9.	TcHMA3	Cd tolerance	*Thlaspi caerulescens*	Ueno et al. (2011)
10.	TaPCS1	Detoxification of heavy metals	*Triticum vulgaris*	Liu et al. (2011)

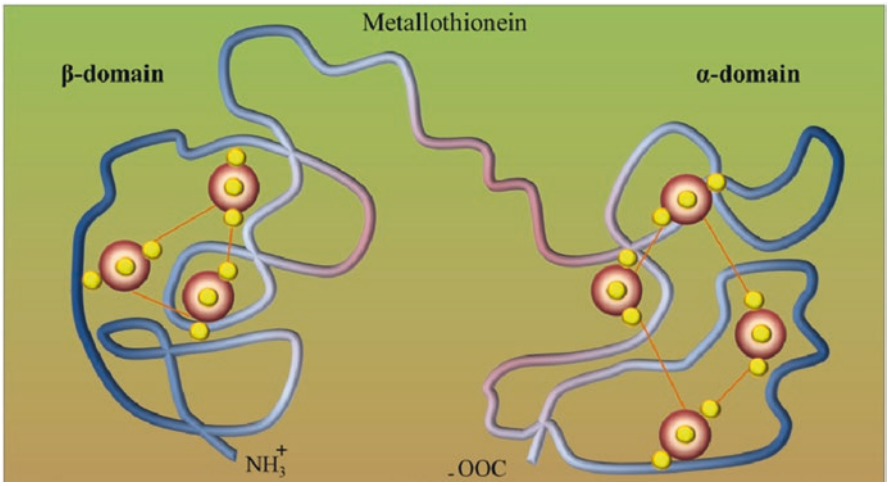

Fig. 2.5 Metallothionein structure which has two domains, NH_3^+ and -OOC, to attach with metal atoms. (Petrlova et al. 2006)

2.6.2 Metallothioneins (MTs)

Metallothioneins (MTs) were first discovered in the horse kidney cells in 1957; it is in the form of metal-binding proteins (Thirumoorthy et al. 2011). MTs are rich in cysteine amino acid; they are generally low-molecular-weight protein, and they have two domain structures (Fig. 2.5). MTs are efficient in metal complexing due to the high affinity of the sulfur molecule present in the cysteine. MTs bind to different metal ions via the thiol group of cysteine or sulfur molecule and exert a major role in heavy metal stress and detoxification. Under heavy metal stress condition, the plant starts synthesizing metalloenzymes complex, activates the transcription genes, and metabolizes metallodrugs (Bractic et al. 2009; Gautam et al. 2012).

2.6.3 Classification of Metallothioneins (MTs)

The metallothioneins (MTs) are classified on the basis of cysteine collection in their structure. There are more than 20 conserved Cys found in mammals and vertebrates which are known for their tolerance toward Cd ions (Shri et al. 2014; Gu et al. 2015). MTs are classified into four classes; first the *MT1*, which was expressed in a plant named *Cicer arietinum* and its subclasses *MT1a* and *MT1c* in *A. thaliana*. The second one is *MT2*; it is also found in the plant *Cicer arietinum*, and its subclasses are *MT2a* and *MT2b*. And the last one is class *MT3*; it is found in *A. thaliana* and *Musa*. Some other classes include *MT4a-Ec-2* and *MT4b-as Ec-1* found in *A. thaliana* and *Triticum aestivum* (Hassinen et al. 2011; Lee 2014). There is strong

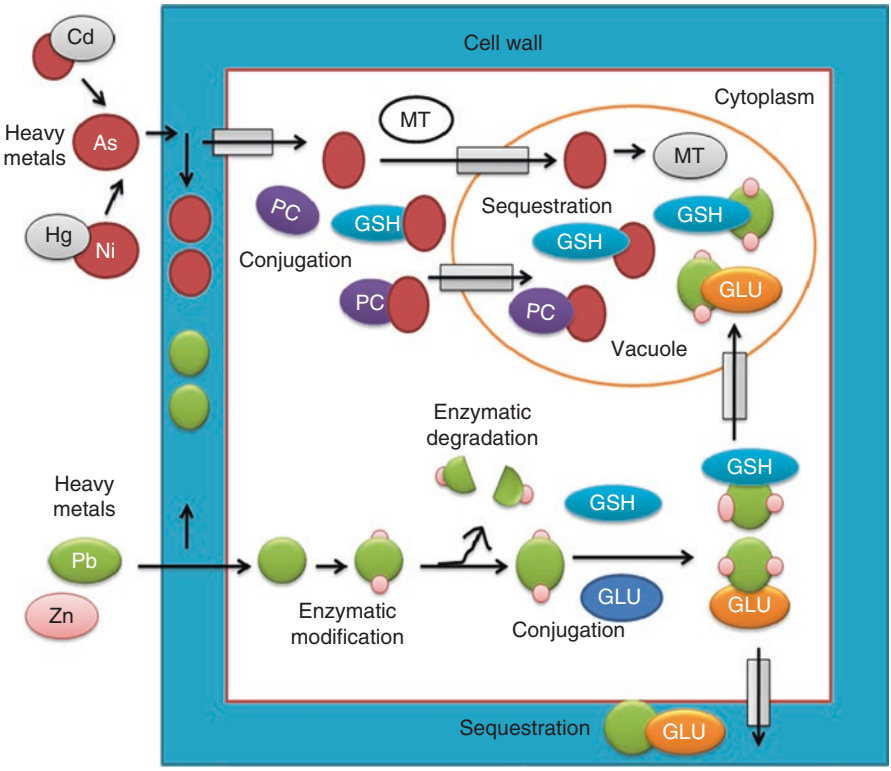

Fig. 2.6 Diagram of detoxification, conjugation, and sequestration in the vacuole where the pollutant can do harm to the cell. (Chelators shown are *GSH* glutathione, *GLU* glutamate, *MT* metallothioneins, *PCs* phytochelatins)

structure similarity between GSH and *MT3* due to their same biosynthesis precursor molecule which is thiol-rich tripeptides. A powerful inhibitor named buthionine sulfoximine inhibits the activity of g-glutamylcysteine synthetase enzyme which leads to the decrease in concentration of g-glutamylcysteine and GSH in cells (Fig. 2.6). In *Silene cucubalus*, *MT3* was found, which is synthesized, formed by the reaction of GSH and g-glutamylcysteine dipeptidyl transpeptidase (Grill et al. 1989).

2.6.3.1 Metallothioneins Action Mechanism

The expression of *MT* genes was regulated and differs from plant tissue to tissue (Yuan et al. 2008). The genes are activated when a plant is under abiotic stress such as cold, heat, salt, drought, heavy metal, and oxidative stress (Usha et al. 2007, 2009; Singh et al. 2011; Gautam et al. 2012). The action mechanism of MTs in stress is unknown in plants in comparison to those MTs which belong to the

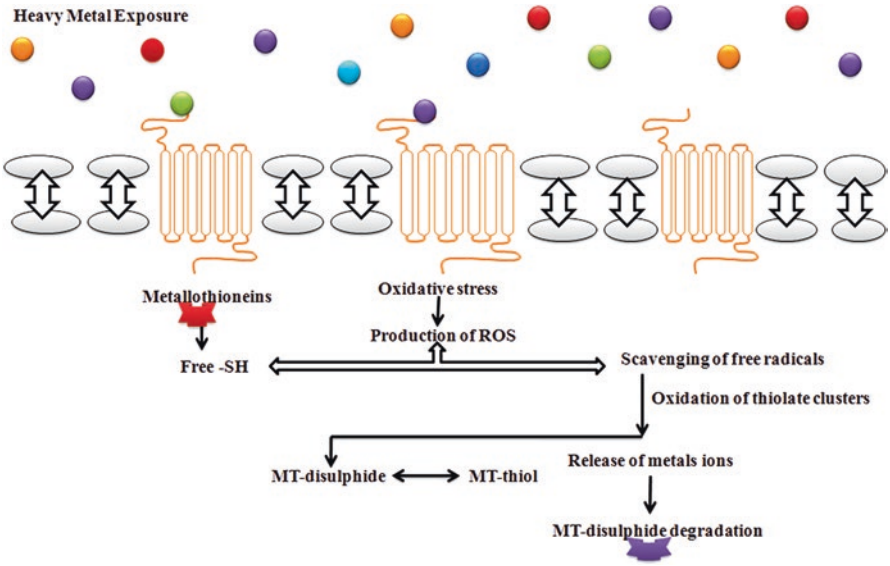

Fig. 2.7 Basic mode of action in metallothioneins under heavy metal stress

mammals. In the case of plant MTs, it is difficult to purify them due to the occurrence of oxygen molecule which makes it to a large extent unstable (Guo et al. 2008; Sharma et al. 2016). The *MT* genes are very specific in nature; they are always expressed in specific organs or tissues; for instance, *MT1* are expressed in the root region, *MT2* are in leaves, *MT3* are in fruits, and other MTs are in the seeds (Huang et al. 2011). When heavy metal ions bind to the MTs, it activates metal response elements in the presence of metal-responsive transcription factor-1. Signal transducers and activators of transcription proteins are activated in the presence of cytokinin signaling molecule, and in the same way, the antioxidant proteins are also triggered by the redox imbalance (Fig. 2.7, Table 2.2) (Huang and Wang 2010; Usha et al. 2011; Hegelund et al. 2012; Lv et al. 2013; Zhang et al. 2014; Liu et al. 2015; Sharma et al. 2016).

2.6.4 *Heavy Metal ATPase Genes (*HMA2*,* HMA3*, and* HMA4*)*

It has been reported that overexpression of phytochelatin (*PC*) synthase and metallothionein (*MT*) in a number of genes increases heavy metal tolerance under stress (Liu et al. 2011). The molecular mechanism in plant stress is response to heavy metals, especially in herbaceous plants such as *Arabidopsis halleri*, *Arabidopsis thaliana*, and *Thlaspi caerulescens* (Meyer and Verbruggen 2012). The genes encoding bivalent cation transporters belonging to *HMAs* (among which *HMA4*) are

Table 2.2 Metallothionein (MT) genes expressed in plant under heavy metal stress

S. No	MT genes	Plant	Effect	References
1.	IIMT2b	*Iris lactea*	Cu concentration increased and reduced H_2O_2 production	Gu et al. (2015)
2.	OsMT2c	*Oryza sativa*	Increased ROS activity and more tolerance to Cu	Liu et al. (2015)
3.	ScMT2-1-3	*Saccharum* spp. L.	Increased the host cells tolerance to Cd	Guo et al. (2013)
4.	TaMT3	*Tamarix androssowii*	Tolerance to Cd and increased activity of ROS	Guo et al. (2013)
5.	BcMT1 and BcMT2	*Brassica campestris*	Enhance tolerance to Cu and Cd and decreased production reactive oxygen species	Lv et al. (2013)
6.	SaMT2	*Sedum alfredii*	Cd accumulation and tolerance	Zhang et al. (2014)
7.	MT2	*Populus alba* L.	Cd, Zn, Cu ions increased tolerance	Lee (2014)
8.	PcMT3	*Porteresia coarctata*	Cd, Zn, Cu tolerance	Usha et al. (2011)
9.	MT3 and MT4	*Hordeum vulgare*	Zn tolerance	Hegelund et al. (2012)
10.	ThMT3	*Tamarix hispida*	Zn, Cd, Cu enhances	Liu et al. (2015)
11.	Am MT2	*Avicennia marina*	Cd, Pb, Cu, and Zn	Huang and Wang (2010)
12.	HbMT2	*Hevea brasiliensis*	Zn and Cu ions tolerance	Lee (2014)

overexpressed in roots and shoots of Zn and Cd hyperaccumulators *T. caerulescens* and *A. halleri* (Papoyan and Kochian 2004; Mils et al. 2003). It has constantly played a very important part in uncovering the molecular mechanism of plant response to pollutants as its genome information is obtainable. The most strongly connected of the *A. thaliana* is P1B-type genes. The *HMA2* is expressed in the translocation of Zn and Cd in *A. thaliana*, barley, rice, and wheat. In *Arabidopsis*, the cellular and subcellular patterns of *AtHMA2* expression were related to the *AtHMA4* gene. The expression of *HMA2p-GUS* gene was observed for the most part in the vascular tissues of the leaf, stem, and root. *HMA2*-GFP proteins were also localized in the plasma membrane of the plant cell.

Recently, results on the characterization of the *HMA2* gene from the different plants for possible request apply in phytoremediation approaches (Chaudhary et al. 2016). The ATPase families of integral membrane transporter proteins that help to uptake transition metals are worried in mediating metal-resistant and metal-hyperaccumulating traits. The plants were expressing 35S promoter *AtHMA4* as well as the metal transporters such as *HvHMA2* (Barabasz et al. 2013). *HMA3* is used in metal detoxification by sequestering Cd into the vacuole, *HMA4* acts as a physiological master switch during the process of hyperaccumulation metal, and

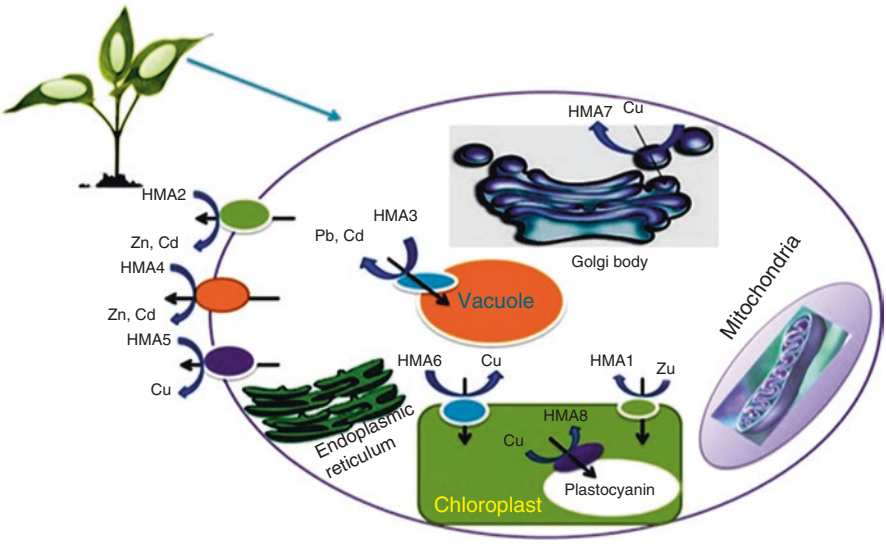

Fig. 2.8 Heavy metal ATPase gene contributes in hyperaccumulation of heavy metals

HMA2 and *HMA4* play roles in root-to-shoot metal translocation (Fig. 2.8). It is hypothesized that the roles of metal transporters in plants will be needed for the growth to genetically modify plants that accumulate definite metals, with subsequent use in phytoremediation process.

The efficiency enhanced of *HMA3* and *HMA4* is a prerequisite for hyperaccumulation and hyper-resistance in hyperaccumulator plants. Through genetic engineering, these genes are able to generate nontoxic food. The overexpression of *CsHMA3* might increase Pb and Zn tolerance and uptake. Also, the transgenic lines showed a wider leaf shape compared with wild-type plant suitable to an inducement of genes, connected to leaf enlargement, and displayed a greater total seed production compared to the wild-type species under heavy metal contamination (Park et al. 2014). *HMA3* is a tonoplast-localized transporter that involves metal detoxification by sequestering specific metals, such as Cd, into the vacuole and that *HMA2* and *HMA4* plasma membrane proteins contribute in translocating Zn and Cd ions from the root to shoot and detoxifying these metals in aboveground tissues

2.7 Advancements of Phytoremediation

Phytoremediation has few advantages over other techniques used for metal contamination removal. It can be used also as an in situ or ex situ application. It is a green technology and eco-friendly. Phytoremediation process is low cost and it is relatively easy to apply. Phytoremediation is its low-cost technology as compared to conventional cleanup technologies which was estimated at $60,000–$100,000

compared to $400,000 for the conventional method. Disposal sites are not necessary, and residual biomass is utilized for papermaking, alcohol production, antimicrobial activity, biosynthesis of nanoparticle, etc. It is more possible to be accepted by the public and avoids excavation for polluted media.

2.8 Demerits of Phytoremediation

Phytoremediation does have a few disadvantages that include the following: it is controlled to the rooting depth of plants, remediation with plants is a lengthy process, and thus it may take several years to clean up hazardous wastes and contaminants. The utilization of contaminated plants by wildlife is also of concern. The harsh climate is one more important concern because it can reduce to plant growth and biomass production.

2.9 Future Prospects

Fast-growing technology focuses on molecular biology of transgenic plant species that can accumulate more contaminants to identify the gene for resistance against such toxic compounds. The researchers should focus more on vacuolar transport which is the main section of metal hypertolerance; this mechanism is essential for hyperaccumulation, improving root uptake, vacuolar transport, xylem loading, and mobilization of heavy metals. This information will also be a help to improve the productivity of crops. However, a lot of plants lack these properties. Thus, the option to produce new plants through transgenic methods is a good choice for outstanding phytoremediation techniques.

2.10 Conclusion

Phytoremediation is an affordable and attractive technique that emerged in recent years. This technology helps to clean environment with the help of metal hyperaccumulator plants. The physiological roles played by PCs, yet MTs still not clear in the plant system. However, we need a better understanding of the pathway inclusive of PCs and MTs in respect to heavy metals. As this transporter genes, increase the heavy metal toleratance in stress condition and successfully clean environment. The records of companies have ongoing their business in phytoremediation process to relate for clean up the environment and also raise the biomass production. Understanding of the signaling mechanism of transporter genes will prove to an important tool to understand the genetics of hyperaccumulation. Under stress condition some specific plants have certain mechanism to uptake the nutrients more than

their limits. In this condition, the plant defense system mechanism plays an important role using metabolic, physiological, and expression pathway. More detailed studies are necessary to know the plants mechanism by which we can enhance their phytoremediation efficiency.

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Chapter 3
Production of Bio-oils from Microbial Biomasses

Laura Bardi

3.1 Introduction

Microbial cells have been considered since the nineteenth century as a potential source of lipids. In fact, it was early discovered that several microorganisms can accumulate great amounts of storage lipids and, even when they cannot quantitatively compete with alternative lipid sources, such as plants or animals, it is possible to obtain compounds with peculiar composition by looking for them in the considerable biodiversity of the microbial world and the extreme variability of the metabolisms that can be found in it. Moreover, microbial metabolism can also be addressed to a specific goal, by acting on the physiological growth conditions, such as modifying the composition of growth medium, or other factors, such as temperature or oxygen availability, or choosing the cells collection time at different growth phases. The same results cannot be easily obtained from plant crops, not even from chemical processing that usually is expensive and not always effective.

The history of the industrial production of lipids from microbial biomasses follows variable trends, depending mainly on the variability of markets and the parallel development of agricultural and industrial technologies. A special interest in microbial lipids persists overtime for high valued compounds, such as oils rich in polyunsaturated fatty acids (PUFA), recommended as dietary supplements for both children and adults. Since the beginning of the twenty-first century, with the increase of need for renewable energy sources, a rapidly growing interest on microbial lipids as biofuels has been worldwide observed as an alternative to plant oils. The main convenience of producing biofuels by microbial biotechnological processes, instead of by agricultural activities, is that microbial biomasses can be produced in factories, therefore without depletion of agricultural land and without competing with produce for food destina-

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tions. Moreover, microbial cells can be grown using wastes and by-products from agriculture and agro-industry as substrates; by this way, the costs of the process can be significantly reduced, improving the market competitiveness. At the same time, the environmental impact of the waste disposal is improved: wastes are converted in sources, disposal costs are reduced and the global added value is further increased.

Many studies conducted on different microorganisms allowed to demonstrate that storage lipids can be produced in remarkable amounts by several strains of yeasts, filamentous fungi, microalgae and bacteria. Conventionally, a microorganism is defined "oleaginous" when it accumulates more than 20% lipids (w/w) in its cell mass, and the lipids produced and extracted by microbial cells are usually defined "single cell oils" (SCO), a term that can be considered as a transposition of "single-cell proteins" (SCP), coined before, when microorganisms were well studied and characterised to develop biotechnological processes to produce proteins (Ratledge 1976) (Table 3.1).

Table 3.1 Lipid content of oleaginous yeasts and fungi

	Lipid content (% w/w)
Yeasts	
Candida sp. *107*	42
Cryptococcus albidus	65
Cryptococcus curvatus	58
Waltomyces lipofer	64
Lipomyces starkeyi	63
Rhodosporidium toruloides	66
Rhodotorula glutinis	72
Rhodotorula graminis	36
Trichosporon beigelii	45
Yarrowia lipolytica	36
Fungi	
Aspergillus oryzae	57
Conidiobolus nanodes	30
Cunninghamella japonica	60
Entomophthora coronata	43
Humicola lanuginosa	75
Mortierella alpina	48
Mortierella isabellina	70
Mortierella ramanniana	50
Mortierella vinacea	66
Mucor alpine-peyron	38
Mucor circinelloides	25
Pythium ultimum	48
Rhizopus arrhizus	57
Aspergillus terreus	57
Fusarium oxysporum	34
Pellicularia practicola	39
Cladosporium herbarum	49
Claviceps purpurea	60

Extracted from Ratledge and Wynn 2002; Meng et al. 2009; Certik and Shimizu 1999

Table 3.2 Lipid content and yields from SCO fermentations with yeasts and fungi

	Lipid content (% w/w)	Productivity (g/l/year)
Yeasts		
Candida curvata	29,2–58,0	315
Cryptococcus albidus	33–43,8	146
Cryptococcus curvatus	25–45,8	1154
Lipomyces starkeyi	61,5–68,0	410
Rhodosporidium toruloides	58,0–68,1	2120
Fungi		
Aspergillus oryzae	18–57	215
Cunninghamella echinulata	35–57,5	134
Mortierella isabellina	50–55	678,8

Extracted from Thevanieau and Licaud 2013

Among oleaginous microorganisms, yeasts are, in general, considered very suitable for industrial applications, because they possess several favourable characteristics: they grow rapidly, and they are capable to use several low-cost substrates, such as agricultural or agro-industrial wastes or by-products, allowing to reach high yields in low time and without land consumption (Table 3.2). Among oleaginous yeasts, the most known and characterised genera are *Lipomyces*, *Rhodosporidium*, *Cryptococcus*, *Candida*, *Rhodotorula*, *Trichosporon* and *Yarrowia*.

Filamentous fungi are also considered very suitable for their great adaptability to a wide range of different, and also "difficult", growth substrates and conditions. In general, moreover, fungi produce lipids with different composition from yeasts: richer in long chain fatty acids and with a highest unsaturation rate; then they are preferred for specific aims, such as the production of PUFA (see Box 3.1) (Papanikolaou and Aggelis 2011a, b). Among the oleaginous fungi, the genera more characterised are *Mortierella*, *Mucor*, *Cunninghamella*, *Rhizopus*, *Thamnidium*, *Conidiobolus*, *Pythium*, *Aspergillus* and *Claviceps.*

Microalgae have also been largely considered and employed for lipid production; their peculiar interest is mainly born from their potentiality to produce organic matter using light as energy source and consuming CO_2 as carbon source. However, microalgae in general grow slowly and with low-yield rate, they are expensive for biomass growth and oil extraction; when cultivated on open ponds, they require wide land consumption and are exposed to high risk of contaminations (Ratledge 2013). Only few bacteria produce oils, and the concentrations reached are usually lower than in eukaryotic microorganisms, but they are easy to be cultivated and usually show high-growth rate; metabolic engineered strains were produced to increase fatty acid synthesis (Shi et al. 2011).

The biochemistry of lipid biosynthesis has been well studied and characterised since long time in yeasts; these unicellular organisms can be cultivated very easily and rapidly, and they represent a useful model for studies on eukaryotic organisms. Nowadays the knowledge in this field is very advanced and well developed, and many in-depth analyses have also been carried out on specific yeasts and fungal strains

Box 3.1 Nomenclature and Description of Fatty Acids

SFA = saturated fatty acids are linear fatty acids without double bonds.

UFA = unsaturated fatty acids are fatty acids with double bonds, usually in *cis* (or *Z*) configuration.

LCFA = long chain fatty acids are fatty acids with chain length longer than 18 atoms of Carbon.

PUFA = poly unsaturated fatty acids are fatty acids with more than one double bond.

The chain length is denoted by C followed by the total carbon number.

The number of double bonds is indicated after the number of Carbon and preceded by colon (i.e.: C18:1 = oleic acid, chain length 18 C, one double bond).

The position of the last double bond is numerated from the methyl-terminal end of the chain and indicated as a number preceded by *n*- or *ω*- (i.e. *n*−3 or *ω*−3).

In polyunsaturated fatty acids, the position of each double bond is indicated starting the numeration from the carboxy-terminal end of the chain (i.e. C18:3(9, 12, 15), *n*−3 = α-linolenic acid)

Below are reported some of the more common fatty acids:

C16:0	Palmitic acid
C18:0	Stearic acid
C16:1(9), *n*−7	Palmitoleic acid
C18:1(9), *n*−9	Oleic acid
C18:2(9, 12), *n*−6	Linoleic acid
C18:3(9, 12, 15), *n*−3	α-linolenic acid (ALA)
C18:3(6, 9, 12), *n*−6	γ-linolenic acid (GLA)
C20:4(5, 8, 11, 14), *n*−6	Arachidonic acid (ARA)
C20:5(5, 8, 11, 14, 17), *n*−3	Eicosapentaenoic acid (EPA)
C22:5(4, 7, 10, 13, 16), *n*−6	Docosapentaenoic acid (DPA ω-6)
C22:6(4, 7, 10, 13, 16, 19), *n*−3	Docosahexaeneoic acid (DHA)

aimed to peculiar industrial applications. Genetic manipulation strategies have also been developed to address metabolic pathways towards the accumulation of specific molecules (Papanikolaou and Aggelis 2011b; Thevenieau and Nicaud 2013).

3.2 Biochemistry and Physiology of Lipid Biosynthesis

The main products of lipid biosynthesis are phospholipids and sterols, the principal structural components of cell membranes. The lipid composition of cell membranes strongly influences the physiological behaviour of the cell, in particular affecting the

membrane permeability and dynamics and the functionality of the membrane-associated proteins. The capability of cells to adapt to changing environmental conditions, and consequently to maintain the viability and the capability to grow, is strictly related to the functionality of cell membranes, therefore to their lipid composition: it is fundamental for the cell to be able to rapidly change it, in particular to maintain the right fluidity, that mainly depends on the unsaturation degree of fatty acids and on sterol content (Henry 1982; Daum et al. 1998; Ratledge and Evans 1989).

Storage lipids are also produced in yeasts and fungi as neutral lipids that are mainly represented by triacylglycerols (TAGs) and steryl esters. Steryl esters are formed by one molecule of a sterol esterified to one molecule of a (frequently unsaturated) fatty acid. The role of sterol esters in cells is not related to energy storage: sterols in esterified form are not included in membrane structures, but in lipid particles, and contribute to sterol homeostasis (Taylor and Parks 1978). Their concentration is usually low in oleaginous yeasts, while they can become dominant in non-oleaginous yeasts, where they can reach amounts higher than 50% of total lipids (Ratledge and Evans 1989).

TAGs (Fig. 3.1) are instead synthesised as energy storage molecules, in general when carbon sources are still available, while another nutrient, usually nitrogen, becomes limiting to cell growth. They are composed of one molecule of glycerol, a polyalcohol, whose three hydroxyl groups are esterified to three fatty acid molecules. TAGs are stored in lipid droplets and can sometimes exceed 70% of the biomass weight. The industrial production of SCO is then based on the capability of oleaginous microorganisms to accumulate TAGs.

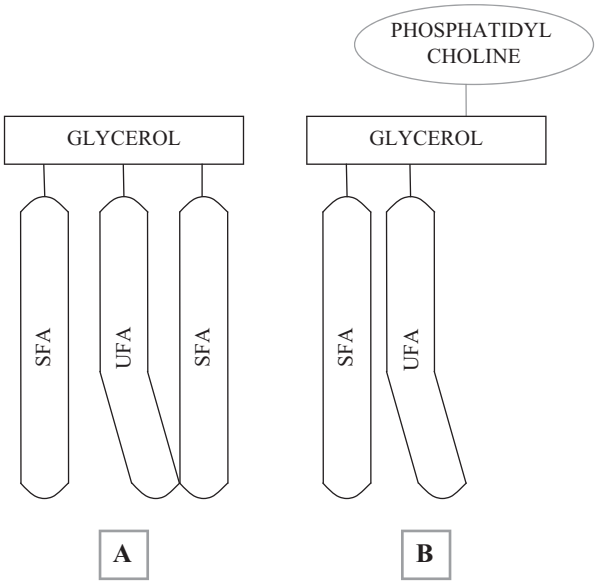

Fig. 3.1 Model of a triacylglycerol (**a**) and a phospholipid (**b**) molecule: triacylglycerol is an apolar molecule, while phospholipid has a polar moiety (esterified to glycerol by a phosphate group in *sn*-3) and an apolar moiety (fatty acyl chains in *sn*-1 and 2)

Lipid biosynthesis is strongly influenced by the growth conditions, in particular by oxygen availability and temperature; in fact, oxygen is necessary to synthesise unsaturated fatty acids and sterols, and the temperature determines the need for specific compounds from which membrane fluidity depends: lower temperatures impose a high unsaturated fatty acid content, while at high temperatures a high sterol content is found (Ratledge and Evans 1989; Bardi et al. 1998, 1999).

Fatty acids can be assimilated from the growth medium, if it contains them, and transferred to anabolic or catabolic pathways; they can also be directly incorporated into phospholipids or TAGs (Ratledge and Evans 1989; Belviso et al. 2004). If growth medium is devoid of lipids, they are synthesised from available carbon sources.

Sterols and fatty acids have acetyl CoA as the common basic precursor unit for biosynthesis. Several studies report that oleaginous microorganisms do not possess hyperactive system of fatty acid biosynthesis, but they produce high quantities of acetyl CoA (Papanikolaou and Aggelis 2011a).

3.2.1 Biosynthesis of Cytoplasmic Acetyl CoA

The main steps of fatty acids and sterols synthesis are cytoplasmic reactions; then suitable amounts of acetyl CoA must be available in the cytoplasm.

The principal pathway of acetyl CoA biosynthesis takes place into the mitochondrion: it is the oxidation of pyruvate, the final product of glycolysis, by the pyruvate dehydrogenase (*PDH*); then acetyl CoA has to be transported from mitochondrion to cytoplasm to become available for lipogenesis. Therefore, glycolysis that is strongly regulated at different steps in determining the glycolytic flux is the crucial step in determining the amount of acetyl CoA produced.

The first step of glycolysis (Fig. 3.2) is the uptake of carbon sources from growth medium into the cell. Hexose uptake is mediated by hexose transporters that are plasma membrane proteins. Transport across the plasma membrane is the first limiting step of sugar metabolism, and the glucose concentration is the main factor that regulates the quantity, types and activity of glucose transporters, both at the transcriptional and posttranslational levels (Ozcan and Johnston 1999). The flux of hexoses through the plasma membrane is determined during the early stages of growth: if a lack of nitrogen happens during this phase, the glycolytic flux can be irreversibly prevented throughout all cell growth phases.

Among glycolytic enzymes, phosphofructokinase (*PFK*) is considered the bottleneck at which level the overall glycolytic flux is determined. This enzyme that catalyses the irreversible conversion of fructose-6-phosphate in fructose-1,6-bisphosphate is allosteric, and it is activated by one substrate of the reaction, fructose-6-phosphate, and inhibited by the other one, ATP. *PFK* is also activated by ammonium, phosphates, AMP and ADP, and it is responsible for the glycolytic oscillation (Wolf et al. 2000). A feedback strong inhibition on *PFK* is also exerted by citrate that is formed into the mitochondrion from acetyl CoA in the Krebs cycle and whose concentration

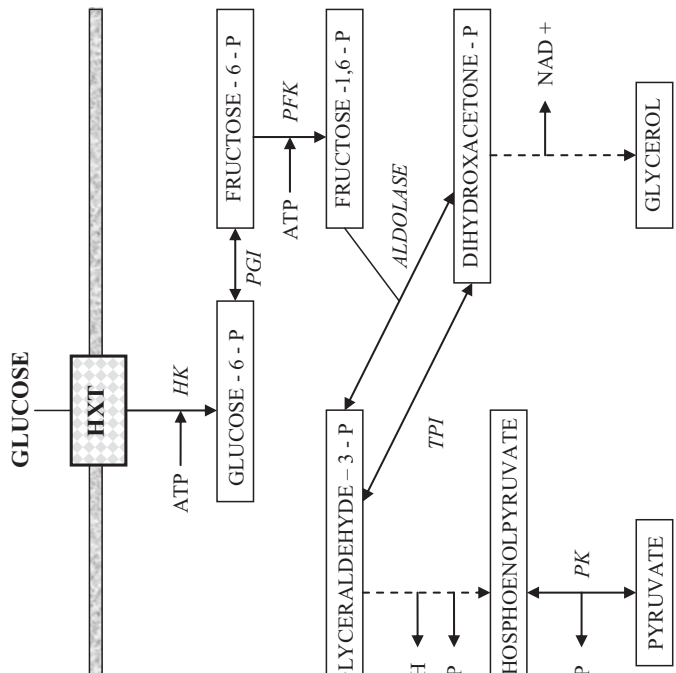

Fig. 3.2 Glycolysis. *HXT* hexose transporter, *HK* hexokinase, *PFK* phosphofructokinase, *PGI* phosphoglucoisomerase, *TPI* triosephosphate isomerase, *PK* pyruvate kinase

into the cell gathers during lipid accumulation. However, the inhibitory effect of citrate is counterbalanced if nitrogen supply is adequate during early growth phases, when nitrogen make *PFK* resistant to citrate inhibition, probably due to the formation of a stable *PFK*-NH_4 complex (Ratledge and Wynn 2002).

Pyruvate kinase (*PK*), releasing pyruvate as the final product, is another important enzyme regulating the glycolysis; it is inhibited by ATP, citrate, acetyl CoA and saturated fatty acids, while it is induced by ammonium, ADP and fructose-1,6-bisphosphate (Ratledge and Wynn 2002).

It is therefore clear that, even at the level of the glycolysis, in oleaginous yeasts and fungi, a suitable availability of nitrogen during early phases of growth is fundamental to reach good yields during lipid accumulation phase.

Pyruvate, as said above, is transported into the mitochondrion, where it is oxidised by *PDH* to acetyl CoA, starting the tricarboxylic acid (TCA) cycle (Fig. 3.3).

In *Saccharomyces cerevisiae*, a typical Crabtree-positive, non-oleaginous yeast, a glucose concentration higher than 2% can rapidly inhibit lipid synthesis. Indeed, the mitochondrial synthesis of acetyl CoA by *PDH* is repressed in these conditions, and the main pathway producing acetyl CoA is from acetate by acetyl CoA synthetase (*ACS*) directly in the cytoplasm (Ratledge and Evans 1989).

When acetyl CoA is produced into the mitochondrion, it must be translocated into the cytoplasm to become available to fatty acid synthetase (*FAS*), an enzymatic complex that produces one molecule of palmitic acid (C16:0) from eight molecules of acetyl CoA.

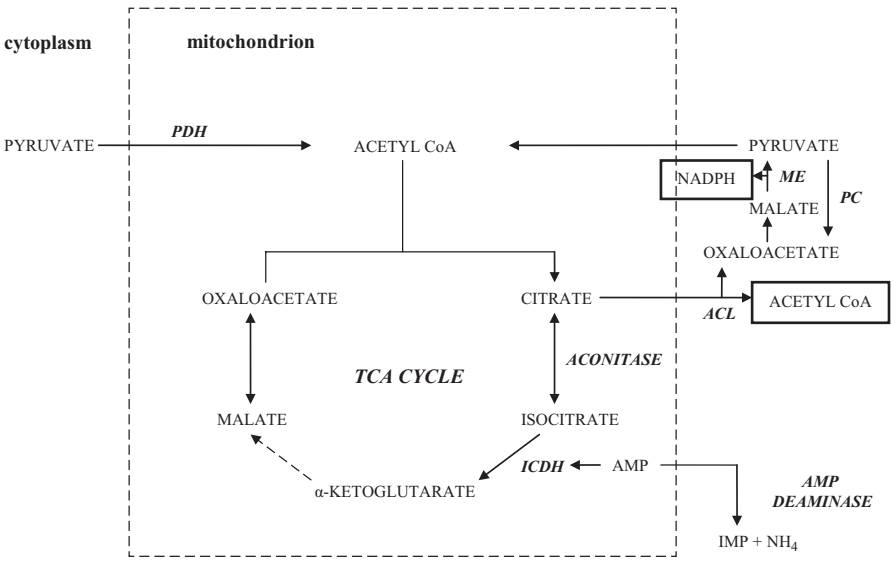

Fig. 3.3 Biosynthesis of acetyl CoA from pyruvate. *PDH* pyruvate dehydrogenase, *ICDH* isocitrate dehydrogenase, *ACL* ATP-citrate lyase, *PC* pyruvate carbxylase, *ME* malic enzyme

The transport of acetyl CoA through the mitochondrial membrane is mediated by the citrate. In the TCA cycle, the citrate is formed from acetyl CoA by the citrate synthase, and it is reversibly transformed to isocitrate by the enzyme isocitrate-aconitase; isocitrate is then decarboxylated to α-ketoglutarate by isocitrate dehydrogenase (*ICDH*), an allosteric enzyme activated by intracellular AMP. In the oleaginous yeasts and fungi, following a nitrogen starvation, the intracellular AMP concentration rapidly decreases, because it becomes a nitrogen source due to the activity of the AMP deaminase, an enzyme that cleaves it to inosine monophosphate (IMP) and ammonium. As a consequence of AMP concentration decrease, the activity of *ICDH* drops, and isocitrate concentration raises in the mitochondrion. As isocitrate is in equilibrium with citrate, the citrate concentration raises too: in oleaginous yeasts and fungi, a citrate release is frequently associated to the lipid accumulation phase (Fig. 3.3).

Citrate is transported through the mitochondrial membrane into the cytoplasm by a citrate/malate translocase; there it is cleaved to acetyl CoA and oxaloacetate by ATP citrate lyase (*ACL*), also stimulated by ammonium.

In non-oleaginous yeasts, this pathway is not active; AMP concentration does not decrease following nitrogen starvation, and there is no *ACL* activity: this enzyme has then also been proposed as the key factor to distinguish oleaginous from non-oleaginous microorganisms. However, whereas *ACL* occurrence in yeasts is variable, this enzyme is always present in filamentous fungi; moreover, significant differences of *ACL* activity have not been found in correlation with different amount of accumulated lipids among different strains, and *ACL* activity does not vary much between the balanced growth phase and the lipid accumulation phase. Therefore, the peculiarity of lipid-accumulating yeasts and fungi seems to be the capability to strongly increase the *AMP* deaminase activity under nitrogen starvation (Ratledge and Wynn 2002).

In non-oleaginous yeasts, also the citrate/malate transport system seems not to be active, and the transport through the mitochondrion membrane is mediated by the carnitine acyl transferase, an enzyme that transforms acetyl CoA into acetyl carnitine; this pathway, however, is usually activated on the opposite way, namely, to transport into the mitochondrion the acetyl CoA produced by β-oxidation during lipid catabolism (Papanikolaou and Aggelis 2011a; Ratledge and Evans 1989) (see also Sect. 3.2.4.3).

Among microorganisms characterised by significant AMP deaminase activity, a distinction can be made into lipid-accumulating and citrate-accumulating strains. *ACL* can show different affinity for citrate in different microorganisms: this could explain why lipogenesis or citrate accumulation can be activated in different microorganisms equipped with *ACL* at the same citrate concentration (Ratledge and Wynn 2002). Citrate accumulated is either excreted into the medium (i.e. *Aspergillus niger* or *Candida* sp. strain) or transformed into intracellular polysaccharides (i.e. *Aureobasidium pullulans*) (Papanikolaou and Aggelis 2011a). Otherwise, *ACL* converts cytoplasmic citrate into acetyl CoA that is addressed to anabolic biosynthetic pathways.

ACL activity is repressed by long-chain acyl CoA; by this way, a loop of lipid biosynthesis and stored lipid consumption is prevented when starvation occurs (Ratledge and Wynn 2002).

Nitrogen availability is then one main factor on which focusing the attention when high yield SCO must be reached; in fact, during early stages of growth, sufficient nitrogen is necessary in order to activate all the enzymes necessary in this pathways; then, at late fermentation phases, nitrogen starvation must intervene, to induce change in direction of carbon flow, from energy metabolism and cell multiplication towards storage lipids accumulation.

3.2.2 Biosynthesis of Fatty Acids

Next step towards the fatty acid synthesis is the carboxylation of acetyl CoA to malonyl CoA, catalysed by the acetyl CoA carboxylase (*ACC*), a biotine-dependent, allosteric enzyme, downregulated by palmitoyl CoA. Repression of the expression of *ACC* by saturated fatty acids avoids the maintenance of lipid biosynthetic pathway when lipids are present in the cell or in the growth medium. In oleaginous yeasts, *ACC* is activated by citrate that induces the aggregation of monomers in a macrostructured active protein: therefore, *ACC* is another step at which level a basic role is exerted by citrate on lipid accumulation in oleaginous yeasts and moulds.

Malonyl CoA is charged on the fatty acid synthetase (*FAS*), a multienzymatic complex, where it is condensed with one molecule of acetyl CoA, forming a C_4 acyl-ACP (*ACP* = acyl carrier protein, a polypeptide that forms an integral part of *FAS*); then the acyl-ACP chain is progressively elongated, with malonyl CoA as the constant C_2 donor, until the synthesis of palmitoyl-ACP, in a cyclical process that involves eight different catalytic sites of *FAS* and that requires 14 molecules of NADPH for one molecule of final palmitoyl-ACP that is transferred to CoA and released from *FAS* as palmitoyl CoA (Ratledge and Evans 1989). Fatty acids longer than palmitic acid (C16), first of all stearic acid (C18), are produced by elongases.

The high amount of NADPH necessary to synthesise fatty acids is generated by several enzymatic activities, such as glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-dependent isocitrate dehydrogenase (Ratledge and Wynn 2002); in oleaginous microorganisms, another important NADPH-generating step is the "transhydrogenase cycle", in which the malic enzyme (*ME*) decarboxylates malate to pyruvate-producing NADPH in the cytosol (Fig. 3.3). *ME* is not ubiquitous, but it is present in several yeasts and fungi and is associated to lipogenic activity. It was thought that the main *ME* function was the contribution to the cytosolic NADPH pool, but also a direct role of this enzyme in fatty acid synthesis and desaturation has been proposed, and above all, the essentiality of *ME* for lipid biosynthesis and accumulation was established (Ratledge and Wynn 2002; Wynn et al. 2001). *ME*, in fact, is not vital, but without *ME* the carbon flux from glucose to lipids is diminished and only essential lipids are synthesised; moreover, in studies carried out on filamentous fungi showing different extents of

lipid accumulation, only *ME* activity was correlated with the pattern of lipid accumulation (Wynn et al. 1999; Ratledge and Wynn 2002). *ME* was downregulated following nitrogen exhaustion from the medium; but as this enzyme is present in different isoforms, characterised by different stability, the final lipid content was determined by the stability of the enzyme, from which the length of time over which lipid accumulation occurs depends (Wynn et al. 1999). Another interesting application of *ME* activity control could be a diversion of acetyl CoA units into secondary metabolites other than storage lipids, consequent to inhibition of *ME* activity; sesamol is a specific inhibitor of *ME* (Jacklin et al. 2000).

A possible source of NADPH alternative to *ME* activity has been proposed by Bellou et al. (2016) as the pentose phosphate pathway, on the basis of the results obtained in fermentations carried out with *Yarrowia lipolytica* under double nitrogen and magnesium limitation, where during lipogenesis a low *ME* activity was observed, and the essential role of magnesium and the requirement of organic nitrogen at low concentrations to reach lipid accumulation emerged.

Unsaturated fatty acids (UFA) are produced from saturated fatty acids (SFA) due to the action of desaturases, molecular oxygen- and NADPH-requiring proteins bound to endoplasmic reticulum membranes. Desaturation is the result of the activity of three proteins: NADPH-cytochrome b_5 reductase, cytochrome b_5 and the terminal cyanidine-sensitive desaturase (Certik and Shimizu 1999). Several desaturases have already been cloned and characterised, and in general it has been observed that they do not show great similarity, neither among different desaturases of the same cell nor for the same desaturase among different species; however, a common and highly conserved feature is the presence of three histidine (*HIS*) boxes, separated by two hydrophobic regions whose function is probably to anchor the enzyme to the endoplasmic reticulum membrane (Stukey et al. 1990), while *HIS* boxes are positioned on the cytosolic face of the endoplasmic reticulum (Shanklin et al. 1994).

There are three types of desaturases: acyl CoA desaturases, acyl-ACP desaturases and acyl lipid desaturases (Certik and Shimizu 1999). Δ^9 desaturase introduces the first double bond between carbons 9 and 10 into the palmitoyl CoA or into stearoyl CoA, producing the mono-unsaturated fatty acids palmitoleoyl CoA or oleoyl CoA, respectively (Ratledge and Evans 1989). The Δ^9 is the only desaturase that acts on saturated fatty acids and that has acyl CoA as its substrate: the others are active on phospholipid-bound acyl groups (Ratledge and Wynn 2002). The expression of Δ^9 desaturase is regulated by temperature, indicating an involvement in cold acclimation, as demonstrated in *Mucor rouxii* (Laoteng et al. 2000), and repressed by exogenous fatty acids with a Δ^9 desaturation (Meesters and Eggink 1996).

Phospholipid (usually phosphatidylcholine)-bound oleic acid is further desaturated to linoleic acid by Δ^{12} desaturase; only a small amount of desaturation occurs of oleyl CoA. A third desaturation can be effected by Δ^{15} desaturase producing linolenic acid (Ratledge and Evans 1989).

In general, the fatty acids found in oleaginous yeasts are palmitic acid, stearic acid, palmitoleic acid, oleic acid, linoleic acid and linolenic acid (Table 3.3 and Box 3.1). Among them, the less abundant are usually palmitoleic acid and stearic acids; linolenic acid can be found in trace, while the most abundant is usually oleic acid.

Table 3.3 SCO fatty acyl profile of selected oleaginous yeasts and fungi

	Major fatty acyl residues (relative % w/w)											
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	18:3	20:4	20:5	22:6	
			(n-7)		(n-9)	(n-6)	(n-3)	(n-6)	(n-6)	(n-3)	(n-3)	Others (%)
Yeasts												
Candida sp. *107*	Trace	44	5	8	31	9	1	–	–	–	–	
Cryptococcus albidus	Trace	12	1	3	73	12	–	–	–	–	–	
Cryptococcus curvatus	Trace	32	–	15	44	8	–	–	–	–	–	
Waltomyces lipofer	Trace	37	4	7	48	3	–	–	–	–	–	
Lipomyces starkeyi	Trace	34	6	5	51	3	–	–	–	–	–	
Rhodosporidium toruloides	Trace	18	3	3	66	–	–	–	–	–	–	23:0 (3%)24:0 (6%)
Rhodotorula glutinis	Trace	37	1	3	47	8	–	–	–	–	–	
Rhodotorula graminis	–	30	2	12	36	15	4	–	–	–	–	
Trichosporon beigelii	Trace	12	–	22	50	12	–	–	–	–	–	
Yarrowia lipolytica	Trace	11	6	1	28	51	1	–	–	–	–	
Fungi												
Conidiobolus nanodes	1	23	–	15	25	1	–	4	4			20:1 (13%) 22:1 (8%)
Cunninghamella japonica	Trace	16	–	14	48	14	–	8	–	–	–	
Entomophthora coronata	31	9	–	2	14	2	–	1	–	–	–	12:0 (13%)
Mortierella alpina	–	19	–	8	28	9	–	8	21	–	–	20:3 (7%)
Mortierella isabellina	1	29	–	3	55	3	–	3	–	–	–	
Mucor alpine-peyron	10	15	–	7	30	9	–	1	5	–	–	20:0 (8%) 20:3 (6%)
Mucor circinelloides	–	22	1	5	38	10	–	15	–	–	–	
Pythium ultimum	7	15	–	2	20	16	1	–	11	14	–	20:1 (5%)
Rhizopus arrhizus	19	18	–	6	22	10	–	12	–	–	–	
Aspergillus terreus	2	23	–	Trace	14	40	21	–	–	–	–	
Fusarium oxysporum	Trace	17	–	8	20	46	5	–	–	–	–	
Pellicularia practicolo	Trace	8	–	2	11	72	2	–	–	–	–	
Cladosporium herbarum	Trace	31	–	12	35	18	1	–	–	–	–	
Claviceps purpurea	Trace	23	–	2	19	8	–	–	–	–	–	12-HO-18:1 (42%)

Extracted from Ratledge and Wynn 2002; Papanikolaou and Aggelis 2011a

However, the relative concentrations can strongly change in relation to yeast species and growth conditions.

Fatty acids with more than three desaturations (PUFA = poly unsaturated fatty acids) and longer than C18 (*LCFA* = long chain fatty acids) can be found in significant amounts in fungi. PUFA and LCPUFA (long chain polyunsaturated fatty acids) are sought after their specific properties mainly for nutritional or nutraceutical exploitations. Their synthesis is due to the subsequent action of elongases, endoplasmic reticulum membrane-bound proteins, that extend the chain with two-carbon units at the carboxyl end and desaturases that catalyse the insertion of further double bonds.

The reaction catalysed by elongases is similar to *FAS* reaction that is composed of four sequential phases: condensation of acyl chain with malonyl CoA forming a ketoacyl compound, reduction of ketoacyl to hydroxyacyl compound, dehydration of hydroxyacyl to enoyl compound and reduction of enoyl to acyl compound. Only the first of these four phases is specific for the substrate. Elongases can be a rate-limiting step of PUFA synthesis (Wynn and Ratledge 2000).

Oleic acid, linoleic acid and α-linolenic acid are the origin of the three pathways from which n-9, n-6 and n-3 PUFA are synthesised, respectively, following the alternate action of specific elongases and different desaturases. Among the most commercially valued, γ-linolenic acid (GLA), arachidonic acid (ARA) and docosapentaenoic acid (DPA) belong to n-6 series and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to n-3 series (see also Box 3.1). Δ^6, Δ^5, Δ^4, Δ^{15} and Δ^{17} desaturases are involved (Certik and Shimizu 1999).

In eukaryotic cells the main UFA synthesis pathway is aerobic. The need for molecular oxygen of desaturases determines the lack of growth in anaerobiosis when exogenous lipids are not present in the growth medium. Yeasts and fungi can easily assimilate fatty acids from the growth medium, inserting them directly into the cell lipids; this also explains the presence of polyunsaturated fatty acids in yeasts, such as *Saccharomyces cerevisiae*, that does not possess desaturases other than Δ^9. Addition of specific exogenous FA to the growth medium can be a possible way to modify the lipid composition of SCO when a specific goal is aimed.

3.2.3 Biosynthesis of TAG

Growing microorganisms use fatty acids foremost to synthesise phospholipids that are the main cell membrane components. Lipid accumulation usually starts if cell growth ceases when carbon sources are still available in growth medium, becoming convertible into storage fats. The main storage lipids in oleaginous yeasts and fungi are the triacylglycerols, formed by three fatty acids whose carboxyl group gives rise to an ester bond with each hydroxyl group of one molecule of glycerol (Fig. 3.1).

Glycerol that is synthesised by NADH-mediated reduction of dihydroxyacetone phosphate produced during glycolysis is activated by glycerol kinase to glycerol 3-phosphate, from which the “Kennedy pathway” starts, producing phosphatidic

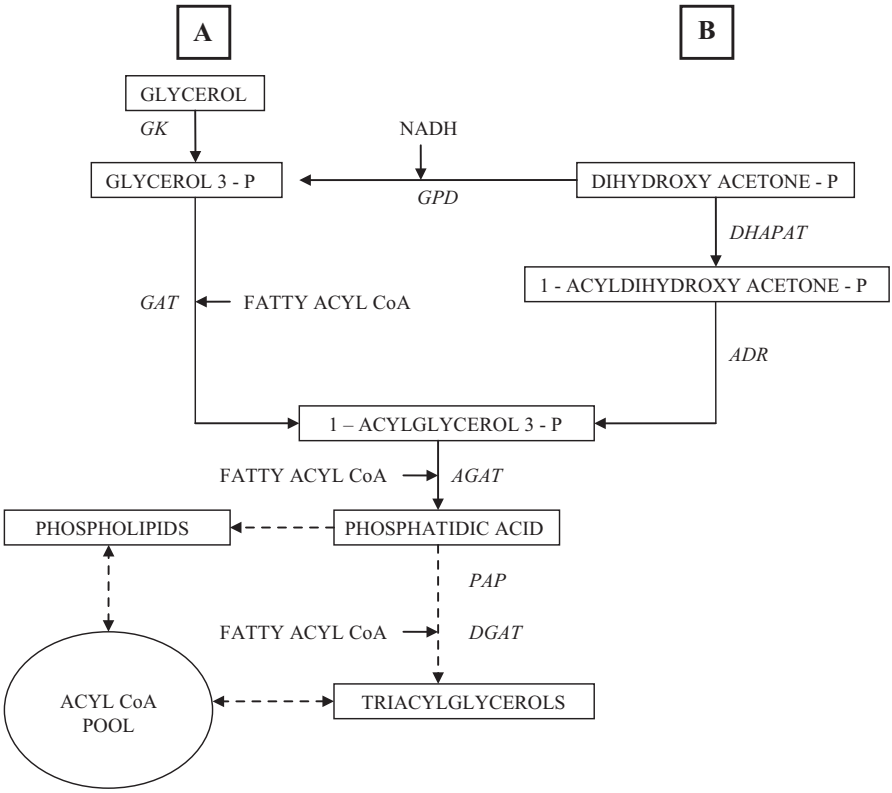

Fig. 3.4 Biosynthesis of triacylglycerols. (**A**) Kennedy pathway: *GK* glycerol kinase, *GAT* glycerol 3-phosphate acyltransferase, *AGAT* lysophosphatidate acyltransferase, *PAP* phosphatidate phosphatase, *DGAT* diacylglycerol acyltransferase. (**B**) Dihydroxyacetone phosphate (DHAP) pathway, *DHAPAT* DHAP acyltransferase, *ADR* 1-acyl DHAP reductase
GPD glycerol 3-phosphate dehydrogenase

acid (1,2-acyl-*sn*-glycerol 3-phosphate) through the action of glycerol 3-phosphate acyltransferase (*GAT)* and lysophosphatidate acyltransferase (*AGAT*) that transfer to glycerol 3-phosphate two fatty acids from fatty acyl CoA (Fig. 3.4). Usually the acyl substituents into the *sn*-1 position are saturated chains, while the acyl substituents into the *sn*-2 position are unsaturated chains. Phosphatidic acid is a common substrate for phospholipids and for triacylglycerols synthesis; for the latter pathway, phosphatidic acid is dephosphorylated by a phosphatidate phosphatase (*PAP*), an enzyme that has been characterised as regulatory in *Saccharomyces cerevisiae*, producing 1,2-diacyl-*sn*-glycerol (*DAG*); then, the third acyl chain from a fatty acyl CoA molecule is bound to 1,2-diacyl-*sn*-glycerol by diacylglycerol acyltransferase (*DGAT*), forming a 1,2,3-triacyl-*sn*-glycerol (TAG) (Fig. 3.4).

Phosphatidic acid can also be synthesised from dihydroxyacetone phosphate (DHAP), acylated by the DHAP acyltransferase (*DHAPAT*) to 1-acyl DHAP, that is then reduced to 1-acyl glycerol 3-phosphate by 1-acyl DHAP reductase (*ADR*).

Fatty acids can also be transferred from phospholipids to triacylglycerols. In particular, as PUFA are usually formed on phospholipids, a "acyl shuttling" can occur between a phospholipid and a diacylglycerol or a triacylglycerol; this can occur through combined reverse and forward reactions of acyl CoA phospholipid acyltransferases that contribute to a common acyl CoA pool or through making the entire diacylglycerol portion of phosphatidylcholine available to TAG synthesis by CDP-choline phosphotransferase (Certik and Shimizu 1999). The phospholipid biosynthetic enzymes are mainly localised on microsomal and mitochondrial membranes (Kuchler et al. 1986), while TAG biosynthetic enzymes are mainly localised in the lipid bodies. For long time it was thought that lipid bodies formation in cytoplasm was the consequence of a physical process of coalescence, due to hydrophobic nature of neutral lipids, and that they were inert structures. Instead, lipid bodies are involved in the regulation of lipid storage and reutilisation, and they are covered by membranes, in which also several proteins are present: among them, there are proteins with probably structural role and enzymes associated to TAG biosynthesis, in particular *DGAT* (Mlickova et al. 2004a, b; Athenstaedt et al. 2006). Interestingly, in *Mortierella ramanniana* it has been observed that phosphatidic acid is preferentially incorporated in lipid bodies for TAG biosynthesis, while phosphatidylcholine is preferentially incorporated in internal membranes, probably endoplasmic reticulum membranes, and then gradually transported into lipid bodies (Kamisaka et al. 1999). It has been proposed that the lipid bodies play a crucial role, in the intracellular organisation, connected to a "lipogenic metabolon", finalised to clustering the enzymes and the substrates involved and needed in the same biosynthetic pathway (Ratledge and Wynn 2002).

3.2.4 Physiological and Environmental Factors Affecting the Lipid Accumulation

3.2.4.1 Temperature, Oxygen Availability, pH

Fatty acyl composition of phospholipids determines the physical properties of the cell membranes, strongly affecting their structure, dynamics and permeability. These properties are strictly related to the temperature: in fact, low temperatures induce stiffening, while high temperatures increase fluidity of membranes; in both cases, the membrane permeability and the activity of the membrane-associated proteins can significantly change, exposing cell to risk of damages, physiological alterations and greater exposure to environmental stresses. The first strategy adopted by the cell to maintain the membrane functionality is changing the fatty acyl residues: in fact, shorter-chain and unsaturated fatty acids increase fluidity, while saturated fatty acids decrease it. Then, when cells are grown at low temperatures, their UFA and MCFA (medium chain fatty acids) content increases; conversely their SFA content increases when grown at high temperatures (Ratledge and Evans 1989; Bardi et al. 1999), with few exceptions (Papanikolaou and Aggelis 2011b).

Fatty acyl composition of phospholipids can be different from that of triacylglycerols: for example, in *Rhodotorula gracilis* a higher unsaturation degree was observed in phospholipids of cells grown under nitrogen limitation than of cells grown under carbon limitation, while triacylglycerols did not show differences (Rolph et al. 1986). Moreover, significant increases of cell lipid content are usually due to triacylglycerol accumulation, because the amount of phospholipids does not usually show significant changes. Then, temperature can mainly affect phospholipids composition than triacylglycerol composition; however, in general, a higher UFA content can be expected when cells are grown and incubated at lower temperatures. Also oxygen availability affects the UFA content, as desaturases are oxygen-dependent enzymes.

Both temperature and oxygen availability influence the total lipid content of cells: in general, it increases with raising aeration rate and decreasing temperature, even if bell-shaped trends have also been observed for lipid accumulation in relation to temperature (Papanikolaou and Aggelis 2011b; Ratledge and Evans 1989).

No significant effects of pH have been reported for lipid biosynthesis; however, a crucial role of pH was observed for lipid accumulation in *Yarrowia lipolytica* grown on stearin (Papanikolaou et al. 2002).

3.2.4.2 Growth Medium Composition

Growth medium composition is fundamental in determining cell lipid synthesis and accumulation. The latter is caused first of all by a nutrient imbalance: when a certain nutrient (usually nitrogen) become insufficient for growth, but carbon sources are still available in growth medium, cells stop multiplication but continue to assimilate carbon, converting it into storage lipids. The leading role of nitrogen in regulation of carbon flow, acetyl CoA and fatty acids biosynthesis has already been described above (2.1). C/N > 20 molar ratio values are considered in general necessary for SCO accumulation. In very few cases, an accumulation of lipids simultaneous to cell growth has been observed in yeasts and fungi (Boulton and Ratledge 1984); instead, it usually happens, for example, in microalgae that assimilate carbon at a speed higher than their ability to use it for cell multiplication. Moreover, under nitrogen-limited conditions, an accumulation of intracellular total sugars has been observed during early phases of fermentation in *Cryptococcus curvatus*, followed by a consumption of these intracellular sugars accompanied to lipid accumulation at the end of fermentation: this demonstrates an interplay between the biosynthesis of intracellular sugars and lipid synthesis (Tchakouteu et al. 2015a).

Fermentation yield of SCO depends on the amount of microbial biomass produced, the amount of lipids accumulated in cells and the growth phase at which the accumulated lipids are at the higher concentration; then, to maximise the SCO production, it is suitable to find the best balance among cell growth and lipid accumulation and to avoid the lipid turnover. Therefore, the initial nitrogen concentration must be quantified taking into account that cell multiplication should not stop too early, but also that lipid accumulation starts, in general, when nitrogen becomes

limiting to cell growth, but cells are still active in the presence of an excess of carbon. Moreover, the C/N ratio has to be chosen taking into consideration the kind of nitrogen and carbon source in relation to the metabolism of the chosen yeast or fungus. Regarding the nitrogen source, many studies have been carried out with ammonium as the preferred mineral nitrogen source favouring lipid accumulation, but in some cases higher lipid content was observed in cells grown with organic nitrogen instead of ammonium (Ratledge and Evans 1989; Certik et al. 1999). If glucose is used as carbon source, a significantly different behaviour is shown by Crabtree-positive and Crabtree-negative yeasts: the latter show higher cellular lipid content at increasing glucose concentrations, while the former, suffering for catabolite repression, reduce their lipid content. The maximum theoretical yield of SCO that can be reached from glucose, calculated on the basis of a stoichiometric complete conversion of glucose into triacylglycerols, is 32%, even if, in real fermentations, yields no higher than 22% have been obtained. The theoretical yield is higher if other carbon sources are used: 54% with ethanol, 34% with xylose, 30% with glycerol (Papanikolaou and Aggelis 2011a). Many studies have investigated the SCO yields that can be reached with different yeasts and fungi species and with different kind of carbon sources. For example, raising lipid content ranging from 39% to 62% with *Rhodotorula gracilis* grown on cellobiose, sucrose, glucose and xylose; from 30% to 49% with *Candida curvata* grown on ethanol, glucose, lactose and xylose, respectively; and of 30% on ethanol has been obtained (Ratledge and Evans 1989). The kind of carbon source can also affect the fatty acyl composition: in *Rhodotorula glutinis* grown on glycerol an unsaturation degree (53%) higher than on xylose (25%) was obtained (Easterling et al. 2009).

Several agricultural and agro-industrial wastes or coproducts, such as cheese whey, molasses, sewage sludge, tomato waste hydrolysate, rice straw hydrolysate, polysaccharides, N-acetyl-glucosamine and starch hydrolysate, have been examined as growth substrate, to explore the opportunity to improve the environmental and economic sustainability of these activities and to increase the added value of the SCO production processes (Papanikolaou and Aggelis 2011b).

Recently, much attention has been paid on glycerol, produced in great amounts as residue of biodiesel industry. A screening of 15 yeasts and fungal strains grown on glycerol showed a better lipid accumulation in fungi (up to about 46% w/w dry biomass) than in yeasts (up to about 22% w/w dry biomass), but yeasts showed to be able to reach higher production of biomass, and a really high SCO yield was obtained with *Thamnidium elegans*: more than 70% w/w dry biomass, corresponding to more than 11 g/L (Chatzifragkou et al. 2011). The opportunity to carry out fermentation in non-aseptic conditions can also be a way to reduce the costs, improving the added value of SCO production process: large amounts of lipids were accumulated in *Zygomycetes* mycelia in these conditions, yielding about 13% w/w of oil per glycerol consumed (Moustogianni et al. 2015). An improvement of SCO yields with yeasts can also be obtained by increasing the initial glycerol concentrations (even 180 g/L); this can be also accompanied by an accumulation of polysaccharides in cells (Tchakouteu et al. 2015b). A lipid-free biomass accumulation was

sometimes observed during lipid accumulation phase of nongrowing cells (Fakas et al. 2009; Papanikolaou and Aggelis 2011a).

Lignocellulosic biomasses are considered a suitable substrate for microbial transformations, in particular to produce biofuels, due to their large availability as residues and waste and to their end use not in competition with food destinations. They are characterised by high C/N ratio that makes them suitable as substrate for microbial conversion into SCO. In fermentations carried out with *Lipomyces starkeyi*, a 38% w/w lipid accumulation was obtained when cells were grown on lignocellulose hydrolysate (Calvey et al. 2016) and a 27,8% w/w lipid content when cells were grown on hemicellulose hydrolysate (Anschau et al. 2014).

Hemicellulose that is a residue of pulp and paper production is a heteropolymeric carbohydrate, whose main monomer is xylose, a carbon and energy source used by many oleaginous yeasts and fungi. To make the monomers forming lignocellulose and hemicellulose available for cell growth, a pretreatment is necessary, during which several toxic compounds are produced, such as acetic acid, furfural and hydroxymethylfurfural, that impede the growth of microorganisms that are not resistant to them. The adoption of a fed-batch fermentation strategy, combining the feeding with hemicellulose hydrolysate to the pH regulation due to co-consumption of xylose and acetic acid by *Lipomyces starkeyi* cells, allowed to produce a lipid accumulation of 51.3% and a complete consumption of xylose (Brandenburg et al. 2016). Direct conversion of cellulose into lipids can be also obtained by cellulolytic fungi. Hui et al. (2010) carried out solid-state fermentations of wheat straw with *Aspergillus oryzae*, obtaining good lipid production; yield was influenced by cellulase secretion and enhanced by the addition of agro-industrial wastes.

Another agro-industrial effluent posing serious environmental problems is the olive mill wastewater, generated during the extraction of the olive oil; this effluent is characterised by a high organic load, associated to the presence of phenolic compounds with phytotoxic and antimicrobial activities that limit the efficacy of biotreatments. *Lipomyces starkeyi* was individuated as able to grow on these effluents accumulating lipids reducing phytotoxicity, total organic carbon and phenolics of residues (Yousuf et al. 2010). Some *Zygomycetes* strains (*Thamnidium elegans* and *Zygorhynchus moelleri*) have also been selected and characterised for their ability to grow on olive mill wastewater, accumulating up to 60% w/w lipids rich in PUFA (Bellou et al. 2014).

Yeasts and fungi can also grow on fatty materials, such as vegetable oils, fish oils, stearin, soap stocks or hydrocarbons, but only few of them are able to accumulate intracellular lipids in these conditions. The main enzymes involved in lipid biosynthesis (such as *FAS*, *ACC* and *ACL*) are downregulated by fatty acids and other aliphatic compounds, while enzymes of lipid catabolism (such as lipases and oxidases) are activated, using lipids as energy and carbon sources for cell growth. Nevertheless, when lipid accumulation occurs, this is independent from nitrogen limitation, but it happens simultaneously to cell growth: this is the case, for example, of *Yarrowia lipolytica*, that was considered for a long time a non-oleaginous yeast because of its inability to accumulate lipids when grown on sugars, whereas up to 60% w/w lipids can be accumulated in its cells grown on fats or oils

(Papanikolaou and Aggelis 2011a). Storage lipids can, in this case, be constituted not only from TAGs but also from other compounds, mainly free fatty acids, as was observed for *Candida lipolytica*, *Amylomyces rouxii* and *Cunninghamella blackesleeana* (Koritala et al. 1987). Moreover, the fatty acid composition of the stored lipids can be different from that of the substrates, because they can be assimilated directly without variations but also modified by cells. For example, some yeasts can consume saturated fatty acids and store unsaturated fatty acids; others have desaturases that transform saturated in unsaturated fatty acids; finally, stored lipids are more unsaturated than substrates. In other cases, unsaturated fatty acids from substrate are dissimilated and saturated fatty acids accumulated, resulting in storage lipids more saturated than substrate. Another possibility is that certain fatty acids are selectively assimilated by cells, leaving others into the growth medium. These specific behaviours can be exploited to obtain SCO with a specific composition requested (Papanikolaou and Aggelis 2011a).

As already said above, lipid accumulation is induced by a nutrient unbalance that is usually due to nitrogen limitation. However, other compounds can play this role, such as phosphate or sulphate. In *Rhodosporidium toruloides* a great production of lipid-rich biomass was obtained in a growth medium with sufficient nitrogen but devoid of phosphorous or sulphate, but interestingly, the fatty acid composition was different, with a high saturated fatty acids percentage when sulphate was the limiting factor (Wu et al. 2011) and an enrichment in C18:1 and a decrease of C18:0 when phosphorous was the limiting factor (Wu et al. 2010). The possibility to grow oleaginous yeasts also in nitrogen-rich substrates devoid of phosphorus allows the employment of substrates rich in N-acetylglucosamine, such as wastes from crustacean-fabricating facilities (Wu et al. 2010).

Also vitamins can influence the cell lipid content: an increase was observed under inositol deficiency, while a decrease was induced by lack of pantothenic acid, thiamine and pyridoxine in *Saccharomyces cerevisiae* (Ratledge and Evans 1989). Biomass and oil content could be improved also by the optimisation of Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} and Ca^{2+} concentration (Li et al. 2006).

3.2.4.3 Lipid Turnover

As explained above, in general, lipid accumulation starts when a nutrient, usually nitrogen, is exhausted become the limiting factor for the cell multiplication, and it lasts until a carbon source is available. When also the carbon source is exhausted, accumulated lipids can be reutilised to synthesise other compounds, such as proteins or nucleic acids, and for maintenance of basal metabolism. This lipid turnover can happen only if lipases are present; then, fatty acids released from triacylglycerols become substrates of β-oxidation process that generates acetyl CoA. This enters in the TCA cycle and in the glyoxylate cycle, upon the transport into the mitochondrion mediated by the carnitine acetyltransferase, then becoming available for several anabolic pathways, from which new non-lipidic biomass is biosynthesised. The activity of the glyoxylate cycle enzymes (i.e. isocitrate lyase that cleaves isocitrate

to succinate and glyoxylate, bypassing the decarboxylation of isocitrate catalysed by *ICDH* in the TCA cycle; see also Sect. 3.2.1) is usually repressed when sugars are available as carbon sources, while they are activated when intracellular or extracellular lipid compounds are available.

During lipid turnover, a preferential consumption of specific fatty acids can occur. As a consequence, the composition of remaining lipids can change with respect to the original composition; for example, *Cunninghamella echinulata* depletes only saturated or monounsaturated fatty acids; then the remaining lipids are rich in polyunsaturated fatty acids (Fakas et al. 2007). On the contrary, the remaining lipids of *Yarrowia lipolytica* are more saturated (Papanikolaou and Aggelis 2003).

In order to avoid lipid turnover, to maintain intact the lipid amount produced during accumulation, several strategies can be adopted. Among them, iron or magnesium starvation showed to be effective in *Cunninghamella echinulata* and *Mortierella isabellina* (Papanikolaou et al. 2004). Strains can be selected as inactive in lipid turnover: for example, *Lipomyces starkeyi* does not reutilise storage lipids (Holdsworth et al. 1988). By a genetic engineering approach, "obese" yeast strains have been obtained by disrupting genes coding for acyl CoA oxidases (Mlickova et al. 2004a).

3.3 Metabolic Engineering Strategies for SCO Production

Genetic manipulation strategies can be pursued to enhance triacylglycerols accumulation or to modify the lipid composition in order to obtain a product with specific characteristics. The possible approaches can be gathered in four types: (1) increasing the biosynthesis of the main precursors (acyl CoA and glycerol 3-P), (2) enhancing the biosynthesis of triacylglycerols, (3) hindering lipid turnover and (4) redirecting fatty acyl elongation and desaturation (Thevenieau and Nicaud 2013).

The lack of molecular tools for genetic engineering prevents the genetic manipulation of the lipid metabolism of many oleaginous yeasts and fungi. Molecular genetic tools have been developed for *Yarrowia lipolytica*, in which associated disruption of *GUT2*, encoding a glycerol 3-P dehydrogenase that converts glycerol 3-P into dihydroxyacetone, and of *POX1-6* genes, encoding acyl CoA oxidases, involved in β-oxidation of fatty acids, resulted in a significant increase of lipid production (Beopoulos et al. 2008).

Tai and Stephanopoulos (2013) engineered *Yarrowia lipolytica* overexpressing acetyl CoA carboxylase (*ACC1*), increasing lipid content twofold over control, and diacylglycerol acyltransferase (*DGA1*), the final step of the triacylglycerol synthesis pathway, which yielded a fourfold increase in lipid production over control; a further increase of lipid content was observed when the two gene were simultaneously overexpressed.

Dulermo and Nicaud (2011) increased glycerol 3-phosphate concentration overexpressing *GPD1* (glycerol 3-P catabolic dehydrogenase) and inactivating *GUT2* (glycerol 3-P anabolic dehydrogenase; see also Fig. 3.4) in *Yarrowia lipolytica*, leading to an increase of triacylglycerols accumulation. Moreover, they created a

deficient β-oxidation pathway by inactivating *POX1-6* or *MFE1* genes, increasing TAG and free fatty acids content. Transcriptional analysis in these strains revealed that the high levels of lipids resulted from the overexpression of genes involved in TAG synthesis (*SCT1*, encoding a *sn*-1 acyltransferase, and *DGA1*, encoding an acyl CoA diacylglycerol acyltransferase) and from the repression of genes involved in the degradation of TAG (*TGL3* and *TGL4*, encoding triacylglycerol lipases).

Mutant strains of *Yarrowia lipolytica* were obtained in which various *Aox* were deleted (Mlickova et al. 2004b). These strains presented significant and comparable growth with the wild strain during growth on pure fatty oleic acid, but in the former case noticeable quantities of microbial lipids were accumulated as high-size intracellular lipid droplets.

Saccharomyces cerevisiae can be considered an established cell factory, as developed tools for molecular manipulation are available and lipid metabolism has been well studied since long time; however, it is not an oleaginous yeast. Strategies to induce lipid accumulation should increase the biosynthesis of acetyl CoA and redirect the carbon flux from production of ethanol towards precursors of lipids. Overexpression of *ALD6* (aldehyde dehydrogenase), a mutated acetyl CoA synthase gene from *Salmonella enterica*, *ADH2* (assimilatory alcohol dehydrogenase) and *ERG10* (acetyl CoA acetyltransferase) produced substantial amounts of acetyl CoA redirection from ethanol and biomass production towards α-santalene; this platform can be also used to produce biodiesel. The synthesis of fatty acid ethyl esters, which could be directly used as biodiesel, would also be very suitable; expression of heterologous wax synthases associated to overexpression of *ACC* allowed to produce them, but at very small concentrations (Passoth 2014).

Several mutant strains of oleaginous yeasts and fungi were constructed by suppression or activation of specific desaturases and elongates to allow the production of tailor-made fatty acids. With these mutants it was possible to modify the degree of fatty acyl unsaturation and/or the chain length, to improve the production of naturally occurring PUFA, and also to produce new PUFA, commonly not found in the wild-type microorganisms. Mutants can be also excellent tools for regulating exogenous fatty acid flow to targeted PUFA (Certik and Shimizu 1999). For example, γ-linolenic acid (GLA) was produced in *Yarrowia lipolytica* overexpressing Δ^{12} and Δ^{6} desaturases from *Mortierella alpina* (Chuang et al. 2010).

3.4 Industrial Applications

3.4.1 Nutrition and Nutraceuticals

3.4.1.1 PUFA

Among the potential industrial applications of SCO, the production of PUFA is characterised by the highest added value. Many unsaturated and polyunsaturated fatty acids are named “essential” because mammals need to assimilate them from

food, as they are unable to biosynthesise them. UFA and PUFA play a fundamental role in cell membranes and in many physiological processes. PUFA demand as dietary supplements is very high for both adult and infant nutrition, and with the progressive ageing of the world population, their demand is still increasing due to their healthy actions against most of the illness of ancient people. They are recommended for the prevention of hearth diseases and for the improvement of retinal, brain and gut functions. The more diffused PUFA sources are vegetal and fish oils, whose consumption can pose in some cases dietary problems and may be incompatible with vegetarian diets; so SCO can represent a precious alternative. Moreover, when a specific fatty acid has to be purified, it is easier to obtain it from a SCO than from vegetable or animal oil.

The first PUFA produced at industrial level as a SCO was γ-linolenic acid (GLA), useful in relief of premenstrual tension and for treatment of eczema; it has also been shown that it aids sufferers of chronic inflammatory arthritis and that it is useful also in diseases such as Alzheimer, cancer, depression, peroxisomal disorders and hyperactivity. The microorganism used was *Mucor circinelloides*, a filamentous fungus that has long been associated with oriental food, so it is universally considered safe for human nutrition.

The second PUFA produced as SCO was arachidonic acid (ARA) that is added to infant feed formula together with docosahexaenoic acid (DHA), produced using *Mortierella alpina*. ARA is traditionally found in eggs and liver.

Eicosapentaenoic acid (EPA) and DHA occur together in fish oils, and their consumption is advocated in older people to prevent cardiac problems; they have also shown to reduce the occurrence of inflammatory disease such as asthma and type I diabetes mellitus. However a need for pure DHA or EPA exists for particular uses, and as it is difficult to separate these two compounds when they are present in the fish oil, SCO is a possible alternative way. DHA is recommended for infant dietary supplementation only if it is associated to arachidonic acid (ARA): the latter can be produced with *Mortierella alpina*. DHA can be produced from marine microorganisms (*Crypthecodinium cohnii* and Thraustochytrids), while EPA, considered efficacious in the treatment of mental disorders, blood pressure, platelet aggregation, hypertriglyceridemia and various inflammatory responses, can be produced from microalgae but also from a genetically modified *Yarrowia lipolytica* strain (Ratledge 2013).

3.4.1.2 Cocoa Butter Substitutes (CBS)

Cocoa butter is a fat commonly used for food and cosmetics applications. It is characterised by a composition reach in saturated fatty acids: about 55–67% as the sum of palmitic and stearic acids. The usual composition is 23–30% palmitic acid, 32–37% stearic acid and 30–37% oleic acid. The typical triacylglycerols of cocoa butter contain palmitic or stearic acid in *sn*-position 1, oleic acid in *sn*-position 2 and stearic acid in *sn*-position 3 (Papanikolaou and Aggelis 2011b). The convenience to find substitutes for cocoa butter depends on its price that can significantly

fluctuate. One way to produce lipids with composition similar to cocoa butter is to employ oleaginous yeasts, enriching the more saturated SCO fraction or addressing their metabolism towards an increase of stearate, whose percentage is usually lower (about 5–10%). The first experimented way was to grow oleaginous yeasts, such as *R. toruloides*, *L. lipofer* or *Rhodotorula graminis*, and to separate the most saturated triacylglycerol fraction by crystallisation, but the achievable yield was rather low. Then, desaturase inhibitors were tested, such as sterculic acid and malvalic acid associated to *cis*-methylene-octadecenoic acid, to prevent the conversion of stearic acid in oleic acid and of oleic acid into linoleic acid, with several yeasts (*Candida* sp. 107, *Trichosporon cutaneum*, *L. starkeyi*, *R. toruloides*); cell growth and lipid accumulation were not altered, and lipids had composition similar to cocoa butter, but this process is not suitable due to high costs and to toxicity of inhibitors. Successful strategies were also the achievement of a Δ^9 defective mutants, but even more convenient was the metabolic manipulation, taking advantage of operating under controlled fermentation conditions. Limitation of oxygen availability, by a completely eco-friendly method, allowed to reach a good biomass and cell lipid content yield with less unsaturated fatty acyl residues, exploiting the need for oxygen of desaturases enzymes. By this way, good results were obtained with both yeasts (*Apiotrichum curvatum*) and fungi (*Mucor circinelloides*). Also the control of temperature allows to modify the unsaturation degree of lipids accumulated, as high temperature favours the accumulation of saturated fatty acids (Papanikolaou and Aggelis 2011b; Wynn and Ratledge 2000).

3.4.2 Biodiesel

The rising demand for renewable energy sources alternative to fossil fuels in third millennium sparked interest towards oleaginous microorganisms as producers of lipids that can be used as such or converted in biodiesel via trans-esterification with an alcohol, usually methanol. Diesel is a mixture of hydrocarbons with an average carbon length of 16; 75% are alkanes (linear, branched and cyclic); 25% are aromatic hydrocarbons. Technical properties of biodiesel should achieve a similar freezing temperature (−9.5 °C), vapour pressure (0.009 psi at 21 °C) and cetane number (50–60). Potential alternative to diesel from renewable sources are fatty acid methyl esters (FAME), fatty alcohols, alkanes and linear or cyclic isoprenoids (Lee et al. 2008).

Yeasts and fungi are able to produce high amounts of lipids in very short times, without allocation of lands and affection by climate and season, as oilseed crops are; on the other hand, process costs are high, unless cheap substrates are used, such as lignocellulosic biomasses or agro-industrial coproducts, effluents and wastes (see also Sect. 3.2.4.2). Another opportunity offered by oleaginous yeasts and fungi for biodiesel production is that trans-esterification can be directly carried out on microbial biomass, avoiding the oil extraction step, that is the most expensive step of SCO production process.

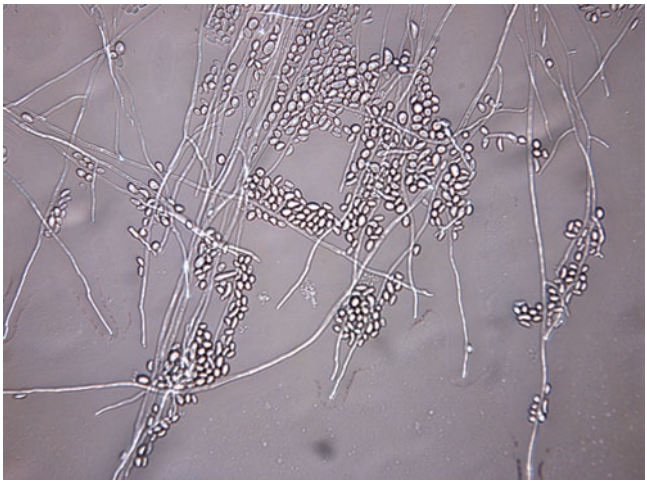

Fig. 3.5 *Gliocladium roseum*

Several factors make yeasts preferable to fungi for biodiesel production: their fatty acyl composition is more suitable, their growth rate is better and they are able to grow and give high yields on many carbon sources, including wastes. SCO produced by oleaginous yeasts are also technically suitable, even more than vegetal oils, for conversion into biodiesel, because of their fatty acyl composition nearest to ideal, for which chain length no higher than C18 and saturated or monounsaturated fatty acids are preferred; higher unsaturation degree, typical of vegetable oils, makes them subject to oxidation, and hydrogenation is necessary to prevent it.

An endophytic fungus, *Gliocladium roseum* (*Ascocoryne sarcoides*) (Fig. 3.5), was signalled as a potential producer of hydrocarbons, named "mycodiesel" (Strobel et al. 2008); even if coculture with *E. coli* seems to increase yields (Ahamed and Ahring 2011), the very low amount of hydrocarbons produced makes it unuseful for fuel production. However, the potential biosynthesis of hydrocarbons with a structure almost equal to crude oil with microorganisms is a very exciting perspective. Several endophytic fungi have been isolated and described that produce compounds, such as mono- terpenoids, alkanes, cyclohexanes, cyclopentanes and alkyl alcohols/ketones, benzenes and polyaromatic hydrocarbons, that are either identical to or are closely related to those specific classes of molecules that are found in diesel (Strobel 2014).

Metabolic engineering and microbial cell factories challenge and include also the biosynthesis of fatty acid esters in great amounts at cellular level, in order to eliminate trans-esterification from the biodiesel process (Peralta-Yahya and Keasling 2010; Shi et al. 2011).

3.5 SCO Extraction and Refining

Extraction of oils from cells is one of the most expensive and hard steps of SCO production. During extraction with solvents, it is necessary to prevent lipolysis and oxidation; if oils are intended to nutritional uses, solvents and other compounds added as anti-oxidant or anti-lipolysis must be chosen based on toxicity, handling, safety and cost. For producing GLA, a two-step extraction with ethanol and hexane has been proposed, reaching high yield of oil but with high contamination, due to undesirable reactions catalysed by enzymes that were not inactivated (Suzuki and Yokochi, US Patent 4 870 001, 1989; 117, 118). At a semi-industrial scale, the supercritical fluid extraction was used to produce GLA from *Cunninghamella echinulata*; quality was similar to oil obtained by conventional hexane/alcohol or chloroform/methanol methods, and yield was slightly higher. Supercritical CO_2 extraction is more expensive but suitable for preparations to be applied in the pharmaceutical, medical and nutritional fields (Certik and Horenitzky 1999).

Significant variations in lipid yield and composition can depend on the extraction method and microbial biomass employed. FTIR spectroscopy applied to intact biomass and biomass residual after lipid extraction has been tested as a tool for evaluating the lipid extraction efficiency: the presence or absence of lipid bands in the IR spectrum of the biomass after extraction can function as a measure for the efficiency of the extraction method; then this method can be also used to identify components that may affect lipid extraction processes, for example, chitin, glucuronans and polyphosphates in fungal biomass (Forfang et al. 2017).

Strategies to force secretion of lipids from cells could be a way to simplify and improve extraction, also allowing the reuse of cells in further fermentations: overexpression of ABC transporters could be attempted to reach this goal. An increased excretion of free fatty acids was observed in *Candida tropicalis* when transferred to oxygen-limited conditions (Passoth 2014).

After the extraction, the oils have to be refined and purified. Several compounds, such as tocopherols and tocotrienols, can give a contribution to oil quality if it is addressed to nutritional or nutraceutical purposes; other compounds are contaminants and must be removed: the processes that can be applied include urea adducts formation, separation on zeolites, solvent winterisation, differential crystallisation, various chromatographic techniques and lipase-catalysed reactions. High-temperature extractions and distillation should be avoided because they can cause double-bond migrations, stereomutations, cyclisations and dimerisations (Certik and Shimizu 1999). Purification of specific fatty acids is, in general, easier from SCO than from vegetable oils; for example, GLA purification from fungal oil is easier than from vegetable oil due to lower content of linoleic acid (Nakajima and Izu 1993).

Modifications can also become necessary for specific applications, such as hydrogenation to avoid oxidation during storage of bio-oils for biofuels.

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Chapter 4
Mycoremediation of Agricultural Soil: Bioprospection for Sustainable Development

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
AmDNT	Amino-dinitrotoluene
CBA	Chlorobenzoic acids
DDT	Dichlorodiphenyltrichloroethane
LiP	Lignin peroxidase
MnP	Manganese peroxidase
PAH	Polycyclic aromatic hydrocarbons
PCBs	Polychlorobenzoic acids
SVOCs	Semi-volatile organic compounds
TNT	Trinitrotoluene
VOCs	Volatile organic compounds

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4.1 Introduction

Soil is a complex interface of biotic and abiotic components comprising of minerals, water, gasses, organisms, etc. Healthy soil nourishes plants, animals, and microbes as well as humans for growth and survival for many centuries till today. However, in the past few decades, widespread exploitation is turning this soil gradually unhealthy and unfit for proper plant nourishment. Intensive use of fertilizers and agrochemicals along with industrialization adds excessive heavy metals and toxic pollutants that become the main obstacle for upbringing and sustaining the life-supporting system in soil. The presence of heavy metals, pesticides, and chemical pollutants in soil and water is the major concern, as they are not degraded easily into nontoxic forms and have long-lasting effects on the environment, which ultimately enter into the agri-food chain. Cancer, kidney failure, mental disorder, and paralysis, along with physical weakness, headaches, diarrhea, and anemia, are the unpleasant consequences of the chronic exposure to these toxic and persistent chemicals. In concern to this alarming situation, many attempts have been made since the mid-1980s to find out the ways to prevent the continuous degradation of soil health due to industrial pollutants and to enhance soil health to a state of nontoxicity.

Although several techniques (viz., chemical precipitation, oxidation-reduction, filtration, ion exchange, dialysis, and electrochemical treatments) are available to remove these heavy metals and pollutants from soil, but none of them is fully effective. Moreover, higher cost, restricted application with limited prospects, and inability to improve inherent soil health make them nearly abandoned. In replacement, bioremediation process emerged as the potential alternative with eco-friendly and cost-effective remediation strategy. Bioremediation is basically a process of reducing contaminant levels to undetectable, nontoxic, or at least acceptable levels (i.e., within limits set by regulatory agencies) by exploiting available natural bioresources, which mostly include plants, microbes, organic amendments, etc. that alter these contaminants.

The term “mycoremediation” was coined by Paul Stamets; and it refers to detoxification of contaminated site by using one or more species of fungi. It is a process of sequestering toxic contaminants from soil and water by using fungi. Biotechnological exploitation of such fungi for in situ and ex situ cleanup and management of contaminated soil and water is considered as mycoremediation (Strong and Burgess 2008). It involves enhancement of soil health using native and/or alien microflora. This type of bioremediation involves specific augmentation of fungal cultures that helps in rapid decomposition. Saprophytic fungi play an important role in the decomposition of organic molecules. These fungi secrete diverse extracellular enzymes and acids that break down natural polymers like keratin, chitin, lignin, pectin, cellulose, and hemicellulose (King et al. 1997; Lamar and White 2001). Besides decomposition, some fungi also produce small amount of biostatic or biocidal compounds that are involved in the bioremediation of complex organic and

inorganic molecules like hydrocarbons and heavy metals. Some approaches involve enriching contaminated soils with manure and compost, while others have focused on the mass application of single organism including flora or fauna on or into the soil. Meanwhile others adopted holistic approach to use consortia of plants, fungi, and bacteria. Many of these applications have shown wonderful results in degrading several toxins, pollutants, and hazardous chemicals (Singh 2006). Overall, inclusion of these approaches in combination may be complementary and may maximize the prospect and effectiveness of bioremediation. Partnering fungi for bioremediation open up the door to accelerate soil healing process. They have great potential in mycoremediation by playing early events of degradation and also by supporting other organisms in the remediation activity. Many fungi through the enzymatic degradation of contaminants supply energy to stimulate aggressive toxin degradation by other microbes present in the soil. Moreover, fungi can also remove heavy metals by channeling them into their fruiting bodies; thereby, they perform excellent job in revitalizing rhizospheric ecosystem (Prasad 2017).

Fungi, the eukaryotic microorganisms, are ubiquitous and represent diverse groups from various environments (Deacon 2006). They are well adapted to almost every habitat, from agricultural land to forest ecosystem and from marine to arctic environment. The natural behavior of fungi is to work within community and support a large number of organisms in ecosystem. Fungal community is not disassembled; it always prefers living in association with thousands of other living beings and plays vital role in most of the ecosystems and is proficient in regulating the flow of nutrients and energy. Fungi are widely known for their skill in breaking down advanced plant cell structures and complex cellular molecules like cellulose, hemicellulose, lignin, etc. They are well equipped with highly advanced mechanisms and digestive enzymes for pulling apart and degrading very complex carbon-based compounds into simpler form (Deacon 2006), and thus, they regulate C:N ratio and participate in nutrient recycling in soil. Besides, some species are blessed with mechanisms for degrading toxins, pollutants, and heavy metals (Pointing 2001) and, hence, can be an essential component of remediation process. The most important bioremediation process of soils involves microbial metabolism (Kearney and Kellog 1985). Probably degrading microorganisms obtain carbon, nitrogen, or energy from the pesticide molecules and other toxicants. Similar to other microbes, fungi are also known to degrade or to deteriorate a wide variety of pollutants and toxicants. Through mycelial networks, they may cover several hectares of land and speed up the process of soil reclamation. They have the inherent capacity to break down molecules, disassembling long-chained toxins into simpler and less toxic chemicals, and, hence, are considered natural ecosystem engineer (Lawton and Jones 1995) and can be used to create pollutants-free soil environment through the process of mycoremediation (Prasad 2017). Although many reviews exist on mycoremediation, but almost all of them focus on reclamation of soil from hazardous pesticides and herbicides. However, in this chapter, our main emphasis is on the various aspects of mycoremediation, specifically bioprospection of fungi and their role in maintenance/improvement of soil health.

4.2 Concepts of Bioremediation

Bioremediation is a natural process of decontaminating soil and groundwater from organic pollutants using living organisms (Rhodes 2014). It involves the techniques of waste management for eliminating pollutants from the environment in effective and eco-friendly way (Kumar et al. 2011). These hazardous wastes can be removed or detoxified by various living organisms mostly, plants, fungi, and bacteria which are also known as bioremediators. The main purpose of bioremediation is to abate or clean up soil environment from contamination and to improve soil health through eco-friendly approaches. The various approaches of bioremediation can be categorized in two types, in situ and ex situ (Azubuike et al. 2016). In situ bioremediation approach involves treatment of intoxicated soils right at the point of contaminated sites itself and provides facility for avoiding excavation and transportation of contaminants; hence, there is no chance of spread of toxicants during excavation (Talley 2005). However, ex situ bioremediation approach involves excavation of contaminated soils and water from sites and subsequent transportation to another site for treatment and makes them free of toxicants and pollutants. Both the approaches include various processes that are briefly discussed below.

4.2.1 In Situ Bioremediation Approaches

It is a purely biological transformation process, in which fungal enzymes act as catalyst for breakdown and cleaning of the hazardous substances present on the subsurface. During the process of in situ bioremediation, organic contaminants are degraded and used by certain microbes for their growth, providing the supply of macro-(nitrogen, phosphorus) and micro-nutrients (sulfur and trace elements), and energy. In most cases, organic compound containing carbon and energy is transformed by the metabolic activities of heterotrophic microorganisms that bring about extensive modifications of the structural and toxicological properties of the contaminants (Joutney et al. 2013). Generally, the techniques used to biodegrade contaminants include mechanism such as abiotic oxidation, dispersion, hydrolysis, dilution, sorption, volatilization, and infiltration of nutrients containing water or other electron acceptors (Gavrilescu 2005). Most often, in situ bioremediation is applied to biodegrade the toxic waste in saturated soils and groundwater. It is a superior, cheaper, and harmless method by using microbial organisms to degrade the toxic chemicals to clean up contaminated environments. The study of in situ bioremediation chemotaxis is important because microbiota with chemotactic abilities can move into an area containing contaminants. Hence, in situ bioremediation could become a safer mechanism by enhancing the cells chemotactic abilities for degradation or transformation of hazardous compounds (Tiwari and Singh 2014). The in situ bioremediation is further divided into following categories:

4.2.1.1 Bioventing

It is the technique of eliminating oxygen-sensitive contaminants from unsaturated zone by blowing air through soil. Aerobically degradable contaminants are degraded due to increased supply of oxygen in the waste, which in turn enhances the activities of native microbes. Since it is mostly aerobic process, it needs sufficient supply of oxygen or air. Sometimes, toxic soils with lower oxygen concentration are reclaimed by supplying nutrients like nitrogen and phosphorus to aid microbial biodegradation (Rockne and Reddy 2003). Oxygen is typically introduced by air injection wells that push air into the subsurface vacuum extraction wells, which draws air through the subsurface. The distribution of these nutrients and oxygen in soil is dependent on soil texture. In bioventing enough oxygen is provided through low airflow rate and amended with nutrients and moisture for microbial growth and transformation of pollutants (EPA 1997; Philp and Atlas 2005). In aerobic bioventing, air is pumped into contaminated soil above the water table through vent well. The pumped air diffuses to contaminated part of soil and removes the toxicants. The removal rate varies from one site to another site depending on soil texture and types of toxicants. However, uniform distribution of oxygen increases the rate of biodegradation. It is mainly used for the removal of hydrocarbons like gasoline, oil, petroleum, etc. (Tiwari and Singh 2014). It is more effective in high-temperature zone where water table is much below the soil surface. The shallow water table and thin soil cover are inefficient for this process of bioremediation (Rayner et al. 2007). On the other hand, very deep soil may have insufficient microbial populations for bioremediation (Frishmuth et al. 1995). In spite of these, due to simplicity, robustness, and low cost, this technique is widely adopted for bioremediation.

Sometimes, bioventing techniques are improvised into various forms depending on the types of contaminants and many other issues; few examples are cold-climate bioventing, cometabolic bioventing, anaerobic bioventing, etc. In cold-climate bioventing, soil warming is essential to speed up bioremediation process in cold-climate situations (EPA 1995), whereas cometabolic bioventing is used for bioremediation of chlorinated contaminants such as trichloroethylene, trichloroethane, and dichloroethene, which cannot be degraded by simple aerobic bioventing. This technique is based on the breakdown of a contaminant by an enzyme or cofactor that is produced during microbial metabolism of another compound (EPA 2000). The process involves the injection of electron donors, mostly volatile organic substrate such as methane, ethane, propane, butane, aromatic hydrocarbons (such as toluene and phenol), and ammonia for aerobic oxidation (EPA 2000) and methanol, glucose, acetate, lactate, sulfate, or pyruvate for anaerobic reduction (Hazen 2010). Supply of an appropriate organic substrate and air facilitates the degradation of contaminants by eliciting the production of enzymes and cofactors like monooxygenases, dioxygenase, dehydrogenase, dehalogenase, etc., which are capable of degrading the organic substrate (Karigar and Rao 2011). Like bioventing, cometabolic bioventing is equipped with similar instruments but different biological mechanism. This cometabolic bioventing technique is very useful for degrading many hydrocarbons and lightly chlorinated contaminants.

Sometimes, degradation of recalcitrant contaminants such as polycyclic aromatic hydrocarbons (PAH) and chlorinated solvents is accelerated under anaerobic conditions. This process of venting substrates other than oxygen for microbial growth is known as anaerobic bioventing (Gibbs et al. 1999; Litchfield 1993). This anaerobic bioventing also involves microbial mechanisms like fermentation and methanogenesis to destroy the contaminants. Here, in place of air, nitrogen and an electron donor are injected to create anaerobic conditions, and the nitrogen replaces oxygen from soil. Small amount of an electron donor (such as hydrogen and carbon dioxide) is produced in reducing state in the subsurface soil zone, thereby facilitating microbial dechlorination. Volatile and semi-volatile compounds may be produced, but slowly they degrade under anaerobic bioventing. These compounds can be treated in two ways. Volatile compounds may disperse into the soils surrounding the treatment zone, where aerobic degradation may occur. Halogenated and nonhalogenated semi-volatile organic compounds (SVOCs) and volatile organic compounds (VOCs) remaining in the treatment zone may be treated using aerobic bioventing followed by anaerobic bioventing. Since aerobic and anaerobic bioventing share similar gas delivery systems, the switch can be made by simply changing the injected gas.

4.2.1.2 Biosparging/Air Sparging

In biosparging, gas (usually oxygen or air) is injected below the groundwater to enhance the concentration of oxygen in the saturated zone, rather than unsaturated zone as in bioventing. It facilitates aerobic biological degradation of pollutants by naturally occurring microbiota (Singh 2006). Volatile pollutants can be eliminated from the saturated zone by air due to increased desorption and volatilization into the air stream. This technology is applied to a known source of gasoline components such as benzene, ethyl benzene, toluene, xylenes, and SVOCs in order to quantify the magnitude of remediation achieved in terms of both mass removed and decline in mass discharged into groundwater. Biosparging is most effectively used to remove light to mid-weighed petroleum-based contaminants (diesel, kerosene, and gasoline) due to their rapid volatilization (Tiwari and Singh 2014). Hence, this technique is commonly recommended for purifying aquifers and underground storage tank sites.

4.2.1.3 Bioaugmentation

It involves introduction of exogenous microorganisms having specific metabolic activity to the contaminated site for enhancing the biological degradation or transformation of organic contaminants (Suthersan 1999). Soil and groundwater contaminated with chlorinated ethane, such as trichloroethylene and tetrachloroethylene, are remedial sites where bioaugmentation is applied. The in situ microorganisms can effectively degrade these toxic contaminants to ethylene and chloride, which are

innocuous or nontoxic. However, specialized microbial population inoculated into the contaminated site may not produce desired extent of transformation of contaminants due to the influence of some biotic and abiotic factors, viz., temperature, moisture, pH, hydrolytic and osmotic pressure in environment, nutrient availability, soil type, and existing microbial composition that influences the survival and activity of microbes alone or in consortia. For bioaugmentation, microbes that have higher affinity toward hydrocarbons and ability to survive in a wide range of environment are most commonly preferred (Mrozik and Piotrowska-Seget 2010). This technique is mostly effective for degradation of light fractionated petroleum hydrocarbons (C_{12}–C_{23}) than that of heavy fractionated petroleum hydrocarbons (C_{23}–C_{40}) (Bento et al. 2005). Therefore, this method is not applicable on a wide scale for soil bioremediation.

4.2.2 Ex Situ Bioremediation Approaches

In this approach, ex situ treatments of contaminated soil or groundwater are given after excavating or pumping out of the original site. Wide range of toxic wastes and soil contaminants can be removed easily by various processes, which are categorized into slurry phase bioremediation and solid phase bioremediation.

4.2.2.1 Slurry Phase Bioremediation

In this process, contaminated site is amalgamated with water and other reagents in a big tank called as bioreactor. The congenial environment in the bioreactor support optimum growth of microbes and amalgamation helps these microorganisms to remain in contact with organic toxicants present in the soil. Further, oxygen and nutrients are incorporated to enhance the breakdown rate of toxic wastes by the native microorganisms (EPA 1990). After completion of the process, water move apart from the soil, and the soil is examined and replaced into the environment. For excellent biodegradation, several biotic and abiotic factors like temperature, pH, agitation, aeration, augmentation, nutrient addition, substrate, and inoculum concentrations are the main limiting factors for biodegradation that can be manipulated easily in a bioreactor. Hence, it has greater advantages for faster bioremediation over other techniques.

4.2.2.2 Solid Phase Bioremediation

In this approach, aboveground treatment of contaminated soil is done in solid phase, which can be made possible by land farming, soil bio-piling, and composting method (Girma 2015). For decontamination, excavated materials are placed in an aboveground enclosure/treatment chamber, which is facilitated with

aeration system ensuring controlled environment for optimum treatment. These methods are very easy to perform but require longer time and space, hence not commonly in use.

4.2.2.3 Land Farming

Land farming is a simple approach and is also known as land treatment, in which polluted soil is excavated and spread over a prepared bed and periodically tilled to allow natural degradation of pollutants. To stimulate native biodegradative microbes and to facilitate aerobic degeneration of contaminants, a sandwich layer of excavated soil between clean soil and clay-concrete is made, where clean soil is placed at bottom and concrete layer at the top. In general, the practice is limited to treat soil of 10–35 cm depth. In land farming, various operations like tillage (for aeration), nutrient application (nitrogen, phosphorus, potassium, lime), and irrigation are performed to stimulate microbial activities for rapid bioremediation. Due to simple design, low-maintenance costs, and least cleanup liabilities, land farming is considered the best disposal alternative, mainly for the removal of pesticide residue from soil (Felsot et al. 2003). However, it is less successful for removing toxic volatiles from soil, especially in hot tropical countries.

4.2.2.4 Compositing

Compositing is a biologically controlled process to treat toxic contaminants in soil by mixing materials like straw. During composting, the waste material is digested at high temperature (55–65 °C) under the influence of thermophilic microbes (NFESC 2005). During the whole degradation process, heat released in compost pit increases the temperature of material which intern leads to more solubility of waste and breakdown of complex compounds due to higher metabolic activity of microbes (Blanca et al. 2007). The soil is transported to a composting pit with a temporary structure to provide containment and protection from weather extremes. In composting, organic ingredients (agricultural wastes, vegetable wastes, sawdust and wood chips, etc.) are added with compost materials to provide supplementary carbon source. There are three designs commonly applied for composting: (1) aerated static piles (compost is formed into piles and aerated with blowers or vacuum pumps), (2) mechanically agitated in-vessel composting (compost is kept in a reactor vessel, in which it is mixed and aerated thoroughly), and (3) windrow composting (soil and organic amendments are layered in long, low, narrow piles (i.e., windrows) and periodically mixed with mobile equipment). In windrow composting, removal of the rocks and other larger particles from excavated contaminated soils becomes easier (FRTR 2003). It is a very cost-effective method and has the potential to remediate larger quantities of volatile components from contaminated soil. Composting has been successfully applicable to soils contaminated with petroleum hydrocarbons (e.g., fuels, oil, and grease), solvents, chlorophenols, herbicides, PAHs, pesticides and nitro-aromatic explosives, etc. (EPA 1998, 2004).

4.2.2.5 Bio-piling

This type of ex situ bioremediation is a composite form of land farming and composting. The biopile system includes a treatment bed, an aeration system, nutrient/irrigation system, and a leachate collection system. For proper degradation there should be control of heat, moisture, oxygen, nutrients, and pH. The irrigation system is buried beneath the soil, and it provides air and nutrient through vacuum. To prevent the runoff, soil is covered with plastic to minimize evaporation and volatilization and to promote solar heating. Biopile treatment takes 20 days to 3 months to complete the degradation of petroleum hydrocarbons. It typically uses the version of land farming that helps to control physical losses of the soil contaminants by leaching and volatilization (Tiwari and Singh 2014). Other added advantage is that ambient environment for native aerobic and anaerobic microbes can easily be regulated in biopiles. Therefore, it gains popularity in comparison to the other ex situ bioremediation techniques, including land farming, in spite of its reserved space requirement.

4.3 Groups of Fungi Involved in Bioremediation

It is very important to understand the significance of fungal interactions and its behavior in diverse ecosystems associated to biodegradation or myco-transformation of various hazardous and toxic components. Despite an estimated 1.5 million and identified 69,000 fungal species worldwide, limited number of fungal species has been identified to be associated with mycoremediation from diverse ecology (Hawksworth 1991). The various fungal species exploited for bioremediation have been classified into various sections along with their functions and mechanisms in Table 4.1. The occurrence and growth of fungal communities depend on abiotic and biotic factors such as availability of carbon, nitrogen, phosphorus, metal ion concentration, temperature, aeration, moisture, and interspecific microbial competition. Sufficient literature exists on fungal ecology, but little information is available on the ecology of fungi associated with mycoremediation. However, based on ecology and functionality, these fungi have been grouped into following sections:

4.3.1 Wood-Rotting Fungi

Wood-rotting fungi represent a diverse group of fungi associated with wood rotting. They have the ability to disintegrate wood tissue to simpler form by employing diverse enzymes to degrade complex molecules like cellulose, hemicellulose, lignin, etc. Besides wood rotting, these fungi also play significant role in bioremediation of organic pollutants. These fungal groups degrade various toxic environmental pollutants by their extracellular lignin-degrading system. Moreover, the extracellular system enables fungi to withstand considerably higher doses of hazardous

Table 4.1 Fungal diversity exploited for mycoremediation

Groups	Fungal spp.	Used in remediation of	Mechanisms	Reference
Wood-decaying white-rot fungi	*Pleurotus ostreatus*	Cadmium	Biosorption of heavy metals	Tay et al. (2011)
	Pleurotus ostreatus	Biodegradable plastic	Degradation of plastic	da Luz et al. (2013)
	Pleurotus sajor-caju	Heavy metal, Zn	Biosorption of heavy metals	Jibran and Milsee Mol (2011)
	Pleurotus tuber-regium	Heavy metals	Biosorption of heavy metals	Oyetayo et al. (2012)
	Pleurotus pulmonarius	Crude oil	Degradation of crude oil	Olusola and Anslem (2010)
	Pleurotus tuber-regium	Crude oil-polluted soil	Enzymatic degradation	Isikhuemhen et al. (2003)
	Bjerkandera adusta	PAHs, PCBs	Enzymatic modification of lignin	Bumpus et al. (1985)
	Irpex lacteus	Dyes, PAHs, lindane, TNT, bisphenol A, nonylphenol, dimethyl phthalate	Enzymatic degradation [laccase, lignin peroxidase (LiP), manganese peroxidase (MnP, versatile peroxidase (VP)]	Novotny et al. (2000)
	Phanerochaete chrysosporium	Synthetic dyes, PAHs, lindane, polychlorophenol, DDT, PCBs	Enzymatic degradation (LiP, MnP)	Singh (2006)
	Phlebia sp.	PAHs, TNT, AmDNT, coal, humic acids	Enzymatic degradation (laccase, LiP, MnP)	Singh (2006)
	Agaricus bisporus, *Lactarius piperatus*	Cadmium (II) ions	Biosorption	Nagy et al. (2014)
	Lentinula edodes	2,4-dichlorophenol	Enzymatic degradation (laccase, MnP)	Tsujiyama et al. (2013)
	Pleurotus platypus, *Agaricus bisporus*, *Calocybe indica*	Copper, zinc, iron, cadmium, lead, nickel	Biosorption	Lamrood and Ralegankar (2013)
	Pleurotus pulmonarius	Radioactive cellulosic waste	Fungal mycelia solidified with Portland cement act as barrier for releasing radio-contaminants	Eskander et al. (2012)
	Coriolus versicolor MKACC 52492	PAH	Enzymatic degradation	Jang et al. (2009)
	Trametes versicolor	PAHs and synthetic dyes	Enzymatic degradation	Novotny et al. (2004), Tanaka et al. (1999)
	Lentinus squarrosulus	Crude oil (1–40%)	Mineralization	Adenipekun and Fasidi (2005)

Wood-decaying brown-rot fungi	*Fomes fasciatus*	Copper (II)	Biosorption	Sutherland and Venkobachar (2013)
	Daedalea dickinsii, *Fomitopsis pinicola*, *Gloeophyllum trabeum*	DDT	Microbial biodegradation via Fenton reaction	Purnomo et al. (2011)
	Flammulina velutipes	Copper	Biosorption	Luo et al. (2013)
	Fomitopsis palustris	Metals (Zn, Co, Cu)	Oxalate production	Gadd et al. (2014)
	Postia placenta, *Gloeophyllum trabeum*	Polysaccharide decomposition	Lignin demethylation	Filley et al. (2002)
	Gloeophyllum striatum	Degradation of polychlorophenol	Mineralization	Singh (2006)
	Schizophyllum commune, *Polyporus* sp.	Malachite green dye	Enzymatic degradation	Rajput et al. (2011)
Leaf-decomposing fungi	*Agrocybe praecox*	PAHs, TNT	Modification by laccase and MnP	Steffen et al. (2000)
	Nematoloma frowardii	Radionuclide C	Enzymatic degradation (MnP)	Hofrichter et al. (1999)
	Stropharia coronilla	Mineralization of ^{14}C-labeled synthetic lignin	Degradation by Ligninolytic enzymes	Singh (2006)
Soil fungi	*Mucor* sp.	Heavy metals (Ni, Cd, Pb, Zn)	Bioadsorption of heavy metals	Yan and Viraraghavan (2000)
	Rhizopus sp.	Heavy metals (Cd, Cu, Zn)	Biosorption of heavy metals	Volesky and Holan (1995)
	Cunninghamella sp.	Heavy metals (Pb, Cu, Zn)	Ions-sequestration	El-Morsy (2004)
	Mortierella sp.	2,4-D (2,4-dichlorophenoxy acetic acid)	Hydroxylation and dechlorination	Nakagawa et al. (2006)
	Mortierella sp. Gr4	Phenylurea herbicides, viz., chlorotoluron, diuron, linuron, isoproturon	N-demethylation and hydroxylation	Badawi et al. (2009)
	Aspergillus niger	Heavy metals (Cd, Zn, Ur, Ag, Cu)	Biosorption of heavy metals	Wang and Chen (2006)
	Aspergillus fumigates	Heavy metal (Ur)	Bioaccumulation of heavy metal	Guibal et al. (1995)
	Trichoderma viride, *Humicola insolens*	Heavy metal (Hg)	Biosorption of heavy metal	Muhammad et al. (2007)
	Paecilomyces sp., *Penicillium sp.*, *Phlebia sp.*	PAH, Endosulfan	Hydroxylation	Anastasi et al. (2009), Tigini et al. (2009)

(continued)

Table 4.1 (continued)

Groups	Fungal spp.	Used in remediation of	Mechanisms	Reference
Mycorrhizal fungi	*Glomus geosporum*	Zn	Enzymatic degradation	Leyval et al. (1997)
	Suillus granulatus	Cresol, catechol	Biotransformation	Singh (2006)
	Scutellospora heterogama	Cu	Enzymatic degradation	Sambandan et al. (1992)
	Gigaspora gigantea	Zn, Cu, Pb, Ni, Cd	Enzymatic degradation	Sambandan et al. (1992)
	Rhizopogon vinicolor	2,4-D	Mineralization	Leyval et al. (1997)
	Hymenoscyphus ericae, *Oidiodendron griseum*	2,4-D, atrazine	Mineralization via enzymatic degradation	Donnelly and Fletcher (1994)
Endophytic fungi	*Coriolopsis gallica*	PAH	Biotransformation	Pickard et al. (1999)
	Ceratobasidum stevensii	Phenanthrene	Enzymatic degradation (MnP)	Dai et al. (2010)
	Phanerochaete chrysosporium	PAH degradation	Enzymatic degradation (LiP, MnP)	Dhawale et al. (1992)
	Bjerkandera sp.	Phenanthrene	Oxidation	Terrazas et al. (2005)
	Phomopsis sp.	PAH degradation	Enzymatic degradation	Tian et al. (2007)
Aquatic fungi	*Nia vibrossa*, *Julella avicinnae*, *Lignincola laevis*	Polymeric dyes	Enzymatic degradation	Pointing et al. (1998)
	Aspergillus sclerotiorum CBMAI 849, *Cladosporium cladosporioides* CBMAI 857, *Mucor racemosus* CBMAI 847	Lignin-based industrial pollutant	Mineralization	Raghukumar et al. (1996)
	Phaeosphaeria spartinicola, *Halosarpheia ratnagiriensis*, *Sordaria fimicola*	Industrial pollutant	Mineralization	Bonugli-Santos et al. (2010)
	Penicillium raistrickii CBMAI 931, *Aspergillus sydowii* CBMAI 1241, *Trichoderma* sp.	Profenofos	Enzymatic degradation	da Silva et al. (2013)

pollutants (McErlen et al. 2006). The added advantage of utilizing these fungi is that they are nonspecific and nonselective in biodegradation. Thus, they do not need any preconditioning prior to transformation of pollutants (Azadpour et al. 1997). Hence, they have wider adaptability and extensive degradative ability. Wood-degrading fungal species significantly differ in their colonization ability and can be characterized as strong competitors (i.e., *Pleurotus* spp., *Phanerochaete* spp., *T. versicolor*) and weak competitors (*Dichomitus squalens* and *Ganoderma applanatum*) (Baldrian 2008). Based on the mode of attack on the woody tissue, these fungi are categorized as white-rot fungi and brown-rot fungi.

4.3.1.1 White-Rot Fungi

Among diverse wood-rotting fungi, only certain fungi have the unique ability to degrade lignin along with cellulose and hemicellulose and result in white bleaching of woods during decay, hence, considered as white-rot fungi. They mainly include fungi of *Basidiomycota*, and few belong to *Ascomycota*, exclusively members of *Xylariaceae* family. These white-rot fungi were the first to be used to study mycoremediation. They produce some enzymes like lignin peroxidase, manganese peroxidase, H_2O_2-generating enzymes, and laccase. Among them laccase initiates ring cleavage, and others generate strong oxidants, which result in the disintegration of lignin/xenobiotic molecules (Anastasi et al. 2009; Kirk and Farrell 1987). The biodegradation of complex molecules by extracellular oxidative ligninolytic enzymes has been studied in detail in *Phanerochaete chrysosporium*. Based on this, Bumpus et al. (1985) proposed use of this fungus in bioremediation studies, and this has emerged as an archetypal model system for mycoremediation. *P. chrysosporium* has the ability to degrade toxic or insoluble compounds to CO_2 and H_2O more efficiently than other fungi or microbes. The diverse oxidative and reductive methods of degradation or biotransformation of recalcitrant compounds make its application magnetic in various matrices. It can be used effectively to remove broad spectrum of aromatic compounds and xenobiotics present in contaminated soil due to nonspecific and robust nature of ligninolytic enzymes (Schauer and Borriss 2004). In addition to *P. chrysosporium*, several other white-rot fungi (e.g., *Pleurotus ostreatus*, *Trametes versicolor*, *Bjerkandera adusta*, *Lentinula edodes*, and *Irpex lacteus*) are also known to degrade these compounds (Singh 2006). Past two decades of literature indicates that the white-rot fungi account for at least 30% of the total research on mycoremediation (Borràs et al. 2010; Singh 2006). It indicates the immense potentiality of this group of fungi in mycoremediation of environmental pollutants and toxicants from soil.

4.3.1.2 Brown-Rot Fungi

This group of wood-rotting fungi is responsible for the degradation of cellulose and hemicellulose present in wood, leaving lignin more or less intact as a brown chemically modified component. Hence, they are known as brown-rot fungi. They mainly

belong to the phylum *Basidiomycota* under class *Agaricomycetes*. Majority of the brown-rot fungi are distributed in *Agaricales*, *Hymenochaetales*, *Gloeophyllales*, and *Polyporales*. They partly modify the lignin via demethylation, partial oxidation, and depolymerization by a nonenzymatic Fenton-type catalytic system. The partially modified lignin gives distinctive dark brown to the decayed wood. Interestingly, only 6% of all the known wood-decay fungi are recognized to cause a brown rot and are preferably associated with conifers (Anastasi et al. 2009). The brown-rot fungi degrade cellulose and hemicellulose by an oxidative process, involving the production of hydrogen peroxide, which helps in the synthesis of free hydroxyl (OH) radicals and that in turn facilitate the degradation and mineralization of synthetic chemotherapeutants. In addition to this, antimicrobial drug tolerance and oxalic acid production increase their metal degrading ability. This potentiality of brown-rot fungi can be exploited in larger scale for bioremediation.

4.3.2 Leaf-Decomposing Fungi

Leaf-decomposing fungi are one of the main components of forest ecology. They actively take part in the decomposition of wood and litter, humification, and mineralization of soil organic matter. Fungal community undergoes rapid successional changes during leaf litter decomposition (Singh 2006). Fungi of *Ascomycota* phylum are predominant during the initial stages of litter decay, but their population gradually decreases with increase in fungi of the *Basidiomycota* phylum during the later stages of decomposition (Osono 2007). Both these fungal group play a vital role in the decomposition of lignocellulolytic materials of plant litters. Basidiomycetous litter fungi produce numerous ligninolytic enzymes like cellulase, laccase, and oxidoreductases that are essential for degradation of plant materials deposited on forest floor (Osono and Takeda 2002). Their enzymes also take part in degradation of organic pollutants, pesticides, and herbicides that are persistent in soil (Aranda et al. 2010; Casieri et al. 2010; Farnet et al. 2009). Therefore, utilization of these fungi will open up new scope for bioremediation of agricultural soil.

4.3.3 Soil Fungi

Soil fungi represent heterogeneous groups, especially *Ascomycota*, *Chytridiomycota*, and *Zygomycota*. They are important components of soil ecology and play a crucial role in organic matter decomposition and carbon and nitrogen cycling in soil. They are saprophytes and have very good cellulose-decomposing ability but are mostly non-ligninolytic in nature. They mostly include different species of genera *Acremonium*, *Allescheriella, Alternaria*, *Aspergillus*, *Beauveria*, *Cladosporium*, *Cunninghamella*, *Engyodontium*, *Fusarium*, *Geomyces*, *Microsporum*, *Mortierella*, *Paecilomyces*, *Penicillium*, *Phlebia*, *Rhizopus*, *Stachybotrys*, and *Trichoderma*

(Anastasi et al. 2009; D'Annibale et al. 2006; Pinedo-Rivilla et al. 2009; Tigini et al. 2009). The extracellular enzymes like monooxygenase produced by non-ligninolytic fungi lead to degradation of PAHs via hydroxylation. They are also tolerant to pollutants such as PAHs, polychlorobenzoic acids (PCBs), chlorobenzoic acids (CBA), and endosulfan, which indicate their potentiality as bioremediation agents in soil (Garon et al. 2000; Tigini et al. 2009; Pinedo-Rivilla et al. 2009). The fungi degrading recalcitrant polymers are often predominant in the later stages of decomposition. The ecological succession of these fungi later in the decomposition sequence is related to their specialized ability to degrade complex polymers such as lignin and keratin that most of the other fungi cannot utilize. Thus, they are often considered as very good xenobiotics-degrading fungi, and their consortia with different species ensure a greater effectiveness in soil bioremediation.

4.3.4 Mycorrhizal Fungi

Mycorrhizae fungi have symbiotic association with plant roots and establish mutualistic relationship by facilitating nitrogen and phosphorus supply to the plants and in turn derive organic carbon from plants for fungal metabolism (Goltapeh et al. 2008; Prasad et al. 2005, 2017). There are several types of plant mycorrhizal associations, viz., ectomycorrhiza, ectendomycorrhiza, ericoid mycorrhiza, arbuscular mycorrhiza, arbutoid mycorrhiza, monotropoid mycorrhiza, and orchid mycorrhiza. They are mainly involved in nutrient supply to plant and provide protection against various environmental stresses including water stress, metal toxicity, etc. They can ameliorate heavy metal toxicity by reducing metal translocation within plant system (Leyval et al. 1997; Kamal et al. 2010); thereby, they help plants to adapt and survive in heavy metal-polluted sites and degraded lands like coal mines and waste sites (Gaur and Adholeya 2004). On the other side, the host plant accommodates selective advantage to fungus for establishing at a contaminated site and metabolizing various polycyclic aromatic hydrocarbons, petroleum, and chlorinated aromatic pesticides, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and atrazine via enzymatic degradation (Donnelly and Fletcher 1994). Thus, it will be very noteworthy to decipher the role of mycorrhizal fungi in the bioremediation of hazardous compounds in soil.

4.3.5 Endophytic Fungi

Plant endophytes are the microbial group including fungi and bacteria that have colonizing ability within plants without causing any negative impact. They mostly reside inside specific plant tissues like root cortex, vascular bundle, apoplastic space, young buds, and even in dead bark cell and perform diverse metabolic activity for methane assimilation, nitrogen fixation, etc. Several of them have

saprotrophic ability to sustain in the dead litter (Osono 2006). They are equipped with an array of enzymes like cellulase, lipase, peroxidase, and protease (Orlandelli et al. 2015; Petrini et al. 1992) for bioremediation of environmental pollutants (pesticides, herbicides, insecticides, petrochemicals, polychlorobiphenyls, polyaromatic hydrocarbons, polyester polyurethane) and biotransformation of heavy metals (Dai et al. 2010; Russell et al. 2011). In this way, they enhance plant tolerance and adaptability to contaminant and metal toxicity. Thus, they can be employed as valuable tools for bioremediation.

4.3.6 Aquatic Fungi

Aquatic environment is very complex, and it supports a wide range of activities that influence the microbial life. Microbial communities are important ecological components in aquatic environments, and among them, aquatic fungi are one of the most predominant groups, essential for various biogeochemical processes. They can be either obligate or facultative in nature and are distributed from freshwater to marine ecosystem. They produce diverse enzymes like laccase, lignin peroxidase, and Mn-peroxidase that are essential for decomposition of lingo-cellulolytic materials as well as lignin-based industrial pollutants. It ensures wider adaptability of these fungi in extreme environment like high salinity, high pressure, low temperature, metal, and oil spill toxicity. This can be evident from the production of ligninolytic enzyme and subsequent mineralization of lignin-based compound to CO_2 by many aquatic fungi like *Aspergillus sclerotiorum* CBMAI 849, *Cladosporium cladosporioides* CBMAI 857, *Mucor racemosus* CBMAI 847, *Phaeosphaeria spartinicola*, *Halosarpheia ratnagiriensis*, *Sordaria fimicola*, and *Flavodon flavus* (Bonugli-Santos et al. 2010; Raghukumar et al. 1996). Similarly, some marine aquatic fungi like *Penicillium raistrickii* CBMAI 931, *Aspergillus sydowii* CBMAI 935, *Aspergillus sydowii* CBMAI 1241, and *Trichoderma* sp. CBMAI 932 have been tested for their biocatalytic potential to degrade profenofos (da Silva et al. 2013). Thus, aquatic fungi are said to possess the potentiality that would open up the scope for mycoremediation of pesticides and industrial effluents.

4.4 Mechanisms of Mycoremediation

Microbes are ubiquitous and have wider distribution in various environmental *milieus*. Among various microorganisms, fungi are very opportunistic and have wide adaptability and quick responsiveness to stress condition, environmental disasters, and extreme climatic situations. They can degrade complex hydrocarbons and chains of hazardous molecules into simpler, nontoxic, biodegradable form to clean up the environment. Many fungi also have excellent capacity to bind with metal ions, which includes the efflux of metal ions outside the cell and

accumulation and formation of metal ion complex inside the cell, and later they reduce the toxic metal ions to a nontoxic state. Several mechanisms have evolved by which they can immobilize, mobilize, or transform metals rendering them inactive or tolerate the uptake of heavy metal ions. The mechanism adopted by fungi for bioremediation include:

(i) exclusion—the metal ions are kept away from the target sites by formation of a permeable barrier
(ii) extrusion—the metals are pushed out of cells by active transport
(iii) fixation-fix metals by forming complex with metal-binding proteins or other cell components like enzymatic detoxification, intra- and extracellular sequestration, dissolution of metal by acid production, chelation, and precipitation through the production of organic bases, extracellular metal precipitation
(iv) biotransformation—toxic metal is reduced to less toxic forms like methylation, demethylation, volatilization, oxidation, and reduction

In general, immobilization, mobilization, biosorption, and biotransformation are considered main approaches used for mycoremediation of hazarders in the agroecosystem in order to avail good air and water quality for future generations.

4.4.1 Immobilization

Microorganisms utilize this approach to alter physical or chemical characteristics of contaminants for reduction of its mobility. This can be accomplished by physically restricting contact between the contaminants or by chemically altering the contaminants. For immobilization of the toxic contaminants, most of the contaminated sites use solidification and stabilization approach. This approach emphasizes mixing of the contaminated material with appropriate amounts of water and stabilizer. The mixture results in the formation of a solidified matrix with the toxic waste. Heavy metals can be precipitated by injecting chemicals to the contaminated soil, which leads to formation of metal hydroxides. The chemical composition of the site, the amount of water present, and temperature are the key factors for the successful use of this mechanism (Gadd 2004). The stabilization and solidification techniques can occur both in situ or ex situ. However, in situ method is preferred for volatile or semi-volatile organics and for treatment of surface or shallow contamination of soil.

4.4.2 Mobilization

Microorganisms can mobilize contaminants through leaching, chelation by microbial metabolites and siderophores, alkylation, methylation, and redox transformations. Leaching occurs when acidification of soil environment takes place through microorganisms by their proton efflux leading to acidification and resulting in

release of free metal cations. In most fungi, leaching is generally mediated through the production of low-molecular-weight organic acids, which upon breakdown provide protons and metal-complexing organic acid anions. *Trichoderma harzianum* is regarded to have the potentiality to solubilize MnO_2, Fe_2O_3, metallic zinc, and rock phosphate through the mechanism of chelation and reduction (Altomare et al. 1999).

Siderophores are low-molecular-weight iron-chelating legends, which are able to bind to other metals such as magnesium, manganese, chromium, gallium, and radionuclide (e.g., plutonium). Alkylation involves the transfer of an alkyl group from one molecule to another, which can be transferred as an alkyl carbocation, a free radical, a carbanion, or a carbene. Methylation involves incorporation of methyl groups that are enzymatically transferred to a metal, forming a number of different metalloids. Redox transformations can allow microorganisms to mobilize metals, metalloids, and organometallic compounds by reduction and/or oxidation processes. In addition, various metal-mobilization techniques can also occur in nature.

4.4.3 Biosorption

Biosorption is a physicochemical approach which involves uptake of toxicants from dead/inactive biological sources using mechanisms like adsorption, chelation, precipitation, reduction, ion exchange, and coordination with suitable functional groups (amine, hydroxyl, carboxyl, phosphate, and sulfhydryl) present on cell surface (Strandberg et al. 1981). Biosorption process can be rapid, reversible, and unique and should encourage sequestration of dissolved metals from very dilute and complex solutions efficiently. The process of biosorption involves a solid phase (biosorbent) and a liquid phase (solvent) containing dissolved material, which is to be sorbed (Dhankhar and Hooda 2011). Fungal biomass receives much attention due to the high percentage of cell wall materials which act as biosorbents. It increases the variety of functional groups involved in metal binding and their sequestration by fungi like *Mortierella ramannianc*, *Rhizopus sexualis*, *R. stolonifer*, *Zygorhynchus heterogamus*, *Z. moelleri*, *Aspergillus niger*, *Mucor racemosus*, *Penicillium chrysogenum*, and *Trichoderma viride* (Azab et al. 1990; Kurek et al. 1982; Ross and Townsley 1986). Thus, biosorption contributes in complementary manner to the overall sequestration of toxic pollutant even from very small concentrations.

4.4.4 Biotransformation

Biotransformation of metal/metalloids and radionuclide can be done by exploiting the microorganisms that can modify the microenvironment near the microbial cell through catalysis, oxidation, and reduction of the solubility/mobility of metal through methylation and/or demethylation (Singh 2006). The microorganisms contribute possible physicochemical mechanism of interaction with metals or metal ions together with other metabolically mediated mechanisms such as bioprecipitation and bioreduction.

4.4.4.1 Bioprecipitation

It helps in modification of the environment through metabolic mediated processes around the microbial cell. Under aerobic conditions, microbes grow by the transfer of electrons available from the electron donor molecule to the oxygen (Remoudaki et al. 2007). Mineralization of organic carbon into carbon dioxide and reduction of oxygen into water increase the alkalinity and pH of the cell microenvironment, and the excess bicarbonate formed favors the precipitation of metal ions as metal hydroxides Me(OH)x or carbonate $Me_2(CO_3)x$.

4.4.4.2 Biological Oxidation/Reduction

Reduction of heavy metals such as Fe(III) to Fe(II), Mn(VI) to Mn(II), Cr(VI) to Cr(III), Se(VI) to Se(IV), As(V) to As(III), Mo(VI) to Mo(IV), and U(VI) to U(IV) can be catalyzed by the enzymes from a wide variety of microorganisms. These reduced elements serve as electron acceptors in alternative microbial respiration or reduced by the enzymes without energy production (Gadd 2004).

Mechanisms of immobilization, mobilization, biosorption, and biotransformation of metals/metalloids, radionuclides, and related organic substances have potential for remediation of contaminated sites with the use of native microorganisms especially fungal biomass.

4.5 Application of Mycoremediation

Mycoremediation is a form of bioremediation in which native fungi are applied to surface soils to remove and degrade toxic contaminants. It involves different practices like bioaugmentation, biostimulation, biotransformation, biosorption, etc. which have several important applications for the management of industrial wastewaters, petroleum hydrocarbons, heavy metals, distillery and brewery wastes, dyes, pesticides, pulp and paper mill effluent, bleach plant effluent, wood preservatives, organochlorines, and many other hazardous contaminants (Ma and Zhai 2012).

4.5.1 Mycoremediation of Soil

Mycoremediation (fungal-based technique) of soil can be accomplished using several strategies depending on site, conditions, and typically involved chemical pollutant. Fungi have also demonstrated the adsorption of heavy metals, degradation and mineralization of phenol/phenolic compounds, petroleum hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, chlorinated insecticides and pesticides, dyes, biopolymers, and other substances in various matrices with

suitable mechanisms. Similarly, fungi have the capacity to break down and disassemble long-chain toxins into simpler and less toxic chemicals. Keeping this in view, role of fungi in mycoremediation for treatment or biodegradation of pesticide residue, heavy metals, and xenobiotic compounds for bioprospection of agricultural soil in sustainable development is being discussed in this section.

4.5.1.1 Biodegradation of Pesticide Residue

Depending upon usage, pesticides are of different types—insecticide, fungicides, nematicides, and weedicides, which are used, respectively, against various pests like insects, fungi, nematodes, and weeds. These chemicals are used frequently and intensively, which lead to the persistence of these harmful pesticides in soil. In nature, the pesticide residues are degraded through physical, chemical, and biochemical means, but because of their high stability and water solubility, the residues persist in the soil as well as environment for longer time. There have been instances of pesticide persistence and their unwanted side effects on the various components of the agroecosystems, thereby bringing risk to the major forms of life (Gurug and Tanabe 2001; Meriel 2005; Sivasankaran et al. 2007). The fungal degradation of pesticides is carried out by lignin-degrading enzymes or by other enzymatic systems in mycoremediation. Oxidoreductases, laccase, and peroxidases are the fungal enzymes which have prominent application in the removal of polyaromatic hydrocarbons (PAHs) contaminants from freshwater, marine water, or terrestrial sites. Among the white-rot fungi group, *Phanerochaete chrysosporium*, *Agrocybe semiorbicularis*, *Auricularia auricula*, *Coriolus versicolor*, *Dichomitus squalens*, *Pleurotus ostreatus*, and *Flammulina velutipes* degrade a wide variety of pesticides such as aldrin, atrazine, diuron, terbuthylazine, dichlorodiphenyltrichloroethane (DDT), gamma-hexachlorocyclohexane, dieldrin, metalaxyl, heptachlor, chlordane, lindane, mirex, etc. (Odukkathil and Vasudevan 2013). For the degradation of persistent chemical substances/pesticides, involvement of enzymatic reactions has been observed to have high bioremediation potential, and it represents one of the most important strategies for the degradation of hazarders. Hence, fungal bioremediation is one of the most promising approaches to overcome the pesticide problem of cultivable soils.

4.5.1.2 Bioremediation of Heavy Metals

Heavy metal pollution of soil is one of the most serious concerns in the agroecosystem. Industrial effluents and disposal of sewage and concentrated metal wastes are mainly responsible for the accumulation of these heavy metals in soil, which has negative impact on yield as well as crop quality (Singh and Gauba 2014). Fungi are cosmopolitan in nature and play a promising role in natural remediation of heavy metals from agricultural land by channeling them to the fungal fruiting bodies (Dugal and Gangawane 2012). Decomposition of hazardous substances in the

ecosystem is one of the primary roles of fungi, performed by its mycelium, which secretes extracellular enzymes and acids that break down the toxic contaminants. Fungal biomass has a high percentage of cell wall material that shows excellent metal-binding properties. Their heavy metal-binding capacity is dependent on the mycelial age and on the culture media composition used for cultivation. Similarly, fungal cell wall also plays a key role in heavy metals sorption, as isolated cell wall fraction contributes about 38–77% of metal uptake and its sorption capacity is 20–50% higher than the overall binding capacity of the mycelium. Fungi are a versatile biosorption group as they can grow under extreme conditions of pH, temperature, and nutrient availability as well as high metal concentration. Mushrooms are also excellent in heavy metal biotransformation and are considered good recyclers. They can be found in all sorts of environment, as they are capable to grow on logs, animal dung, agricultural wastes, lawns, etc. (Demirbas 2001). However, for mycoremediation of heavy metals, it is important to determine the right fungal species to target a specific heavy metal toxicant because they should be able to absorb the soluble substances so formed. Therefore, there is a need of extensive exploration and bioprospection for fungal species for removal of heavy metals in the field, because mycoremediation is a potential tool which can expedite the process of sequestration or degradation of contaminants like heavy metals from soil.

4.5.1.3 Degradation of Xenobiotics

Accumulation of recalcitrant xenobiotics is one of the major concerns of high input-based agricultural systems. Major xenobiotics include alkanes, polycyclic hydrocarbons (PAHs), synthetic azo dyes, fuels, solvents, antibiotics, pollutants (dioxins and polychlorinated biphenyls), chlorinated, polyaromatic, and nitro-aromatic compounds (Sinha et al. 2009). They are thermodynamically stable, can persist (via micropore entrapment and soil accumulation) in the environment for several decades, as well as pose toxicity to soil health, public health, and environmental health. Therefore, physicochemical and biological methods have been employed for the biodegradation of xenobiotics. Physicochemical methods are expensive, whereas biological methods include microorganisms (fungi, aerobic and anaerobic bacteria) and are comparatively safer to the environment. This method of biodegradation has high ecological and eco-friendly significance as it depends on the indigenous microbial diversity participating in mineralization of hazardous contaminants and their transformation or immobilization into less harmful or nonhazardous compounds. In degradation of such compounds, enzymes like oxygenase play major role by breaking down the ester, amide, or ether bonds and in some cases the aliphatic chains and cyclic rings in aromatic compounds. However, presence of a suitable site is required for accurate action of this enzyme. In addition, its concentration and favorable reaction conditions are required for induction of breakdown of such compounds. Sometimes, xenobiotic compounds are often utilized as a source of energy, nitrogen, carbon, or sulfur by various microorganisms. *Aspergillus* and *Penicillium* are one of the most predominant organisms in soil that show high oil degrading

capability (Hamsavathani et al. 2015). The degradation of xenobiotic compound can take place through white-rot fungi with certain enzymes, whereas degradation of trinitrotoluene (TNT) has been reported by non-ligninolytic strains of *P. chrysosporium* (Singh 2006). Bioremediation is one of the useful cleanup processes that exploit the catabolic abilities of microorganisms to degrade harmful and toxic xenobiotics. Moreover, it maximizes the potential benefits of microbial community in combating pollution problems from xenobiotic compounds.

4.5.2 Mycofiltration of Water

Mycofiltration is the pioneering technique of using fungi to filter out pollutants from agricultural runoff, estuaries, storm water, and gray water. This technique was first developed in the USA by Paul Stamets in the late 1980s. Mycelium is the vegetative structures of fungi which are able to hold soils together, absorb nutrients, and, in certain species, consume harmful bacteria. Fungi perfecti is identified as a creator of an innovative, low-cost bacterial removal system called mycofiltration—using mycelium to treat contaminated storm water runoff. For example, the mycelium of *Stropharia rugosoannulata* ("garden giant" mushroom) can survive under harsh environmental stresses and remove large amounts of *E. coli* from flowing water. Use of fungal biomass to filter water is one of the most efficient applications of mycoremediation, where the network of fungal cells produces a living micron filter to trap or inactivate the pathogens physically as well as biologically (Tiwari and Singh 2014). Chemical degradation in water can be accomplished using enzyme harvesting systems and in-line filters of biomass to conclude the extent of contact time required to achieve a needed safety level to release the output into the environment (Bernasconi et al. 2006). Living swales and rain gardens lined with mycelium offer this type of biological and chemical activity. It should be designed on the basis of trial to determine the amount of biomass and fungal species needed for most effective application of bioremediation. The mycofiltration research should be blended with the application of fungal biotechnology, which provides an innovative and interdisciplinary knowledge for remediation of contaminants from water sources.

Agricultural industry is reported to produce approximately 40 billion metric tons of organic wastes worldwide, which involve wastes produced by human, livestock, fishery, forestry, and crop residues; so its disposal has become a prime global priority in eco-friendly manner (Suthar 2007). Therefore, much attention is being paid recently to develop low-input, effective, and efficient technologies to convert such nutrient-rich organic wastes into value-added products for sustainable land uses. However, these can be managed through composting (through the action of lignocellulolytic microorganisms) and vermicomposting (joint action between the earthworms and microbes where microbes help in degradation of organic matter and earthworms drive the process, condition the substrate, and alter the biological activity) (Gaur 1999). Composting of agricultural residues becomes easier with the help

of several fungi like *Trichoderma harzianum*, *P. ostreatus*, *Polyporus ostriformis*, and *P. chrysosporium*. These recycle the lignocellulosic waste with high economic efficiency (Singh and Nain 2014). The recycled material plays important role when applied to soil and improves soil fertility and health. Hence, bioconversion of agro-wastes may be utilized as a useful resource for production of food, feed, biofuels, and sustainability of the agroecosystems.

4.6 Conclusion and Future Prospects

Advancement in modern agriculture has made tremendous progress in supply of food to the growing populations. However, increased population, industrialization, and urbanization are responsible for environmental contamination and degradation. These contaminants in agriculture are divided into four main groups: agriculture and allied waste, pesticides and its residues, fossil fuel and its combustion products, and heavy metals and xenobiotics. Fertilizers and agro-waste are subjected to the common recycling processes constantly operating in nature, whereas fuel, pesticide, and heavy metal pollution are often long-term and cause persistence problems due to their recalcitrance, xenobiosis, and potential toxicity. Degradation of such pollutants can be done by chemical, physical, or biological means. However, biological methods are performed due to low-cost and minimal adverse effect on the environment. The biological approach of remediating hydrocarbon, organic wastes, and heavy metals with the help of fungal agents to reclaim contaminated soils and water resource is a necessity in order to have a safe and sustainable environment. Fungi or fungal mycelia are among the major decomposers and play major role as natural remediators. They have the ability of enzymatic degradation and mineralization, release and store various elements/ions, and accumulate toxic materials, which facilitate energy exchange between the aboveground and belowground systems. It has proven the modification of soil permeability, ion exchange, and detoxification of contaminated soil. It has become an economically and environmentally attractive alternative, as it does not leave behind products that are comparatively more toxic to the environment than the substrates.

The knowledge of methodologies and ecological tools to sustain fungal biomass and their activity and enzymatic production into contaminated matrices is still limited and is considered as a great hindrance in the process of mycoremediation. Further, greater emphasis is essential for the application of this technology on large-scale projects, which demand advanced research along with integration of biotechnological, microbiological, and engineering tools to streamline the methodologies to achieve enhanced success of mycoremediation. Although endophytes and aquatic fungi play an indispensable role in mycoremediation, limited findings are available regarding nutritional profiles and enzyme activities of aquatic fungi and endophytes. Greater attempts are needed to employ aquatic fungi and endophytes in the mycoremediation of contaminated water and soil resources. Extensive research is needed to develop application methods of

mycofiltration and degradation of xenobiotics and agricultural waste that may turn more reliable and helpful in the understanding of degradation mechanisms as well as physiological and enzymatic regulators involved in the mineralization of contaminants particularly for remediation of agricultural soils.

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Chapter 5
Bioremediation and Decolorization of Textile Dyes by White Rot Fungi and Laccase Enzymes

Ozfer Yesilada, Emre Birhanli, and Hikmet Geckil

5.1 Introduction

Remediation is the removal, degradation, or remediation of pollutants such as xenobiotics and wastewaters from environment by physical, chemical, and biological systems or their combinations. Bioremediation, as the term implies, uses biological systems for remediation (Table 5.1). Mycoremediation, which is the cover topic of this book, is a process of fungal remediation.

Textile and dyeing wastewaters contain various dyes and other inorganic/organic compounds. However, dyes in these wastewaters are the most important pollutants. They have serious effects on environment especially on organisms. In addition, many dyes may be toxic and genotoxic. Textile dyes profoundly affect the color of the receiving water body such as rivers and lakes. Even minute quantities of these dyes can change the color of water bodies dramatically, resulting in the inhibition of photosynthetic activity and reduced dissolved oxygen concentration creating an anaerobic condition.

Physical and chemical methods are the common ones used for treatment of dye-containing wastewaters (Table 5.2). However, these methods are often very costly, and accumulation of concentrated sludge creates a disposal problem after dye removal. Some methods may also produce more serious by-products such as toxic aromatic amines (Robinson et al. 2001). Since synthetic dyes are designed to resist degradation, conventional biological systems are not efficient for their bioremediation and decolorization. While some anaerobic microorganisms have the potential to

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Table 5.1 Types of bioremediation depending on the biological systems

Bioremediation	Biological system
Mycoremediation (fungal remediation)	Fungi
Bacterial remediation	Bacteria
Algal remediation	Algae
Phytoremediation	Plants

Table 5.2 Some chemical and physical methods of dye removal (Robinson et al. 2001)

Chemical methods	Physical methods
Fenton reagents	Adsorption by activated carbon
Ozonation	Adsorption by peat
Photochemical	Adsorption by wood chips
Sodium hypochlorite	Adsorption by fly ash and coal
Electrochemical destruction	Separation by membrane filtration
	Ion exchange
	Irradiation
	Electrokinetic coagulation

decolorize various dyes, such microorganisms can produce toxic aromatic amines which may have more devastating effect.

There have been great efforts to solve the textile dye pollution problems by effective biological systems. Fungi and their enzymes have promising potential for bioremediation of various xenobiotics and wastewaters. Especially, white rot fungi are the most efficient microorganisms in decolorization and degradation of textile dyes. Therefore, these fungi and their enzymes laccase, lignin peroxidase, and manganese peroxidase may prove an alternative to traditional bioremediation of textile dyes (Prasad 2017).

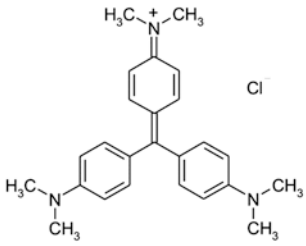
In this chapter, bioremediation, decolorization, and detoxification abilities of white rot fungi and their biotechnologically important enzyme laccase are reviewed. Laccases are the main enzymes in bioremediation of textile dyes. Therefore, the mechanism and role of these enzymes in bioremediation and decolorization are discussed. The roles of immobilization, enzyme mediators, and conditions for bioremediation are also discussed. The advantage and disadvantage of treatment methods are reviewed.

5.2 Textile Dyes

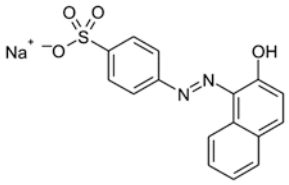
Colored organic compounds contain chromophores which absorb radiation in the visible part of the electromagnetic spectra. The wavelength spectrum of absorbed light is affected by variations in the chromophoric system and other components called auxochromes`. Some dyes contain metals, which enhance both the color and the strength of the dyes for binding to textiles. Chemical structure determines the

color, property, and their use and provides the only rational basis for the classification of these compounds (Kiernan 2001).

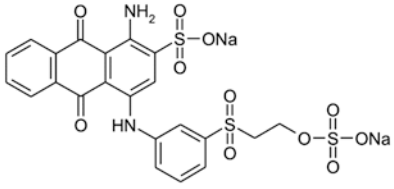
Dyestuffs are classified based on the origin, chemical and/or physical properties, and also characteristics related to application process (Wesenberg et al. 2003). There are various dyes with different chemical structures (Fig. 5.1).



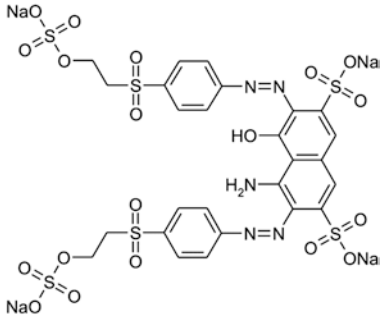
1. Crystal Violet (triarylmethane dye)



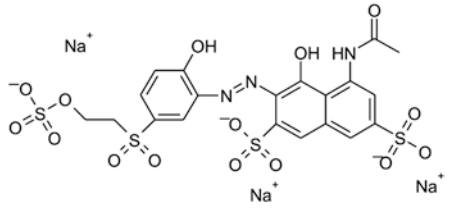
2. Orange II (monoazo dye)



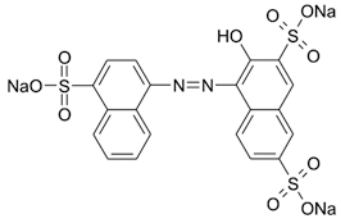
3. Remazol Brilliant Blue R (anthraquinone dye)



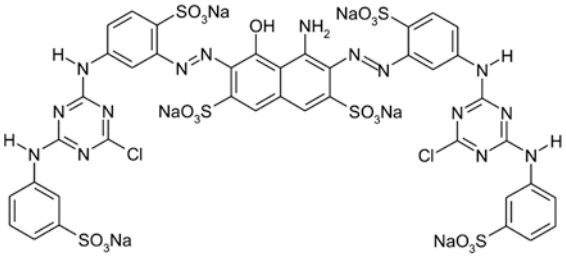
4. Reactive Black 5 (diazo dye)



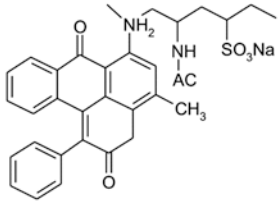
5. Reactive Violet 5 (monoazo dye)



6. Amaranth (monoazo dye)



7. Reactive Blue 171 (diazo dye)



8. Poly R-478 (polymeric dye)

Fig. 5.1 Structure of some chemically different dyes

5.3 White Rot Fungi

In the classification system introduced by Carolus Linnaeus, all organisms were grouped in two kingdoms, namely, Animalia and Plantae. In the late 1960s, Whittaker proposed a five kingdom classification system: Animalia, Plantae, Fungi, Protista, and Monera. In 1977, Carl Woese proposed a three-domain system where whole life based on the phylogenetic relationship of the organisms is divided into three domains: Archaea, Bacteria, and Eukarya. Eukarya includes Animalia, Plantae, Fungi, and Protista.

The kingdom *Fungi* are subdivided into three divisions as *Gymnomycota*, *Mastigomycota*, and *Amastigomycota*. *Amastigomycota* includes four classes: *Zygomycetes*, *Ascomycetes*, *Basidiomycetes*, and *Deuteromycetes*. The members of fungi are chemoheterotrophic organisms, being parasitic or saprophytic. Some are unicellular and many are filamentous and have cell walls. Fungi are important for the health and sustainability of the ecosystem. The three types of fungi are molds, mushrooms, and yeasts. The former two are generally filamentous; the latter are unicellular. Those organisms are biotechnologically important. They can be grown with batch, continuous, and semicontinuous methods.

There are several wood-decaying fungi classified according to their decay types, such as white rot fungi, brown rot fungi, and soft rot fungi. They produce different enzymes and can degrade different wood materials. White rot fungi are *Basidiomycetes* and contain several important genera such as *Trametes*, *Pleurotus*, *Phanerochaete*, *Ganoderm*a, *Lentinus*, etc. These fungi could degrade the lignin part of the woody plants, resulting in the bleaching of wood. They produce enzymes such as laccase, lignin peroxidase, and manganese peroxidase, all important in the degradation of wood and various recalcitrant and xenobiotics such as dyes.

5.4 Dye Decolorization by White Rot Fungi

Large amounts of chemically different dyes are used in textile and dyeing industries. The annual production is about 7×10^5 tonnes, and 5–10% of dyes are lost in effluents (Wong and Yu 1999; Park et al. 2007). Discharge of such effluents to water bodies reduces the sunlight penetration and decreases both photosynthetic activity and dissolved oxygen concentration. Thus, color removal from dye-containing wastewaters is often more important than the removal of colorless organic substances (Banat et al. 1996). Therefore, textile dyes are the major pollutants in textile/dyeing wastewaters, and they have to be decolorized, degraded, or removed prior to discharge. Because of their synthetic origins and complex structures, textile dyes are resistant to microbial degradation or decolorization (Banat et al. 1996). Conventional secondary treatment systems are insufficient for completely decolorizing this type of wastewaters and cause serious pollution problems. Thus, specific biological systems must be used to solve such problems.

White rot fungi are also good candidates for decolorization of textile dyes. Given the high oxidative potential of their enzymes, these fungi are able to decolorize various textile dyes with different chemical structures. Laccase enzymes are the main enzymes responsible for dye decolorization activity (Birhanli and Yesilada 2006; Erkurt et al. 2007). Due to the differences in their physiological characteristics, white rot fungal strains differ in their potential of dye decolorization. A simplistic way of looking to decolorization activity of a fungal strain could be done by agar plate screening method using solid media containing the respective dye. Such decolorization activity could be determined by visual disappearance of color during fungal growth (Fig. 5.2).

There are many studies on textile dye decolorization and degradation activity of white rot fungi in liquid media where decolorization of dyes is possible by growing cells or whole pellets. When growing cells are used, fungi are grown in the test media containing the respective dye. However, the dye may have toxic effects on growing cells. High Remazol Brilliant Blue R (RBBR) dye concentrations are toxic to fungal growth (Zeng et al. 2011). In the pellet method, however, whole fungal pellets are used. Given their reusability and maintenance of high and long-term decolorization abilities, pellets are more advantageous than growing cell method. Pellets also tolerate high concentration of dyes, which are toxic to growing cells. Some studies also tested the dye decolorization activity of white rot fungi under solid-state fermentation conditions in which substrate is a moistened solid. This substrate is also an attachment place and a source of nutrient for fungi (Rodriguez-Couto et al. 2002; Boran and Yesilada 2011) (Fig. 5.3).

5.4.1 Dye Decolorization in Liquid Media by Growing Cells of White Rot Fungi

Growing cells of white rot fungi can decolorize and degrade various dyes in liquid media. There are various studies on bioremediation activity of these cells (Table 5.3).

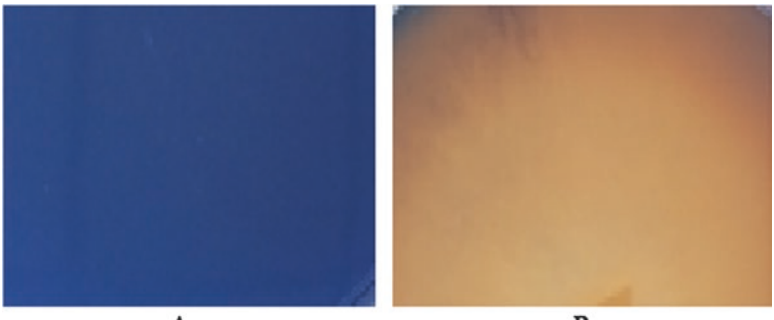


Fig. 5.2 Dye decolorization on Sabouraud Dextrose Agar containing Remazol Brilliant Blue R (100 mg/L). (**a**) Before and (**b**) after fungal decolorization (unpublished data)

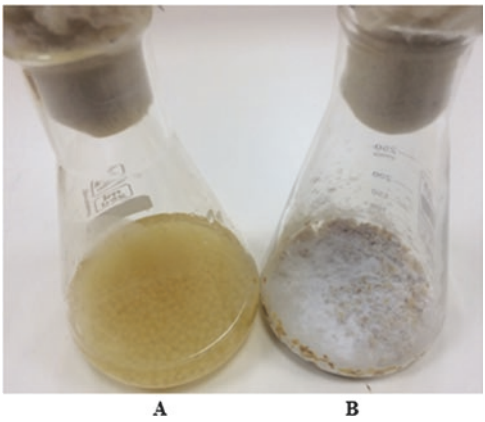


Fig. 5.3 Liquid-state (**a**) and solid-state (**b**) cultures of white rot fungus

Table 5.3 Dye decolorization in liquid media by growing cells of white rot fungi

Incubation mode	Microorganism	Dye	Reference
Static batch	*F. trogii* ATCC 200800, *T. versicolor* ATCC 200801, *P. chrysosporium*	Crystal Violet	Yesilada et al. (1995)
Static batch, shaking batch	*D. squalens*	Orange G, RBBR	Eichlerová et al. (2007a)
Shaking batch	*T. versicolor* strain 1	Reactive Blue 4	Yemendzhiev et al. (2009)
Shaking batch	*P. ostreatus* MTCC 142	Crystal Violet	Kunjadia et al. (2012)
Shaking batch	*C. plicatilis*	Turquoise Blue HFG	Akdogan and Topuz (2014)
Shaking batch	*Coriolopsis* sp. (1c3)	Crystal Violet, Methyl Violet, Cotton Blue, Malachite Green	Chen and Ting (2015)
Shaking batch	*Curvularia* sp.	Congo Red	Senthilkumar et al. (2015)
Shaking batch	*P. ostreatus*, *P. sapidus*, *P. florida*	Coralene Golden Yellow, Coralene Navy Blue, Coralene Dark Red	Kunjadia et al. (2016)
Shaking batch	*Ganoderma* sp. En3	RBBR, Indigo Carmine, Methyl Green	Lu et al. (2016)

Yesilada et al. (1995) studied Crystal Violet (triarylmethane dye) decolorization activity of various white rot fungi such as *Funalia versicolor* ATCC 200801, *Funalia trogii* ATCC 200800, and *Phanerochaete chrysosporium* during static batch studies, and it was reported that the effective decolorization could be obtained by using suitable fungi. In this respect, *T. versicolor* ATCC 200801 and *T. trogii* ATCC 200800 were also used to decolorize azo dye Orange II (Acid Orange 7, monoazo dye) (Sam and Yesilada 2001).

Heinfling et al. (1997) found that liquid cultures of *T. versicolor* DSM11309 and *Bjerkandera adusta* DSM 11310 could efficiently decolorize dyes such as hydrolyzed Reactive Violet 5 (azo dye) and hydrolyzed Reactive Blue 38 (phthalocyanine dye). These two fungi were reported to be more efficient in dye degradation than *P. chrysosporium* K3, and the toxicity of hydrolyzed Reactive Blue 38 on *Vibrio fischeri* was also significantly reduced. The degradation of the azo dye Reactive Violet 5 by *B. adusta* resulted nontoxic metabolites. These dyes could also be decolorized in a H_2O_2-dependent manner by crude extracellular enzymes, indicating the oxidation capacity of the peroxidases of these fungi (Heinfling et al. 1997).

In the study, the decolorization ability of *Dichomitus squalens* against eight chemically different synthetic dyes, Orange G (azo dye), Amaranth (monoazo dye), Orange I (monoazo dye), Remazol Brilliant Blue R (reactive anthraquinone dye), Cu-phthalocyanine (phthalocyanine dye), Poly R-478 (polyaromatic anthraquinone dye), Malachite Green (triarylmethane dye), and Crystal Violet, was tested on agar plates (Eichlerová et al. 2006). Malachite Green and Crystal Violet caused a strong mycelial growth reduction even at 0.05 g/L concentration. The growth of the fungus was also completely inhibited in the medium containing 0.1 g/L Malachite Green dye. The presence of the dyes negatively affected the mycelial growth. In liquid media, this fungus decolorized Orange G and RBBR dyes, and decolorization varied depending on conditions. It was reported that Orange G and RBBR dyes were efficiently decolorized by the static and shaking cultures of *Dichomitus squalens* and the toxicity of the dyes decreased after the fungal decolorization (Eichlerová et al. 2007a).

The decolorization activity of some white rot fungi on various dyes on agar plate and in liquid static culture was also studied. *Bjerkandera adusta* showed high decolorization ability, and the majority of the dyes used, except RBBR, were highly decolorized on solid media. This strain was capable of decolorizing various chemically different dyes such as Orange G, Amaranth, Remazol Brilliant Blue R, Cu-phthalocyanine, and Poly R-478 even at relatively high concentrations (Eichlerová et al. 2007b).

In another study by Erkurt et al. (2007), *T. versicolor* ATCC 200801, *T. trogii* ATCC 200800, and *Pleurotus ostreatus* were used for decolorization of anthraquinone under static batch conditions. Drimaren Blue CL-BR (Reactive Blue 19) showed the toxic effect on *P. ostreatus*, while only concentration above 60 mg/L had inhibitory effect on the growth of *T. versicolor. T. trogii* was detected as the best decolorizing fungus among the fungi tested. Activity staining showed that laccase was the main enzyme responsible for such decolorization.

Revankar and Lele (2007) studied the decolorization potential of *Ganoderma* sp. WR-1 for structurally different dyes: Reactive Orange 16 (monoazo), Orange II, Acid Red 106 (monoazo), Cibacron Brilliant Red 3B-A (reactive monoazo), RBBR, and Amaranth. *Ganoderma* sp. WR-1 could efficiently degrade Amaranth and Cibacron Brilliant Red 3B-A dyes. The maximum dye decolorization reported for Amaranth and Cibacron Brilliant Red 3B-A were 96% and 50%, respectively. Agitation was detected as an important parameter, and starch and yeast extract were the most effective carbon and nitrogen sources, respectively, for Amaranth

decolorization. The other dyes were also partially decolorized after 8 h incubation by the strain WR-1. RBBR (anthracene derivative that is frequently used as starting material in the production of polymeric dyes) was also significantly decolorized (73%) by this strain. Furthermore, *Ganoderma* sp. WR-1 effectively decolorized the industrial effluent containing a mixture of reactive dyes. However, the time required for complete decolorization of single dye was shorter than the time required for the effluent.

The decolorization activity of white rot fungal strain L-25 for 12 different azo, diazo, and anthraquinone dyes showed that high level of decolorization could be obtained (Kariminiaae-Hamedaani et al. 2007). Reactive Blue 4 (anthraquinone-based chlorotriazine dye) decolorization activity of growing cells of *Trametes versicolor* strain 1 with varied concentrations of glucose under agitation reported a direct correlation between laccase activity and the efficiency of color removal. The highest laccase production and also color removal occurred in medium containing 125 mg/L dye and 3% glucose (Yemendzhiev et al. 2009).

The decolorization potential of agitated liquid batch cultures of *P. ostreatus* IBL-02 and *P. chrysosporium* IBL-03 against the reactive dye 222 (diazo dye) was reported by Kiran et al. (2012). The highest decolorization ratios under the optimum fermentation conditions were 92% and 86% for *P. ostreatus* IBL-02 and *P. chrysosporium* IBL-03, respectively. The most effective carbon source, nitrogen source, mediator, and metal ion in terms of the enzyme activities and removal of the dye were detected as rice bran (2%), ammonium oxalate (0.1%), MnSO4 (1 mM), and CuSO4 (1 mM), respectively. It was also shown that the highest decolorization values obtained from *P. ostreatus* IBL-02 and *P. chrysosporium* IBL-03 in the presence of 1 mM $CuSO_4$ were 96% and 96%, respectively (Kiran et al. 2012).

The extracellular enzyme production potential of *Pleurotus ostreatus* MTCC 142 for decolorization of Crystal Violet dye under submerged conditions showed that the decolorization in the medium containing 20 mg/L Crystal Violet was 92%, and it decreased to 32% in the presence of 50 mg/L of the dye after 10 days. When the dye concentration increased to 100 mg/L and above, the decolorization was less than 10% after 10 days of incubation. This showed the inhibitory effect of Crystal Violet at higher concentrations. While the dye induced the production of laccase and manganese peroxidase, lignin peroxidase activity was unaffected. This showed that the decolorization of Crystal Violet was assisted by laccase and manganese-dependent peroxidase (Kunjadia et al. 2012).

Of the 42 white rot fungi tested for their decolorization activity of the azo dye Congo Red, *T. pubescens* Cui 7571 exhibited the highest performance (Direct Red 28, diazo dye) (Si et al. 2013). *Pleurotus eryngii* F032 showed the highest Reactive Black 5 (diazo dye) decolorization activity (94%), and an increase in the initial dye concentration decreased the decolorization activity. This was attributed to the toxicity of the dye on fungal growth. Tween 80 assisted high color removal due to its effect on solubility of the dye (Hadibarata et al. 2013).

The decolorization potential of *Phanerochaete chrysosporium* on synthetic dye bath effluent containing Amido black 10B (diazo dye) was examined in a study by

Senthilkumar et al. (2014). The researchers investigated the effects of different concentrations of azo dye, glucose, and manganese sulfate on the decolorization. Because the growth of fungus decreased with an increase in dye effluent concentration, the decolorization decreased when the concentration of effluent was raised. Glucose acted as the primary nutrient in decolorization of dyes, and the growth of this fungus increased with the increase in glucose concentration. The production of manganese peroxidase was induced by manganese sulfate. The decolorization increased up to a concentration of 0.5%, but a further increase in concentration decreased the rate of decolorization. Addition of 0.5 g starch or lignin to dye bath effluents increased the color removal as starch increased the laccase production. It was shown that lignin increased the production of enzymes such as laccase, lignin peroxidase, and manganese peroxidase.

Coprinus plicatilis decolorized Turquoise Blue HFG (reactive dye) at 10 and 25 mg/L concentrations almost completely in liquid media under shaking condition. This activity was decreased to 63–77% at the concentrations above 50 mg/L. The presence of the dye could induce the extracellular laccase and manganese peroxidase activity, and these enzymes could have a significant role in decolorization (Akdogan and Topuz 2014).

The decolorization of triphenylmethane dyes such as Crystal Violet, Methyl Violet (Basic Violet 1), Cotton Blue, and Malachite Green by *Coriolopsis* sp. (1c3) displayed good efficiencies: 94% of 100 mg/L of Crystal Violet within 7 days, 97% of 100 mg/L of Methyl Violet within 7 days, and 91% of 50 mg/L of Cotton Blue within a day. Malachite Green (100 mg/L) was detected as the most recalcitrant dye, and only 52% was decolorized after 9 days of incubation. The decolorization efficiency of the fungus was better at lower initial dye concentrations (50 and 100 mg/L) compared to higher concentration (200 mg/L). The decolorizations of Crystal Violet (82%), Methyl Violet (82%), and Cotton Blue (86%) in the presence of oxygen were higher than the decolorization of Crystal Violet (46%), Methyl Violet (74%), and Cotton Blue (66%) in the absence of oxygen. However, the decolorization efficiency on Malachite Green was more effective in the absence of oxygen with 48% compared to 40% in aerobic conditions. Therefore, the decolorization efficiency of this isolate was influenced by fungal biomass, initial dye concentrations, and also oxygen requirement (except for Malachite Green) (Chen and Ting 2015). The optimum parameters for efficient color removal of Congo Red by *Curvularia* sp. by using Box-Behnken design were determined as 60 mg/L, pH 5, and 32.5 °C under agitated conditions (Senthilkumar et al. 2015).

The Coralene Golden Yellow (CGY), Coralene Navy Blue (CNB), and Coralene Dark Red (CDR) azo dye decolorization and ligninolytic enzyme production abilities of *P. ostreatus*, *P. sapidus*, and *P. florida* were tested under agitated conditions. Although all *Pleurotus* species had decolorizing capacity for these dyes, *P. florida* was found as the best decolorizing organism. The increase in dye concentration negatively affected the color removal potential of these fungi. Above 100 mg/L concentration, total decolorization efficiency was decreased because of the toxicity of the dyes. The removal of the dyes was sharply reduced under strongly

acid or alkaline conditions depending on the optimum pH. The order of laccase and manganese peroxidase efficiency of the white rot fungi was *P. florida* > *P. ostreatus* > *P. sapidus* and *P. ostreatus* > *P. sapidus* > *P. florida*. In addition, no lignin peroxidase activity was reported, and it was reported that laccase was the major extracellular ligninolytic enzyme produced by these fungi (Kunjadia et al. 2016).

Also, the decolorization capacity of *Ganoderma* sp. En3 was investigated by using different types of dyes: anthraquinone dye RBBR, Indigo Carmine (indigoid), and triphenylmethane dye Methyl Green. High concentrations of RBBR (1000–6000 mg/L), Indigo Carmine (800–2000 mg/L), and Methyl Green (600–4000 mg/L) were used in 5-day cultures. The fungus efficiently decolorized all three dyes (Lu et al. 2016).

5.4.2 *Dye Decolorization in Liquid Media by Immobilized White Rot Fungi*

Textile dyes especially at high concentrations inhibit the growth of fungi. Therefore, the primary problem of white rot fungal decolorization is to adapt fungi in media containing high amounts of textile dyes (Apohan and Yesilada 2005). Papinutti and Forchiassin (2004) reported that while *P. chrysosporium* was unable to grow on solid media containing 64 μM of Malachite Green, *F. sclerodermeus* was more resistant. However, in liquid media both fungi were more sensitive. This toxicity (growth inhibition) problem could be overcome by using whole pellets or immobilized cells. Various methods can be used for immobilization of biological systems. The main immobilization methods are attachment and entrapment. During the attachment, biological system is immobilized onto support material such as polymers, activated carbon, or lignocellulosic materials. On the other hand, the biological system is entrapped in a matrix such as agar, gel, or other synthetic polymers (Papinutti and Martínez 2006). Dye decolorization in liquid media by immobilized white rot fungi is presented in Table 5.4.

Acid Violet 7 (monoazo dye) decolorization ability and stability of *T. versicolor* pellets immobilized on activated carbon were higher than the free pellets. The immobilized pellets in a fluidized bed reactor with a repeated-batch feeding gave higher and more stable decolorization efficiency than the continuous flow feeding. The reason for this was the poor production of extracellular enzymes in a continuous flow feeding fluidized bed reactor (Zhang and Yu 2000). The immobilized forms of *T. versicolor* ATCC 200801 and *T. trogii* ATCC 200800 in alginate beads could be used repeatedly as much as three times, and still a high Orange II dye decolorization activity could be obtained (Sam and Yesilada 2001). Immobilized *T. hirsuta* on stainless steel sponge decolorized Indigo Carmine and Lanset Marine dyes, and it was possible to operate this system with high efficiency in a bioreactor at 1 L (Rodriguez-Couto et al. 2004). The growth inhibition effect of Reactive Black 5 on *T. trogii* ATCC 200800 under static batch conditions was reported. This inhibition

Table 5.4 Dye decolorization in liquid media by immobilized white rot fungi

Immobilization	Microorganism	Dye	Reference
Attached on activated carbon	*T. versicolor*	Acid Violet 7	Zhang and Yu (2000)
Entrapped in alginate beads	*F. trogii* ATCC 200800, *T. versicolor* ATCC 200801	Orange II	Sam and Yesilada (2001)
Attached on stainless steel sponge	*T. hirsuta*	Indigo Carmine, Lanset Marine	Rodriguez-Couto et al. (2004)
Attached on *Luffa cylindrica* sponge	*F. trogii* ATCC 200800	Reactive Black 5	Mazmanci and Unyayar (2005)
Attached on crushed orange peelings	*T. hirsuta*	Indigo Carmine, Bromophenol Blue, Methyl Orange, Poly R-478	Rodriguez-Couto et al. (2006)
Attached on pine wood chips, attached on palm oil fiber	*T. versicolor*, *P. chrysosporium*	Levafix Blue, Remazol Brilliant Red	Boehmer et al. (2006)
Attached on polyurethane foam cubes	*T. pubescens*, *P. ostreatus*	RBBR, B49, R243	Casieri et al. (2008)
Entrapped in alginate beads	*C. gallica*, *B. adusta*, *T. versicolor*, *T. trogii*	Lanaset Grey G	Daâssi et al. (2013)
Entrapped in alginate beads	*T. versicolor* U97	Reactive Green 19	Sari et al. (2016)

was minimized when the fungus immobilized on a cheap and nontoxic sponge. Immobilized *T. trogii* ATCC 200800 on *Luffa cylindrica* sponge effectively decolorized the dye (Mazmanci and Unyayar 2005).

In another related study, *T. hirsuta* immobilized on crushed orange peelings were used to decolorize four structurally different dyes, Indigo Carmine, Bromophenol Blue (sulfonephthalein), Methyl Orange (Acid Orange 52, monoazo dye), and Poly R-478, in an expanded-bed bioreactor under solid-state conditions during both batch and continuous modes. High Indigo Carmine and Bromophenol Blue decolorization percentages were obtained in a batch mode. The system employed was reported to be suitable for application in dye decolorization in a continuous mode (Rodriguez-Couto et al. 2006).

Tavčar et al. (2006) investigated the azo dye Reactive Orange 16 decolorization activity of immobilized *Irpex lacteus* in three different reactors: small trickle-bed reactor (STBR), large trickle-bed reactor (LTBR), and rotating disc reactor (RDR). The decolorization rate decreased in order STBR (11.6 g DW/L) > LTBR (8.3 g DW/L) > RDR (4.9 g DW/L). The decolorization rates (around 90%) were obtained after 3, 4, and 6 days, respectively. In the column reactors, the flow was clogged by extensive growth of this strain. Therefore, rotating disc reactors could be suitable for the degradation of pollutants. Similarly, Novotný et al. (2012) showed

that *D. squalens* effectively decolorized 99% RBBR, 93% Methylene Blue, and 59% Azure B (latter two being phenothiazine dyes) in an RBC-type reactor, within 7, 40, and 200 h, respectively.

Levafix Blue (reactive dye) and Remazol Brilliant Red (reactive dye) decolorization activities of *Trametes versicolor* and *Phanerochaete chrysosporium* were tested in a study by Boehmer et al. (2006). Both fungi were immobilized separately either on pine wood chips or palm oil fiber and cultivated in the temporary immersion RITA system (fungal bioreactor). After 24 h of incubation, the color of Levafix Blue was removed more than 80% by both fungi. The maximum decolorization of Remazol Brilliant Red within 4 days by *Trametes versicolor* immobilized on palm oil fiber and also pine wood chips was about 50%. Laccase enzymes were also produced in this study from the cultures of immobilized *Trametes versicolor* (Boehmer et al. 2006).

Immobilized *T. pubescens* and *P. ostreatus* on polyurethane foam cubes successfully decolorized and detoxified three reactive dyes, B49 dye (industrial anthraquinone), R423 (industrial azo), and RBBR, in a bioreactor. These fungi were able to remove about 97% of the anthraquinone dyes (RBBR and B49) and 65% of the azo dye (R243) during five sequential cycles (Casieri et al. 2008).

The azo dyes Direct Violet 51 (DV), Reactive Black 5 (RB), and Ponceau Xylidine (PX) were almost completely decolorized by *P. chrysosporium* immobilized into alginate beads. Bismark Brown R (BB) was more resistant to decolorization, and it was not completely decolorized (87% in 144 h). Here manganese peroxidase played the main role, and the activity of the immobilized cells after storage showed their reusability potential (Enayatzamir et al. 2010).

Coriolopsis gallica, *Bjerkandera adusta*, *Trametes versicolor*, and *Trametes trogii* immobilized into Ca-alginate beads were used to decolorize metal complex dye Lanaset Grey G. The decolorization obtained by the immobilized pellets after three cycles was about 75%, 70%, 60%, and 68% for immobilized *C. gallica*, *B. adusta*, *T. versicolor*, and *T. trogii*, respectively. Laccase was reported as the main enzyme involved in decolorization (Daâssi et al. 2013).

Another study investigated the decolorization potential of *Trametes polyzona* LMB-TM5 and *Ceriporia* sp. LMB-TM1 biofilms, developed on polyester cloth using dyes Levafix Yellow E-3RL (Reactive Orange 30), Remazol Brilliant Red 3BS (Reactive Red 239), Remazol Brilliant Blue R (Reactive Blue 19), Cibacron Deep Red S-B (Reactive Red 278), Synozol Yellow HF-4GL 150% (reactive dye), and Synozol Turquoise Blue HFG 133% (reactive dye) and also simulated and real textile effluents. *T. polyzona* LMB-TM5 was detected being more efficient than *Ceriporia* sp. LMB-TM1. Azo dyes were moderately decolorized as compared to Remazol Brilliant Blue R and Synozol Turquoise Blue HFG (phthalocyanine) dyes. The latter two dyes were highly decolorized (97% and 80%, respectively) by *T. polyzona* LMB-TM5. Simulated effluents prepared with six dyes were moderately decolorized by both strains, while a real textile effluent was almost completely (98%) decolorized by *T. polyzona* LMB-TM5 (Cerrón et al. 2015).

Reactive Green 19 decolorization activity of free cells in flasks and also alginate beads of *T. versicolor* U97 in a bioreactor was reported by Sari et al. (2016). While free cells showed 44% dye decolorization within 72 h, immobilized fungus in a bioreactor system efficiently decolorized the Reactive Green 19, and the decolorization determined within 72 h was 70% (Sari et al. 2016).

5.4.3 *Dye Decolorization in Liquid Media by White Rot Fungal Pellets (Whole Cells)*

Some immobilization methods may be uneconomical. Therefore, instead of immobilized forms, whole fungal pellets which are self-immobilized forms could also be used (Wang et al. 2005). Whole fungal pellets have many advantages as they could be stored and repeatedly used. Furthermore, they can be easily separated from the liquid medium. The fungal pellets could effectively decolorize various dyes (Fig. 5.4) (Table 5.5).

As the required most important condition for an economical and practical process is the use of pellets repeatedly, Knapp et al. (1997) showed that with repeatedly used fungal pellets, the decolorization of Orange II dye can achieve up to 98%. The high percentage of decolorization was due to biodegradation. A report showed that agitated cultures resulted higher decolorization than static cultures (Swamy and Ramsay 1999). This was attributed to physiological state as pellets have increased mass and oxygen transfer. Another study showed the Acid Orange 7 decolorization by self-immobilized pellets of *C. versicolor* and the pellets could be used continuously and repeatedly. The pellets could also decolorize the wastewaters from printing and dyeing industry (Lin et al. 2003). Yesilada et al. (2003) reported high Astrazon dye decolorization activity of various white rot fungal pellets (*T. versicolor* ATCC 200801, *T. trogii* ATCC 200800, *P. chrysosporium* ME446, *Pleurotus florida*, *Pleurotus ostreatus*, and *Pleurotus sajor-caj*u) under repeated-batch mode. Live pellets of *Trametes trogii* ATCC 200800 could effectively decolorize Astrazon

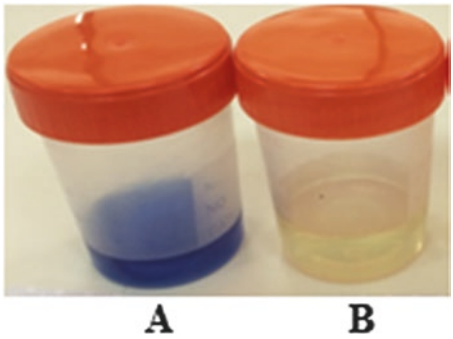


Fig. 5.4 Color of reactive blue 171 dye (100 mg/L) before (**a**) and after (**b**) decolorization by *T. trogii* pellets

Table 5.5 Dye, mixed dyes, and wastewater decolorization in liquid media by white rot fungal pellets (whole cells)

Incubation mode	Microorganism	Dye	Reference
Repeated-batch	*T. versicolor*	Acid Orange 7	Lin et al. (2003)
Repeated-batch	*F. trogii* ATCC 200800, *T. versicolor* ATCC 200801, *P. chrysosporium* ME446, *P. florida*, *P. ostreatus*, *P. sajor-caj*u	Astrazon Red FBL, Astrazon Blue	Yesilada et al. (2003)
Shaking batch, repeated-batch	*T. trogii* ATCC 200800	Reactive Blue 19, Reactive Blue 49, Acid Violet 43, Reactive Black 5, Reactive Orange 16, Acid Black 52	Park et al. (2007)
Shaking batch	*P. sanguineus*	Crystal Violet	Sulaiman et al. (2013)
Repeated-batch	*Ganoderma weberianum* TZC1	Indigo dye, Indigo dye-containing textile wastewater	Tian et al. (2013)
Shaking batch	*Ganoderma* sp. En3	Reactive Orange 16, Indigo jean dyeing real wastewater	Ma et al. (2014)

Red FBL (monoazo dye C.I. Basic Red 46) and Astrazon Blue dyes (mixture of azacyanine/oxazine dyestuffs, contains and azo dye C.I. Basic Blue 159 and also C.I. Basic Blue 3) in dye containing distilled water with no supplements (Yesilada et al. 2002, 2003). It was reported that the decolorization performance and stability of the pellets could be induced with the addition of cheese whey even at high dye concentration. The dye decolorization activity of free and heat-killed pellets suggested that high decolorization activity is mainly due to microbial metabolism, not biosorption (Yesilada et al. 2003).

The live pellets of *Phanerochaete chrysosporium* ME446 had a 95% Indigo dye decolorization activity within 24 h of incubation. They also investigated the textile wastewater decolorization activity of live pellets and reported 61% and 95% decolorization after 2 h and 24 h of incubation. This decolorization involved microbial metabolism, and a comparison of live pellets and dead pellets showed that live pellets had a higher and more stable decolorization activity for 5 days. However, the wastewater was determined to be not suitable for growing the fungal cells, indicating the inhibitory activity of wastewater on fungal cells (Cing et al. 2003). Cing and Yesilada (2004) compared the Astrozon Red dye decolorization activity of *T. trogii* ATCC 200800 with the decolorization activity of growing fungal cells. The pellet method was more advantageous than the growing cell method, and the immobilized pellets on activated carbon had a stable dye decolorization activity during repeated-batch studies. While 97% decolorization could be obtained with pellets in the medium containing 264 mg/L dye, this was only 16–24% with the growing cells in

medium containing 50 mg/L dye. High amounts of dyes showed a toxic effect on growing cells, and a concentration of 150 mg/L completely inhibited the growth of cells and their decolorization activity (Cing and Yesilada 2004). Apohan and Yesilada (2005) investigated the toxicity of Astrazon Red and Astrazon Blue dyes on bacterium and fungus before and after the pellet treatment. The toxicity of these dyes on cells was significantly decreased after decolorization by *T. trogii* ATCC 200800 pellets. This decolorization and detoxification activity of fungal pellets could also be advantageous to integrate the biodecolorization process before or after conventional process.

T. trogii ATCC 200800 cultivated on either solid (yeast-malt-peptone-glucose agar medium) or in a liquid phase (batch and repeated-batch) decolorized the blue dyes more rapidly than the black dyes. The decolorization rate can be affected by small differences in dye structures, including steric effect and redox potential. The complete decolorization took 10 days by solid culture, while it occurred in 3 days by batch liquid culture. Similar to repeated-batch cultures, the anthraquinone-based dyes (Reactive Blue 19, Reactive Blue 49, and Acid Violet 43) were decolorized faster than the azo-based dyes (Reactive Black 5, Reactive Orange 16, and Acid Black 52) in liquid batch cultures (Park et al. 2007).

Whole cells of *P. ostreatus* and *P. chrysosporium* could be used for the decolorization of model acid, direct, and reactive dye wastewaters. These fungi could not decolorize reactive dye wastewater both in the presence and absence of added nutrients. While *P. chrysosporium* caused an effective decolorization of direct dye wastewater with nutrients, *P. ostreatus* decolorized acid dye wastewater with and without nutrients (e.g., malt extract). Therefore, these fungi showed different specificities in decolorizing the dye wastewaters. Laccases reported as the main enzyme for the wastewater decolorization by *P. ostreatus.* This strain reduced the toxicity of the acid dye wastewater both in the presence and absence of nutrients. The differences in activity could be due to the differences in the enzyme activity profiles of fungi, mainly laccases in *P. ostreatus* and manganese peroxidase in *P. chrysosporium* (Faraco et al. 2009).

Industrial effluents may contain a mixture of dyes, and it was reported that the pre-grown pellets had higher mixed dye decolorization activity than growing cells in batch culture. While the pellets showed 85% decolorization in 24 h, this figure was 32% for growing cells. This pointed to the efficiency of the pellet method in decolorization of mixed dyes (Yesilada et al. 2010).

The process time and agitation had significant effect on Crystal Violet decolorization potential of *Pycnoporus sanguineus* pellets. The optimum conditions for dye decolorization in shake flasks were reported as 2 days, 128 rpm, and dye amount of 40 ppm. A stirred tank reactor with 180° curved blade impeller resulted in high decolorization (Sulaiman et al. 2013).

Ganoderma weberianum TZC1 decolorized the Indigo dye and Indigo dye-containing textile wastewater repeatedly and effectively in a repeated-batch process. Native polyacrylamide gel electrophoresis showed that the main enzyme in Indigo dye decolorization was laccase (Tian et al. 2013).

Ma et al. (2014) reported the efficient decolorization and detoxification of the sulfonated azo dye Reactive Orange 16 and its simulated wastewater by *Ganoderma* sp. En3 actively growing 5-day-old submerged agitated cultures. The strain showed strong adaptability and tolerance to high concentrations of Reactive Orange 16 and also showed a strong ability to decolorize high concentrations of this dye in submerged cultures. Similarly, the whole cultures of *Ganoderma* sp. En3 effectively decolorized and detoxified high concentrations of Reactive Orange 16. However, the crude laccase enzyme could not efficiently decolorize high concentrations of this dye without natural phenolic compound, syringaldehyde (a redox mediator). The use of fungus instead of crude laccase was more economical. Furthermore, decolorization reduced the phytotoxicity of the dye. The indigo jean dyeing real wastewater was also decolorized up to 85% within 8 days with actively growing mycelium pellets of *Ganoderma* sp. En3.

5.4.4 Dye Decolorization by Semisolid-State and Solid-State Fermentation

RBBR and Remazol Black 5 decolorization activity of white rot fungus *Ganoderma lucidum* KMK2 (*G. lucidum*) was investigated under solid-state fermentation by Murugesan et al. (2007). The maximum laccase and MnP activities were obtained from 7-day cultures of this fungal strain. Laccase was reported as the major lignino-lytic enzyme of *G. lucidum* obtained from solid-state culture containing wheat bran. Polyacrylamide gel electrophoresis (PAGE) results also showed that laccase was the major enzyme participating in color removal of both dyes (Murugesan et al. 2007).

RBBR decolorization activity of *T. pubescens* grown on sunflower seed shells under solid-state fermentation conditions in temporary immersion bioreactors during five successive batches was also reported, and the decolorization of simulated textile wastewater based on RBBR by *T. pubescens* grown on various supports under semisolid-state fermentation conditions was studied by Rodriguez-Couto (2011, 2012). The mechanisms responsible for decolorization process were reported as biodegradation by laccase and adsorption.

The decolorization of Congo Red, Aniline Blue, and Indigo Carmine dyes by *Trametes* sp. SYBC-L4 during solid-state fermentation on cassava residue was also investigated. The effect of pH and HBT concentration on decolorization of the dyes was monitored. The decolorization ratios of the dyes tested were not enhanced by HBT. After 10 days of fermentation, decolorization ratios of Congo Red, Aniline Blue, and Indigo Carmine were 58%, 93%, and 97% at pH 4.5, respectively (Li et al. 2014).

Adsorption of dyes on various lignocelluloses may be an alternative method for removing dyes. However, dye-adsorbed lignocelluloses create another pollution problem. Nigam et al. (2000) proposed the two-step method, adsorption, and then solid-state fermentation, for removing and bioremediation of dyes from

wastewaters. They demonstrated that the dye-adsorbed lignocelluloses (wheat straw, corncobs, and wood chips) are suitable substrates for solid-state fermentation by *T. versicolor* and *P. chrysosporium*. This fermented substrate could be used as fertilizer or soil conditioner. It was possible to decolorize and use dye-adsorbed lignocelluloses for the production of biotechnologically important enzymes. Mixed dyes (Cibacron Yellow C-2R, Cibacron Red C-2G, Cibacron Blue C-R, Remazol Black B, and Remazol Red RB) adsorbed onto barley husks were 53% decolorized under solid-state fermentation condition by *B. adusta*. Because the fermented material is rich in ligninolytic enzymes and biomass, it could be utilized as soil conditioner. This method may be an economically viable process for the remediation of textile wastewater (Robinson and Nigam 2008). Rodriguez-Couto et al. (2009) reported that dye-adsorbed sunflower seed shells could be used for production of high titers of laccase by *T. pubescens* under semi-solid-state conditions. This system was efficiently scaled up to static tray reactor. Ozmen and Yesilada (2012) tested the laccase production and Astrazon dye decolorization activity of *T. versicolor* ATCC 200801 and *T. trogii* ATCC 200800 using this two-step method. While *T. trogii* showed 80% Astrazon Black and 69% Astrazon Blue decolorization activity against dye adsorbed onto wheat bran, *T. versicolor* showed 86% and 84% Astrazon Black and Astrazon Blue dye decolorization, respectively, under same conditions. This process maybe is an economic and easy way of bioremediation of textile wastewaters.

5.5 Dye Decolorization by Laccase Enzymes

5.5.1 Laccases

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are the members of a multicopper oxidase family (Solomon et al. 1996). These enzymes are glycosylated in structure (Bligny et al. 1986; Yaropolov et al. 1994; Madhavi and Lele 2009). The laccase enzymes have approximately 500 amino acids (Yaropolov et al. 1994). These enzymes may be monomeric, dimeric, or tetrameric with a molecular weight between 60 and 80 kDa (Thurston 1994; Majeau et al. 2010). Four copper atoms are located in the active site, and one-electron oxidation of various substrates is carried out at the T1 copper site. The electron is transferred to the trinuclear copper cluster T2/T3 after this oxidation where oxygen is reduced to water (Solomon et al. 2008; Jones and Solomon 2015; Pardo and Camarero 2015).

Different organisms such as some higher plants, insects, bacteria, and fungi produce laccases (Claus 2004). The fungal laccases are produced extracellularly by various fermentations such as submerged, semisolid-state, and solid-state fermentation (Birhanli and Yesilada 2013; Mota et al. 2015). Most fungi produce more than one laccases (Janusz et al. 2013). The number of laccase isoenzymes depends on the laccase-producing organisms, and the molecular weight, optimum

pH value, and substrate specificities of these laccases may vary. Production of laccases may be stimulated under different methods and conditions such as submerged, semisolid-state, and solid-state fermentations, various carbon and nitrogen sources, and inducers. However, due to low substrate specificity, laccases are able to catalyze the oxidation of a wide variety of substrates by using molecular oxygen as the final electron acceptor instead of hydrogen peroxide (Revankar and Lele 2006; Madhavi and Lele 2009; Birhanli and Yesilada 2013; Vantamuri and Kaliwal 2016). These enzymes could also be used for removing phenolic contaminants, pesticide transformation, dye and textile wastewater decolorization, sewage treatment, transformation of lignin-related compounds, dechlorination of polychlorinated phenols, wood pulping and pulp bleaching, enzyme immunoassay, laccase-based biosensors, and production of organic materials (Kahraman and Yesilada 2001; Desai and Nityanand 2011; Vantamuri and Kaliwal 2016). These enzymes are also used for the stabilization and processing of beverages, for the production of anticancer drugs, and even as ingredients in cosmetic products (Madhavi and Lele 2009).

The mediators act as electron shuttles. Natural mediators such as 3-hydroxyanthranilate, syringaldehyde, and p-coumaric acid and artificial mediators like 2 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1-hydroxybenzotriazole (HBT), N-hydroxyfthalimide (NHPI), and violuric acid (VLA) further expand the biotechnological, industrial, environmental, and other application areas of laccases (Kunamneni et al. 2007; Cañas and Camarero 2010; Desai and Nityanand 2011).

5.5.2 Dye Decolorization by Crude, Purified, and Immobilized Laccases

White rot fungi and their enzymes are being increasingly used in medicinal, biotechnological, and environmental applications. These fungi are able to depolymerize lignin using laccase, lignin peroxidase, and manganese peroxidase. However, laccases are the main enzymes responsible for dye decolorization, and white rot fungi are the best laccase producers. Many studies for laccase enzyme production under different culture conditions and methods have been reported. Growing cells on solid medium containing compounds such as ABTS, guaiacol, or syringaldazine as an indicator for laccase is the preliminary and easy way to detect the laccase-producing organisms. In the solid medium containing ABTS, the organism forms green to dark purple zone depending on the oxidation activity of laccase enzyme (Fig. 5.5). If the organism has no laccase enzyme (no oxidation), there is no change in color, while low oxidation is represented by green color and high oxidation by dark purple color.

Dye-containing wastewaters have deep color. The color has a negative effect on the photosynthetic activity and thus dissolved oxygen concentration of receiving water bodies. Therefore, the elimination of color from these types of wastewaters is

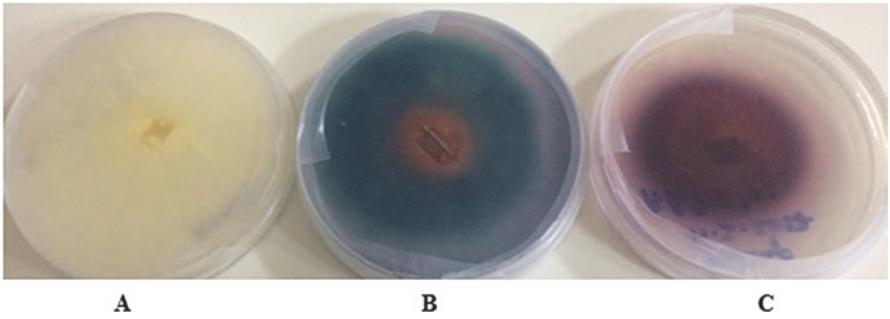


Fig. 5.5 Laccase activity of white rot fungus after production on Sabouraud Dextrose Agar with and without ABTS. (**a**) Without ABTS, (**b**) with ABTS (green color), and (**c**) with ABTS (dark purple color)

very important. The match between the chemical structure of the dye and the active site of the enzyme and also the redox potential of the dye are important for dye decolorization (Tavares et al. 2008). The decolorization process also depends on the source and form of enzyme. Different fungal laccases may have different redox potentials (Li et al. 1999; Soares et al. 2001). For this reason, there are many studies on decolorization of various dyes by the enzymes obtained from the culture filtrates from different strains and different fermentation types such as liquid batch, liquid repeated-batch, and solid-state fermentations. Laccase represents a promising enzyme for application in colored wastewaters decolorization. It is the primary enzyme responsible for textile dye decolorization. Free or immobilized forms of the crude culture filtrate, semi-purified, and purified enzymes could be used to decolorize and degrade textile dyes (Table 5.6).

Decolorization potential of laccases even on the same dye shows variations depending on the microorganisms. It was reported that dye decolorization activity of crude laccase from repeated-batch culture could also be enhanced (Gomashe and Dharmik 2014). Some studies showed that laccase could only decolorize dyes with a mediator, while others reported that decolorization activity could be enhanced by using mediators (Camarero et al. 2005; Murugesan et al. 2007). There are also some reports that show laccases with high decolorization activity without mediators (Erkurt et al. 2007; Yesilada et al. 2014). Some mediators have some disadvantages such as their hardly degradable character and toxicity to organisms. In addition, mediator could also inactivate the enzyme in decolorization process (Roriz et al. 2009).

Yesilada and Ozcan (1998) reported Bromophenol Blue, Orange II, and Remazol Brilliant Blue R decolorization activity of static batch culture filtrates of *T. versicolor* ATCC 200801, *T. trogii* ATCC 200800, and *P. sajor-caju*. The culture filtrates of white rot fungi could be used to decolorize the dyes used without mediator. The culture filtrate of *T. trogii* ATCC 200800 from solid-state fermentation also achieved

Table 5.6 Dye decolorization by crude, purified, and immobilized laccases

Laccase form	Microorganism	Dye	Reference
Crude	*F. trogii* ATCC 200800, *T. versicolor* ATCC 200801, *P. sajor-caju*	Bromophenol Blue, Orange II, RBBR	Yesilada and Ozcan (1998)
Crude	*F. trogii* ATCC 200800	Drimarene Blue X3LR	Unyayar et al. (2005)
Crude	*T. trogii* ATCC 200800, *T. versicolor* ATCC 200801	Congo Red, Indigo Carmine, Orange II, RBBR	Birhanli and Yesilada (2006)
Partially purified	*T. trogii*, *T. versicolor*, *P. ostreatus*	RBBR, Drimarene Blue CL-BR	Erkurt et al. (2007)
Crude	*G. lucidum* KMK2	RBBR, Reactive Black 5	Murugesan et al. (2007)
Crude, purified laccase isoforms (Lacc 1 and Lacc 2)	*C. unicolor*	Direct Black 22, Acid Red 27, Reactive Blue 81, Acid Blue 40, Acid Blue 62	Michniewicz et al. (2008)
Crude, purified	*Polyporus* sp. S133	RBBR	Hadibarata and Tachibana (2009)
Purified	*Trametes* sp. SQ01	Congo Red, Fast Blue RR Salt, Acid Red, Amido Black 10B, Orange G, Malachite Green, Crystal Violet, Cresol Red, Coomassie Brilliant Blue G250, Bromophenol Blue, RBBR	Yang et al. (2009)
Crude	*T. trogii* BAFC 463, *T. villosa* BAFC 2755, *T. versicolor* BAFC 266	Gentian Violet, Xylidine, Congo Red, Malachite Green, RBBR, Indigo Carmine, Anthraquinone Blue	Levin et al. (2010)
Crude, purified	*T. trogii* BAFC 463	RBBR, Indigo Carmine, Xylidine, Malachite Green, Gentian Violet, Bromophenol Blue	Grassi et al. (2011)
Crude	*T. versicolor* ATCC 200801	Reactive Red 198, Rem Blue RR, Dylon Navy 17, Rem Red RR, Rem Yellow RR	Sasmaz et al. (2011)
Crude	*T. trogii*	Reactive Blue 4, RBBR, Acid Blue 129, Acid Red 1, Reactive Black 5	Zeng et al. (2011)
Crude	*F. trogii* ATCC 200800	Reactive Blue 171, Indigo Carmine	Birhanli et al. (2013)
Crude	*L. polychrous*	Reactive Black 5, Reactive Orange 16, Reactive Green 19, Methyl Orange, Acid Blue 80, Water Blue	Ratanapongleka and Phetsom (2014)

(continued)

Table 5.7 (continued)

Laccase form	Microorganism	Dye	Reference
Crude	*Trametes* sp. SYBC-L4	Congo Red, Aniline Blue, Indigo Carmine	Li et al. (2014)
Crude	*Peniophora* sp. (NFCCI-2131)	Amido Black, Crystal Violet, Brilliant Green, Methyl Orange, Methylene Blue	Shankar and Nill (2015)
Immobilized concentrated laccase	*C. bulleri*	Simulated effluent containing Acid Red 27 or Basic Green 4 or Acid Violet 17	Chhabra et al. (2015)
Crude	*P. acaciicola* LA 1	RBBR	Adak et al. (2016)
Crude	*Ganoderma* sp. En3	RBBR, Indigo Carmine, Methyl Green	Lu et al. (2016)
Immobilized, free laccase	*T. versicolor* ATCC 200801	Reactive Red 3	Ilk et al. (2016)
Immobilized purified laccase	*T. versicolor* IBL-04	Reactive Violet 1, Reactive Blue 21, Reactive Yellow 145A, Reactive Black 5, Reactive Red 195A	Asgher et al. 2017

the Drimarene Blue X3LR dye decolorization without mediator (Unyayar et al. 2005). Optimum decolorization was obtained at 50 °C and pH 4 after incubation for 2 min without any mediator. Laccase is the main enzyme responsible for decolorization.

Birhanli and Yesilada (2006) obtained high amount of laccase enzyme produced by *T. versicolor* ATCC 200801 and *T. trogii* ATCC 200800 under repeated-batch mode and used the crude culture filtrates of these two fungi to decolorize various dyes such as Congo Red, Indigo Carmine, Orange II, and Remazol Brilliant Blue R without mediator. These crude culture filtrates were able to decolorize the dyes without mediator and hydrogen peroxide. Laccase was indicated as the main enzyme responsible for decolorization.

Erkurt et al. (2007) also tested Remazol Brilliant Blue R and Drimarene Blue CL-BR decolorization activity of partially purified laccase from the static liquid cultures without mediator by activity staining and reported that the enzyme responsible for dye decolorization was laccase.

Dye decolorization with crude laccase from solid-state culture of *Ganoderma lucidum* was also described. Murugesan et al. (2007) reported that crude laccase from solid-state culture of *Ganoderma lucidum* KMK2 could decolorize RBBR without a redox mediator. However, the effective mediator, hydroxybenzotriazole (HBT), could enhance the decolorization activity. While the crude laccase without HBT decolorized 40% of RBBR after 2 h of incubation, the presence of 1 mM HBT induced the decolorization activity with a 92% decolorization. On the other hand, Reactive Black 5 could only be decolorized in the presence of mediator. In the presence of 1 mM HBT, 62% and 77% RB5 decolorization activities were detected

within 1 and 2 h, respectively. This study demonstrated the high thermostability of this crude laccase from solid-state fermentation.

Michniewicz et al. (2008) investigated the decolorization of azo (Direct Black 22, Acid Red 27, and Reactive Blue 81) and anthraquinone dyes (Acid Blue 40, Acid Blue 62) by crude and purified laccase isoforms (Lacc I and Lacc II) obtained from the liquid cultures of *Cerrena unicolor*. The enzyme activity, the chemical structure of the dye, and the initial dye concentration were the determinative factors of decolorization efficiency. The optimum pH value was 3.5 for decolorization, and both purified and crude enzymes degraded all the dyes tested except Acid Red 27 in a short time. Acid Red 27 dye was decolorized completely by using a 50-fold higher amount of the purified enzyme within 24 h. All the decolorization studies were performed without any redox mediator.

The decolorization of RBBR by *Polyporus* sp. S133 culture filtrate and its purified laccase was investigated. The crude laccase decolorized RBBR, but the decolorization of RBBR could be increased in the presence of HBT. However, RBBR at 200 mg/L initial concentration could be completely decolorized by the purified laccase (1.5 U/mL) without any mediator. The most suitable decolorization parameters were pH 5 and 50 °C (Hadibarata and Tachibana 2009).

In a study by Yang et al. (2009), five azo dyes (Congo Red, Fast Blue RR salt (FBRR), Acid Red, Amido Black 10B, and Orange G), five triphenylmethane dyes (Malachite Green, Crystal Violet, Cresol Red, Coomassie Brilliant Blue G250 (CBB), and Bromophenol Blue), and an anthraquinone dye (RBBR) are treated with purified laccase from *Trametes* sp. SQ01. Orange G, Amido Black 10B, FBRR, Bromophenol Blue, Malachite Green, and RBBR were completely degraded by the purified laccase (0.5 U/mL). RBBR was the best-removed dye among the dyes tested, and the purified laccase (0.5 U/mL) could decolorize 80% of this dye (50–400 mg/L) in 30 min at 25 °C, pH 4.5. When the dye concentration increased to 1 g/L, the decolorization decreased to 30%. However, the effectiveness of color removal could be raised by increasing the laccase activity. Among all the dyes tested, the least decolorized dyes were Acid Red and CBB G250, and the color removal of these dyes was only 21% and 30%, respectively. In addition, Congo Red and Crystal Violet dyes were decolorized by 47% and 65%, respectively, but they were not completely degraded by the purified laccase.

Decolorization of structurally different dyes such as Congo Red, Remazol Brilliant Blue R, Poly R-478, and Lanaset Grey by crude laccase (500 U/L) from solid-state culture of *Cerrena unicolor* PM 170798 and submerged culture of *Trametes hirsuta* BT 2566 was also described (Moilanen et al. 2010). All dyes were decolorized by *C. unicolor* laccase without mediator, but only two of them were with *T. hirsuta* crude laccase without mediator. The maximum color removal percentages of *C. unicolor* crude laccase were reported as 91%, 80%, 69%, and 48% after 19.5 h for Congo Red (12.5 mg/L), Remazol Brilliant Blue R (100 mg/L), Poly R-478 (50 mg/L), and Lanaset Grey (75 mg/L), respectively. However, *T. hirsuta* crude laccase decolorized only Congo Red (91%) and RBBR (45%) after 19.5 h.

Addition of mediators enhanced the decolorization potential of *T. hirsuta* crude laccase. Thus, the addition of ABTS induced the Poly R-478 and Remazol Brilliant Blue R dye decolorization of crude laccase, and they were decolorized at 78% and 91% after 25.5 h, respectively (Moilanen et al. 2010).

Levin et al. (2010) investigated the dye decolorization activity of crude culture filtrates from static batch cultures of *T. trogii* BAFC 463, *T. villosa* BAFC 2755, and *T. versicolor* BAFC 266, and they reported that *T. trogii* crude culture filtrates decolorized 13%, 23%, 40%, 46%, 82%, 94%, and 95% of Gentian Violet, Xylidine, Congo Red, Malachite Green, Remazol Brilliant Blue R, Indigo Carmine, and Anthraquinone Blue dyes, respectively, at pH 4.5 and 30 °C in half hour. *T. trogii* was reported as the most efficient strain in decolorization, and small structural differences between the dyes could significantly affect decolorization.

Grassi et al. (2011) also used the culture filtrate of *T. trogii* BAFC 463 and the purified laccase to decolorize six dyes. The preparations highly decolorized RBBR, Indigo Carmine, Xylidine, Malachite Green, Gentian Violet, and Bromophenol Blue dyes. Culture filtrate could decolorize various dyes without mediator. These mediators could exist in crude culture filtrate naturally, and manganese peroxidase could also assist laccase in decolorization. The efficiency of mediators depended on the type of the dye. Synthetic mediator 1-hydroxybenzotriazole (HBT) was detected as the most effective mediator for dye decolorization. Natural laccase mediator *p*-hydroxybenzoic acid (*p*HBA) was also very effective on decolorization activity of the culture filtrate. Purified laccase also decolorized the dyes, without mediators, but not at the same extent. The partially decolorized dyes were almost completely decolorized in the presence of 0.5 mM HBT. Redox potentials of laccases vary depending on the enzyme source. HBT could destabilize the laccase. Therefore, using culture filtrate instead of purified laccase could reduce the cost of decolorization and destabilization of the enzyme.

Sasmaz et al. (2011) investigated the decolorization efficiency of Reactive Red 198, Rem Blue RR, Dylon Navy 17, Rem Red RR, and Rem Yellow RR dyes by using crude laccase obtained from the submerged culture of *Trametes versicolor* ATCC 200801. The crude laccase could decolorize Rem Blue RR and Dylon Navy 17, while the other dyes could not. Therefore, dimethoxyphenol, phenol, vanillin, guaiacol, veratryl alcohol, and L-tyrosine were tested as the mediators. Because vanillin is a low-cost and nontoxic material and was the most effective mediator tested, it was selected for further studies. However, neither crude laccase nor laccase-mediated system could decolorize Rem Yellow RR. The decolorization values of Rem Blue RR and Dylon Navy 17 by the use of crude laccase were detected as 65 and 75%. However, Reactive Red 198 and Rem Red RR dyes were decolorized 62% and 68% by the combination of laccase and vanillin, respectively. The types of mediator and dye structure were important for decolorization ability.

Zeng et al. (2011) investigated the synthetic dye decolorization by crude laccase (crude extracellular culture filtrate containing only laccase) from solid-state culture (on wood shavings and soybean cake) of *T. trogii*. The crude enzyme

showed good decolorization activity against anthraquinone dyes (Reactive Blue 4 (70%), RBRR (85%), and Acid Blue 129 (46%)) without any mediator. Redox mediator, HBT, had no effect on anthraquinone dye decolorization. However, HBT enhanced decolorization of azo dyes, and high amounts of Acid Red 1 (90%) and Reactive Black 5 (65%) decolorization occurred in the presence of this mediator. Native polyacrylamide gel electrophoresis and also laccase inhibitor such as NaN_3 studies showed the involvement of laccase in decolorization. The enzyme concentration, pH, and temperatures were the important parameters for decolorization. The use of immobilized or purified enzymes could increase the cost of decolorization, and involvement of natural laccase mediators and various metabolites in culture filtrate could induce the dye decolorization activity and also stabilize the enzyme.

Birhanli et al. (2013) reported that immobilization of *F. trogii* ATCC 200800 on copper-impregnated apricot stone-based activated carbon induced the laccase production under repeated-batch studies. Dye decolorization activity of the obtained crude laccase was determined using single-step detection method on native gels by staining the gels with Reactive Blue 171 and Indigo Carmine after electrophoresis. The crude laccase could decolorize dyes without mediator. This method could be used to show the decolorization activity and also the responsible enzyme for decolorization.

It was also possible to decolorize azo dyes such as Reactive Black 5 and Reactive Blue 171 by crude repeated-batch culture filtrate of *T. trogii* ATCC 200800 without mediator. The study showed that the temperature for dye decolorization was significantly related to pH of the decolorization medium. Native polyacrylamide gel electrophoresis results showed the role of laccase enzyme in decolorization. Given the high price and toxicity of some mediators, the decolorization potential of this crude culture without mediator may be advantageous (Yesilada et al. 2014).

Ratanapongleka and Phetsom (2014) tested the decolorization potential of crude laccase of *Lentinus polychrous* on some synthetic dyes: azo dyes Reactive Black 5, Reactive Orange 16, Reactive Green 19, Methyl Orange, indigoid and anthraquinone dyes Acid Blue 80, and Water Blue. The anthraquinone Acid Blue 80 and Water Blue were decolorized up to 85% and 30%, respectively, by the crude laccase (125 U/L), whereas Indigo Carmine and all azo dyes tested were decolorized only 20% and less than 10%, respectively.

Peniophora cinerea cultivated in a medium containing sucrose, corn steep liquor, copper, and other minor components could only produce laccase enzyme. Eight salt-tolerant laccase isoenzymes were detected after purification. The decolorization rates of Reactive Blue 19 dye by purified laccases (1 U/mL final activity) were compared in the presence of five mediator (syringaldehyde, 1-hydroxybenzotriazole, 4-hydroxybenzoic acid, 3,4-dihydroxiphenilacetic acid, and 2,3-dihydroxybenzoic acid). Syringaldehyde was the best mediator since it increased dye decolorization almost threefold when compared to the decolorization by using laccase alone (Moreira et al. 2014).

Li et al. (2014) showed the decolorization of Congo red, Aniline Blue, and Indigo Carmine by crude laccase enzyme of *Trametes* sp. SYBC-L4 cultivated under solid-state fermentation on cassava residue for 10 days. The decolorization ratios of dyes differed considerably with pH changes and enhanced with the increasing of HBT concentration. Some concentrations of Congo Red, Aniline Blue, and Indigo Carmine were decolorized by laccase at 68%, 95%, and 99%, respectively, with 2.5 mM HBT at 36 h of incubation (Li et al. 2014).

Zapata-Castillo et al. (2015) isolated three laccase isoenzymes (Lac I, II, and III) from the culture of *Trametes hirsuta* Bm2 grown in liquid media containing 2% wheat bran. Laccases were resistant or partially resistant to metal ions. Crude laccases and isoenzymes had high resistance to solvents. While the isoenzymes did not decolorize Indigo Carmine, 100% decolorization could be obtained when syringaldehyde (0.2 mM) was added. Therefore, it significantly increased the dye decolorization activity of isoenzymes. Crude laccase decolorized the dye more extensively in a relatively short time. Thus, laccases and other enzymes such as manganese peroxidase were involved in the decolorization of this dye. The use of crude enzyme instead of purified forms was reported as a cost-effective process.

Shankar and Nill (2015) investigated the effect of various concentrations of metal ions and also redox mediators on both *Peniophora* sp. (NFCCI-2131) crude laccase from liquid static culture and Amido Black, Crystal Violet, Brilliant Green, Methyl Orange, and Methylene Blue decolorization activity of crude laccase. The crude laccase tolerated metal ions and decolorized these dyes in the absence of metal ions and mediators. Among the five dyes tested, it efficiently decolorized Crystal Violet, and decolorization reached to 96% and 86.0% in the presence of 1 mM ABTS or 0.1 mM HBT, respectively.

Laccase obtained from *Cyathus bulleri* was immobilized in poly(vinyl alcohol)-boric acid or polyvinyl alcohol-nitrate beads (Chhabra et al. 2015). Thanks to this method, which takes place with 90% efficiency, the original activity of the immobilized laccase was conserved more than 70% even after 5 months of storage at 4 °C. The immobilized enzyme was tested for the decolorization of simulated effluent containing Acid Red 27 (acidic monoazo dye) or Basic Green 4 (Malachite Green) dyes under batch mode in a 25 mL flask. This immobilized laccase was also tested for the decolorization of the simulated effluent containing Acid Red 27 under continuous mode in a packed bed column. While the simulated effluent containing 100 μM Acid Violet 17 with 100 μM ABTS was decolorized 90% by laccase entrapped in PVA-nitrate up to 10 cycles, this enzyme decolorized 95% of the other simulated effluent containing 100 μM Basic Green 4 with 100 μM ABTS up to 20 cycles under batch mode. The immobilized laccase decolorized the simulated effluent containing 100 μM Acid Violet 17, 2.5 g/L sodium sulfate, and 100 μM ABTS more than 70% up to 120 h under continuous mode in a packed bed column (Chhabra et al. 2015).

Adak et al. (2016) investigated the laccase production of *Pseudolagarobasidium acaciicola* LA 1 under solid-state fermentation on various agro wastes (rice straw,

wheat straw, sugarcane bagasse, and *Parthenium* biomass). *Parthenium* biomass was selected as a suitable substrate for laccase production. It was reported that this was a thermostable fungal laccase and it functioned optimally at pH 4.5 and temperature of 60 °C with ABTS. This crude laccase decolorized 90% of RBBR within 4 h and 33% of RB5 within 48 h without mediator.

Sayahi et al. (2016) studied that the decolorization of Reactive Black 5 (RB5) and Reactive Violet 5 (RV5) and also the mixture of RB5 and RV5 and RBBR by purified laccase from *T. trogii*. The highest decolorization of RB5 was about 93%, and this color removal occurred in the presence of 25 mg/L dye, 1 U/mL enzyme, and 1 mM HBT. On the other hand, the maximum decolorization of RV5 (100%) was obtained with 25 mg/L dye, 0.5 U/mL enzyme, and 0.5 mM HBT. RBBR was also acted as a mediator and increased the decolorization of these two dyes. This purified laccase also removed the color (55%) of mixed dyes (RBBR, RB5, and RV5) without HBT after 24 h of incubation.

Lu et al. (2016) reported that high concentrations of RBBR, Indigo Carmine, and Methyl Green efficiently decolorized by crude enzyme from liquid cultures of *Ganoderma* sp. En3. Studies showed that the decolorization efficiency decreased as the concentration of Indigo Carmine increased. However, various redox mediators (syringaldehyde, ABTS, acetosyringone, and acetovanillone) significantly increased the decolorization activity. The presence of mediators could also enhance the decolorization efficiency of the crude enzyme. Laccase was reported as the main enzyme for this decolorization.

In a study performed by Ilk et al. (2016), the laccase of *Trametes versicolor* ATCC 200801 was immobilized onto the poly(MA-alt-MVE)-g-PLA/ODA-MMT nanocomposite by adsorption or covalent coupling and then, the decolorization of Reactive Red 3 (monoazo dye) by this immobilized laccase was investigated comparatively with free enzyme. The dye decolorization potential of the immobilized laccase (65%) was much higher than free laccase (33%) under the optimum conditions, and also immobilized laccase retained more than 77% activity even after ten uses (Ilk et al. 2016).

The laccase enzyme purified from the crude supernatant of the solid-state culture of *Trametes versicolor* IBL-04 was immobilized onto chitosan microspheres. The immobilization provided the enzyme with a higher catalytic efficiency and also higher thermal and storage stability. Reactive Red 195A (monoazo dye), Reactive Blue 21 (phthalocyanine dye), Reactive Yellow 145A (monoazo dye), Reactive Black 5, and Reactive Violet 1 (monoazo dye) decolorization activities of this immobilized laccase were higher than the free laccase. After 4 h incubation in the presence of 1 mM ABTS as a redox mediator, the immobilized enzyme resulted in 100, 99, 98, 97, and 89% color removal of Reactive Red 195A, Reactive Violet 1, Reactive Yellow 145A, Reactive Black 5, and Reactive Blue 21, respectively. Furthermore, the immobilized enzyme retained its 80% activity after ten applications (Asgher et al. 2017).

5.6 Future Prospects

Textile and dyeing wastewaters, two important environmental pollutants, contain various dyes. Physical and chemical methods for dye removal are common methods. However, they pose various disadvantages. Conventional biological treatment systems such as activated sludge are not efficient for bioremediation and decolorization. Further, anaerobic treatment can produce toxic aromatic amines. White rot fungi are a group of the most efficient microorganisms in decolorization and degradation of textile dyes. Different strains of white rot fungi may have different bioactivities, making them highly popular for the bioremediation of a broad range of dyes. However, given that textile dyes cause growth inhibition in fungi, there is still a need to obtain new fungal strains with novel physicochemical characteristics that can withstand these dyes and have better decolorization and detoxification activities. As they are used continuously and repeatedly, whole pellets (self-immobilized forms) or immobilized cells can be used to overcome this toxicity (growth inhibition) problem. In this regard, fungal bioreactors may prove suitable for the application of whole pellets or immobilize cells. Moreover, the efficiency of these systems is also high in bioreactors. Two-step method, adsorption and subsequent solid-state fermentation, may also be an economic and easy way for the bioremediation of textile wastewaters. White rot fungi have various enzymatic activities, particularly as the source of oxidoreductases. Laccase enzymes are the main ones for dye decolorization activity. Due to their low substrate specificity, these enzymes have the potential to catalyze the oxidation of a wide variety of dyes with or without mediators. In such activity, the redox potential of the dye and its conformational match to the active site of the enzyme is of paramount importance. Depending on strains, laccases may have different properties and redox potentials. Crude culture filtrate, semi-purified, and purified enzymes may be used to remediate the textile dyes. Immobilized laccases or culture filtrates may also be used for the bioremediation of dye-containing wastewaters. Developing new immobilization supports and immobilized enzymes/cells may decrease the final cost application. The major disadvantage of free enzyme preparations is that they cannot be used repeatedly and for a long duration. Various bioreactors are used in bioremediation of dyestuffs, and the yield obtained from these systems can change according to the type of the bioreactor and the fungal species or strains used. The use of efficient strains in various bioreactors will provide an important approach to solving the existing problems. The future research should also focus on the development of new and effective bioreactors for long-term operation with whole pellets, immobilized cells, or enzymes. Some studies have shown that the toxicity is significantly reduced after mycoremediation process. Accordingly, similar to the decolorization of dyestuffs, the use of fungi or their enzymes for elimination of the dyestuff toxicities is promising. The toxicity arising as a result of decolorization processes should be controlled by different toxicity methods. Thus, there is a need for more

concentrated research effort in this area, especially on decolorization and detoxification of real wastewaters by white rot fungi or enzymes. Finally, decolorization and detoxification of textile dyes by fungal pellets or enzymes could also be advantageous to integrate such bioremediation process before or after a conventional process.

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Chapter 6
Mycoremediation of Common Agricultural Pesticides

Chitra Pandey, Deepti Prabha, and Yogesh Kumar Negi

6.1 Introduction

Pesticides are bewildering variety of organic compounds that either by design or by accident eventually winds up in the environment. Since the ancient time, pesticides are being used either to kill unwanted insects, pathogens, plants, etc. or otherwise reduce their adverse impact on agriculture crops and their production. The sulfurous rock, salt, tobacco extract, red pepper, wooden ash, etc. were the common pesticides of choices in the ancient time. Chemical pesticides predominately replaced these all authentic versions of pesticides in the world's agricultural system due to their instant effect on crop protection from pest infestation, thereby minimizing crop losses. Agro-pesticides are classified as insecticides, fungicides, and herbicides or weedicides. A total of 1175 pesticides including 335 insecticides, 410 fungicides, and 425 herbicides were registered for use in the United States till 1975. Whereas, in India 261 pesticides are registered for use under section 9(3) of the Insecticide Act, 1968. According to a survey, approximately 50,000 species of plant pathogens, 8000 species of weeds, and 9000 species of insects and mites are known to smash up crops and reduce crop productivity which at the end results in reduced food availability. After the introduction of benzene hexachloride (BHC), dieldrin, 2, 4-dichlorophenoxyacetic acid (2, 4-D), and dichlorodiphenyltrichloroethane (DDT), a new advancement was achieved in pesticide application.

Being more efficient and feasible to use, these chemicals got admired in all agricultural systems, and over the time, they became indispensable in agricultural production. With the continuous use of the pesticides, crop protection and production

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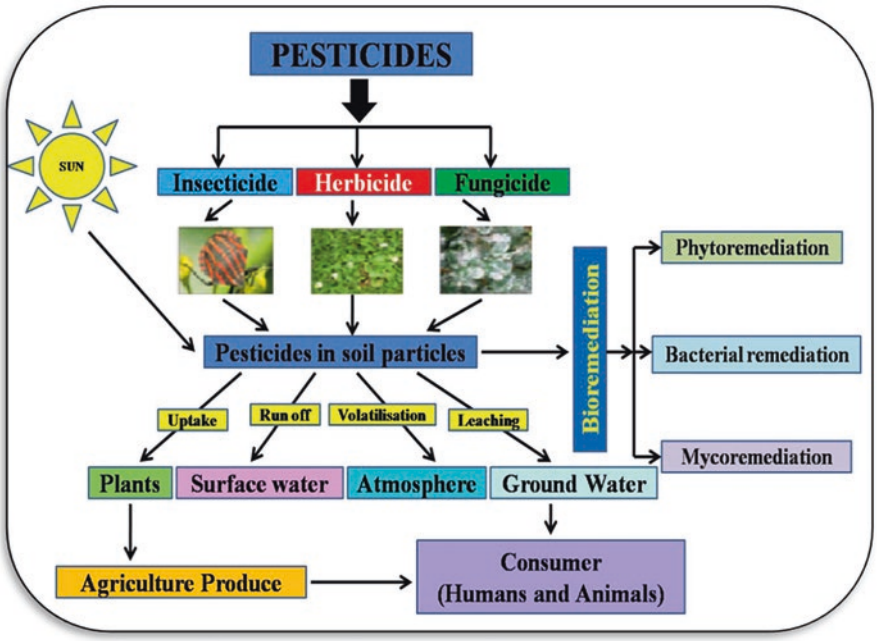

Fig. 6.1 Fate of increased use of pesticides on environment

became dependent on these chemicals. It was found that in their absence, fruit, vegetable, and cereal productions were reduced and around 78%, 54%, and 32% losses, respectively, were recorded by the attack of different pests (Pimentel 2009). Though they are reported harmful to human beings and animals, their abrupt discontinuation would surely cause depletion in crop productivity. At the same time, their anarchic, frequent inexpedient use has developed resistance in some pests, and their increasing concentration started causing harm to nontarget organisms too. Their residues remain in arable land and other unexpected places as well (Damalas 2009). In all over the world, Europe is the prevalent consumer of pesticides followed by Asia, while the United States, France, China, Brazil, and Japan are among the largest manufacturers and traders of pesticides (Zhang et al. 2011). Around 4 million tons of pesticides are consumed by global agricultural sector to reduce food losses and to enhance food production substantially. However when high doses of pesticides and other harmful chemicals are used, they get accumulated in the ecosystem raising their residual effect in soil and consequently enter in the food chain (Bartha 1980). Once the pesticides are exposed in the environment, their fates vary according to their half-life, amounts of pesticide applied, physicochemical reactions in the soil, and climatic factors. Pesticides are exposed in the environment by volatilization, leaching, and absorption in soil particles and then contaminate the groundwater too. They also get accumulated in plants and thereby in agriculture produce and finally reach to consumer (Fig. 6.1).

In all over world including India, pesticide consumption has increased over the time. This increase in pesticide use is either because of resistance in pests or otherwise infestation of new pests. Since residues of these pesticides get accumulated in the agriculture produce, they may pose adverse effect on consumer's health. Pesticide residues have been detected from various leafy vegetables including spinach, fenugreek, mustard and cabbage (~21.5 ppm), tomato (~17.5 ppm), and cauliflower (~1.70 ppm) which unfortunately are above the maximum acceptable daily intake (ADI) as prescribed by WHO in India (Bakore et al. 2002). The subsequent investigation revealed the presence of significant amounts of pesticide residues in the groundwater resources and tropic levels at a magnitude (Maloney 2001). These residues may induce carcinogenic, teratogenic, and other serious health hazards to the humans and animals because of their bioaccumulation and fat-soluble nature (Agrawal et al. 2010). Several hypersensitive reactions like eczema, dermatitis, allergic respiratory diseases, etc. are also caused by pesticides. They have been examined as mutagens and reported to cause mutations in chromosomes of humans and animals, thereby inducing carcinoma of the lung and liver (Chauhan and Singhal 2006). From the point of view of environmental sustainability and public health, pesticide degradation is an indispensable prerequisite.

Due to all aforementioned dilemmas, techniques are required for their degradation into nontoxic and ecologically safe products, which is beneficial for the environment as well as for the human health. Different methods have been developed for the degradation of pesticides in contaminated soil and water, e.g., heterogeneous photocatalysis with TiO_2, physical treatment (adsorption and percolator filters), high-temperature incineration, etc. According to FAO assessment, 3000–4000 USD/ton is the cost of these maneuvers (Ortiz-Hernandez et al. 2013). These methods are not only expensive but may also have serious disadvantages such as toxic emissions, hazardous ash formation, and production of dioxin which is carcinogenic (Vidali 2001). An alternative method for the treatment of pesticides is use of biological methods in which pesticides can be degraded with the help of microbes and plants without causing any harmful effect to the environment (Atlas and Pramer 1990). In a variety of organic and inorganic chemicals, pollutants are naturally transformed into their simpler non-harmful or less harmful forms by different microorganisms and certain plant species which is termed as "bioremediation." Various parameters such as soil moisture, temperature, and physicochemical properties of the soil persuade the rate of bioremediation of harmful chemicals in soil.

Most of the pesticides are synthetic and are similar to naturally occurring compounds to be subjected to microbial degradation. The degradation of chemical compounds depends on their structure and chemical bonds. Biochemical processes occurring in the environment are responsible to abate pesticides. Synthetic pesticides have a bewildering variety of chemical structure enclosing aliphatic and aromatic chains (Fig. 6.2). Aliphatic carbon chain containing pesticides are generally degraded by β-oxidation process, and the resulting carbon fragments are further metabolized by tricarboxylic acid cycle. Pesticides having aromatic chains can be degraded by dihydroxylation and ring cleavage. Bioremediation is therefore should be promoted as an effective alternative approach to remove or otherwise minimize the adverse effects of such hazardous chemicals.

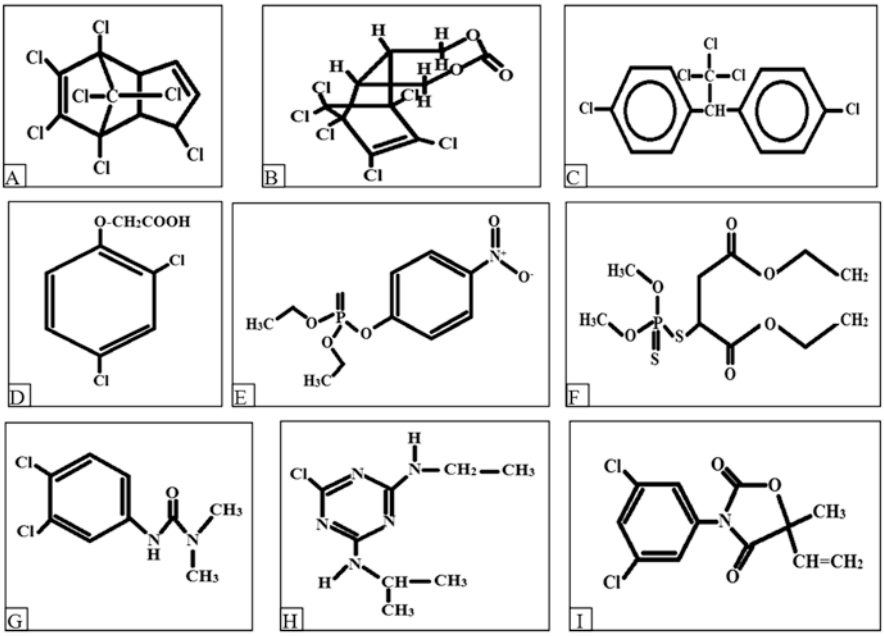

Fig. 6.2 Structures of some common agricultural pesticides. (**a**) Heptachlor, (**b**) Endosulfan, (**c**) DDT (Dichlorodiphenyltrichloroethane), (**d**) 2,4-D (2,4-Dichlorophenoxy acetic acid), (**e**) Parathion, (**f**) Malathion, (**g**) Diuron, (**h**) Atrazine, (**i**) Vinclozolin.

6.2 Bioremediation

Bioremediation is a process of degradation of biological wastes, chemical fertilizers, pesticides, etc. into nontoxic or less toxic form using certain microbes or plants. In some of the plants, bacteria and fungi have been identified to detoxify the substances which are perilous to the environment and human health. Plants and microorganisms used for bioremediation must enzymatically attack the pollutants and convert them into non-harmful end products. Similar to other biological processes, bioremediation requires suitable environmental conditions which permit the growth of the candidate microbe or plant to achieve effective degradation of pollutants at a higher rate. It often involves modification in environmental conditions to promote the growth of the candidate organism which simultaneously enhances degradation of the pollutants which is known as bioaugmentation. Bioremediation is safe, feasible to use, and more economical than traditional methods. However, some chlorinated organic or high aromatic hydrocarbons cannot be degraded using bioremediation and showed resistance particularly toward microbial actions. Since bioremediation process takes place in soil, therefore, use of plants (phytoremediation) and microbes (microbial remediation) for the purpose is more feasible than any other technique (Vidali 2001).

6.2.1 Phytoremediation

Phytoremediation is the process in which plants are used to degrade or transform the pollutants. Plants perform this reaction by different mechanisms including phytoextraction, phytodegradation, and rhizofiltration. The completion time of this process depends on the length of time taken by the plant to grow and type of the pollutant. Phytoremediation involves implantation of trees and grasses so it is environment-friendly and sometimes cost-effective too. It is a good technique for remediation of the pollutants with minimal maintenance, but it has its limitations that the pollutant should be in the reachable zone of plant roots (Trapp and Karlson 2001). In addition, if the pollutant is too water soluble, it will not be accessible to the root system, and no degradation will take place (Ghosh and Singh 2005). Other than these, high concentration of pesticides can be toxic to plants.

6.2.2 Microbial Remediation

Microbes are employed in this process for safe and effective remediation of environmental pollutants, pesticides, and other hazardous chemicals. Both bacteria and fungi are involved in different processes. Among the different bacterial genera, *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus*, and *Mycobacterium* have been found promising to degrade pesticides and hydrocarbons (Sahinkaya and Dilek 2007; Caliz et al. 2011). Some of the anaerobic bacteria (*Dehalobium chlorocoercia* DF1 and *Dehalococcoides mccartyi*) are used in the bioremediation of polychlorinated biphenyls (PCBs) in river sediments (He and Bedard 2016; Payne et al. 2013). On the other hand, different fungi are also involved in remediation of pollutants in waste water, soil, organic wastes, etc. It has been reported that many filamentous fungi including *Penicillium* spp., *Fusarium* spp., white-rot fungi, mushrooms, etc. are entangled in the remediation of various chemical fertilizers and pesticides, and the process is known as "mycoremediation." Since a large number of fungi produce resting bodies, they can survive longer in adverse conditions and induce their beneficial impact. Therefore, use of fungi is more common in remediation processes of different chemicals and organic waste.

6.2.3 Mycoremediation

Fungi play important role in human welfare because they are used by humans in various aspects like edible fungi which are being used to fulfill food requirements, waste decomposition, and production of industrially important products (enzymes, organic acids, alcohol, etc.). On the other hand, certain fungi are also used for the remediation of hazardous chemicals including pesticides (Prasad 2017). Numerous

fungi including white-rot fungi have been reported to degrade various chemical compounds such as DDT, endosulfan, heptachlor, etc. (Kullman and Matsumura 1996; Singh and Kuhad 1999; Nwachukwu and Osuji 2007). *Pleurotus ostreatus*, *Trametes versicolor*, *Lentinula edodes*, *Bjerkandera adusta*, etc. also play a vital role in the process of bioremediation (Singh 2006). Similarly, *Aspergillus flavus*, *A. niger*, and *Trichoderma harzianum* have been found responsible for the degradation of chlorpyrifos and endosulfan (Katayama and Matsumura 1993; George et al. 2014). *Rhizoctonia solani*, *Sporothrix cyanescens*, *Mortierella*, etc. can degrade chloroneb fungicides and other pesticides (Hock and Sisler 1969). Fungi have good potential to degrade pesticides including different insecticides, fungicides, and herbicides. Major classes of pesticides with their representative compounds and different fungi involved in their remediation are elaborated in Table 6.1.

6.3 Insecticide Degradation

Insects such as beetles, butterflies, moths, and grasshoppers widely destroy the crops and thereby decrease the crop productivity resulting in economic losses. *Phyllophaga* spp., *Aphis* spp., *Prostephanus truncatus*, *Callosobruchus maculatus*, *Helicoverpa armigera*, *Pyrilla perpusilla*, and *Spodoptera litura* are among the major pests of the major crops such as wheat, maize, sugarcane, etc. and are reported to cause heavy losses in their respective host crop. Therefore, to prevent crop losses and productivity, their control is necessary. A number of insecticides is therefore applied in different crops to eradicate these pests. Combination of copper arsenite and copper acetate (Paris green) was firstly used to control Colorado potato beetle. Different types of insecticides are recommended for different insects. Among them chlorinated insecticides are broad spectrums, less toxic with large residual effect on soil. From soil, these residues either get accumulated in agricultural produce or leach down and contaminate the groundwater. Consumption of such contaminated water by human and other animals induce health hazards in them. Major examples of chlorinated insecticides include aldrin, dieldrin, DDT, endosulfan, endrin, heptachlor, and lindane. The role of fungi in the remediation of some hazardous insecticides is discussed briefly in the following section of this chapter.

6.3.1 Heptachlor (1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methano-1H-indene)

This is a compound with pure white crystals and was first introduced in 1948 to kill termites. Later on it was also introduced in the agricultural system to protect the crops (e.g., maize, small grains, and sorghums) from ants, termites, wireworms,

Table 6.1 Classes of pesticides with representative examples and fungi involved in their mycoremediation

Class	Group	Major representative	Fungus involved in degradation	References
Insecticides	Organochlorine	Aldrin, lindane, DDT, endosulfan, heptachlor, dieldrin	*Aspergillus terreus*, *Trichoderma*, *Phlebia lindtneri*, *Fusarium ventricosum*, *Cladosporium*, *Phanerochaete chrysosporium*	Purnomo (2017), Kamei et al. (2011), Mougin et al. (1996) Siddique et al. (2003), Xiao et al. (2011c)
	Organophosphates	Chlorpyrifos, malathion, parathion, ethion	*Aspergillus oryzae*,	Rao and Sethunathan (1974), George et al. (2014)
			A. niger, *A. flavus*, *Penicillium waksmanii*, *Acremonium* sp.	
	Pyrrole	Fipronil	*Trametes versicolor*	Wolfandqa et al. (2016)
	Pyrethroids	Bifenthrin, cypermethrin, fenvalerate	*Cladosporium* spp., *T. versicolor*	Chen et al. (2011), Mir-Tutusaus et al. (2014)
Herbicides	Bipyridyl derivatives	Diquat, paraquat	*Mucor hiemalis*, *Zygorhynchus heterogamous*, *A. niger*, *Penicillium frequentans*	Smith et al. (1976)
	Amides	S-metolachlor and all chloro and propanol	*Aspergillus flavus*	Sanyal and Kulshrestha (2002)
	Urea	Diuron, isoproturon	*Rhizoctonia solani*, *Pestalotiopsis versicolor*, *Cunninghamella echinulata*, *Mortierella*	Ronhede et al. (2005), Ellegaard-Jensen et al. (2013)
	Triazines	Atrazine, propazine	*Aspergillus*, *Rhizopus*, *Fusarium*, *Penicillium*, *Phanerochaete chrysosporium*	Mougin et al. (1994)
	Cholorophenoxy compounds	2, 4-D, glyphosate	*P. chrysosporium*	Ferreira-Guedes et al. (2011), Bastos and Magan (2009), Ronhede et al. (2005)
			Trichoderma, *Trametes versicolor*, *Penicillium*, *Aspergillus*, *Rhizopus*, *Fusarium*	

(continued)

Table 6.1 (continued)

Class	Group	Major representative	Fungus involved in degradation	References
Fungicides	Phenyl amide	Tetraconazole, mefenoxam, metalaxyl	*Rhizopus stolonifer*, *Gongronella* sp.	Martin et al. (2013)
	Morpholine	Piperalin,	*Bjerkandera adusta*	Ermakova et al. (2008)
	Phthalimides	captafol, folpet, captan	*R. stolonifer*, *Gongronella* sp.	Martin et al. (2013)
	Dicarboximide	Vinclozolin, iprodione	*Cunninghamella elegans*, *Stereum hirsutum*	Pothuluri et al. (2000), Bending et al. (2002)
	Ethylenebisdithiocarbamates	Mancozeb, azithiram, thiram	Button mushroom, *Rhizoctonia solani*	Ahlawat and Singh. (2011), Chatrath and Raju (1986)

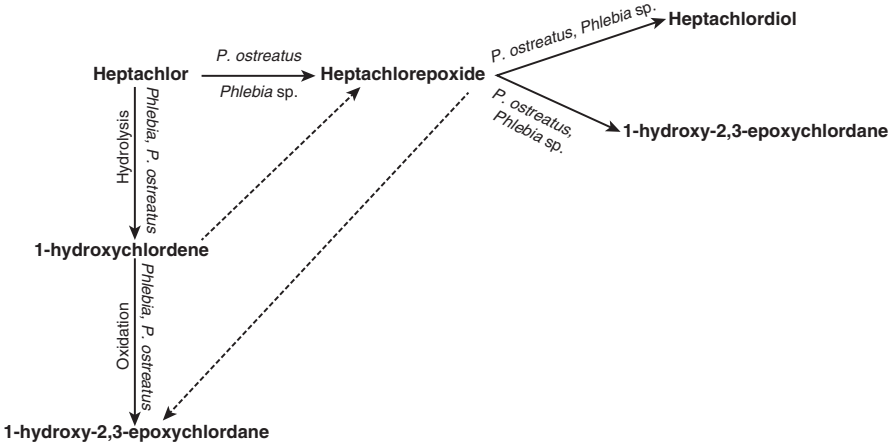

Fig. 6.3 Heptachlor degradation pathway. (Adapted and modified from Leon-Santiesteban and Rodriguez-Vazquez (2017))

cutworms, maggots, etc. Due to its carcinogenic effect on human beings, it has been banned in some countries including India. However, some countries like Algeria, Brazil, etc. are still using heptachlor. The half-life of heptachlor has been reported 2 years to several years (Maloney 2001). Because of its long persistence, several adverse effects including liver damage, carcinogenicity, irritability, muscle tremors, and convulsions have been reported in humans and animals (Dadey and Kammer 1953). Different, fungal species found in soil have been isolated and evaluated to degrade this molecule. Some of these fungi include *A. niger*, *Lentinus subnudus*, and *P. chrysosporium* and are found effective to degrade this pesticide (Nwachukwu and Osuji 2007). Heptachlor is transformed in heptachlor epoxide by *Penicillium*, *Fusarium*, *Rhizopus*, and *Trichoderma* spp. (Miles et al. 1969; Nwachukwu and Osuji 2007) which is however more toxic than its parent compound. *Pleurotus ostreatus* has been reported as a potential transformer of heptachlor into heptachlor epoxide along with two metabolites in nitrogen-deficient and nitrogen-rich medium which have been identified as 1-hydroxychlordene and 1-hydroxy-2,3-epoxychlordane (Purnomo et al. 2013). Xiao et al. (2011b) have reported that heptachlor can be degraded in heptachlor epoxide by the fungus *Phlebia* sp. and also hydrolyzed into 1-hydroxychlordene and was subsequently oxidized into 1-hydroxy-2,3-epoxychlordane (Fig. 6.3).

Phlebia aurea, *Phlebia brevispora* (Xiao et al. 2011b), and *Pleurotus ostreatus I* (Purnomo et al. 2013) use heptachlor epoxide as a substrate and further transform into 1-hydroxy-2, 3-epoxychlordane and heptachlor diol (2,3-dihydroxyheptachlor) by oxidative dechlorination and hydrolysis of the epoxide ring. These metabolites were found less toxic than the parent compound (Xiao et al. 2011b; Leon-Santiesteban and Rodriguez-Vazquez 2017). Among the different fungi involved in the remediation of heptachlor, *Phlebia aurea* and *Pleurotus ostreatus* were found most effective for the transformation of heptachlor epoxide and degrade heptachlor

into the less toxic metabolite heptachlor diol (Fig. 6.3). Therefore, such degaradative fungi can be useful to reduce the harmful effects of heptachlor on environment, animals, and human beings as well.

6.3.2 *Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9ahexahydro-6,9-methano-2,4,3-benzodioxa-thiepin-3-oxide)*

The world is using endosulfan since its introduction in 1950s. It is used to control a wide variety of insects such as flea beetle, cabbageworm, peach tree borer, and leafhopper that attack on a wide range of food crops including vegetables, cereals, pulses, and fruits. This is also used in wood industries for the preservation of wood. It is recommended to control pests like *H. armigera* and *S. litura* in India (Vendan 2011). This is a chlorinated hydrocarbon insecticide of the cyclodiene subgroup and has been categorized under the category of persistent organic pollutant (POP). Endosulfan is a mixture of two stereoisomers alpha and beta-endosulfan in a ratio of 7:3. This has been reported that the endosulfan is rapidly degraded in water within 3–7 days but persists in soil for a longer period (Zhulidov et al. 2000). The residues of endosulfan have been detected in mammals, fishes, soil, and other food stuffs and even in human breast milk (Golfinopoulos et al. 2003). Consumption of contaminated food and water has been considered as major mode of transportation of this molecule to humans and other animals. Higher residual effects have been reported to induce adverse effects on human health such as cancer and infertility.

Due to its lots of side effects, it has been banned in many countries of the world. However, in some countries like Australia, Thailand, Canada, the United States, and India, its restricted use is permitted. Among the different countries, India is the largest producer and consumer of endosulfan. Since several adverse effects on human and animals are reported, complete degradation of this pesticide is needed. Many microorganisms have been investigated for this purpose, among which some fungi such as *A. niger* (Mukherjee and Gopal 1994), *T. harzianum* (Katayama and Matsumura 1993), *P. chrysosporium* (Kullman and Matsumura 1996), and *Mucor thermohyalospora* MTCC 1384 (Shetty et al. 2000) have been found effective for its mycoremediation. *Fusarium ventricosum* and *F. oxysporum* have been effective for the complete degradation of α and β-endosulfan (Siddique et al. 2003, Mukherjee and Mittal 2005). Singh (2006) reported *Phanerochaete chrysosporium* BU-1 as very effective fungus for the remediation of endosulfan (Fig. 6.4).

Mycoremediation of endosulfan by *P. chrysosporium* is catalyzed by the oxidation of endosulfan to endosulfan sulfate and then to endosulfan diol. Endosulfan sulfate formed by fungal oxidative metabolism is as much toxic as endosulfan is, whereas endosulfan diol is less toxic than the endosulfan. Further endosulfan diol is converted into less toxic endosulfan ether, endosulfan monoaldehyde, endosulfan dialdehyde, endosulfan hydroxyether, or endosulfan lactone (Awasthi et al. 2003;

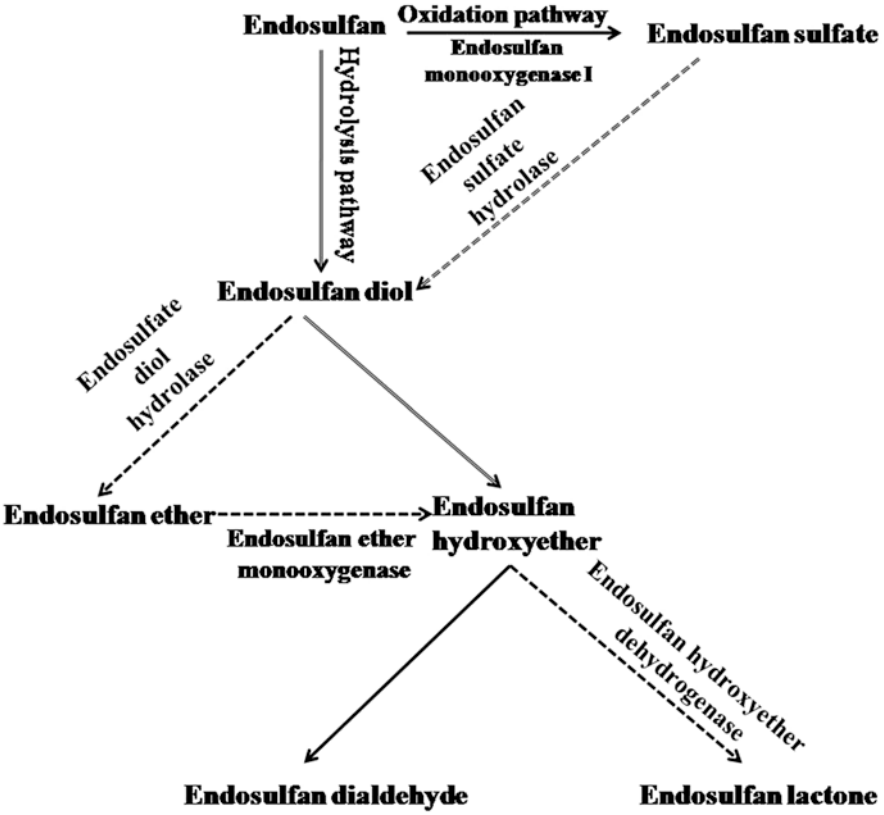

Fig. 6.4 Flow chart showing metabolism of endosulfan by *Phanerochaete chrysosporium* BU-1. (Adapted and modified from Singh (2006)). Dashed arrows indicate minor metabolic pathway; solid arrows indicate major metabolic pathway

Goswami et al. 2009). Therefore, these fungi could be effective in significant removal of endosulfan from the environment to reduce their effects on human and animal health.

6.3.3 DDT (Dichlorodiphenyltrichloroethane)

In 1939, a Swiss entomologist Paul Muller discovered DDT which was effective against the pests resistant to arsenic insecticides. In 1949, DDT was recommended to control pests such as Indian meal moth, European corn borer, potato leafhopper, and caterpillars (Yu 2008). DDT is however now banned in various countries such as China, the United States, and other developed countries due to a vast array of side effects, but still this is being used to control mosquitoes in many countries. In Africa

and some developing countries, this is still in use due to its low cost. It has been widely reported that DDT exposure causes liver damage, endocrine disruption, sex hormone obstruction, and even cancer (Persson et al. 2012). Moreover, this is a pesticide with slow rate of degradation in environment. A vast array of bacteria and fungi degrade DDT into DDE (1,1-dichloro-2,2-bis (4-chlorophenyl) ethylene), DDD (1,1-dichloro-2,2-bis (4-chlorophenyl) ethane) (less toxic to humans), and DBP which have one or two benzene ring present and toxic to human beings and animals. The degradation of DDT has been reported by several mechanisms in deferent fungi. Some fungi transform DDT via reductive dechlorination, whereas others initiate degradation through hydroxylation prior to dechlorination. White-rot fungus, *Phanerochaete chrysosporium* has been reported widely for the degradation of DDT and its metabolites (Bumpus et al. 1985) into carbon dioxide; along with that some brown-rot fungi able to degrade DDT by the hydroxyl radicals produced via the Fenton reaction.

The common degradation pathway of DDT is known as Fenton reaction after the name of H.J.H. Fenton who gave the basic pathway in 1894. In this reaction, DDT is first dehydrochlorinated into DDE (1,1-dichloro-2,2-bis(p-chlorophenyl ethylene) by *Gloeophyllum trabeum* and then hydrogenated into DDD, followed by oxidative dechlorination to form DBP (4,4 dichlorobenzophenone). A fungus *Daedalea dickinsii* transforms DDT into DDE by dehydrochlorination and then transformed into DDD by hydrogenation. DDD is further dechlorinated and is converted to DDMU (di-1,1-chlorophenyl-2-chloroethene). However in case of *Fomitopsis pinicola*, the end product of the degradation of DDT is DDD (Purnomo et al. 2010). Some fungi degrade DDT into DDE by dehydrochlorination reaction and further into DDD by reductive dechlorination. DDD is further degraded into DDA (bis (4-chlorophenyl) acetic acid), DBP, DBH (4,4-dichlorobenzhydrol), and DDM (di-1,1-chlorophenyl-2-chloroethane). DBP is further transformed into 4-chlorobenzophenone (CBP) and 4-chloromethylbenzophenone by *Aspergillus niger*. Another pathway of DDT degradation was proposed by Bumpus and Aust (1987) and reported *P. chrysosporium* to be the most preferable organism to yield different metabolites after degradation of DDT. *P. chrysosporium* is able to renovate DDT into different by-products and afterward oxidize them to end-product carbon dioxide (CO_2) (Fig. 6.5). In this pathway, first of all, a reductive dechlorination of DDT to DDD was carried out. After that dicofol [1,1-bis(4-chlorophenyl)-2,2,2-trichloroethanol] and FW-152 [2,2-dichloro-1,1-bis(4-chlorophenyl) ethanol] were produced by hydroxylation of DDT and DDD. Another product of the transformation of FW-152 to DBP may be the results of reductive dechlorination, oxidation, decarboxylation, and the ring cleavage of DBP which however has not been fully described by them. These aforementioned fungi can be used to reduce the residual effects of DDT which still is causing harm in some countries.

Another group of insecticides is organophosphate; the insecticidal property of this group was firstly described by Gerhard Schrader in 1989. A total of 100 types of organophosphorus insecticides are used in agricultural system, and many of them have been banned now due to their toxic effects on animals, birds, and mammals. Major side effect of these insecticides is the damage to the nervous system by

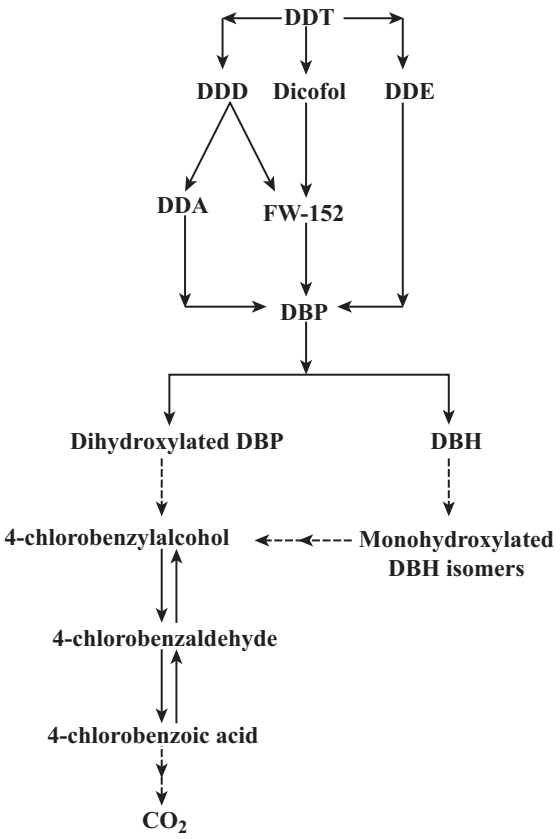

Fig. 6.5 DDT degradation pathway by basidiomycetes. (Adapted and modified from (Bumpus et al. 1985; Bumpus and Aust 1987; Xiao et al. 2011c))

phosphorylation of acetylcholine esterase enzyme (Ach E) resulting in the excess of acetylcholine in the system. This was found responsible to induce impairment of diaphragm, respiratory depression, etc. Among them, malathion and parathion have long been used widely in past.

6.3.4 Malathion

Malathion is used to protect agricultural crops, stored products, and home garden and also to kill mosquitoes in surroundings. A maximum of eight parts per million (ppm) of malathion as residue in food crops are permissible as per the food and drug administration (FDA) standards. Usually malathion is broken down from few weeks to several months after application in field by soil bacteria and fungi, but its extensive use has caused contamination of food and water reservoirs. From these resources it enters in human body and reaches to many organs and tissues through blood stream. In the liver, malathion is broken down in various metabolites, which

are unfortunately more harmful than malathion. It causes vomiting, cramps, diarrhea, watery eyes, headache, loss of consciousness, and even death. Therefore, scientists started working toward its effective removal from the environment. It is transformed into β-monoacid and dicarboxylic acid by carboxylesterase activity of *Aspergillus oryzae* and is subsequently converted into inorganic phosphate. According to Massoud et al. (2008), malathion degradation by fungi is faster than the bacteria. He suggested that this may be due to the fungal chitinases which act faster than bacterial esterases. Other fungi such as *F. oxysporum* and *Candida cylindracea* are also reported to degrade malathion. Ester hydrolysis of malathion by cutinase of *F. oxysporum* results in the degradation of malathion monoacid (MMA) and malathion diacid (MDA). *Candida cylindracea*, however, degrade malathion into MMA by detoxification (Kim et al. 2005).

6.3.5 Parathion

It is another insecticide of this group that was firstly described by Schroder in 1944. This is generally used to control bollworms, armyworms, and aphids in a large variety of crops. Similar to such other compounds, its exposure to humans is associated with cancer and adrenal cortical adenoma, malignant pancreatic tumors, etc. In addition, parathion induces toxicity in nervous system and also affects adversely the synthesis of macromolecules like DNA, RNA, and proteins (Eaton et al. 2008). Therefore, proper degradation of this pesticide molecule is quite necessary. *Penicillium waksmanii* has been found effective to degrade parathion into amino-parathion (Rao and Sethunathan 1974). Amino-parathion is less toxic as compared to its original form. It has been reported by scientific community that reductive transformation of parathion reduces its toxicity.

6.4 Herbicide Degradation

Weeds are the undesirable plants which grow in association with the main crops and share foremost part of nutrients, light, place, water, and CO_2 from the main crop. Major weeds of the agricultural fields in India are fine leaf fumitory (*Fumaria parviflora*), yellow pea (*Lathyrus aphaca*), Mexican prickly poppy (*Argemone mexicana*), small-seeded canary grass (*Phalaris minor*), wild oats (*Avena fatua*), wild onion (*Asphodelus tenuifolius*), wild green amaranth (*Amaranthus viridis*), and beggarweed (*Desmodium triflorum*). These weeds reduce the crop stand and productivity. Some weeds such as *Agropyron repens*, *Lantana camara*, *Sorghum halepense*, etc. have shown allelopathic effects on different crops. To control such unwanted plants, a vast array of chemicals known as herbicides are being used. The use of synthetic herbicides has begun at the time of World War II, and 2, 4-D was the first herbicide that was introduced. In India, first attempt to control weeds by using

synthetic chemicals was carried out in the year of 1937. Just like other chemicals, herbicides have also been found effective for weed management. But, their extensive and many a time indiscriminate use has developed resistance in many weed varieties.

This increasing resistance has led to an increase in resilience of weeds which ultimately increased the use of such chemicals. Market value of herbicides has been increased by 39% during 2002–2011 (Gianessi 2013). However, their excessive use has also induced harmful effects on environment and simultaneously on human health. The toxicity and perseverance of herbicides in environment depend on its chemical properties and quantity used. Herbicides that are used commonly include atrazine, metolachlor, clodinafop propargyl (CF), 2, 4-dichlorophenoxyacetic acid (2, 4-D), diuron, paraquat, and glyphosate (GP). They are harmful to birds, animals, and humans even at very low concentrations (in μg). Therefore, their proper degradation is required, and the microbes have shown a great hope to degrade them. Many fungi are involved in their degradation in simple non-harmful compounds.

6.4.1 2, 4-D (2, 4-Dichlorophenoxyacetic Acid)

This is a selective chlorinated acidic phenoxy herbicide and has been widely used to control broadleaf weeds of different crops such as wheat, oats, barley, rye, and corn. This herbicide was introduced in India in 1946. In most of the environmental conditions, 2, 4-D amine salts and esters convert to the 2, 4-D acid (pKa = 3.11) which is highly water soluble and therefore contaminate rivers, streams, and lakes (Muller and Babel 2004). It is classified as a hormonal herbicide with level II toxicity according to WHO and gets absorbed easily into the human and animal organs. Due to its toxicity, it exerts teratogenic, neurotoxic, carcinogenic, immunosuppressive, and hepatotoxic effects on humans (Singh and Singh 2016). In addition, 2, 4-D is responsible to interrupted energy (ATP) production in mammals and also related to the soft tissue sarcoma and Hodgkin's and non-Hodgkin's disease. Moreover, it is reported to induce lung toxicity in humans (Ganguli et al. 2014). Higher concentration/dose of 2, 4-D also causes toxicity to plants and increases multipolar cells with chromosomal aberration and decreases mitotic index (Gui et al. 2011). Due to its high consumption and very high toxicity, researchers are working toward effective degradation of such chemicals. This is a well-known herbicide, which mimics the effect of plant growth-regulating hormone and ultimately increases plant growth and results in plant death. According to one study, 2, 4-D at a higher concentration (100 mg/L) transforms into 2,4-dichlorophenol, which has been reported more toxic than the 2, 4-D.

Numerous studies have reported that *Mucor genevensis*, *Phoma glomerata*, *Chrysosporium pannorum*, *Aspergillus penicillioides*, *Aspergillus niger*, and *Fusarium oxysporum* are useful to degrade 2, 4-D and its other derivates (Fournier and Catroux 1980; Vroumsia et al. 2005). However, 2, 4-dichlorophenol degradation rate is higher than that of 2, 4-D. *Mortierella* sp. and *P. chrysosporium* are involved

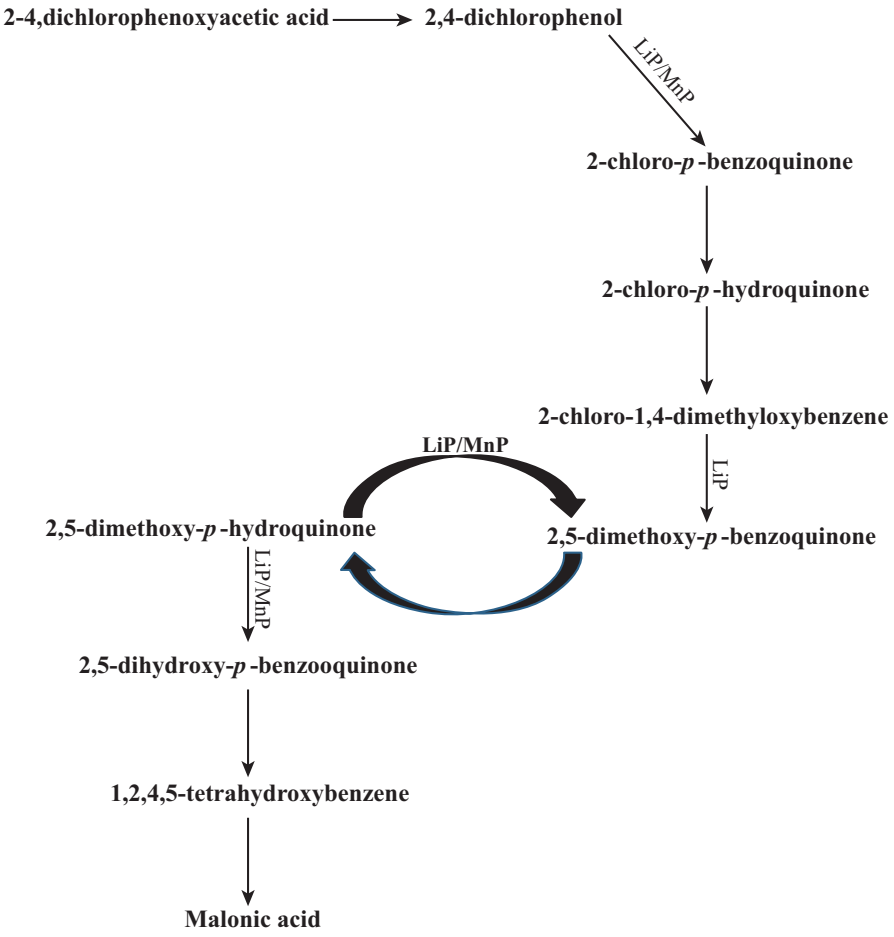

Fig. 6.6 Degradation pathway of 2,4-D and 2,4-dichlorophenol. (Adapted and modified from Buswell (2001)). LiP, Lignin peroxidase; MnP, Manganese peroxidase.

in the degradation of dichlorophenol (DCP) (Nakagawa et al. 2005). 2, 4-D is first converted into 2, 4-dichlorophenol which is further transformed in other metabolites by several reactions such as peroxidative dechlorination, quinone reduction, methylation, etc. (Fig. 6.6). These are the extracellular and cell-associated reaction of *P. chrysosporium*. 2, 4-dichlorophenol is oxidized either by LiP (Lignin peroxidase) or MnP (Manganese peroxidase) into 2-chloro-*p*-benzoquinone in which 4-chlorine atoms are removed to yield *p*-quinone. 2-chloro-*p*-benzoquinone is further reduced into 2-chloro-*p*-hydroquinone (Buswell 2001). After reduction of 2-chloro-*p*-benzoquinone, methylation reaction takes place in which 2-chloro-1,4-dimethoxybenzene is yielded. 2-chloro-1,4-dimethoxybenzene serves as a substrate for LiP-catalyzed oxidative dechlorination and yields 2,5-dimethoxy-*p*-hydroquinone and then to 2,5-dihydroxy-*p*-benzoquinone by subsequent oxidative dechlorination and

reduction reaction which ultimately yields 1,2,4,5-tetrahydroxybenzene, and further oxidation and aromatic ring cleavage of 1,2,4,5-tetrahydroxybenzene yields to malonic acid which is the end product of this reaction.

6.4.2 Diuron [N-(3, 4-dichlorophenyl)-N, N-dimethylurea]

Diuron is one of the phenylurea herbicides and is most commonly used in cotton crop. Surprisingly, diuron has been recovered from groundwater resources in the limits exceeding threshold levels (Ruberu et al. 2000). A slow degradation of phenylurea herbicides are reported in various laboratory studies (Bozarth and Funderburk 1971; Zablotowicz et al. 2000). According to various studies on diuron, it is reported to leach down deeper in soil easily and pollutes the water bodies (Tworkoski et al. 2000). Diuron containing antifouling paint has also been reported as a pollutant of aquatic environment of Japanese and Dutch coastal areas (Okamura et al. 2003). Thus, appropriate degradation of this chemical is required. Some fungi are involved in its degradation such as *Rhizoctonia solani*, *Pestalotiopsis versicolor*, *Sporothrix cyanescens*, *Cunninghamella echinulata*, *Mortierella*, etc. Ellegaard-Jensen et al. (2013) reported the pathway for the degradation of diuron (Fig. 6.7). Diuron is first converted into DCPMU (1–3,4-dichlorophenyl-3-methylurea) that is further transformed into DCPMDU (1-3,4-dichlorophenyl-3-methylideneurea) and DCPU (1-3,4-dichlorophenyl urea). The DCPU is further converted into 3,4- DCA (3,4 dichloroaniline) that is comparatively much safer.

6.4.3 Atrazine (2-Chloro-4-ethylamino-6-isopropylamino-S-triazine)

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a selective herbicide which kills only target weeds. It is frequently used to protect crops such as pineapple, sugarcane, pearl millet, sorghum, maize, etc. Its average life in water and

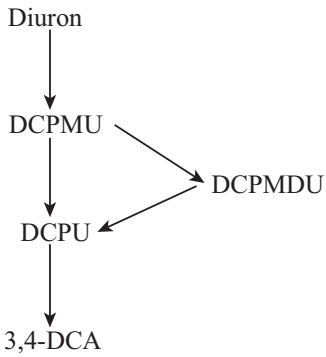

Fig. 6.7 Diuron degradation. (Adapted and modified from Ellegaard-Jensen et al. (2013))

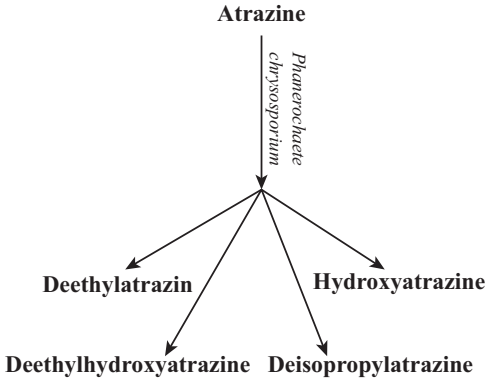

Fig. 6.8 Degradation of Atrazine given by Mougin et al. (1994)

soil is about 13–261 days (Abigail and Das 2012). Atrazine has benzene-like aromatic structure and high water solubility (Garcinuno et al. 2003). High persistence of atrazine has been detected in groundwater and also in drinking water that causes many hazardous effects on human health and ecosystem. It has been reported to be responsible for inhibition of testosterone production (Friedmann 2002) and is also responsible for the removal of MHC-I molecules from the dendritic cell surface (Pinchuk et al. 2007). This was also found responsible for various birth defects in human beings. Use of soil microorganisms has been found an inevitable approach to degrade atrazine into carbon dioxide and ammonia (Rousseaux et al. 2001). Many fungi such as *Aspergillus*, *Rhizopus*, *Fusarium*, *Penicillium*, *Trichoderma*, and *Phanerochaete* are proficient to degrade atrazine (Mougin et al. 1994). Among these, *Phanerochaete chrysosporium* efficiently degraded atrazine into deethylatrazine, deethylhydroxyatrazine, hydroxyatrazine, and deisopropylatrazine (Fig. 6.8). Another fungus, *P. pulmonarius*, is involved in the dealkylation and hydroxylation of the side chain of the atrazine, and the enzymes P-450 monooxygenases and chloroperoxidases somehow affect the dealkylation process (Abigail and Das 2012).

6.5 Fungicide Degradation

Numerous fungi have been reported to induce a variety of diseases in different crops and subsequently are responsible for heavy crop losses worldwide. *F. oxysporum* (fusarium wilt), *Claviceps fusiformis* (ergot), *Rhizoctonia solani* (root rots), *Puccinia graminis tritici* (black rust of wheat), *Ustilago tritici* (loose smut), *Sclerospora sorghi* (downy mildew), etc. are some common deadly fungal pathogens. Fungicides are the chemicals which are frequently used to protect crops from the infectious fungal diseases. Vast array of fungicides is being used in agricultural system to protect crops and thereby to enhance their productivity. Fungicides such as benomyl, vinclozolin, mefenoxam, metalaxyl, azoxystrobin, etc. are frequently used to protect crops.

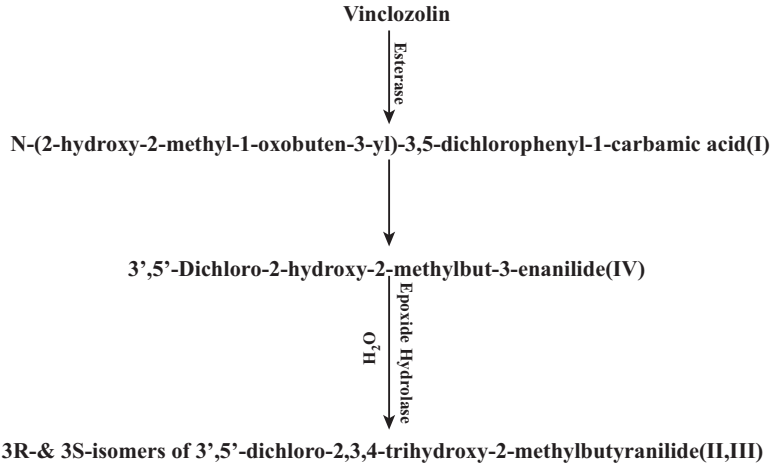

Fig. 6.9 Biotransformation of vinclozolin by *Cunninghamella elegans*. (Adapted and modified from Pothuluri et al. (2000))

6.5.1 Vinclozolin

It is a dicarboximide fungicide that is widely used in various fruit and vegetable crops. Excessive use of vinclozolin has shown residual effects on ecosystem and has been found in groundwater near to threshold concentration (Cova et al. 1990). This has been listed as endocrine disrupter and causes reproductive defects such as reduced prostate weight and vaginal pouching in rats (Gray et al. 2001). Pothuluri et al. (2000) have reported that *Cunninghamella elegans* can transform vinclozolin into four different metabolites (Fig. 6.9). Vinclozolin is transformed into metabolite I (*N*-(2-hydroxy-2-methyl-1-oxobuten-3-yl)-3, 5-dichlorophenyl-1-carbamic acid (I) by esterase enzyme; around 50% of the total metabolism is accounted by this metabolite. Metabolite IV (3′, 5′-dichloro-2-hydroxy-2-methylbut-3-enanilide) is formed by the decarboxylation of the oxazolidine portion of vinclozolin. Epoxide hydrolase reaction via ethylene dihydroxylation of metabolite IV results in the formation of metabolite II and III (3R- and 3S- isomers of 3′, 5′-dichloro-2, 3, 4-trihydroxy-2-me thylbutyranilide). Though vinclozolin is degraded in simpler molecule by fungi, their toxicity is still unknown and needs further research on these compounds.

6.5.2 Chloroneb (1,4-dichloro-2,5-dimethoxybenzene)

Chloroneb (1,4-dichloro-2,5-dimethoxybenzene) is generally insoluble in water and stable at temperature up to 267.78° C. It efficiently controls soilborne fungal diseases in plants including food crops and ornamental plants (Hock and Sisler 1969). It is also used to treat seeds of sugar beets, soybeans, cotton, and beans to control

fungal attacks. Exposure of this fungicide causes vomiting, tremors, and convulsions in human beings. *R. solani* has been reported to transform chloroneb into a nontoxic metabolite 2,5-dichloro-4-methoxyphenol (Hock and Sisler 1969). In addition to *R. solani*, complete degradation of chloroneb can be achieved by using *P. chrysosporium*.

6.5.3 *Metalaxyl [Methyl N-(2, 6-dimethylphenyl)-N-(methoxyacetyl)-D, L-alaninate]*

Another fungicide metalaxyl is a phenylamide and was introduced in 1977 for the first time for seed treatment and foliar sprays. This is a photostable compound and soluble in water at 20 °C (Sukul and Spiteller 2001). This fungicide is commonly used to control plant diseases such as late blight, downy mildew, damping off, etc. This has a broad-spectrum activity and is used on a wide range of agricultural crops and ornamental plants. Due to its high mobility and low absorption, it exists in soil for a long time and thereby contaminates groundwater (Martin et al. 2013). Metalaxyl adversely affects the environment by suppressing soil fungi and actinomycetes (Penttila and Siivinen 1996). This has also been reported as a mutagen (Hrelia et al. 1996). Therefore, methods for its proper degradation are required. Some fungi such as *R. stolonifer* and *Gongronella* sp. have been identified for the bioremediation of metalaxyl though the degradation pathway is still unknown (Martin et al. 2013). As only few organisms of choice are found so far, extensive research to find more organisms for its safer removal should be given a priority.

6.6 Conclusion

Pesticides being very effective to control a variety of insects, pathogens, and weeds have become an important component of agricultural system worldwide. But, unfortunately, their extensive and many a times irrelevant use has resulted in resistance among the target pest populations. Also, the persistence of these pesticides in the environment has been reported detrimental in the long run. Remediation of these pesticides by microbes is economically and environmentally feasible. Mycoremediation is proven to be an effective approach for the remediation of harmful chemicals and thereby to shield the environment against the hazardous effects. Numerous fungi including *P. chrysosporium*, *Phlebia aurea*, *A. niger*, *Phoma glomerata*, *Chrysosporium pannorum*, etc. have shown potential to transform or degrade harmful pesticides to non-harmful or less harmful compounds. These remediation processes are extremely advantageous to safeguard not only environment but the living organisms including humans as well.

6.7 Future Prospects

Mycoremediation has emerged as the best technique for the removal of pesticides. Although various fungi involved in remediation of pesticides have been identified and being used but for some of the pesticides only. However the complete degradation pathway for few pesticides is not available yet. Also in some cases, the toxicity of end product is not validated. Therefore there is a need to make efforts and comprehensive research to define the appropriate remediation pathway and the properties of the end product(s). Being natural inhabitants of diverse agroecosystems, fungi have an added advantage to transform or degrade the hazardous pesticides wherever they have been applied. No extra efforts or cost of transportation and processing of the sample is required, henceforth, if a suitable organism is identified and applied in agroecosystem that can make it safe for all living beings.

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Chapter 7
Bioremediation of Insecticides by White-Rot Fungi and Its Environmental Relevance

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7.1 Introduction

Insecticides are used extensively in agriculture, horticulture, medicine and industry and by household consumers. Increased agricultural productivity and reduced infield and postharvest crop loss in the twentieth century have been attributed to the increased insecticide application (Van Emden and Peakall 1996). The environmental impacts of agriculture arise from such field applications of nutrients and insecticides. Almost all insecticides have the potential to significantly alter ecosystems by negatively affecting the ecosystem functions. Most of them are toxic to humans and some even get concentrated along the food chain. Major concerns are the systemic insecticides, which have strong and prolonged residual activity. Insecticides often harm other micro- and macro-organisms beyond their target resulting in significant loss to the biodiversity of an ecosystem (Palmer et al. 2007). Sprayed insecticides, especially when sprayed aerially, may drift far from the area where it is sprayed (to wildlife areas) and impose nontarget toxicities of varied magnitudes (Palmer et al. 2007).

There are a number of possible mechanisms in practice for degradation of insecticides in soil. Chemical treatment, volatilization and incineration are in use worldwide for clean-up of these insecticides and pesticides in highly contaminated soils. However, these methods have serious environmental concerns limited to be useful to contamination of small proportion. Further, chemical treatment produces large volumes of acids and alkalis, which need subsequent disposal. Incineration, although reliable, is costly and produces toxic emissions. Overall most of these physicochemical cleaning techniques are expensive and inefficient because of the unavailability of effective degradation and disposal mechanism of the residues. These environmental concerns associated with the insecticides have led to the development of safe, convenient and economically feasible methods of pesticide remediation by

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existing and introducing native and engineered organisms (Zhang and Chiao 2002). Hence several biological techniques involving biodegradation of organic compounds by microorganisms have been developed (Benning et al. 1994; Lai et al. 1994; Mohapatra et al. 2003; Schoefs et al. 2004).

A variety of microorganisms belonging to diverse taxonomic groups (algae, fungi and bacteria) are in use in bioremediation to degrade pesticides (Pointing 2001). These microbes can be either naturally occurring or introduced into the degradation system. Microbial metabolism is probably the most important insecticide degrading process in soils (Kearney and Wauchope 1998) and is the basis for bioremediation, as the degrading microorganisms obtain C, N and/or energy from the insecticide molecules (Gan and Koskinen 1998). The primary aim of bioremediation is to at least reduce pollutant levels to undetectable, non-toxic or acceptable levels, i.e. within limits set by regulatory agencies. This technology offers the potential for onsite treatment of contaminated soil and groundwater (Balba et al. 1998; Kearney and Wauchope 1998). The most attractive feature of bioremediation is the reduced impact on the natural ecosystems (Zhang and Chiao 2002).

The complexity of microbial mechanisms for degradation of organopollutants as well as the time period before microbial degradation starts, requiring weeks to months, has made the technology slow to emerge as a viable method of remediation (Nerud et al. 2003). It becomes apparent that more detailed studies of the principles of biodegradation and the development of efficient methods of decontamination are needed to solve the insecticide contamination problem (Nerud et al. 2003). For the purpose there is consistent attempt to upgrade the native strains and develop designed strains of algae, fungi and bacteria for efficient degradation of contaminants. Though bacterial bioremediation processes developed so far are on practice for past two decades, fungal bioremediation is comparatively new and utilizes the ascomycetes and basidiomycetes fungi. There are numerous reports of the use of white-rot fungi (WRF), although not on a commercial scale, for degradation of insecticides with wide chemical diversity. These filamentous organisms, however, offer advantages over bacteria in the variety of compounds they are able to oxidize (Pointing 2001). In addition, they are robust organisms and are generally more tolerant to high concentrations of polluting chemicals than bacteria. Therefore, WRF represent a powerful prospective tool in soil bioremediation of insecticides and pesticides (Sasek 2003). However, the knowledge on the potential of WRF to degrade insecticides is sporadic, and there is no condensed report on the use of WRF for decontamination of insecticides. The present work reviews hitherto information on the ability of WRF to degrade the insecticides and the mechanisms of such degradation.

7.2 Biodegradation Potential of White-Rot Fungi (WRF)

The structural and functional characteristics of microbial community have been found to be affected by the presence of pesticides alone and in combination resulting in reduction in the soil productivity and retarded nutrient cycling (Cycon et al. 2012). The growth and metabolic activities of sensitive soil microbes, which do not

possess the enzymatic system to degrade particular pesticide, are more affected as compared to the microbes capable of degrading the pesticides (Mohapatra and Schiewer 1996). Moreover, activity of several enzymes including esterases, urease, peroxidase, dehydrogenase, antioxidant enzymes, GSH-associated enzymes, lignolytic enzymes, phosphatase and catalase is also affected by the presence of some pesticides (Cycon et al. 2012). However, a number of recent studies indicate that microbial biodegradation of pesticides is one of the most studied methods for bioremediation of pesticides from different components of the environment (Yin and Lian 2012). Numerous bacteria (Hussain et al. 2009, 2011), fungi (Badawi et al. 2009; Sene et al. 2010; Peng et al. 2012; Peter et al. 2015), actinomycetes (Eizuka et al. 2003), yeast (Salam et al. 2013) and microalgae (Megharaj et al. 1994; Mohapatra et al. 2003; Chandrakala and Mohapatra 2012) have been isolated and characterized for biodegradation of pesticides.

Although microbial biodegradation of pesticides has mostly been studied using bacteria, a number of fungal strains belonging to different genera including *Aspergillus* (Sene et al. 2010), *Trichoderma* (Sene et al. 2010), *Penicillium* (Peng et al. 2012), *Fusarium* (Sene et al. 2010), *Phanerochaete* (Sasek 2003; Reddy and Mathew 2001; Chrinside et al. 2011), *Rhizopus* (Sene et al. 2010), *Trametes* (Bastos and Magan 2009), *Lentinus* (Nwachukwu and Osuji 2007) and *Mortierella* (Badawi et al. 2009) have also been isolated and characterized for biodegradation of different pesticides.

In 1985, white-rot species, *Phanerochaete chrysosporium*, was reported to metabolize a number of important environmental pollutants (Sasek 2003). This finding opened up the broad scope of the use of WRF in cleaning up of environmental pollutants. Consequently different white-rot fungal strains such as *Trametes hirsutus*, *Pleurotus ostreatus P. sordida*, *Pleurotus* spp. (*P. florida*, *P. sajor-caju*, *P. eryngii*), *Lentinus subnudus* and *Cyathus bulleri* have been reported for their ability to degrade recalcitrant pesticides (Mougin et al. 1996; Singh and Kuhad 1999; Singh et al. 2000; Jauregui et al. 2003; Nwachukwu and Osuji 2007; Sagar and Singh 2011; Purnomo et al. 2014) (Table 7.1). Similarly different species of *Phlebia* (*P. acanthocystis*, *P. brevispora* and *P. aurea*) were able to cause biodegradation of a variety of pesticides (Xiao et al. 2011). Nyakundi et al. (2012) reported the potential of five WRF cultures and their mixture for degradation of pesticides of different chemical groups. It was observed that both mixture of various WRF and pure cultures are efficient degraders but the fungal consortium was more effective than pure cultures.

Use of WRF in bioremediation has a number of advantages over the use of other microbes in remediation because of their spreading mycelial network. They can metabolically and cometabolically degrade insoluble and sparingly soluble chemicals (Barr and Aust 1994). The growth of mycelia allows rapid colonization of substrate, and hyphal extension enables penetration of soil reaching pollutants (Reddy and Mathew 2001). As a result, physical, chemical and enzymatic contact with the surrounding environment increases (Maloney 2001). Additionally WRF can tolerate a wide range of environmental extremes such as temperature, pH and moisture variations (Maloney 2001). As the degradative system is induced by nutrient deprivation, they do not require preconditioning to a particular pollutant (Barr and Aust 1994).

Table 7.1 Important taxa of white-rot fungi employed so far for bioremediation of insecticides

S. no.	Name	Family	Reference
1	*Agrocybe aegerita*	*Strophariaceae*	Aranda et al. (2010)
2	*Auricularia fuscosuccinea*	*Auriculariaceae*	Escobar et al. (2002)
3	*Bjerkandera adusta*	*Meruliaceae*	Rivero et al. (2012)
4	*Coriolopsis gallica*	*Polyporaceae*	Pickard et al. (1999)
5	*Coriolopsis polyzona*	*Polyporaceae*	Pointing (2001)
6	*Cyathus bulleri*	*Nidulariaceae*	Singh and Kuhad (2000)
7	*Flammulina velutipes*	*Physalacriaceae*	Fan et al. (2013)
8	*Fusarium oxysporum*	*Nectriaceae*	Peter et al.(2015)
9	*Ganoderma austral*	*Ganodermataceae*	Hussaini et al. (2013)
10	*Ganoderma lucidum*	*Ganodermataceae*	Kaur et al. (2016)
11	*Hypholoma fasciculare*	*Strophariaceae*	Bending et al. (2002)
12	*Lentinus subnudus*	*Polyporaceae*	Nwachukwu and Osuji (2007)
13	*Marasmiellus chamaecyparidis*	*Marasmiaceae*	Suhara et al. (2011)
14	*Phanerochaete chrysosporium*	*Phanerochaetaceae*	Bumpus et al. (1993), Mougin et al. (1996), Pointing (2001) and Fragoeiro and Magan (2005)
15	*Phanerochaete laevis*	*Phanerochaetaceae*	Bogan and Lamar (1996)
16	*Phanerochaete sordida*	*Phanerochaetaceae*	Wang et al. (2012a, b)
17	*Phlebia acanthocystis*	*Meruliaceae*	Purnomo et al. (2017)
18	*Phlebia aurea*	*Meruliaceae*	Xiao et al. (2011)
19	*Phlebia brevispora*	*Meruliaceae*	Xiao et al. (2011)
20	*Phlebia lindtneri*	*Meruliaceae*	Mori and Kondo (2002)
21	*Phlebia tremellosa*	*Meruliaceae*	Xiao et al.(2011)
22	*Pleurotus eryngii*	*Pleurotaceae*	Arisoy (1998)
23	*Pleurotus florida*	*Pleurotaceae*	Arisoy (1998)
24	*Pleurotus ostreatus*	*Pleurotaceae*	Baarschers and Heitland (1986), Jauregui et al. (2003) and Purnomo et al. (2014)
25	*Pleurotus pulmonarius*	*Pleurotaceae*	Hernández-Rodríguez et al. (2006)
26	*Pleurotus sajor-caju*	*Pleurotaceae*	Arisoy (1998)
27	*Stereum hirsutum*	*Stereaceae*	Bending et al. (2002)
28	*Trametes hirsute*	*Polyporaceae*	Kamei et al. (2011)
29	*Trametes hirsutus*	*Polyporaceae*	Singh and Kuhad (1999)
30	*Trametes versicolor*	*Polyporaceae*	Pointing (2001) and Bending et al. (2002)
31	*Trichoderma harzianum*	*Hypocreaceae*	Helal and Abo-El-Seoud (2015)
32	*Trichoderma viride*	*Hypocreaceae*	Helal and Abo-El-Seoud (2015)

WRF primarily utilize three main components of woody tissue, i.e. cellulose, hemicellulose and lignin, for metabolism. Biodegradation of lignin results in destruction of the matrix it forms, so that the microorganism can gain better access to the real substrates, i.e. hemicellulose and cellulose (Field et al. 1992; Canet et al. 2001). Energy required by these fungi is mainly obtained by degradation of hemicellulose and cellulose. Lignin-degrading ability of WRF also enables the fungi to target numerous organopollutants causing their partial and/or complete degradation (Field et al. 1992). Due to its molecular size, it is impossible for lignin to be absorbed and degraded by intracellular enzymes. Therefore ligninolytic enzymes are extracellularly excreted by the degrading fungi, initiating the oxidation of substrates in the extracellular environment. Thus, WRF have developed very non-specific mechanisms to degrade lignin extracellularly and in the process possessing the ability to target various synthetic toxicants (Barr and Aust 1994).

There are three major families of lignin-modifying enzymes which are involved in lignin degradation, viz. laccases, lignin peroxidases and manganese peroxidases (Reddy and Mathew 2001). The key step in lignin degradation by laccase or the ligninolytic peroxidases involves the formation of free radical intermediates that are highly reactive and trigger oxidation or reduction of neighbouring compounds. These radicals can carry out a variety of reactions including benzylic alcohol oxidation, carbon-carbon bond cleavage, hydroxylation, phenol dimerization/polymerization and demethylation, each of which is effective to detoxify pesticides (Pointing 2001). Different WRF appear to be able to achieve the same effect with different combinations of enzymes with respect to organopollutant degradation. Consequently, many toxic compounds, which have an aromatic structure, are also highly susceptible to degradation by WRF, which makes them the pivotal players in bioremediation (Barr and Aust 1994). Aside from the lack of specificity, the ligninolytic system also enables WRF to degrade pollutants at relatively low concentrations that may be lower than that required to induce the synthesis of biodegrading enzymes in other microorganisms. Moreover, repression of enzyme synthesis does not occur when the concentration of a chemical is reduced to a level that is ineffective for enzyme induction (Bumpus and Aust 1987).

7.3 Fungal Enzyme Systems for Degradation of Insecticides

Plenty of information are available on the genes and enzymes involved in fungal-mediated transformations of pesticides (Nagpal et al. 2008; Karas et al. 2011; Jain et al. 2014). WRF variously secrete one or more of three extracellular (lignin degrading) enzymes that are known to play a key role in insecticide degradation. They are together referred to as lignin-modifying enzymes (LMEs). The three enzymes comprise two glycosylated heme-containing peroxidases, lignin peroxidase (LiP, EC 1.11.1.14) and Mn-dependent peroxidase (MnP, EC 1.11.1.13) (Orth and Tien 1995), and a copper-containing phenoloxidase, laccase (Lac, EC 1.10.3.2) (Eggert et al. 1996). In addition two more recently described peroxidase groups

found in fungi (but not exclusively in WRF), namely, aromatic peroxygenases (APOs, EC 1.11.2.1), a versatile peroxidase (VP; EC 1.11.1.16) and dye-decolorizing peroxidases (DyPs, EC 1.11.1.19), have shown important degradative potential for organopollutants. A group of catalases are also found to have a role in tolerance of fungi to pesticides by scavenging the free radicals formed by the pesticide stress and thereby playing an indirect role in fungal bioremediation of pesticides. Cytochrome P450-mediated detoxification of insecticides has also been reported in fungi (Guengerich and Munro 2013; Morel et al. 2015).

7.3.1 Peroxidases

Among the peroxidases of fungal origin LiP and MnP are heme peroxidases, which require the presence of hydrogen peroxide and manganese for activity and are mostly reported for degradation of toxic compounds by WRF. On the other hand, VP enzymes are broad substrate-specific enzymes capable of oxidizing both phenolic and non-phenolic compounds and widely applied in biodegradation of environmental pollutants. MnP catalyses a H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}, and the Mn^{3+} ions are stabilized by chelation with organic acids. These chelated ions act as diffusible redox mediators facilitating the attack on diverse molecules and giving MnP a versatile oxidative ability (Hofrichter 2002). On the other hand, LiP, in the presence of H_2O_2, catalyses the oxidation of veratryl alcohol (a natural fungal secondary metabolite that serves as a redox mediator), which then carries out oxidations of non-phenolic aromatic residues or their partial degradation products to generate aryl cation radicals. These radicals, after several reactions, subsequently undergo complete degradation (Pointing 2001).

Two other peroxidase groups, APOs and DyPs, have shown important degradative potential for organopollutants. APOs belong to the superfamily of heme-thiolate peroxidases and have been described in agaric basidiomycetes (*Agrocybe aegerita* and *Coprinellus radians*), but there is molecular evidence of their wide occurrence in other fungi (Aranda et al. 2010). These enzymes share catalytic properties with peroxidases, catalases and cytochrome P450 monooxygenases attacking organic pollutants (aliphatic, aromatic and heterocyclic compounds) by transfer of oxygen from peroxides to the substrate (Ruiz-Duenas et al. 2011). The catalysis results in reactions such as hydroxylation or epoxidation of aromatic rings and benzylic compounds, phenol oxidation, sulphoxidation of tricyclic heterocycles, oxidation of pyridine derivatives and cleavage of esters (Barkova et al. 2011).

The physiology of LMEs has shown that the production of the enzymes occurs during secondary metabolism and is induced by limited nutrient levels, particularly nitrogen. Some taxa have, however, been demonstrated to produce LiP, MnP and Lac under conditions of nitrogen sufficiency (Buswell et al. 1984). Production of LiP and MnP is generally optimal at high oxygen tensions but is repressed by agitation of fungi grown in submerged liquid culture (conversely, Lac production is generally enhanced by agitation). Studies have shown that nutrient nitrogen levels,

mediator compounds and required-metal (i.e. Mn^{2+} for MnP, Cu^{2+} for Lac) concentrations affect transcription levels of LiP (Li et al. 1994), MnP (Ruiz-Duenas et al. 1999) and Lac (Palmieri et al. 2000).

7.3.2 Laccase

Laccases are copper-containing extracellular enzymes of blue oxidases group, which use copper as cofactor and molecular oxygen as co-substrate. Laccases are capable of oxidizing most of the phenolic and non-phenolic compounds, produced during the degradation of pesticides, and their activity has been observed to be more than 20 times greater in WRF such as *Trametes versicolor* compared to other organisms (Margot et al. 2013). Verma et al. (2012) and Vishwanath et al. (2014) for the first time reported the marine fungal laccase-mediated detoxification. The non-specific nature of their activity on a variety of substrates makes them ideal catalyst for metabolism of a variety of insecticides (Donoso et al. 2008; Pizzul et al. 2009; Kadimaliev et al. 2011). In the presence of molecules that act as electron-transfer mediators, laccases are able to oxidize many compounds (Atalla et al. 2013), and some of these laccase mediators are produced during normal metabolic activity of WRF (Asgher et al. 2008).

A number of recent studies have shown the involvement of peroxidase and laccase enzymes together in the biodegradation of different pesticides (Donoso et al. 2008; Pizzul et al. 2009; Kadimaliev et al. 2011). For example, Donoso et al. (2008) documented the involvement of peroxidase and laccase activity in the degradation of tribromophenol by *Trametes versicolor*. Similarly, Kadimaliev et al. (2011) measured the biodegradation of phenol through *Lentinus tigrinus* in liquid medium with the combined action of laccase and peroxidase enzymes that attacked the substrates in tandem.

Bioremediation application of laccases is unfortunately restricted by their low shelf life. Immobilization of the enzyme on nanoparticles and tailoring of these enzymes through site-directed mutations are seen as effective ways for the activities over a broad range of pH and temperature (Patel et al. 2014). Immobilized laccase enzyme was reported to degrade a wide range of pollutants quite for a long time (Wang et al. 2013; Jorenek and Zajoncova 2015). Likewise, laccase immobilized on nanoporous silica beads showed around 90% degradation of 2,4-dinitrophenol within 12 h of treatment. The enzymes were very stable and retained almost 85% of initial activity even after 30 days of treatment (Dehghanifard et al. 2013). Wang et al. (2012a) reported a twofold increase in phenol degradation with immobilized laccase enzyme. The enzyme retained 71.3% of its initial degradation activity even after ten successive batch treatments. Rodríguez-Delgado et al. (2016), in their study, degraded diclofenac (50%), β-naphthol (97%) and 2,4-dichlorophenol (71%) with laccase cocktail. Huifang et al. (2013) observed high efficiency of recombinant laccase system from *Pichia pastoris* for biodegradation of chlorpyrifos.

7.3.3 Catalase

Production of reactive oxygen species (ROS) is a general toxic response in many biological systems. The accumulation of ROS results in damage to cellular macromolecules, which is deleterious for cellular integrity. Primary defence mechanism to ROS in fungi consists of monofunctional catalases and bifunctional peroxidase/catalase. Inhibition of catalase in the presence of pesticides causes increased ROS generation and hence ROS-mediated damage resulting in inhibition of growth and metabolic efficiency (Pita et al. 2013). Mitra et al. (2014) have confirmed stress tolerance of fungi by increased activities of catalase among other enzymes. Experiments with many other fungi have suggested that catalase activity could be used as monitoring tool for quantification of bioremediation efficiency (Lin et al. 2009; Thippeswamy et al. 2014).

7.3.4 Cytochrome P450 Enzyme Systems

Cytochrome P450 (CYP) is a large family of cysteinato-heme enzymes widely distributed in nature, which participate in the oxidative transformation of many endogenous and exogenous molecules through insertion of an oxygen atom (from molecular oxygen) into a substrate and the subsequent reduction of the second oxygen atom to a water molecule (Ichinose 2013). Fungal CYP possess complex oxidative and hydrolytic enzymatic systems for detoxifying toxic compounds in the environment. Besides these systems, certain fungi possess intracellular networks, which constitute the xenome, consisting of cytochrome P450 monooxygenases and the glutathione transferases for dealing with diverse range of pollutants. The members of the detoxification pathways, which generally belong to multigenic families such as cytochrome P450 monooxygenases and glutathione transferases, together constitute the xenome (Morel et al. 2015). The fungal CYP system can serve as versatile catalyst for region- and stereospecific oxidation of nonactivated hydrocarbons and can be ideal substitutes for chemical catalysts (Urlacher and Girhard 2012).

Separate cytosolic and mitochondrial isoforms of CYP are employed by fungi in degradation of organopollutants (Guengerich and Munro 2013; Wang et al. 2015). A cytochrome P450 monooxygenase (CYP) was shown to be involved in the initial transformation of organochlorine compounds. Besides, CYP was also involved in initial oxidation on degradation of pyrene, anthracene, fluorene and dibenzothiophene by WRF by producing epoxidation products (Bezalel et al. 1996). CYP63A2 P450 monooxygenase from WRF (*P. chrysosporium*) oxidized crude oil aliphatic hydrocarbon n-alkanes and endocrine-disrupting long-chain alkylphenols (APs) and mutagenic/carcinogenic fused-ring high-molecular-weight PAHs (HMW-PAHs) (Syed et al. 2013). Preinduction of the P450 monooxygenase, before application in degradation studies, could result in enhanced PAH removal (Bhattacharya et al. 2013). Analysis of *P. chrysosporium* genome has shown that cytochrome P450 monooxygenases represent the largest and most important group of P450 genes in

any fungal species and that induction of these gene clusters is differentially expressed depending on xenobiotic type and nutrition (Yadav and Loper 2000; Yadav et al. 2006).

Many other fungal enzymes are also found to be involved in combination with degradation and detoxification of insecticides. While studying the degradation of simazine, trifluralin and dieldrin by *T. versicolor* and *P. chrysosporium*, Fragoeiro and Magan (2008) observed the action of extracellular enzymatic production with higher activity of cellulase/dehydrogenase. Similarly, the degradation of an organophosphate insecticide monocrotophos by three fungal strains *Aspergillus flavus*, *Fusarium pallidoroseum* and *Macrophomina* sp. was linked with extracellular release of alkaline phosphatase, inorganic phosphates and ammonia (Jain et al. 2014). It was observed that the secretion and activity of laccase enzyme were more at the earlier stage of biodegradation, while peroxidase more actively took part in biodegradation at later stage. Pizzul et al. (2009) used two mediators ($MnSO_4$ and Tween 80) in order to assess the capability of lignin peroxidase (LiP), manganese peroxidase (MnP), horseradish peroxidase (HRP) and laccase for the degradation of glyphosate and other pesticides. Results revealed that, in the presence of $MnSO_4$ and Tween 80, MnP showed complete degradation of glyphosate with and without H_2O_2. Degradation was also observed in the presence of 2,2′-azino-bis(3-ethylbenzthiazo-line-6-sulphonic acid) (ABTS), $MnSO_4$ and Tween 80 alone as well as in combination.

Mir-Tutusaus et al. (2014) observed rapid degradation of imiprothrin by *T. versicolor* in a laccase mediator system and concluded that rapid degradation was mainly associated with extracellular production of laccase by WRF. Gao et al. (2012) characterized the first chlorpyrifos hydrolase enzyme purified from *C. cladosporioides*. It was observed that this purified enzyme hydrolysed various organophosphorus insecticides with P-O and P-S bond with optimal activity at 40 °C but chlorpyrifos was the preferred substrate. Similarly, Xie et al. (2010) used *Fusarium* sp. to extract a free enzyme that has the ability to degrade chlorpyrifos. The authors also compared the properties of immobilized enzymes to the free enzyme recording high rate of degradation by immobilized enzyme at varying range of pH and temperature. Nguyen et al. (2014) investigated the potential of a laccase crude enzyme extract originating from *T. versicolor* for biodegradation of different pesticides including atrazine, ametryn, fenoprop, etc. They found that degradation potential of laccase crude enzyme extract was improved in the presence of 1-hydroxybenzotriazole and syringaldehyde redox mediators.

7.4 Pathways of Degradation in White-Rot Fungi

Different degradative pathways have been investigated in fungal bioremediation of insecticides, herbicides, polychlorinated organics and aromatic compounds. Under ideal conditions the degradation of the hydrophobic molecules is initiated with the removal of active groups by hydrolysis, dealkylation, dehalogenation, etc., resulting

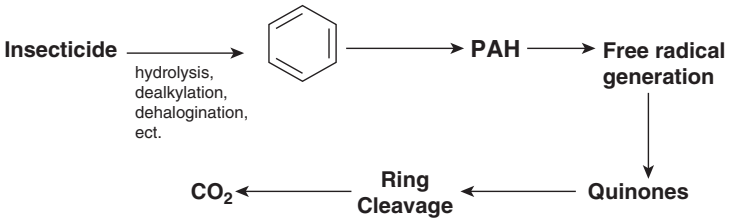


Fig. 7.1 A general scheme of fungal degradation of organopollutants

in the formation of aliphatic or aromatic compounds (Fig. 7.1). The product(s) then is subjected to the action of a number of existing and/or induced enzymes to complete the degradation. In general the biodegradative ability of WRF is thought to be related to their ability to degrade lignin (Tien and Kirk 1984). However, a purely oxidative, peroxidase-based system cannot be used to completely degrade lignin. Some chemicals must first be reduced before they can be oxidized by the lignin peroxidases. There are several mechanisms by which this can occur, but most significantly, this must be the manner in which WRF mineralize a number of highly oxidized chemicals such as TNT and DDT. One method is the indirect oxidation via the cation radical of veratryl alcohol (Fig. 7.2). For compounds that may have sufficiently low oxidation potential but are apparently without access to the heme of lignin peroxidases, indirect oxidation occurs upon the inclusion of veratryl alcohol (Kurek et al. 1990; Aust 1995). Reduction seemed to occur by a membrane-dependent reduction potential that WRF apparently use to initiate the degradation pathway (Bumpus and Aust 1987). Therefore, studies on other pathways are also of extreme importance for understanding of downstream pathways for bioremediation of pollutants and the mechanisms involved in the reactions.

Absorption onto the fungal biomass has been considered to be one mechanism of pollutant removal in addition to action of several enzymes extracellularly as well as intracellularly. For example, laccases are shown to be very effective during transformation of endosulfan to endosulfan sulphate in the presence of WRF *T. versicolor* and *P. ostreatus* (Ulcnik et al. 2013). Similarly transformation of anthracene by *Armillaria* sp. F022 occurred through two alternative routes, which were laccase-mediated ring cleavage reactions, first consisting of oxidation of anthracene to anthraquinone and benzoic acid and second converting anthracene to other products (2-hydroxy-3-naphthoic acid and coumarin) (Hadibarata et al. 2013). Study of the fungal degradation of PAH has shown that 3-chloro-*cis,cis*-muconate was produced as an intermediate, but uncommon compounds from 4-chlorocatechol and 3-chlorocatechol degradation pathways were also produced yielding 3-chlorodienelactone and catechol, respectively (Xiao et al. 2014). *Phlebia* was studied for dieldrin degradation which was attributed to hydroxylation reactions in the pathway leading to three hydroxylated metabolite products. These fungi were also found to degrade aldrin by attacking methylene moiety leading to formation of new metabolites like 9-hydroxyaldrin and two carboxylic acid products. Further, application of proteomics, gene expression studies and the use of gene-replace-

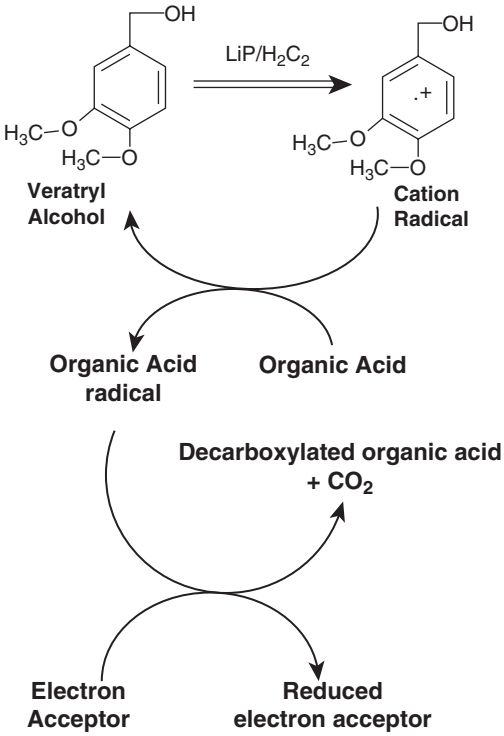


Fig. 7.2 Ligninolytic enzyme-mediated degradation of organopollutants by WRF

ment mutants have assigned most of the steps in well-known but less understood 3-oxoadipate pathway of aromatic compound degradation to particular genes. The study showed the formation of catechol from salicylate either directly or through 2,3-dihydroxybenzoate. Additionally, the study indicated successive muconate isomerization reactions in the catechol branch (Martins et al. 2015; Deshmukh et al. 2016). This indicated that the degradation of insecticides with aromatic carbon skeleton follows the aerobic catechol pathway after the initial removal of the active groups.

7.5 Degradation of Insecticides

7.5.1 Degradation of Organophosphate

The biodegradation of organophosphorus insecticides (OPs) has been studied in soils and in bacterial cultures (Benning et al. 1994; Lai et al. 1994). Less attention has been given to the metabolic removal of these compounds by fungi in general and WRF in particular especially considering the catalytic efficiencies of LMEs. *P. chrysosporium* is the most studied WRF proving its ability to degrade a variety of

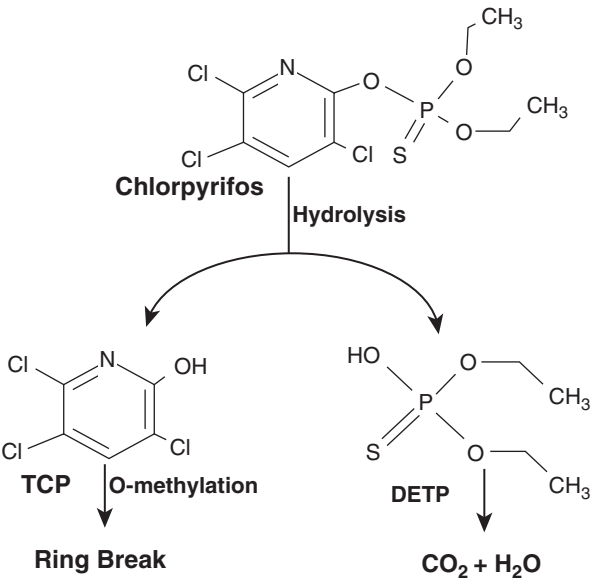


Fig. 7.3 Proposed pathway degradation of chlorpyrifos (Modified from Chen et al. 2012). *Abbreviation*: *TCP* 3,5,6-trichloro-2-pyridinol and *DETP* diethylthiophosphoric acid

pesticides. The fungus has been demonstrated to mineralize 12.2–27.5% of ^{14}C-radiolabeled chlorpyrifos, fonofos and terbufos to CO_2, which has been attributed to LMEs. Proposed pathway for degradation of chlorpyrifos is given in Fig. 7.3. Hernandez et al. (1998) demonstrated that chloroperoxidase from the fungus *Caldariomyces fumago* was able to oxidize seven of ten OPs assayed, although no oxidation was detected when other heme proteins such as lignin peroxidase, horseradish peroxidase or cytochrome c were used. On the other hand, a fungal laccase produced by *Pleurotus ostreatus* has been reported as able to perform the oxidative degradation of two OP nerve agents, VX and RVX, with a mediator-assisted reaction. Hydrolytic cleavage of fenitrothion and fenitrooxon by the non-ligninolytic fungi showed that OP degradation by fungi was not necessarily by LMEs, rather by many other metabolic pathways (Baarschers and Heitland 1986). WRF including *Coriolopsis polyzona*, *P. chrysosporium*, *P. ostreatus* and *T. versicolor* are known to cause significant removal of pesticides (as measured by substrate disappearance) in vivo (Pointing 2001). These suggest that the ability to degrade OP insecticides may be widespread or at least relatively common among the fungi, though the metabolic system varies.

Fragoeiro and Magan (2005) demonstrated that *P. chrysosporium* was able to rapidly degrade a mixture of pesticides under osmotic stress. In a similar study, Jauregui et al. (2003) showed that *P. ostreatus* was the most efficient WRF in the degradation of OP pesticides among the *Pleurotus* species studied. Kulshrestha and Kumari (2011) recorded the utilization of chlorpyrifos as a partial source of carbon/nitrogen by mixed fungal cultures isolated from different types of soils (alluvial, red and black) when growth was studied in nutrient media containing 0.1% sodium nitrate. Degradation as high as 78.2% could be achieved by the authors in broth with

normal carbon and nitrogen nutrients showing the ability of the mixed culture to degrade chlorpyrifos by cometabolism. Utilization of chlorpyrifos as a limited nitrogen source by *P. chrysosporium* has also been reported (Wang et al. 2005). Opening of pyridyl ring by WRF is considered to be the initiation of the degradation and production of a number of metabolites. The metabolite 2,4-bis(1,1-dimethylethyl) phenol is formed as a dominant intermediate before the ring cleavage (Fulekar 2012). Yu et al. (2006) observed that cometabolic removal of chlorpyrifos by the fungal consortium is faster than of metabolic removal.

Inoculation with *Acremonium* sp. strain (GFRC-1) in Czapek Dox medium containing chlorpyrifos (300 mg L^{-1}) has shown that the insecticide is partially metabolized to desmethyl chlorpyrifos (3,5,6-trichloropyridyl-2-phosphorothioate) (Kulshrestha and Kumari 2011). The degradation mechanism might have involved an inducible enzyme chlorpyrifos hydrolase, an organophosphorus ester-hydrolysing enzyme, to hydrolyse chlorpyrifos to a non-toxic metabolite (Xu et al. 2008). Pant and Tripathi (2010) reported that the rot fungi *T. versicolor* and *Oligoporus placentas* effectively degraded chlorpyrifos to 3,5,6-tricholorpyridinol (TCP) at comparatively high concentrations (4.9%). This indicated that WRF are more efficient in degrading chlorpyrifos than other fungi. Synergism between bacteria and WRF for chlorpyrifos degradation has also been reported (Virag et al. 2007).

Karas et al. (2011) observed a rapid loss of chlorpyrifos within the first 2 days after application with more than 50% loss observed in all fungal cultures and building up of TCP as a transit metabolite. In *P. ostreatus* chlorpyrifos caused initial inhibition of laccases and MnP, but the fungus showed rapid adaptation (5 days after exposure) and recovery of the enzyme activities (Karas et al. 2011). This metabolic pattern of chlorpyrifos implies that its initial degradation proceeded via an unknown intermediate metabolite, which was subsequently hydrolysed to TCP. However, TCP also possesses strong antimicrobial activities, and its accumulation leads to complete inhibition of degradation of the parent compound (Singh et al. 2003). Laccase-mediated degradation of chlorpyrifos was reported by Huifang et al. (2013). Significant degradation (up to 99%) of chlorpyrifos could also be achieved by magnetic nanoparticles of fungal-based laccase (Das et al. 2017) indicating that laccase-mediated degradation is primarily responsible for removal of chlorpyrifos. Catalytic activity of such particles not only remained stable but also active at chlorpyrifos concentrations up to 750 mg/l.

Taking a mixture of pesticides, Bending et al. (2002) reported that WRF degraded 80% of the applied combination in liquid culture. Fragoeiro and Magan (2005) showed that *T. versicolor* and *P. chrysosporium* were also able to degrade a mixture of pesticides both in liquid and soil environments. Nyakundi et al. (2012) reported that five isolates of WRF were found effective degrader of diazinon. The degradation products of diazinon included diazoxon and oxypyrimidine as observed with bacterial isolates (Sethunathan and Pathak 1972). The comparison of bacterial and fungal degradation of diazinon showed that though the rate of degradation varied, the pathways of degradation were more or less the same and were initiated by activity of CYP to oxon.

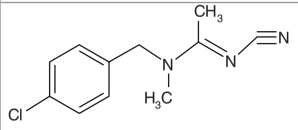
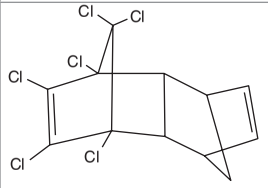
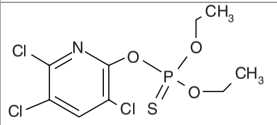
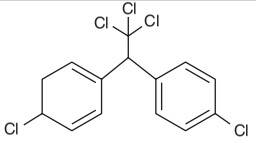
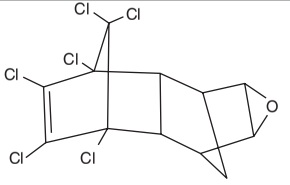
Cytochrome P450-mediated degradation of OPs has also been reported in bacteria and fungi though the process is initially considered as an activation by the formation of more toxic oxons. Mougin et al. (1996) were the first to suggest that cytochrome P450 system is involved in the pesticide degradation in WRF. Transformation of azinphos-methyl was achieved either through the cleavage of the S-C bond or through oxidative desulfuration. Further dealkylation of 2-methyleneazide benzaldehyde or methylation of the azinphos-methyl oxon yielded 4-ketobenzotriazine and O,O,S-trimethyl dithiophosphate, respectively. Similarly cleavage of the S-C bond of terbufos yielded 3,3-dimethyl-2-thiobutane and O,O-diethyl phosphorodithioic acid as products. C-N bond cleavage of phosmet has also been reported to be performed by cytochrome P450 system. Malathion was enzymatically converted to O,O,S-trimethyl phosphorodithioic acid and butanedioic acid (dimethoxyphosphinothioyl)-4-ethyl ester. At least two different reactions seemed to be involved: the cleavage of the S-C bond as observed for the transformation of azinphos-methyl and hydrolysis of one of the ethyl ester bonds, with the concomitant release of one ethanol molecule. Different insecticides along with their structure and degrading WRF have been presented in Table 7.2.

Several studies have shown that diverse white-rot fungi are capable of PAH mineralization (Sack et al. 1997). Experiments with purified cell-free enzyme extracts have confirmed the role of LMEs in PAH degradation. Extracellular preparations of LiP from *P. chrysosporium* were among the first to be shown as capable of PAH oxidation (Hammel et al. 1986; Bumpus 1989). The purified LiP of *Nematoloma forwardii* has also been shown to oxidize anthracene and pyrene in the presence of veratryl alcohol which acted as a mediating substrate (Guenther et al. 1998). Similarly purified MnP of *P. chrysosporium* has been shown to oxidize 12 3–6 ring PAH (Bogan and Lamar 1995). Lipid-peroxidation-coupled MnP-mediated PAH oxidation was also observed for *Phanerochaete laevis*, which produced predominantly polar products with no significant quinone accumulation (Bogan and Lamar 1996). By contrast, MnP of *N. forwardii* (in a lipid-peroxidation-coupled reaction) oxidized anthracene and pyrene to produce quinone products (Guenther et al. 1998). These were not further metabolized, but some transformation of hydroxylated products occurred. PAH transformation by MnP has also been shown to be limited by Mn^{2+} availability (Bogan and Lamar 1996). Purified Lac from *Trametes versicolor* has been shown to oxidize a range of 3–5 ring PAH in the presence of the chemical mediators HBT and ABTS (Collins et al. 1996; Majcherczyk et al. 1998). A similar PAH substrate range was found for purified Lac of *Coriolopsis gallica* (Pickard et al. 1999).

7.5.2 Degradation of Organochlorines

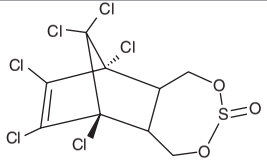
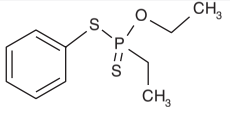
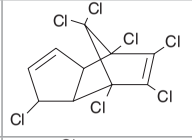
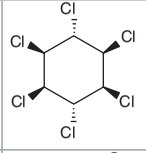
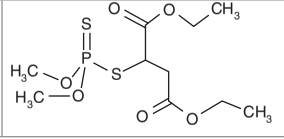
Degradation of OC insecticides by WRF has been more extensively studied than of any other insecticide. In white-rot fungi and brown-rot fungi, DDD and DDE were converted to DBP by reductive dechlorination and hydroxylation (Bumpus and Aust

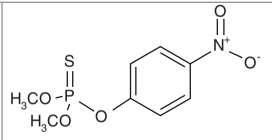
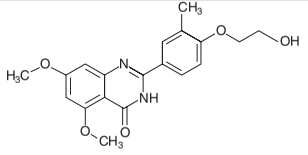
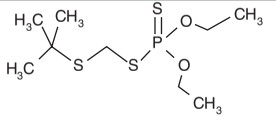
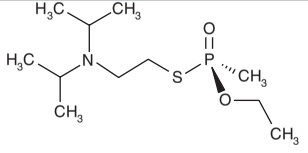
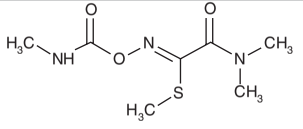
Table 7.2 Different insecticides, their structure and corresponding degrading WRF

S. no.	Insecticide	Structure	Degrading organism	Reference
1	Acetamiprid		*Phanerochaete sordida* YK-624	Wang et al. (2012b)
2	Aldrin		*Pleurotus ostreatus*BM9073, *Phlebia acanthocystis*, *Phlebia brevispora*, *Phlebia aurea*, *Phanerochaete chrysosporium*	Kennedy et al. (1990), Xiao et al. (2011) and Purnomo et al. (2017)
3	Chlorpyrifos		*Trametes versicolor*, *Hypholoma fasciculare*, *Stereum hirsutum*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus*	Bending et al. (2002), Karas et al. (2011) and Kulshrestha and Kumari (2011)
4	Dichlorodiphenyltrichloroethane (DDT)		*Marasmiellus chamaecyparidis*, *Flammulina velutipes*, *Phanerochaete chrysosporium*	Corona-Cruz et al. (1999), Suhara et al. (2011) and Fan et al. (2013)
5	Dieldrin		*Pleurotus ostreatus* BM9073, *Phlebia acanthocystis*, *Phlebia brevispora*, *Phlebia aurea*, *Phanerochaete chrysosporium*	Fragoeiro and Magan (2008), Xiao et al. (2011) and Purnomo et al. (2017)

(continued)

Table 7.2 (continued)

S. no.	Insecticide	Structure	Degrading organism	Reference
6	Endosulfan		*Trametes hirsuta*, *Pleurotus pulmonarius* ECS-0190, *Bjerkandera adusta* (CCM0379)	Hernández-Rodríguez et al. (2006), Kamei et al. (2011) and Rivero et al. (2012)
7	Fonofos		*Phanerochaete chrysosporium*	Bumpus et al. (1993)
8	Heptachlor		*Pleurotus ostreatus*, *Phlebia acanthocystis*, *Phlebia tremellosa*	Xiao et al. (2011) and Purnomo et al. (2014)
9	Lindane and β,α,γ and δ- HCH		*Ganoderma lucidum*, *Ganoderma australe*, *Phanerochaete chrysosporium*, *Trametes hirsutus*, *Bjerkandera adusta*	Mougin et al. (1996), Singh and Kuhad (1999), Quintero et al. (2008), Hussaini et al. (2013) and Kaur et al. (2016)
10	Malathion		*Fusarium oxysporum*	Peter et al. (2015)

11	Parathion		*Bjerkandera adusta*, *Pleurotus ostreatus*	Jauregui et al. (2003)
12	RVX		*Pleurotus ostreatus*	Baarschers and Heitland (1986)
13	Terbufos		*Phanerochaete chrysosporium*	Bumpus et al. (1993)
14	VX		*Pleurotus ostreatus*	Baarschers and Heitland (1986)
15	Vydate		*Trichoderma viride*, *Trichoderma harzianum*	Helal and Abo-El-Seoud (2015)

1987; Purnomo et al. 2008; Xiao et al. 2011), but degradation of DDT to DBP is still unknown though such degradation has been reported with the ectomycorrhizal fungi, *Xerocomus chrysenteron* (Huang and Wang 2013). Purnomo et al. (2008, 2010), recorded that less than 0.25% of the initial DDT was mineralized to CO_2 by *G. trabeum* but effective transform of DDT by *P. australis* of wetland plant species has been reported (Chu et al. 2006)

Three strains of *P. chrysosporium* and the species *Pleurotus ostreatus*, *Phellinus weirii* and *Polyporus versicolor* were able to mineralize DDT, dicofol and methoxychlor over 30 days under ligninolytic growth conditions (Bumpus and Aust 1987). Laccase derived from WRF could degrade DDT in soil by about 50% in 25 days of incubation. The increase order of half-life of DDTs was in red soil (24.71 days) > latosol soil (26.61 days) > lateritic red soil (27.68 days) (Zhao et al. 2010). DDT was also metabolized under aerobic conditions by the P450 enzyme to DDD, dicofol and 2,2-bis(4-chlorophenyl) acetic acid (DDA) (Joußen et al. 2008). Furthermore, *P. ostreatus* was found to metabolize some aromatic compounds by hydroxylation that does not depend on ligninolytic enzymes (Bezalel et al. 1996). It can be assumed that the intracellular P450 has also an important role in the metabolism of persistent aromatic compounds, alongside ligninolytic enzymes.

Suhara et al. (2011) observed degradation of DDT by *P. chrysosporium*, and upon increasing the duration of incubation, degradation occurred up to 35% in 28 days. Interestingly the fungus performed the metabolism by two distinct pathways, which are uncommon to other fungi: first mediated by the P450 system that produces monohydroxy-DDTs (minor pathway) and DDT dechlorinated to DDD by an unidentified system (major pathway). Most of the products produced during the metabolism are more or less toxic, thus requiring larger time for complete removal of the insecticide (Fig. 7.4). Another set of study on the biodegradation ability of *Flammulina velutipes* along with laccase enzyme to degrade DDT in soil showed higher degradation (an increase rate by synergism) rather than using only WRF or laccase (Fan et al. 2013).

A study to test the ability of spent mushroom waste (SMW) from *P. ostreatus* to DDT showed that 48% of the chemical was degraded in 28 days incubation (Purnomo et al. 2010). The degradation in artificial DDT-contaminated soil could be as high as 80% in 28 days on inoculation with WRF, and the microbial cooperation in soil condition was very prominent almost doubling the degradation rate of the insecticide (Purnomo et al. 2010). A study on the degradation ability of *P. chrysosporium* at different oxygen conditions showed the degradation process of the sequence of anaerobic-aerobic fermentation and the use of *P. chrysosporium* over the aerobic mixed culture (Corona-Cruz et al. 1999).

Fig. 7.4 (continued) *DDE* 1,1-dichloro-2,2-bis(4-chlorophenyl)ethylene, *DDD* 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane, *DDA* 2,2-bis(4-chlorophenyl) acetic acid, *DBP* 4,4-dichlorobenzophenone, *DBH* 4,4-dichlorobenzhydrol, *DICOFOL* 2,2,2-trichloro-1,1-bis(4-chlorophenyl) ethanol, *FW-152* 2,2-dichloro-1,1-bis(4-chlorophenyl)ethanol, *DDMU* 1-chloro-2,2-bis(4-chlorophenyl)ethylene, *DDMS* 1-chloro-2,2-bis(4- chlorophenyl)ethane, *DDNU* 2, 2-bis (4 -chlorophenyl)ethylene, *DDOH* 2,2-bis(4- chlorophenyl)ethanol and *DDM* bis(p-chlorophenyl)methane

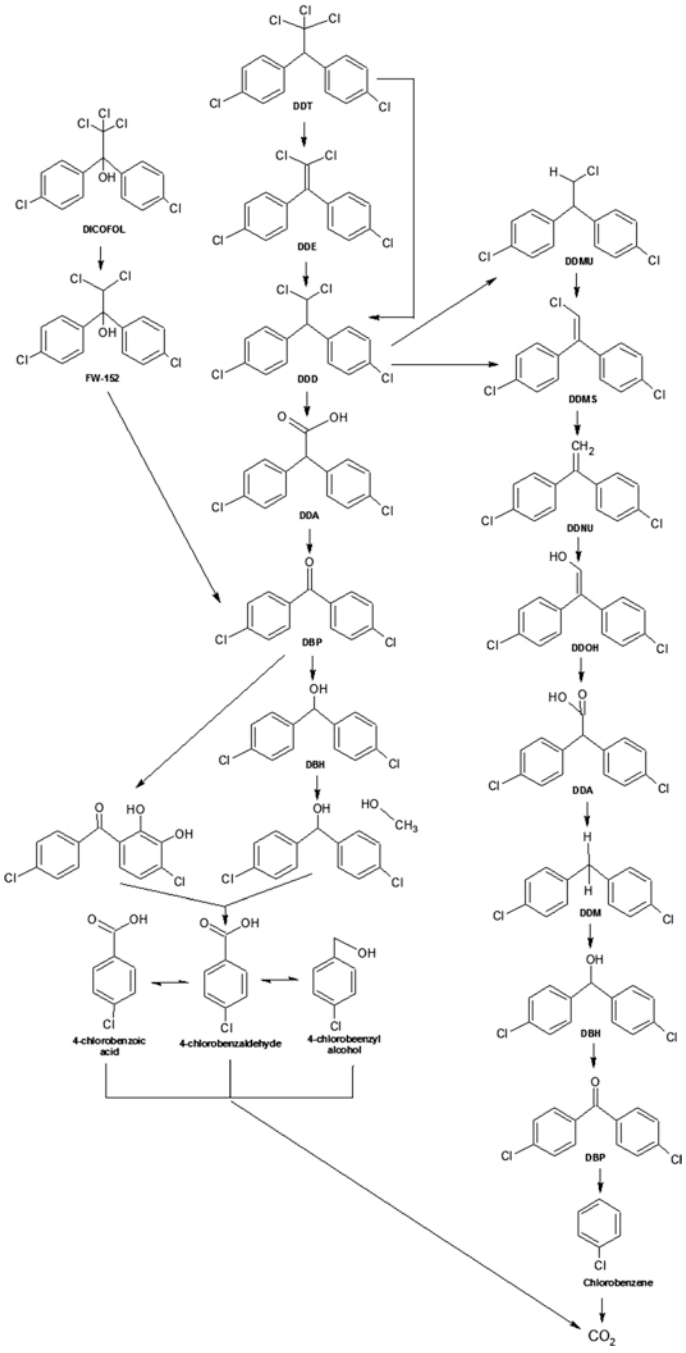


Fig. 7.4 Fungal degradation and complete mineralization of DDT and its metabolites (Modified from Maqbool et al. 2016) *Abbreviation*: *DDT* 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane,

The WRF *P. sordida* YK-624 degraded acetamiprid in liquid media, which was enhanced under ligninolytic conditions compared to that achieved under non-ligninolytic ones. The degradation of the insecticide was more in nitrogen-limited media than in the potato dextrose broth medium though the reverse was reported with respect to growth (Wang et al. 2012b). This showed that for the degradation of acetamiprid, ligninolytic enzymes (LiP and MnP) were not utilized, as it was also eliminated under the non-ligninolytic conditions provided by the PDB medium (Wang et al. 2012b).

Mori and Kondo (2002) reported that the *Phlebia lindtneri* could mineralize 2,7-dichlorodibenzo-*p*-dioxin through hydroxylation. It was also reported that *P. lindtneri* and *Phlebia brevispora* are capable of hydroxylating and methoxylating 2,3,7-trichlorodibenzo-*p*-dioxin, 1,2,8,9-tetrachlorodibenzo-*p*-dioxin, 1,2,6,7-tetra chlorodibenzo-*p*-dioxin and 3,6,8-tetrachlorodibenzo-*p*-dioxin (Kamei and Kondo 2005; Kamei et al. 2005). Kamei et al. (2010) also described the bioconversion of dieldrin by WRF producing 9-hydroxydieldrin as a metabolite by a strain YK543, which was closely related to the fungus *P. brevispora* Nakasone TMIC33929. Some studies have demonstrated that several species of WRF metabolized lindane via hydroxylation reactions but there is a significant difference in the rate of degradation of different isomers, when applied in combination (Mougin et al. 1996; Mori and Kondo 2002; Kamei et al. 2005). Mougin et al. (1996) observed that the degradation of γ-HCH on polluted soils by WRF was limited to levels below 25%, while in liquid phase, this isomer was degraded in a range between 34% and 90%. This indicates that the transfer of the pollutants from the soil to the aqueous phase has a limiting role in degradative removal.

P. ostreatus BM9073 was used as a bioremediation agent for degradation of aldrin and could cause almost 25% and 72% degradation of the initial amount in LN and HN media, respectively, whereas complete degradation was achieved in PDB medium during the 14-day incubation period (Purnomo et al. 2017). On the other hand, slow rate of degradation of dieldrin was observed with WRF though PDB medium effectively supported the fungus (Purnomo et al. 2017). This suggested that systems other than the ligninolytic system were responsible for degradation of aldrin and dieldrin. *P. chrysosporium* has also been shown to mineralize 9.4–23.4% of aldrin and dieldrin over 30 days (Kennedy et al. 1990). In an experiment on 20 white-rot fungi belonging to genus *Phlebia* to test their ability to degrade aldrin, Xiao et al. (2011) found that three *Phlebia* strains *P. acanthocystis*, *P. brevispora* and *P. aurea* were able to degrade aldrin. They exhibited higher levels of degradation activity (over 90%) forming several new metabolites including 9-hydroxyaldrin and two carboxylic acid products. Similarly the fungal species were also able to degrade about 50% of applied dieldrin, which was considered to be due to hydroxylation of the parent compound. These results indicate that the methylene moiety of dieldrin molecules might be prone to enzymatic attack by WRF. Proposed pathway of degradation of aldrin and dieldrin by WRF is presented in Fig. 7.5.

Diverse types of microbes, such as *Pseudomonas* sp. KS-2P, *Aspergillus* sp., *Trichoderma harzianum*, *P. chrysosporium*, *Trametes hirsute*, *Pleurotus* spp., etc., contribute to the degradation of endosulfan (Ang et al. 2005). Endosulfan is first

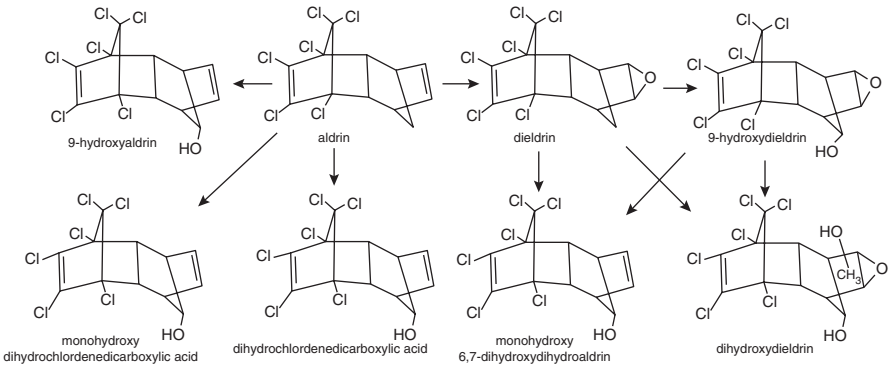


Fig. 7.5 Proposed pathway of degradation of aldrin and dieldrin by WRF. The figure represents important metabolites only (Modified from Xiao et al. 2011)

metabolized to endosulfan sulphate or endosulfan diol, followed by further degradation from endosulfan diol to endosulfan ether (EE) and endosulfan lactone (Fig. 7.6). Yang et al. (2002) studied the degradation of eight insecticides (endosulfan, malathion, diazinon, dimethoate, permethrin, captan, propiconazole and chlorothalonil) during composting and found that factors such as temperature and pH affect the stability of these insecticides. Kullman and Matsumura (1996) detected endosulfan sulphate as a metabolite of endosulfan biotransformation as found in bacterial degradation. Kataoka et al. (2010) isolated *Mortierella* sp. which could degrade α- and β-endosulfan by more than 70% and 50% of, respectively, over 28 days at 25 °C.

Study on the degradation power of white-rot fungi *Trametes hirsute* showed that the organism is able to degrade endosulfan quite effectively as compared to other species (Kamei et al. 2011) The biggest problem with the degradation of these chemicals, however, was the accumulation of endosulfan sulphate which inhibit the degradation process, but in case of *T. hirsute*, the accumulation rate of this intermediate product was very low (almost 2% after 7 days of inoculation with the organism). During 10 days of incubation, the organism was able to degrade almost 70% of the parent chemical with a very low accumulation of the degradable product. There were three degradation pathways used by *T. hirsute* for the degradation of endosulfan. One of the degradation pathways is the hydrolytic production of endosulfandiol from endosulfan. Secondly the hydrolytic production of endosulfandiol followed the oxidative production of endosulfan sulphate. The third and final pathway is the production of endosulfan dimethylene from endosulfan sulphate, although the enzyme(s) involved in the formation of endosulfan dimethylene are still unknown. However, the result obtained suggests an additional degradation pathway resembling the oxygenation of endosulfan sulphate by *Arthrobacter* sp. strain KW whose evidence are obtained from the non-detection of endosulfan dimethylene in culture with the hydrolytic metabolite endosulfandiol. This makes *T. hirsute* a very good agent for the degradation of endosulfan (Kamei et al. 2011). A study on the edible basidiomycete *Pleurotus pulmonarius* ECS-

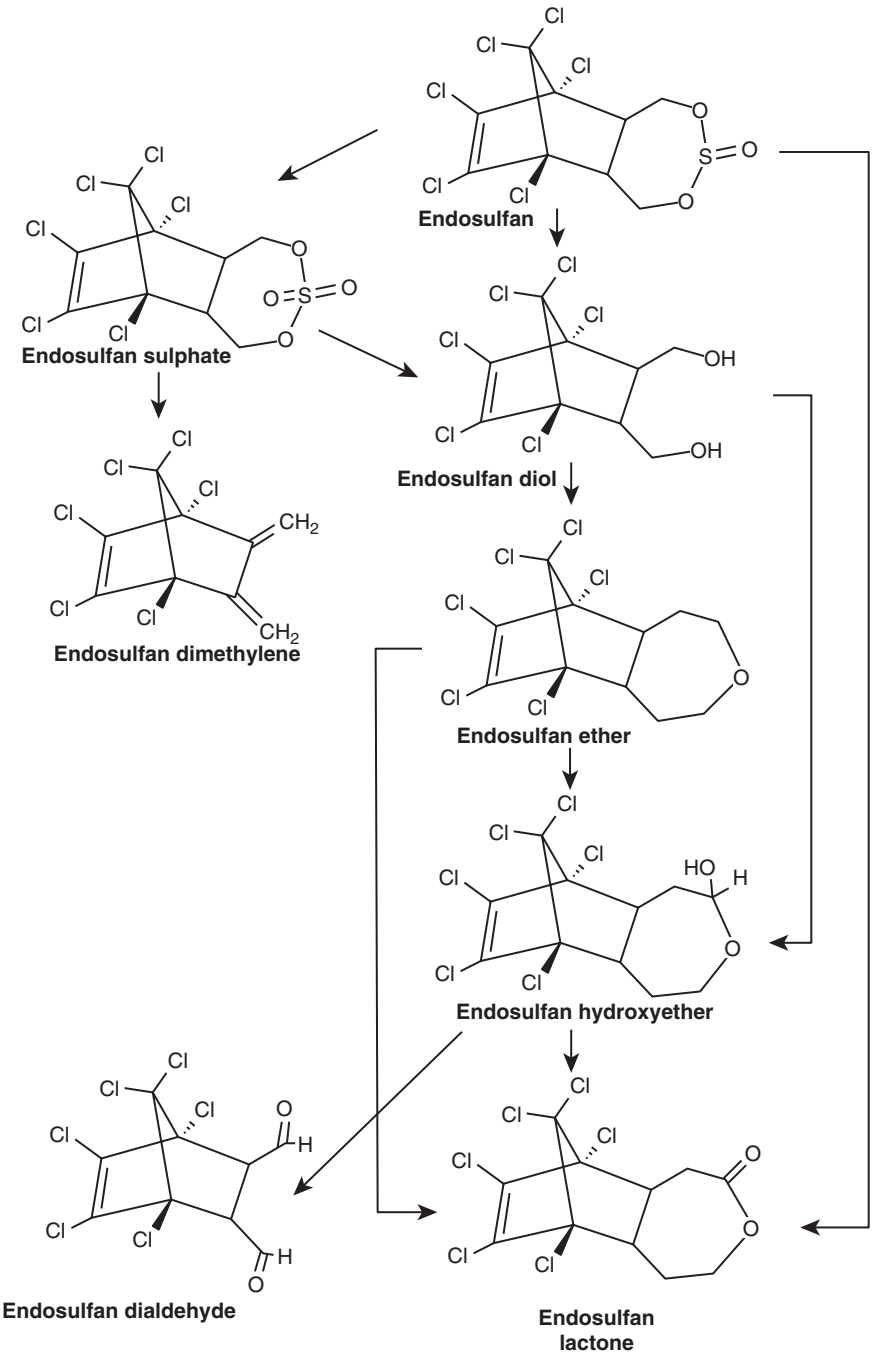


Fig. 7.6 Proposed pathway of degradation of endosulfan and endosulfan sulphate by WRF (Modified from Maqbool et al. 2016)

0190 showed that the fungus has degradation ability for endosulfan. The degradation mostly took place during the colonization of substrate during the incubation period of 1–15 days as more than 95% of the pesticide was degraded during this period and it reached 99% during fruiting. Final study showed degradation of 99.85% of the 65 mg/kg of endosulfan initially added to the grass as substrate (Hernández-Rodríguez et al. 2006). Similarly *Bjerkandera adusta* (CCM0379) was able to degrade 83% of (α+β) endosulfan after 27 days of incubation producing endosulfan diol, endosulfan ether and endosulfan sulphate, which proved the biodegradation ability of the organism to degrade the chemicals and its residues (Rivero et al. 2012).

Degradation of lindane has been very well studied under different growth conditions and nutrient regimes using different species of WRF. Laccase and manganese peroxidase have been associated with optimum biodegradation of lindane by *P. ostreatus*, and temperature optimum was considered as the important regulating factor (Rigas et al. 2005). Rigas et al. (2007) have reported the latent potential of *Ganoderma* spp. in degrading lindane in liquid-agitated cultures and found the species to be more or less effective to remove lindane. In their study, lindane incorporation was found to be inhibitory for production of ligninolytic enzymes in terms of the specific activity by *G. lucidum* when supplemented at higher concentrations. In *Ganoderma australe*, the growth was a function of temperature, moisture and inorganic nutrients such as nitrogen and phosphorus; however, the incorporation of lindane had a toxic effect (Rigas et al. 2007). On the other hand, Dritsa et al. (2009) observed that the optimum lindane degradation obtained for *G. australe* was 3.11 mg lindane/g biomass when the system was provided with 1.28 gL^{-1} nitrogen content and 7.0 mg/L lindane at 18 °C for 5 days. Similarly in the same fungus grown at 28 °C on PDA medium supplemented with 0.5% lindane (w/w), the lindane was degraded by 61% (Hussaini et al. 2013). Quintero et al. (2008) reported that among the various WRF tested, *Bjerkandera adusta* showed 42.4% and 87% degradation of γ-lindane in liquid cultures and sandy soil system, respectively.

G. lucidum strain GL-2 grown on rice bran as substrate for ligninolytic enzyme induction showed sufficient amounts of ligninolytic enzymes in liquid-state fermentation for maximum lindane degradation (Kaur et al. 2016). Mougin et al. (1996) reported *P. chrysosporium* degrading lindane and partially mineralizing the molecule, and for the first time, the main lindane metabolites were characterized in liquid medium, but Kennedy et al. (1990) opined that LMEs are not involved in lindane degradation. Singh and Kuhad (1999) compared *Trametes hirsutus* with *P. chrysosporium* to degrade lindane in liquid culture and found the former to be more effective though the mechanism of degradation appears to be the same. Two metabolites identified in both fungi were tetrachlorocyclohexane and tetrachlorocyclohexanol.

Several WRF belonging to the genus *Phlebia* have been reported to be capable of degrading heptachlor and heptachlor epoxide (Xiao et al. 2011). Heptachlor epoxide, the most predominant metabolite of heptachlor, is more stable than heptachlor and the other metabolites (Lu et al. 1975) (Fig. 7.7). *Phlebia aurea*, *Phlebia brevispora*, *Phlebia acanthocystis* and *Phlebia lindtneri* showed the highest abilities to degrade heptachlor among 18 species of *Phlebia* (Xiao et al. 2011) and *Pleurotus*

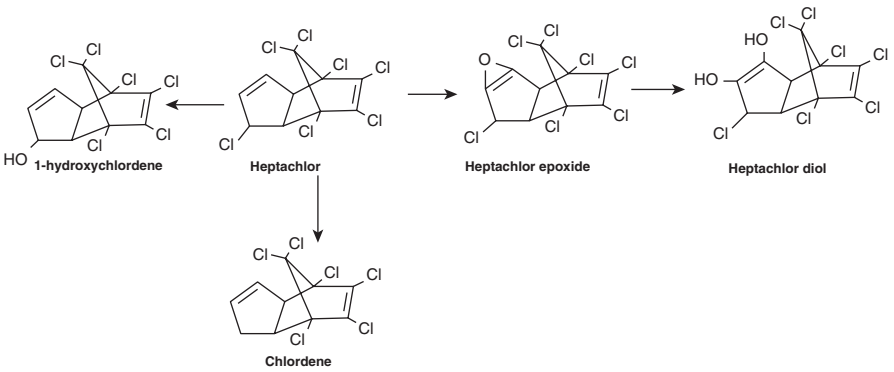


Fig. 7.7 Proposed pathways of degradation of heptachlor and heptachlor epoxide (Modified from Purnomo et al. 2014)

ostreatus (Purnomo et al. 2013, 2014). Specifically, *Phlebia acanthocystis* and *Phlebia aurea* were most effective in degrading heptachlor and heptachlor epoxide, respectively. Different heptachlor epoxide degradation amounts among the *Phlebia* fungi were not significantly different (Purnomo et al. 2014). Xiao et al. (2011) reported that *Phlebia aurea* more readily degraded heptachlor epoxide than heptachlor, while the ability of *Phlebia acanthocystis* to degrade heptachlor epoxide was poor. Further investigations have shown that *Pleurotus ostreatus* completely eliminated heptachlor in Potato Dextrose Broth (PDB) and high-nitrogen (HN) media, while approximately 31% of heptachlor epoxide was eliminated in PDB medium during a 14-day incubation time (Purnomo et al. 2013). This showed that inocula of WRF are highly capable of degrading heptachlor in soil as well as in liquid media (Xiao et al. 2011; Purnomo et al. 2013). Purnomo et al. (2013, 2014) reported that *Pleurotus ostreatus* readily degrades heptachlor into the less toxic metabolite chlordane and could degrade heptachlor epoxide into the less toxic metabolite heptachlor diol.

7.6 Conclusion

Recently, bioremediation conducted on a commercial scale has utilized bacteria, with only few attempts to use WRF for their ability to tolerate high concentrations of polluting chemicals than bacteria. The research achievements presented in this chapter clearly indicate that quite a good number of efficient WRF are available to cause metabolic and cometabolic degradation of insecticides, even at environmentally high concentrations. Therefore, WRF represent a prospective tool in environmental bioremediation. Further to our knowledge, bioremediation processes using bacteria and WRF in sequence have been proved more effective with the syntrophy between the microbial groups. Such protocols can be optimized and upgraded to commercial scale.

During the recent days, upgradation of fungal strains through molecular approach has been found as an important tool for inducing and improving the pesticide-degrading potential. Transformation through restriction enzyme-mediated integration with different types of plasmids has been found to improve the potential of fungal species for biodegradation of organophosphate pesticides. Observations on the *P. chrysosporium* genome have revealed that induction of cytochrome P450 gene clusters is differentially expressed depending on xenobiotic type and nutrition and significantly increased the degradation ability of the fungal cultures. Such approaches in strain upgradation may encourage the use of fungal species singly or as consortium in commercial bioremediation of insecticides.

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Chapter 8
An Overview of Fungal Applications in the Valorization of Lignocellulosic Agricultural By-Products: The Case of Two-Phase Olive Mill Wastes

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Abbreviations

AA	Auxiliary activity
AAO	Aryl-alcohol oxidase
ADOR	Aqueous extract of DOR
AO	Alcohol oxidases
CAZy	Carbohydrate active enzymes
CBM	Carbohydrate-binding modules
CDH	Cellobiose dehydrogenase
CE	Carbohydrate esterase
CiP	*Coprinopsis cinerea* peroxidase
CMC-ase	Carboxymethylcellulase
CytP450	Cytochrome P450 monooxygenases

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DyP	Dye-decolorizing peroxidases
DOR	Dry olive mill residue
GH	Glycoside hydrolase
GLOX	Glucose oxidase
GLX	Glyoxal oxidase
GR	Glutathione reductase
GST	Glutathione-S-transferase
GT	Glycosyltransferase
Lac	Laccase
LiP	Lignin peroxidase
LME	Lignin-modifying enzyme
LPMO	Lytic polysaccharide monooxygenase
MnP	Manganese peroxidase
OXO	Oxalate oxidase
PL	Polysaccharide lyase
PODs	Class II peroxidases
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SF	Submerged fermentation
SSF	Solid-state fermentation
TPOMW	Two- phase olive mill waste
UPO	Unspecific peroxygenase
VP	Versatile peroxidase
WRF	White-rot fungi

8.1 Introduction: Lignocellulose

Lignocelluloses, the principal component of the cell wall, represent a class of polymers of considerable structural diversity composed by three polymeric constituents: the recalcitrant lignin, the highly crystalline cellulose and the short and branched hemicellulose, as well as a small amount of extractives (McKendry 2002). The amount of each of these components varies, depending on the origin of the biomass. The lignin complex forms a matrix surrounding the cellulose in woody cell walls which protects both hemicellulose and cellulose from microbial oxidation. These components are strongly intermeshed and bonded through covalent or non-covalent bonds forming the lignocellulosic matrix and account for half of the carbons fixed by photosynthesis (Sun et al. 2016).

Cellulose is the main component of plant biomass and, therefore, the most abundant compound on Earth. It consists of a fibrous and water-insoluble substance made of parallel linear chains of β-D-glucopyranose units linked by (1→4) glycosidic bonds. These glucans fit perfectly to form a crystalline microfibril that is mechanically strong and highly resistant to enzymatic attack (Cosgrove 2005). Cellulose is found in the protective cell walls of plants, particularly in woody portions (O'Sullivan 1997). Hemicellulose is the second most abundant polysaccharide

on lignocellulosic biomass. It is a heterogeneous polymer of pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose) and sugar acids (Mosier et al. 2005). Hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses principally contain glucomannans. Hemicellulose varies in quantity and composition between the different cell types and between the tissues of an individual organism. Lignin is linked to both hemicellulose and cellulose, forming a physical seal that is an impenetrable barrier in the plant cell wall (Cosgrove 2005). On the whole, it is a heterogeneous three-dimensional network macromolecule mainly constructed by the oxidative combinatorial coupling of three phenylpropanoid alcohol monomers, that is, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, which provide rigidity to cell walls (Eudes et al. 2014). Lignin is a complex macromolecule as a result of the variety of random linkages among the phenylpropanoid subunits (Ralph et al. 2004). The heterogeneity and complexity of the lignocellulose components and the rigidity and compatibility of the resulting cell wall structure are the main factors that contribute to its recalcitrance. However, the degradation of lignocellulose constitutes a natural process of organic matter recycling in which different fungi and bacteria are involved (Daniel 2003).

8.2 Lignocellulosic Agricultural By-Products

Approximately 90% of the global plant biomass production (200×10^9 tons/year) consists of lignocelluloses, with approximately $8–20 \times 10^9$ tons of this primary biomass being potentially accessible (Kuhad and Singh 1993). The correct management of this biomass is especially relevant in today's global economy, in which agro-industrial activity, including forestry, pulp and paper industry and food industry, has generated a serious environmental problem in producing countries, resulting in the generation of enormous amounts of underused lignocellulose wastes that, in most cases, are either left in fields or burned (Sánchez 2009). In Spain, the agricultural commodities produced in largest quantities include barley, olives, wheat, grapes, maize, wine, tomatoes and oranges (http://faostat3.fao.org/home/E). The production of these commodities generates large amounts of lignocellulose residues.

In the present decade, one of the main challenges for researchers globally has been the search for effective waste management solutions. One possible solution is based on the usage of lignocellulosic by-products as feedstock, source of chemicals, an amendment and/or an energy resource, given the large quantities of lignocellulose available and its renewable nature (Sánchez 2009).

8.3 "Alpeorujo" Dry Olive Residue (DOR)

The olive oil industry is of great social, economic and ecological importance in Mediterranean countries, with approximately 98% of global olive oil production being concentrated in this region. Within this area, European Union countries

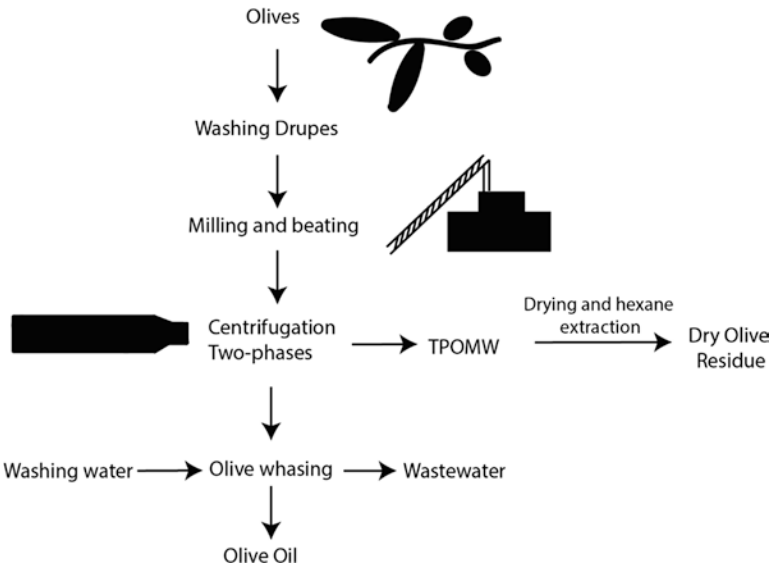

Fig. 8.1 Diagram of the olive oil extraction process using the two-phase olive oil extraction system

produced 2,322,000 tons of olive oil in 2015/2016 [International Olive Council (http://www.internationaloliveoil.org/)]. These data clearly show the massive challenge the industry is facing with respect to waste management. In the early 1990s, the two-phase olive oil extraction system was introduced, replacing the traditional three-phase system, which required higher water consumption levels. This technology yields 200 kg of oil per 1000 kg of olives and generates 800 kg of a two-phase olive mill waste (TPOMW), also known as "alpeorujo" (Fig. 8.1).

TPOMW has high moisture content, slightly acidic pH values and very high organic matter content, mainly composed of lignin, hemicellulose and cellulose, as well as a considerable amount of fats, proteins, water-soluble carbohydrates and a small but active fraction of hydrosoluble phenolic substances (Alburquerque et al. 2004). The phenolic composition can vary depending on the campaign, extraction and analytical methods employed, but the major phenolic acids that are present in TPOMW include tyrosol, oleuropein, catechin and phenolic acids such as *trans*-cinnamic, caffeic and 3,4-dimethoxibenzoic acids (Priego-Capote et al. 2004). The harmful effects of TPOMW have been attributed to the phenols including phytotoxic effects. Phenol compounds are known to have mutagenic, genotoxic and cytotoxic effects at low concentrations in a wide range of microorganisms. Due to their composition, TPOMW also produce short-term negative alterations in the physicochemical properties of soil (Aranda et al. 2006; Justino et al. 2012; Di Bene et al. 2013; Siles et al. 2015).

8.4 Valorization of Olive Mill Wastes

Recent studies have focused on the treatment and valorization of phytotoxic wastes such as DOR by regarding them as renewable resources instead of as residues.

TPOMW undergoes a drying process after which residual oil is extracted using organic solvents such as hexane, leading to the generation of a low-quality oil and dry olive residue (DOR) (López-Piñeiro et al. 2008). With its high caloric strength (400 kcal kg^{-1}), DOR is used to generate thermal or electric energy by means of combustion (Fig. 8.2). In most cases, this energy is used to operate olive oil plants (Roig et al. 2006). However, current international regulations place a limit on the use of this type of combustible material since it produces environmentally harmful gases (Sampedro et al. 2009). Thus, there are two main valorization approaches to DOR.

The first approach is the extraction of valuable phytochemical compounds with beneficial properties. Examples include phenolic compounds such as hydroxytyrosol, tyrosol and caffeic acid, which hold promising potential given their antioxidant, anti-inflammatory, cardioprotective, antihypertensive, antimicrobial and antitumoral properties and could therefore be used in the pharmaceutical, cosmetic and food industries (Araújo et al. 2015). DOR has also been considered as a source of valuable products such as dietary fibres, polymer fatty acids, squalene, tocopherols, mannitol and surfactin (Roselló-Soto et al. 2015). New approaches are currently being developed in order to make the extraction of these components more effective (Mateo and Maicas 2015).

The second valorization approach consists of bioconversion procedures based on the use of by-products to grow microorganisms and benefit from them. In this sense, the aforementioned by-products are suitable substrates for obtaining biofuels (e.g. bioethanol, biomethane), prior to adequate microbiological treatment (Dermeche et al. 2013). Another strategy proposed for DOR is its use as a form of high-quality, low-cost substrate for cultivation of the edible mushroom *Agaricus bisporus*

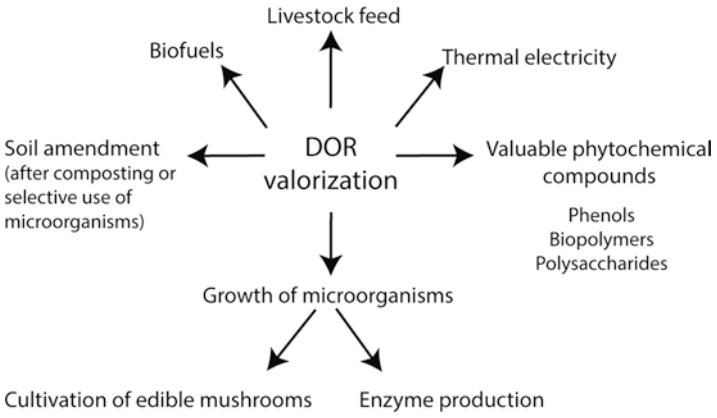

Fig. 8.2 Valorization of dry olive mill residue

(Parati et al. 2011). DOR is also suitable for livestock feed after being mixed with protein supplements or pretreatments (Moumen et al. 2008). In addition, it is interesting to highlight that this residue could be used as a source of substances of biotechnological interest, such as enzymes, which are produced during fungal secondary metabolism (Reina et al. 2014). By using this low-cost fermentation substrate for the growth of the fungi, it is possible to obtain dephenolized and depolymerized products useful in agriculture (Reina et al. 2013) or biopolymers and microbial biomass which could be used in the food industry (Morillo et al. 2009) (Fig. 8.2).

In an alternative agronomic valorization scenario, these bioconverted residues could be added as an organic amendment to soil or act as biosorbent of heavy metals (Hovorka et al. 2016; Reina et al. 2017). This approach could be beneficial to soil due to the high organic matter content of the residues, the absence of heavy metals and their content in some cations such as K, P and Ca (López-Piñeiro et al. 2008). Composting processes using different mixture combinations have been used to obtain safe amendments which require long time for stabilization (Tortosa et al. 2012). However, some studies suggest that the potential phytotoxicity of these residues can be reduced by fungal treatment shortly after fermentation, when these toxic wastes can be transformed into natural fertilizers (Sampedro et al. 2009; Aranda et al. 2012).

The ligninolytic and hydrolytic enzymes seem to be a promising tool for solving numerous environmental problems. However, their industrial application is limited by the high cost, limited operational stability and low output of these enzymes when synthetic media are used. Several soiled renewable substrates have been used to enhance enzyme production, including olive oil residue (Díaz et al. 2010; Sampedro et al. 2012). All these alternatives could be regarded as involving biorefinery processes (Hasunuma et al. 2013).

In this section, we have addressed the participation of wood-rotting fungi in the transformation of by-products and their use for enzyme production as well as organic soil amendments. We discuss the possible trends, challenges and future prospects for the use of fungi in an environmentally sustainable scenario.

8.5 Fungi in the Degradation of Lignocellulose

8.5.1 Wood-Decay Fungi

Wood-decay fungi are among the few organisms which are able to hydrolyse lignocellulose into simple sugars by means of enzymatic and non-enzymatic mechanisms. According to the decay patterns observed during the deconstruction of lignocellulose, they are commonly classified into brown-rot, soft-rot and white-rot fungi. Brown-rot fungi have been considered capable of degrading all the polysaccharides of plant cell walls and partially depolymerizing lignin with the aid of enzymatic and mainly non-enzymatic mechanisms. The latter include free radical systems which can act as oxidants driven by Fenton reactions (e.g. hydroxyl

radicals) (McKendry 2002; Arnstadt et al. 2016). Soft-rot fungi, which included several decay *Ascomycota*, are characterized by extensive degradation of carbohydrates mediated by hydrolytic enzymes (Liers et al. 2011). By contrast, white-rot fungi are known to degrade all polymers of the lignocellulosic complex involving a battery of lignin-modifying enzymes such as peroxidases and laccases, as well as a large set of (hemi)cellulases (Van Den Brink and De Vries 2011). The classification of white-/brown-/soft-rot fungi in terms of their degradation mechanisms has been used extensively (Liese 1970). However, the advent of a large number of sequenced fungal genomes has led to the appearance of complex molecular phylogenetic analyses which have shown that this classification is not sufficiently related to the different fungal metabolisms (Floudas et al. 2015).

8.5.2 Enzymatic Mechanisms for the Degradation of Lignocellulose

The diversity of enzymes involved in the assembly and cleavage of complex carbohydrates are included in the carbohydrate-active enzymes (CAZymes) classification (http://www.cazy.org/). CAZymes classification has become the standard of the field (Lombard et al. 2014). It describes structurally related families of enzymes that cleave or build complex carbohydrates: glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE), glycosyltransferases (GT) and their appended non-catalytic carbohydrate-binding modules (CBM) (Box 8.1). The deconstruction of lignin remains essential since it is intimately associated with plant cell wall polysaccharides. This classification includes eight families of ligninolytic auxiliary activities (AAs) and peroxide-producing enzymes (Lombard et al. 2014) (Box 8.2). Recently, four additional families of lytic polysaccharide monooxygenases (LPMOs), which are responsible for the oxidative breakdown of cellulose polysaccharides, have been added. The finding of these new catalysts has opened the debate of whether the degradation of polysaccharides is only caused by hydrolytic enzymes (Hemsworth et al. 2014).

In addition, there are heme peroxidases which do not fit in the CAZy classification since their role in ligninolysis has not yet been well defined. These are unspecific peroxygenase (UPO; EC 1.11.2.1) and dye-decolorizing peroxidase (DyP; EC 1.11.1.19). UPOs can act as functional hybrids of peroxidases and cytochrome P450 monooxygenases (CytP450) (Ullrich et al. 2004). UPO genes are widely found in various orders of agaricomycetes and also in ascomycetes (Hofrichter et al. 2015). However, to date, the purification of wild-type UPOs has only been achieved in few agricultural fungi: *Coprinellus radians*, *Coprinus verticillatus* (Anh et al. 2007), *Marasmius rotula* (Gröbe et al. 2011) and several *Cyclocybe* sp. (formerly *Agrocybe* sp.) (Ullrich et al. 2004). DyPs are very different from class II peroxidases and lack the typical heme-binding region. In fungi, they have so far been isolated from *Auricularia auricula-judae*, *Bjerkandera adusta*, *Exidia glandulosa*, *Irpex lacteus*, *Marasmius scorodonius*, *Mycena epipterygia* and *Termitomyces albuminosus* cul-

Box 8.1 Hydrolytic CAZymes and Associate Modules

Glycoside hydrolase (GH; EC 3.2.1)

Glycoside hydrolases are responsible for catalysing the glycolytic cleavage of O-glycosidic bonds. The activities represented in this group are endo-β-1,4-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21) and endo-β-1,4-xylanase (EC 3.2.1.8). Some GH families contain enzymes characterized by different hydrolytic activities: for instance, GH1 have up to 17 different enzyme activities. Moreover, the number of gene models for each GH family varies in white-rot fungi (Várnai et al. 2014).

Polysaccharide lyase (PL; EC 4.2.2)

Polysaccharide lyases are enzymes that cleave uronic acid-containing polysaccharides via a β-elimination mechanism to generate an unsaturated hexenuronic acid residue and a new reducing end at the point of cleavage. Eliminative cleavage complements the GH hydrolysis strategy; PLs can be found in a wide range of different microorganisms and are involved in processes such as biomass degradation, tissue matrix recycling and pathogenesis (Lombard et al. 2010).

Carbohydrate esterase (CE; EC 3.1)

Carbohydrate esterases catalyse the O- or N-deacylation of substituted saccharides, e.g. esters or amides in which sugars play the role of alcohol and amine. These enzymes exhibit great substrate specificity. Within the CE family, activities, such as feruloyl esterases or pectin methylesterases, are represented (Biely 2012).

Glycosyltransferase (GT; EC 2.4)

Glycosyltransferases are implicated in the biological synthesis of complex carbohydrates and polysaccharides (Bertozzi and Kiessling 2001). GTs transfer monosaccharide fractions from activated donor molecules to biological molecules. They can be classified as either retaining or inverting enzymes according to the stereochemistry of the substrates and reaction products.

Carbohydrate-binding module (CBM)

CBMs are additional modules which can be appended to the catalytic module of cellulolytic and hemicellulolytic enzymes. Their role consists in binding complex carbohydrates (Medie et al. 2012). The majority of the GHs carry a CBM attached to the catalytic core domain by a flexible linker (Teeri et al. 1992). These modules were thought to potentiate the activity of the catalytic modules by targeting the enzyme to a specific component of the cell wall (Valadares et al. 2016).

Box 8.2 Auxiliary Activities

AA1. Laccase (Lac; EC 1.10.3.2)

Laccases belong to the group of blue oxidases and represent the main subgroup of multicopper oxidases. They have a variable range of oxidable substrates including phenols, aromatic amines and ascorbate (Madhavi and Lele 2009). Lacs are ubiquitous enzymes which can be found in almost all fungi that produce wood decay (Baldrian 2006) and, more commonly, in higher basidiomycetes such as *Trametes* sp., *Funalia floccosa (*formerly *Coriolopsis rigida)*, *Lentinus tigrinus* and *Pleurotus* sp., among others (Yaver et al. 1996; Collins and Dobson 1997; Muñoz et al. 1997; Leitner et al. 2002; Dong and Zhang 2004; Cadimaliev et al. 2005; Lyashenko et al. 2006; Polyakov et al. 2009; Díaz et al. 2010).

AA2. Class II peroxidases (PODs; EC 1.11.1)

Three high-redox peroxidases belong to this group: manganese peroxidase (MnP; EC 1.11.1.13), lignin peroxidase (LiP; EC 1.11.1.14) and versatile peroxidase (VP; EC 1.11.1.16) and other low-redox potential peroxides, the so-called generic peroxidases such as *Coprinopsis cinerea* peroxidase (CiP; EC 1.11.1.7). These enzymes are oxidized by an organic peroxide or H_2O_2 to form the compound I, a Fe^{4+}-oxo-porphyrin radical which undergoes to consecutive electron reductions to recover its native state. The electron donors differ in PODs: for MnPs, they are Mn^{2+} ions; for LiPs, they are aromatic compounds; and for VPs, they are either Mn^{2+} ions, phenolics or non-phenolic aromatic compounds (Camarero et al. 1999).

MnPs have a conserved solvent-exposed Mn^{2+}-binding site consisting of three acidic amino acid residues: Glu35 and Glu39 and Asp179. The oxidation of Mn^{2+} ions leads to an indirect Mn^{3+}-chelate-driven oxidation of phenols, non-phenolics and dyes. There are typical long- and short-type MnPs. The latest lack the exposed tryptophan (Hofrichter et al. 2010). Long MnPs can be found in the majority of the white-rot species, whereas short MnPs only appear in a few fungi such as *P. radiata* or *Cyclocybe praecox* (Hildén et al. 2005; Hildén et al. 2014).

LiPs have the oxidation site located in a tryptophan residue: Trp171 (Choinowski et al. 1999). These enzymes are the strongest oxidizers, and they are mainly secreted by higher white-rot species, for example, *Trametes versicolor* (Johansson and Nyman 1993), *Phlebia radiata* (Lundell 1993) and *Phanerochaete sordida.* They are able to directly convert phenolics, high-redox potential methoxylated aromatics and dyes (Sugiura et al. 2003).

VPs combine the catalytic properties of MnPs and LiPs (Camarero et al. 1999; Salvachúa et al. 2013b). They contain aTrp164 (equivalent to Trp171 residue in LiPs) and in the Mn^{2+}-binding site (Ruiz-Dueñas et al. 2008). To date, they have only been described in a limited number of *Basidiomycota*

(continued)

Box 8.2 (continued)

species from the genera *Bjerkandera* and *Pleurotus* (Ruiz-Dueñas et al. 2001; Moreira et al. 2005).

AA3 and AA5. Peroxide-producing enzymes (EC 1.1.3, 1.2.3 and 1.1.1.9)

Peroxide generation is necessary for the ligninolytic PODs activity and also for the formation of oxy-radicals that participate in non-enzymatic ligninolytic mechanisms (Roy et al. 1994). These families of enzymes include glyoxal oxidase (GLX; EC 1.1.3), aryl alcohol oxidase (AAO; EC 1.1.3.7), glucose oxidase (GLOX; EC 1.1.3.4), oxalate oxidase (OXO; EC 1.2.3.4) and cellobiose dehydrogenase (CDH; EC 1.1.99.18) (Lombard et al. 2014).The latest is the only extracellular fungal flavocytochrome known to date. This enzyme enhances cellulose degradation by coupling the oxidation of cellobiose to the reductive activation of copper-dependent lytic polysaccharide monooxygenases (LPMOs) (Sygmund et al. 2013).

AA9, AA10, AA11 and AA13 lytic polysaccharide monooxygenases (LPMOs; EC 1)

Formerly known as a GH61, these enzymes participate in cellulose degradation via oxidative mechanisms (Jung et al. 2015). LPMOs are widely distributed in the genome of most ascomycetes and basidiomycetes.

tures (Kim and Shoda 1999; Johjima et al. 2003; Scheibner et al. 2008; Liers et al. 2010, 2013; Salvachúa et al. 2013a). They are able to directly oxidize hardly convertible phenols (e.g. p-nitrophenol), azo dyes (e.g. Reactive Black 5), ascorbic acid, veratryl alcohol and even lignin model dimers (Kim and Shoda 1999; Scheibner et al. 2008; Liers et al. 2010; Yoshida et al. 2012; Salvachúa et al. 2013a; Linde et al. 2015). However, their participation in the transformation of plant biomass and/or soil organic matter remains unclear.

The fungal degradation of lignocellulose is accomplished by the synergistic action of a batch of hydrolases and oxidoreductases enzymes. The breakdown of the lignin barrier by oxidative mechanisms is a necessary precondition for the cleavage of the cellulose polymer by hydrolytic or oxidative enzymatic systems (Fig. 8.3).

8.6 Valorization of Dry Olive Waste for the Recovery of Enzymes of Biotechnological Interest

Lignocellulolytic enzymes have been attracting a great interest regarding their potential environmental and biotechnological applications such as the clean-up of xenobiotics (e.g. lacs, UPOs) or for obtaining biofuels. Cellulases, hemicellulases,

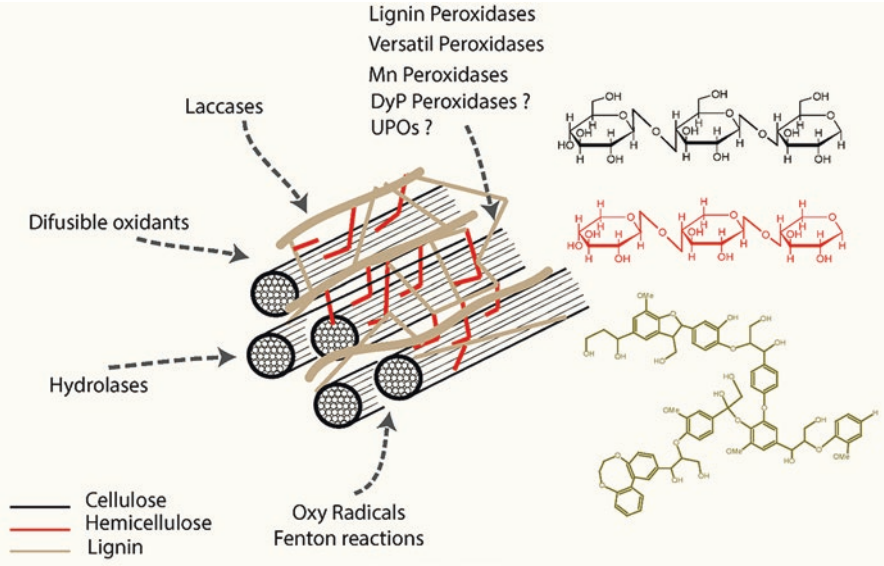

Fig. 8.3 Fungal degradation of lignocellulose

ligninases and pectinases are interesting for biorefinery processes since they perform saccharification reactions for the subsequent production of ethanol by fermentation (Salgado et al. 2014). Therefore, the optimization of integral and affordable strategies for the production of lignin-modifying and carbohydrate-active enzymes is a major environmental issue (Kües 2015). The production of ligninolytic enzymes occurs during the second metabolism and is usually triggered by nitrogen or carbon depletion. When the secretion is regulated by carbon limitation, it is usually done via the cAMP mechanism: significant increases in intracellular cAMP concentrations precede the production of MnPs and LiPs in *Phanerochaete chrysosporium* (Mac Donald et al. 1984).

There is a lot of evidence supporting the use of lignocellulosic materials as elicitor substrates for lignocellulolytic enzyme secretion in filamentous fungi. The addition of agricultural by-products to fungal cultures may reflect complex growth conditions close to nature and can stimulate the secretion of various of these enzymes (Girard et al. 2013). The recently discovered bifunctional GH78 of *Xylaria polymorpha*, which is capable of hydrolysing ester and glycosidic bonds, is only secreted in wheat straw and in liquid orange peel-containing media (Nghi et al. 2012). The presence of DyPs in cultures enriched with lignocellulosic and agro-waste ingredients has also been shown by using wheat straw and eucalypt pulp (Salvachúa et al. 2013a; Yu et al. 2014). DyP from *A. auricula-judae* has only been purified when this fungus was cultivated in tomato juice media and *Cyclocybe aegerita* UPO from soybean meal suspensions (Ullrich et al. 2004; Liers et al. 2010). Lacs from both basidio- and ascomycetes have been produced in complex plant-based media and are inducible by the addition of aromatic substances (e.g.

Table 8.1 DOR composition (Sampedro et al. 2007b)

Chemical properties	DOR
pH	4.69 ± 0.006
CEC (nmoles kg)	646.84 ± 20.35
C (g kg^{-1})	46.35 ± 0.54
N (g kg^{-1})	1.39 ± 0.09
C/N	33.34 ± 2.08
TOC (g kg^{-1})	149.83 ± 3.10
Phenols (g kg^{-1})	73.92 ± 1.50
Ergosterol (μM)	n.d.
P (mg kg^{-1})	938.66 ± 38.58
K (mg kg^{-1})	7121 ± 111.72
Mg (mg kg^{-1})	47.6 ± 2.97
Ca (mg kg^{-1})	1247 ± 90.50
Lignin (%)	25
Hemicellulose (%)	10
Cellulose (%)	18

n.d. non-detected

veratryl alcohol, xylidine) or copper salts (Ullrich et al. 2005; Liers et al. 2006; Michniewicz et al. 2006).

The transcriptional regulatory mechanisms of CAZymes hydrolytic genes have traditionally received much attention in ascomycetes, thanks to the potential industrial applications of cellulases, mainly in next-generation biofuel production (Sun et al. 2012; Tani et al. 2014). In this context, inducers of cellulase gene expression have been identified as cellulose, sophorose and xylose, among others in *Aspergillus niger* and *Hypocrea jecorina*, and cellulolytic substrates in *Neurospora crassa* (Coradetti et al. 2012). These inducers are mainly produced by hydrolysis of cellulose and hemicellulose by small amounts of constitutively expressed cellulases and hemicellulases.

DOR possesses a high rate of C/N and a low concentration of soluble sugars (Table 8.1), conditions which are in some cases required for LME production (Keyser et al. 1978; Lechner and Papinutti 2006). The aqueous extract of DOR contains most of the phenolic compounds responsible of the phytotoxicity (García-Sánchez et al. 2014a, b). These phenolic compounds have been shown to be responsible for the induction of Lac in some fungal species such as *Rigidoporus lignosu*s (Cambria et al. 2011). Experiments have been carried out in which DOR and its aqueous extracts (ADOR) were added to *T. versicolor* cultures under liquid submerged fermentation (SF) and in solid-state fermentation (SSF) in soybean meal, barley meal and eco-tomato juice media, thereby showing that Lac can be stimulated by the addition of DOR and ADOR (Reina et al. 2016). This fact was also observed for *Funalia floccosa* (formerly *Coriolopsis rigida*) in ADOR-supplemented cultures in which Lac activity was enhanced tenfold (Díaz et al. 2010). Previously published studies have also shown an enhancement of Lac secretion in complex liquid media such as eco-tomato juice in *Xylaria polymorpha*,

Table 8.2 Effect of olive mill waste enrichment in the expression of LMEs

Fungal species	Taxonomic group (order/class)	Substrate	Key enzymes	Residue effect	Reference
Aspergillus niger	*Eurotiomycetes*	TPOMW/ winery wastes	Feruloyl esterase	Upregulation	Salgado et al. (2014)
Aspergillus ibericus	*Eurotiales*		Cellulase		
Aspergillus uvarus			Xylanase		
Auricularia auricula-judae	*Agaricomycetes*	DOR	MnP	None	Reina et al. (2013)
	Auriculariales		Lac	Upregulation	
			DyP	None	
Bjerkandera adusta	*Agaricomycetes*	DOR	MnP	Upregulation	Reina et al. (2013)
	Polyporales	ADOR	VP	Upregulation	Reina et al. (2014)
			AAO	Downregulation	
			DyP	None	
Chondrostereum purpureum	*Agaricomycetes*	DOR	Lac	None	Reina et al. (2017)
	Polyporales	ADOR	UPO		Reina et al. (2017)
Cyclocybe aegerita	*Agaricomycetes*	DOR	UPO	Upregulation	Reina et al. (2017)
	Agaricales				
Coprinellus radians	*Agaricomycetes*	DOR	UPO	Upregulation	Reina et al. (2013)
	Agaricales				
Funalia floccosa	*Agaricomycetes*	DOR	Lac	Upregulation	Sampedro et al. (2007b) and Díaz et al. (2010)
	Polyporales	ADOR			
Lentinus tigrinus	*Agaricomycetes*	ADOR	MnP	Upregulated	Sampedro et al. (2012)
	Polyporales				
Mycetinis alliaceus	*Agaricomycetes*	DOR	DyP	None	Reina et al. (2017)
	Agaricales		Lac	Upregulation	
			MnP	Upregulation	
Phlebia sp.	*Agaricomycetes*	ADOR	MnP	Upregulated	Sampedro et al. (2012)
	Polyporales				
Pleurotus sp.	*Agaricomycetes*	OMW (30%)	Lac	Upregulation	Ruiz-Rodríguez et al. (2011)
	Agaricales		MnP	Upregulation	
Trametes versicolor	*Agaricomycetes*	DOR	MnP	Up-regulation	Reina et al. (2016)
	Polyporales	ADOR	Lac	Upregulation	
			LiP	None	Reina et al. (2016)
Yarrowia lipolytica	*Saccharomycetes*	TPOMW	Lipase	Upregulated	Lopes et al. (2016)
	Saccharomycetales				

Agaricus blazei and *Cerrena unicolor* (Ullrich et al. 2005; Michniewicz et al. 2006; Liers et al. 2007). The stimulation of this ligninolytic activity could also be attributed to the phenolic fractions (e.g. flavonoids or tannic acids) which are present in plant extract-based media (Carbajo et al. 2002). Further evidence suggests that DOR can also stimulate MnP and VP secretion (Sampedro et al. 2012; Reina et al. 2013, 2014, 2016).

The role of DOR in the enhancement of LME activities during SSF has been studied with fungi such as the auricularial *A. auricula-judae*; the polyporal *B. adusta*, *Lentinus tigrinus* and *Phlebia* sp.; and the agaric fungi *C. radians*, *C. aegerita*, *Marasmius alliaceus* and *Chondrostereum purpureum* (Reina et al. 2013, 2014, 2016, 2017) (Table 8.2). The analyses of *B. adusta* secretome revealed that the upregulation of several VPs and MnPs mediated by ADOR occurred concomitantly to the downregulation of AAO (Reina et al. 2014).

The stimulation of DyPs by DOR remains unclear, and no general conclusion could be drawn about whether the phenolic elicitors could stimulate the secretion of DyPs. The natural-inducing compounds of DyPs, as well as their natural function (e.g. their probable degradative capabilities), are not yet fully understood. Larger amounts of these enzymes are reportedly produced in liquid cultures, e.g. in the presence of tomato juice (Liers et al. 2010, 2013) which is also a phenol-rich medium. Nevertheless, tentative evidence suggests a potential role of DyPs in natural carbon turnover (Kellner et al. 2014). UPOs are also oxidoreductases whose expression is influenced by the addition of ADOR. Studies on the effects of inducing substrates were mainly carried out for nitrogen-rich compounds like soybean meal, but the effects of lignocellulosic substrates on UPO secretion have not been investigated in great detail. Some studies suggest that DOR was able to stimulate the secretion of UPO in *C. radians* and *A. aegerita* (Reina et al. 2014, 2017), two enzymes which have already been characterized (Ullrich et al. 2004; Anh et al. 2007).

In the case of hydrolytic enzymes, in some ascomycetes, the induction of lipases, cellulases, xylanases and feruloyl esterases has been observed in mixtures of TPOMW and winery wastes by *Aspergillus* species (Salgado et al. 2012, 2014). More concretely, the presence of fats in TPOMW before hexane extraction improves lipase induction in *Yarrowia lipolytica* (Lopes et al. 2016). Apart from this study, not much additional information regarding this particular residue could be found.

Since the cooperation or antagonism between the regulatory elements of these enzymes appears to be specific to each fungal strain, no general statements can be assumed for filamentous fungi. Recently published transcriptomes and proteomes of white- and brown-rot fungal species have underlined the role of cellulose-containing media in the stimulation of CAZymes (Wymelenberg et al. 2009; Yang et al. 2012; Marx et al. 2013). Reina et al. (2014) used ADOR to analyse the lignocellulolytic profile in *B. adusta* with a proteomic approach. In this study, GHs, such as GH15 and GH16, were clearly downregulated by ADOR, whereas peptidases were slightly upregulated. ADOR-enriched media seem to be appropriate for the induction of LMEs (e.g. Lacs and MnPs), as well as cellulolytic enzymes.

8.7 Valorization of Dry Olive Mill Waste as Soil Organic Amendment Using Fungi

The use of DOR as an organic fertilizer has been well-established due to its large content of organic matter and nutrients (Sampedro et al. 2007b). However, its free discharge into the environment needs to be reduced because of its ecological impact on soil microorganisms as the result of the high amount of phenolic compounds, lipids and not stabilized organic matter. The bioremediation with saprobe fungi represents one of the most promising approaches for DOR detoxification (Sampedro et al. 2004b, 2007b). The mechanism involved in the removal of phenolic compounds is primarily based on the polymerization of monomeric phenols through the action of LMEs. The specific enzymatic mechanisms of each fungus establish different polymerization profiles, which influence the toxicity of these residues (Reina et al. 2013, 2017).

Microtoxicity bioassays have been carried out to evaluate the effect of fungi in the depletion of DOR microtoxicity, such as the microtox bioassay with *Vibrio fischeri* (Linares 2003), in which the use of *Phanerochaete flavido-alba* for DOR detoxification in combination with pine chip mixtures decreases the EC_{50}. In this line, studies conducted by Saparrat et al. evaluate the survival of *Azospirillum brasiliensis* in the presence of an aqueous extract from DOR before and after treatment with *F. floccosa*, confirming the microtoxicity reduction (Saparrat et al. 2010).

Most of the studies conducted on DOR's toxicity have focused on the germinability index through the use of cress (*Lepidium sativum* L.), radish (*Raphanus sativus*), lettuce seeds (*Lactuca sativa*, L.) and sunflowers (*Helianthus annuus* L.). Indeed, the use of saprobe fungi such as *F. floccosa*, *T. versicolor*, *P. cinnabarinus* and *Penicillium chrysogenum* has been reported to be effective in increasing the Zucconi germination index of the water soluble fraction of DOR (ADOR) (Aranda et al. 2006, 2007).

The impact of applying DOR on plants by monitoring the biomass decrease of soybean (*Glycine maximum* Merr.), tomato (*Solanum lycopersicum*, L.), alfalfa (*Medicago sativa* L.) and lettuce plant (*L. sativa* L.) has been widely studied (Sampedro et al. 2004a, 2007a). DOR exhibits a high residual toxicity towards lettuce (*L. sativa* L.). However, tomato (*Solanum lycopersicum* L.) has been used as model plant due to its high sensitivity to phenolic compounds, enabling an improved detection of small changes in phytotoxicity (Sampedro et al. 2004a, b, 2007a; Reina et al. 2013, 2016). Nevertheless, the subsequent mycoremediation of DOR through the use of different fungal species has shown a positive correlation between the increase in plant biomass and the decrease in phenolic compounds (Table 8.3). Ascomycetes, such as *Fusarium oxysporum* and *Fusarium lateritium*, have shown to be efficient fungi in reducing the phytotoxicity of DOR after 20 weeks of incubation, under the reported test conditions (Sampedro et al. 2005, 2008). In fact, *F. oxysporum* was able to degrade the lignin, hemicellulose, cellulose and fats of DOR under solid-state fermentation in concentrations of 16%, 25%, 25%, 71% and 13%, respectively. However, enzymatic activities are not shown by the author

Table 8.3 Mycoremediation of olive mill waste and effects on plant toxicity

Fungi	Residue	Ecotoxicological test	Phenol removal (%)	Effects	Reference
Auricularia auricula-judae	DOR	Tomato plants	100	Decrease phytotoxicity	Reina et al. (2013)
Bjerkandera adusta	DOR	Tomato plants	100	Decrease phytotoxicity	Reina et al. (2013)
Chondrostereum purpureum	DOR	Tomato plants	100	Increase plant growth	Reina et al. (2017)
Coprinellus radians	DOR	Tomato plants	100	Decrease phytotoxicity	Reina et al. (2013)
Cyclocybe aegerita	DOR	Tomato plants	80	Decrease phytotoxicity	Reina et al. (2017)
Funalia floccosa	ADOR	Tomato plants	73	Decrease phytotoxicity	Aranda et al. (2006a) and García-Sánchez et al. (2014a, b)
		Tomato and sunflower seedlings	93	Reduction of germinability inhibition and production of ROS (H_2O_2 and O_2^{-}). Increases in the enzymatic antioxidant response of defence (SOD, POD, CAT, APX, GST and GR)	
	DOR	Tomato plants	89	Decrease phytotoxicity	Sampedro et al. (2008)
	DOR	*Azospirillum brasiliensis*	73	Decrease microtoxicity	Saparrat et al. (2010)
Fusarium lateritium	DOR	Tomato plants	60	Increase plant growth	Sampedro et al. (2005)
Fusarium graminearum	ADOR	Tomato plants	1	Decrease phytotoxicity	Aranda et al. (2004)
Flammulina velutipes BAFC 1763	DOR	*Raphanus sativus* seeds	84.3	Decrease phytotoxicity and increase GI	Rugolo et al. (2016)
Fusarium oxysporum	DOR	Tomato and soybean plants	88	Decrease phytotoxicity	Sampedro et al. (2004a)
Mycetinis alliaceus	DOR	Tomato plants	90	Decrease phytotoxicity	Reina et al. (2017)
Mucor racemosus	ADOR	Tomato plants	1	Decrease phytotoxicity	Aranda et al. (2004)
Panus tigrinus	DOR	Tomato and lettuce plants	75	Decrease phytotoxicity	Sampedro et al. (2007b)

(continued)

Table 8.3 (continued)

Fungi	Residue	Ecotoxicological test	Phenol removal (%)	Effects	Reference
Penicillium chrysogenum	DOR	Tomato and soybean plants	92	Decrease phytotoxicity	Sampedro et al. (2004a)
	ADOR	Tomato plants	1	Decrease phytotoxicity	Aranda et al. (2004)
	ADOR	Tomato and sunflower seedlings	1	Reduction of germinability inhibition and production of ROS (H_2O_2 and O_2^-). Increases in the enzymatic antioxidant response of defence (SOD, POD, CAT, APX, GST and GR)	García-Sánchez et al. (2012)
Phanerochaete flavido alba	DOR/ pine chips	Alfalfa, rape, maize and tomato	65	Decrease phytotoxicity	Linares (2003)
Phlebia radiata	DOR	Tomato and lettuce plants	85	Decrease phytotoxicity	Aranda et al. (2006)
Pycnoporus cinnabarinus	ADOR	Tomato plants	73	Decrease phytotoxicity	Aranda et al. (2004)
	ADOR	Tomato and sunflower seedlings	94	Reduction of germinability inhibition and production of ROS (H_2O_2 and O_2^-). Increases in the enzymatic antioxidant response of defence (SOD, POD, CAT, APX, GST and GR)	García-Sánchez et al. (2012) and Garrido et al. (2012)
	DOR	Tomato plants	89	Decrease phytotoxicity	
Trametes versicolor	ADOR	Tomato plants	98	Decrease phytotoxicity	García-Sánchez et al. (2014a, b)
	ADOR	Tomato and sunflower seeds	98	Reduction of germinability inhibition and production of ROS (H_2O_2 and O_2^-). Increases in the enzymatic antioxidant response of defence (SOD, POD, CAT, APX, GST and GR)	García-Sánchez et al. (2012) and Garrido et al. (2012)
	DOR	Tomato plants	75	Decrease phytotoxicity	Reina et al. (2016)

(Sampedro et al. 2007a). Other ascomycetes such as *Mucor racemosus*, *Fusarium graminearum* and *Penicillium chrysogenum* have been studied in detoxification of ADOR, which showed a decrease of phytotoxicity after 15 days of incubation, not related to the decrease of phenolic content (Aranda et al. 2004). In the same line, similar studies were carried out to compare the efficiency of white-rot fungi such as *Phlebia radiata*, *F. floccosa*, *P. chrysosporium*, *Pycnoporus cinnabarinus*, *Poria subvermispora* and *P. pulmonarius* in terms of DOR transformation after 2 and 20 weeks of incubation (Sampedro et al. 2007b). This study shows that *F. floccosa* was the most effective fungus in the degradation of phenolic compounds and therefore in removing the toxicity on tomato plants. Further studies have focused on the reduction of the incubation time necessary for removing phytotoxicity by *Panus tigrinus* and *Phlebia* sp. by immobilizing them in different support media such as chopped maize stalks or polyurethane sponge (Sampedro et al. 2009). These techniques have shown the removal of phenols and phytotoxicity of DOR after 4 weeks of incubation. This study found that *Phlebia* sp. immobilized in polyurethane sponge was able to reduce the phenol content up to 85% and reduce the germinability inhibition of *Lepidium sativum* and *L. sativa* by 80% and 71.4%, respectively. In the same line, studies based on solid-state fermentation of DOR using a barley-based medium by wood- and dung-dwelling agaricomycetes fungi, such as *A. auricula-judae*, *B. adusta* and *C. radians*, have shown a complete removal of phenolic compounds (100%) after their incubation of 4 weeks (Reina et al. 2013). As a consequence of this decrease, a concomitant decline in the phytotoxicity was observed as well as a certain degree of tomato plant growth caused by the plant growth stimulation effect of mycoremediated DOR. The novelty and relevance of this study resides in the use of inocula carriers, such as barley seeds, and the subsequent addition of DOR, which increases the secretion of different peroxidases (DyP, MnP and UPO), thereby accelerating the degradation rate of phenolic compounds. Similarly, a recent study conducted by Reina et al. has shown that DOR toxicity was removed entirely after 1 week of incubation with the fungi *C. aegerita* as the result of the reduction of half of the phenolic fraction, involving enzymes such as the novel UPO. The fermentation of DOR through the inoculation of *C. purpureum* in a barley-based medium significantly decreases the phenolic composition of DOR after 1 week, when significant differences between the dry weight of tomato shoots and roots with respect to tomato plant growth in the presence of non-mycoremediated DOR were observed. In addition, a stimulating effect of the tomato plant growth was observed. Conversely, the fungus *Mycetinis alliaceus* achieved a toxicity depletion of the DOR after 5 weeks of incubation, reducing DOR's toxicity in tomato plants significantly. The fungus *Flammulina velutipes* has been cultivated in the presence of DOR, showing a clear reduction of phenolic content with the concomitant production of Lac, MnP and hydrolytic enzymes (Rugolo et al. 2016).

Despite the studies on geminating index inhibition and plant growth on shoots and roots, there is a need to understand if other physiological processes, different from seed germination and plant biomass, may be affected as a consequence of the exposure to phenolic compounds. In this regard, some studies, using mostly tomato as model plant, were carried out by applying the water-soluble fraction of

DOR. Additional studies in tomato and sunflower seeds have suggested that the presence of water-soluble phenolic compounds could be involved in the generation of a burst oxidative and the subsequent triggering of reactive oxygen species (ROS). This situation produces an oxidative stress in plants which can result in a reduction of the root length, fresh weight and the number of layers forming the cortex (García-Sánchez et al. 2012; Garrido et al. 2012). Likewise, the analysis of some enzymes involved in the counteracting of the oxidative stress, such as class III peroxidases and superoxide dismutase (SOD), revealed a significant increase in their activities in the presence of non-mycoremediated ADOR. Nevertheless, the mycoremediation of ADOR by fungi, such as *F. floccosa*, *T. versicolor*, *P. cinnabarinus* and *P. chrysogenum*, partially recovered normal levels of ROS, morphological characteristics and antioxidant activities. In the same line, studies conducted by the same author (García-Sánchez et al. 2012) reported that the ADOR mycoremediated by these fungi significantly reduced the germination of tomato seeds and were also able to reduce the lipid peroxidation and the production of O_2^- and H_2O_2. However, among all the fungi used, *T. versicolor* was found to be the most effective in inducing important changes in the composition of phenols counteracting the oxidative stress in terms of ROS production. Likewise, important antioxidant enzymes involved in the defence response, such as SOD, glutathione reductase (GR) and glutathione-S-transferase (GST), were triggered in tomato seedlings germinated in the presence of non-mycoremediated ADOR, suggesting ADOR has the capacity of inducing oxidative stress. These results were also supported by the findings reported by García-Sánchez et al. (2014a, b). Moreover, the efficiency of the photo system PSII was also affected as the result of the ADOR exposure, and a decline in the content of chlorophyll a and b was found. These symptoms may be related to an increase in phenolic compounds which was able to induce an oxidative stress in tomato plants. Interestingly, when tomato plants were grown in the presence of ADOR mycoremediated by *F. floccosa*, *T. versicolor*, *P. cinnabarinus* and *P. chrysogenum*, a significant decline in phenol content was observed, and therefore an alleviation of oxidative stress occurred. These results highlight the potential capacity of mycoremediation of ADOR as a useful strategy for its reuse and thus its potential use as an organic fertilizer. However, all experiments reported in this section were conducted using the water-soluble fraction of ADOR. Therefore, there is an urgent need to test the physiological impact of DOR in agricultural crops.

8.8 Future Prospects

Since the introduction of the two-phase extraction system for olive oil processing, several mycoremediation technologies for the treatment of generated olive wastes have been studied. These approaches are mainly focused on the removal of toxicity by filamentous fungal species and the subsequent abstention of organic soil amendments. Recently, studies have been carried out into the use of dry olive mill residues as enhancers of some fungal CAZymes and their auxiliary activities, finding

variations among enzymes and fungal species. The development of analytical technologies in genomics could shed light on this new approach for dry olive mill valorization by studying the regulation of the complete enzymatic battery of each fungal species. The capability of this residue to regulate the expression of enzymes opens up a new spectrum of possibilities to gain a deeper understanding of the role of elicitors in enzyme expression as well as to encourage the development of novel biotechnological applications in the enzyme and biofuel production industries.

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Chapter 9
Fungal Conversion and Valorization of Winery Wastes

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9.1 Introduction: The Vine and Wine Sector

The grape is the fruit of the vine, a plant of Vitaceae family. It is often used to make juice, jam, raisins, and wine or to eat as fresh fruit. Grapes used in wine production belong to the *Vitis vinifera* L. species. Besides its economic value and important role in the organization of the territory, the winemaking activity has a deep-rooted cultural dimension. Based on century-old traditions, the vineyards and wine production have been part of the history, culture, and local identity of many countries.

Nowadays, the winemaking sector is one of the most important sectors of the agricultural production in the European Union (Italy, France, Spain, Germany, and Portugal), being followed by America (the USA, Argentina, and Chile), Australia, China, and South Africa (Table 9.1). Currently, the vineyards of the European Union account for about 60% of the world wine production volume, and Portugal, with a production of about 7 MhL per year, occupies the 11th place in the list of best world producers and the 5th place among European countries (Table 9.1).

Outside Europe, China presents a continued grow of both the vineyard surface and grape production. Hence, China is currently the world leader in producing grapes, even though only 12% of grapes are aimed at winemaking (OIV 2016). There is an upward trend in the percentage of world grape production used to make non-fermented products. The percentage of total production of grapes used for other purposes than winemaking (namely, raisin and table grapes) has been slowly increasing (Aurand et al. 2012).

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Table 9.1 Principal worldwide producers of grape and wine in 2015 (OIV 2016)

Grape production (Mt)[a]	Wine production (Mhl)[a]
Worldwide: 75.1	Worldwide: 277
China: 12.6	Italy: 50.0
Italy: 8.2	France: 47.4
USA: 7.0	Spain: 37.3
France:6.3	USA: 22.1
Spain: 6.0	Argentina: 13.4
Turkey: 3.6	Chile: 12.9
Chile: 3.1	Australia: 11.9
India: 2.6	China: 11.5
Argentina:2.4	South Africa: 11.2
Iran: 2.1	Germany: 8.8
South Africa: 2.0	Portugal: 7.0

[a]Values expressed on fresh weight basis; *MhL* millions of hectoliters, *Mt* millions of tonnes

9.2 Vineyard and Winery By-Products

Modern industrial concepts integrate principles as circular economy in order to achieve a "zero-waste" society and industry, where wastes of one sector may be used as raw materials of another sector. As a consequence, many countries are adopting new policies and developing a so-called bio-based economy. The agri-food industry, which includes transformation of agricultural products, produces high amounts of wastes and raises several problems from the environmental and economic point of view (Mateo and Maicas 2015).

Wine production is one of the most important agricultural activities in the world (Devesa-Rey et al. 2011), spread over three continents. However, this activity generates high amounts of by-products and wastewaters. It has been estimated that 18–20% worldwide production of grapes for winemaking remains as residue (mainly grape pomace), which could generate about 13×10^6 t of fresh weight winery-derived biomass yearly (Corbin et al. 2015). Thus, given the amount of residues produced, these require innovative solutions of management and disposal to develop a sustainable industry (Mateo and Maicas 2015) according to a circular economy vision.

The major solid by-products and residues generated from winemaking activity (Fig. 9.1) are grape leaves, grape stalk, grape pomace or marc (which comprises the pulp, seeds, and skins), wine lees, and also wastewaters. Indeed, solid residues consist of plant remains derived from the de-stemmed grapes, the sediments obtained during clarification, bagasse left after the crushing and pressing, and lees that are accumulated in the bottom of grape juice or fermentation tanks after different decanting steps. The wastewaters come from cleaning and wine production, containing grape pulp, skins, seeds, and dead yeasts used in alcoholic fermentation and cooling processes (Jin and Kelly 2009; Devesa-Rey et al. 2011).

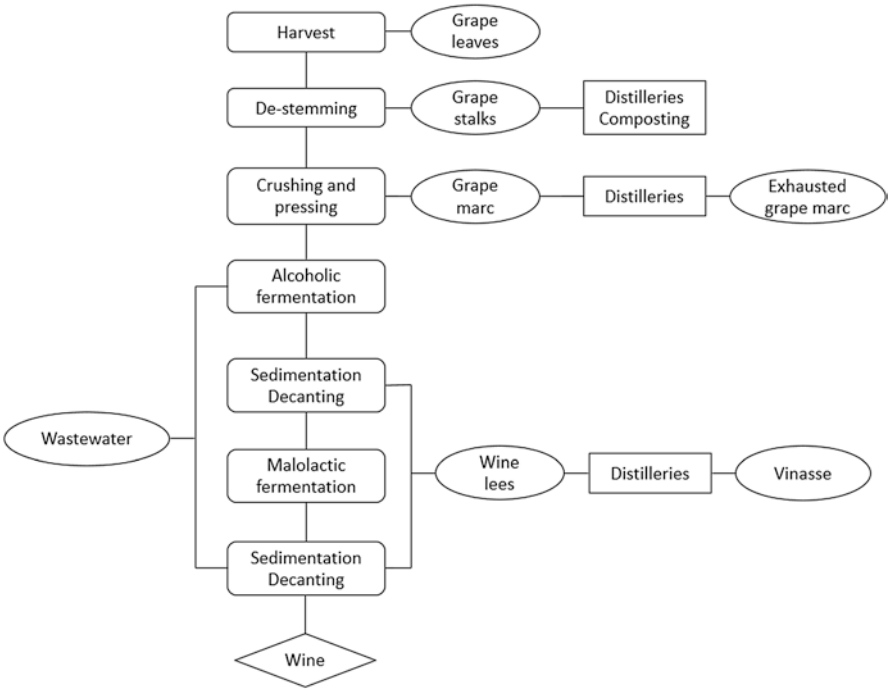

Fig. 9.1 Diagram of wastes and wastewater generation during grape harvest and winemaking. (Adapted from Jin and Kelly 2009; Devesa-Rey et al. 2011)

The composition of by-products produced during winemaking depends on grape variety and the vinification method, which affects their physicochemical properties and determines its further use and valorization (Mateo and Maicas 2015). Indeed, winemaking generates different residues characterized by high contents of biodegradable compounds and suspended solids (Devesa-Rey et al. 2011), which are rich in nutrients suitable for microbial growth (Carreira et al. 2011).

9.2.1 Winery Wastewater

Winery wastewaters originated from various unit operations along winemaking, such as washing procedures of press equipment for grapes crush, fermentation tanks and other containers used to store wine, and other equipment. Per liter of wine produced, 0.3–0.7 L (Bories and Sire 2010) or even up to a volume of 4 L (Welz et al. 2016) of wastewater is generated, depending on grape variety and the vinification process. Winery wastewater is characterized by a high organic load and acidic pH. The high chemical oxygen demand (COD) and biochemical oxygen demand

(BOD) which are mainly due to the presence of sugars, organic acids (including short-chain volatile fatty acids formed during pond storage), alcohols, polyphenols, tannins, and structural polymers (Jin and Kelly 2009; Welz et al. 2016) turn these effluents into an environmental problem. Also, other dissolved compounds such as cleaning chemicals, pesticide residues, and metals may be present (Thassitou and Arvanitoyannis 2001).

9.2.2 Grape Leaves

Grape leaves are characterized by the presence of several organic acids, phenolic acids, flavonols, tannins, anthocyanins, lipids, enzymes, polyisoprenoids (carotenoids and other terpenoids), and reducing and nonreducing soluble carbohydrates, which appears to be a promising source of compounds with nutritional properties, contributing to their biological potential (Orhan et al. 2009; Xia et al. 2010). In fact, *V. vinifera* leaves showed an array of bioactivities, such as hepatoprotective, hypoglycemic, vasorelaxant, antimicrobial and antiviral action, anti-inflammatory, and antioxidant properties (Fernandes et al. 2013). Therefore, these findings have led to a considerable interest in the use of *V. vinifera* leaves for production of natural value-added compounds.

9.2.3 Grape Stalks

Grape stalks, one of the major by-products of winemaking, are a lignocellulosic fibrous material essentially made up of cellulose, hemicellulose, lignin, and tannins (Egüés et al. 2013). Since polysaccharides are the major fraction of grape stalk components, these could represent a cheap source of non-edible material for production of biofuels or papermaking/biocomposites (Prozil et al. 2012). Grape stalks also contain several nutrients and high percentage (more than 10% in some cultivars) of extractable polyphenolic compounds, such as phenolic acids, flavonols, and flavanonols (Llobera and Cañellas 2007; Prozil et al. 2014).

Currently, grape stalks released by distilleries are disposed of to landfills or in rural areas. However, other alternatives have been investigated, such as composting, the use for removal of heavy metals from metal-containing effluents (Villaescusa et al. 2004), including vinasses and winery wastewaters, which have been characterized by several authors for their higher metal concentrations. In addition, the possibility of using grape stalks for the production of biosorbents, cellulosic pulps, or enzymes, using different types of fungi, has been demonstrated (Spigno et al. 2008; Ferreira et al. 2016).

9.2.4 Grape Pomace

Grape pomace or grape marc is the residue left after juice extraction by pressing grapes in the wine industry and accounts for about 20–25% of the weight of the grape crushed for wine production (Yu and Ahmedna 2013). It is the main waste generated in the winemaking process and is mainly constituted of berry skin (45%), seeds (30%), and grape stalks (25%) (Levin et al. 2012). The huge volume of grape pomace generated annually puts in evidence that this by-product has an important environmental impact. In general, most grape pomace is processed in distilleries in order to recover alcohol, tartrates and grape tannins, and in some cases antioxidant compounds (Bustamante et al. 2008; Jin and Kelly 2009). Other processes for recovery, valorization, and reuse of this solid waste include yeast production, extraction and recovery of phenolic compounds, production of grape seed oil, and the use of adsorption material for decontamination of metal-containing effluents (Bustamante et al. 2008).

Grape pomace is regarded as an excellent source of polyphenolic compounds, such as phenolic acids, flavonoids, procyanidins, resveratrol, and anthocyanins (Yu and Ahmedna 2013). Grape skins are particularly rich in anthocyanins, hydroxycinnamic acids, and flavonol glycosides, whereas flavanols were mainly present in the seeds (Kammerer et al. 2004). Although variable and highly dependent on the cultivar analyzed, total polyphenolic content has been positively correlated with the potential antioxidant capacity of winery by-products (Katalinić et al. 2010; Barros et al. 2014; Tournour et al. 2015). The high phenolic content supports its viable economic extraction to be further used in cosmetic, pharmaceutical, and food industry as additives with proven antioxidant activity (Conde et al. 2011). Regarding grape seeds, these are mainly constituted by a lipid fraction (oil content between 13% and 19%), protein (about 11%), nondigestible fiber, and non-phenolic antioxidants such as α-tocopherols and β-carotene (Yu and Ahmedna 2013). The extraction of oils, particularly from grape seeds, has been also a focus of interest to valorize winery residues, especially grape marc, where grape seeds are more abundant (Mateo and Maicas 2015). The grape seed oil is a high-value product with potential for use in pharmaceutical, cosmetic, and food industries exhibiting health-promoting effects (Toscano et al. 2013).

9.2.5 Wine Lees and Vinasses

Wine lees are the sediments that deposit in the bottom of recipients containing wine, after fermentation, during storage, or after authorized treatments. This residue is also obtained after filtration or centrifugation of wine must. Wine lees may represent 2–6% of the total volume of wine produced (Dimou et al. 2015) and may contain grape stalks, pomace, skins, seeds, and yeasts. Owing to the high polyphenolic content, this by-product also exhibits a strong antioxidant activity, but the concentration of these secondary metabolites makes its use as animal feed unsuitable (Devesa-Rey et al. 2011).

Wine mixed with grape marc is sent to distilleries for the production of spirits. To those exhausted products which were given the name vinasses, their composition and amounts produced are very similar to lees but with lower ethanol content (Devesa-Rey et al. 2011; Zacharof 2016). The first tested reuse for these residues was as supplement for animal feed, but it was considered not suitable due to its low nutritional value. Further studies on characterization of the vinasses revealed high contents in vitamins and organic and inorganic nitrogen compounds, which would make them suitable for microbial growth. Besides this application, lees were also investigated for the production of lactic acid and xylitol (Liu et al. 2010; Salgado et al. 2010). The recovery of tartaric acid from lees (before distillation) and vinasse also seemed to be profitable, with very attractive extraction yields (Salgado et al. 2010).

Apart from alcohol and tartrate recovery from grape pomace and wine lees, the product leftovers from the distilleries (exhausted grape pomace and vinasse) are normally used for feedstuff production, agronomic utilization (land farming and composting), methane production, and extraction of antioxidant for pharmaceutical, cosmetic, and nutraceuticals (Toscano et al. 2013).

9.2.6 Vineyard Pruning

The paring of vine branches although not accounting for winery residues is a by-product resulting from grapevine industry. Usually they are burnt on field, but nowadays there has been a great effort to minimize the negative effects of agro-industrial by-products, and so, the valorization and/or safe disposal of vineyard pruning were not forgotten.

Many authors tried approaches (enzymatic or acid hydrolysis) to extract renewable sugars, similarly to what can be performed with grape stalks, to convert by fermentation into other valuable products. Extraction of other interesting compounds from stems includes polyphenols and hydroxycinnamic and hydroxybenzoic acids, particularly ferulic acid and gallic acid, respectively (Makris et al. 2007).

9.3 Bio-recycling of Winery Wastes

Several methods have been proposed in order to recycle the wastes generated by the winery and distillery industries, minimizing their environmental impact. Among the proposed processes, biological treatment occupies a relevant position and consists in waste utilization as substrate for its conversion into value-added products in a "waste biorefinery" concept. Thus, bioconversion of waste materials is an eco-friendly approach that can contribute to the sustainable development of wine industry (Ferreira et al. 2016; Jin et al. 2016).

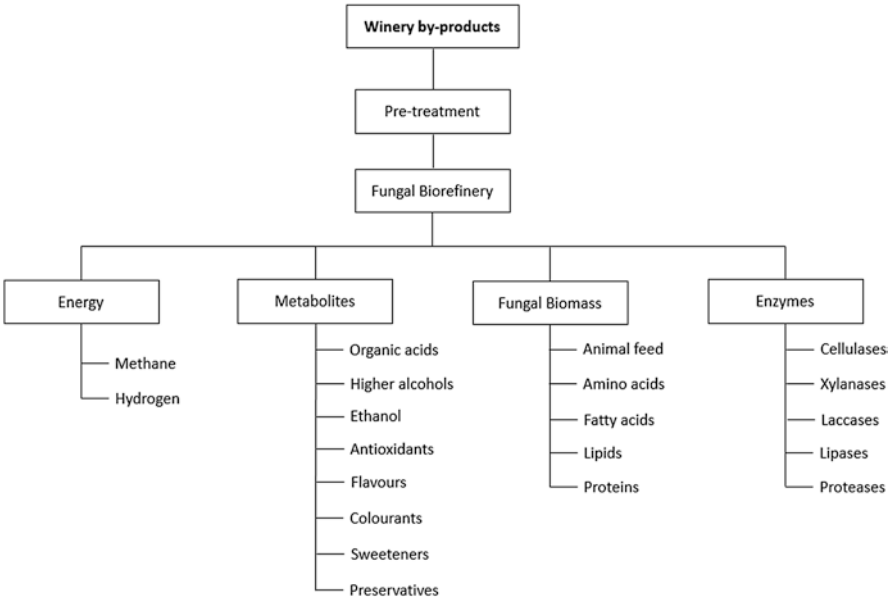

Fig. 9.2 Flow diagram of winery by-product conversion into value-added products in fungal-based biorefinery. (Adapted from Santana-Méridas et al. 2012; Ferreira et al. 2016; Zacharof 2016)

In this context, filamentous fungi or molds are extremely valuable for recycling and pollution abatement of lignocellulosic agro-industrial wastes due to their metabolic versatility, rendering the production of a wide range of value-added products (Fig. 9.2) such as enzymes, antibiotics, ethanol, tartrates, organic acids, grape seed oil, hydrocolloids, and dietary fibers (Ferreira et al. 2016; Sharma et al. 2016).

9.3.1 Fungal Bioremediation of Winery Wastewater

Relatively few studies report fungal treatments of effluents generated in wine production activities. Molds, especially white-rot fungi, have shown effectiveness for the treatment of dark-colored, phenolic-rich effluents such as olive mill (Aggelis et al. 2003) and winery (Zhang et al. 2008) wastewaters due to their extracellular lignocellulolytic system. *Trichoderma viride*, *Aspergillus niger*, and *A. oryzae* were used in a hybrid process aiming the treatment of winery wastewater with the production of fungal biomass protein (Zhang et al. 2008). Besides filamentous fungi, yeasts can also be used for winery wastewater treatment. The basidiomycetous yeast *Cryptococcus laurentii* AGG726 proved to be a useful strain for combined biological/chemical processes. Treatment of winery wastewaters using this yeast strain and Fenton reagent achieved a reduction of 98% and 96% for COD and polyphenols, respectively (Santos et al. 2014).

9.4 Fungal Fermentation of Solid Residues

There are some examples where filamentous fungi have been successfully applied to convert winery-derived biomass into valuable products (Table 9.2). Saprophytic ascomycetes have been previously reported for their ability to degrade plant cell wall components due to their ability for production of extracellular cellulase, hemicellulase, and pectinase enzymes (Dashtban et al. 2010; van den Brink and de Vries 2011). The mold mycelial morphology facilitates its penetration in solid and insoluble substrates, being more effective than bacteria or yeasts in the hydrolysis of polysaccharides (Jin et al. 2016). White-rot fungi basidiomycetes, especially *Phanerochaete*, *Trametes*, *Bjerkandera*, and *Pleurotus* genera, are known for their ability to produce several lignocellulolytic extracellular enzymes necessary to breakdown lignin and cellulose. In general, the conversion of lignocellulosic biomass includes a pretreatment step aiming the removal of protective lignin seal surrounding structural polysaccharides, followed by enzymatic hydrolysis and subsequent fermentation of released soluble sugars. Thus, the reducing sugars from hydrolysis of cellulose and hemicellulose fractions could be metabolized by other microorganisms producing value-added compounds such as alcohols, flavonoids, organic acids, and phenolics (Sánchez 2009; Mateo and Maicas 2015).

9.4.1 Production of Food and Animal Feed

Fungal bioconversion, namely, through fermentative processes, has shown to be an eco-friendly biotechnological approach for the sustainable development of protein-rich animal feedstock (Salgado et al. 2015; Jin et al. 2016). A mixture of both grape marc and wine lees could constitute a substantially cost-effective substrate for fermentation and protein enrichment (Bai et al. 2008; Jin et al. 2016). Among 13 fungal strains of *Aspergillus*, *Rhizopus*, and *Trichoderma*, 3 (*A. oryzae* DAR 3699, *A. oryzae* RIB40, and *T. reesei* RUT C30) yielded the highest protein enrichment (18–23%) and digestibility (~50%) of the grape marc and lees submitted to SSF, in both mono- and cocultivations (Jin et al. 2016).

Grape marc seems to be a suitable support substrate for SSF, while lees contain a wide variety of useful nutrients for microbial growth. Wine lees from red and white wine have been also considered as a less expensive source of essential microbial nutrient for fermentative media. For example, biotin and nicotinic acid contents of wine lees are adequate for most yeast strains, and these may also cover the vitamin and growth factor levels required by lactic acid bacteria, namely, *Lactobacillus* strains such as *L. rhamnosus* (Bustos et al. 2004).

Furthermore, viticulture residues such as vineyard pruning and grape pomace could be used for mushroom production (*Pleurotus ostreatus* and *Pleurotus pulmonarius*), in SSF with 100% vineyard pruning or marc or in a mixture of these two. Fungal mycelium grew more slowly and scarcely in all treatments with 100% grape

Table 9.2 Fungal remediation and/or bioconversion of winery wastes into value-added products

Winery wastes	Treatment	Fungi species	Aim or products	Reference
Vineyard pruning	SSF (sole or mix with marc)	*Pleurotus ostreatus*, *Pleurotus pulmonarius*	Edible mushrooms production animal feed	Sanchez et al. (2002)
Grape stalks	SSF	*Trametes trogii*, *Stereum hirsutum*, *Coriolus antarcticus*	Laccase; Mn-dependent peroxidase; endoglucanase; endoxylanase	Levin et al. (2012)
	Fermentation (after acid hydrolysis)	*Debaryomyces nepalensis*	Ethanol (max. 20.8 g L^{-1}), lactic acid, xylitol, galacturonic acid	Egüés et al. (2013)
Grape pomace	SSF (mixt with olive mill waste)	*Aspergillus uvarum*	Cellulase; xylanase pretreatment for animal feed	Salgado et al. (2015)
	SSF	*Aspergillus awamori*	Cellulase; xylanase; pectinase	Botella et al. (2005)
	SSF	*Aspergillus*, *Rhizopus*, *Trichoderma* spp.	Increasing the protein content	Jin et al. (2016)
Grape seeds	SSF	*Trametes hirsuta*	Laccase	Couto et al. (2006)
	Grape seed pre-cultured followed by submerged culture	*Phanerochaete chrysosporium*	Lignin peroxidase; Mn-dependent peroxidase	Moredo et al. (2003)
	Grape seed pre-cultured followed by submerged culture	*Trametes versicolor*	Laccase	Moredo et al. (2003)
Distilled lees (vinasse)	Fermentation using vinasse and xylose	*Debaryomyces hansenii*	Xylitol	Salgado et al. (2010)
	Submerged culture	*Trametes pubescens*	Bioremediation (COD, TPP, color removal)	Strong and Burgess (2008)
	SSF	*Aspergillus niger*, *A. ibericus*, *A. uvarum*	Endocellulases; endoxylanases; feruloyl esterases	Salgado et al. (2014)

(continued)

Table 9.2 (continued)

Winery wastes	Treatment	Fungi species	Aim or products	Reference
Mixed grape residues	SSF (marc plus lees)	*Trichoderma viride* WEBL0703	*T. viride* conidia; chitinase; β-glucanase; pectinase	Bai et al. (2008)
	SSF	*Pleurotus djamor*	Biosurfactant	Velioglu and Ozturk Urek (2015)
	SSF (dried post-fermentation grape wastes)	*Trichoderma harzianum*, *Aspergillus niger*, *Penicillium chrysogenum*, and *P. citrinum*	Cellulase; β-glucosidase	Karpe et al. (2015a)
	SSF (dried post-fermentation grape wastes)	*Penicillium chrysogenum*	Food and medicinal compounds (arabitol, xylitol, syringate)	Karpe et al. (2015b)
Mixed residues	SSF (olive mill and grape marc)	*Aspergillus niger*, *Aspergillus ibericus*, and *Aspergillus uvarum*	Lipase	Salgado et al. (2014)
Winery wastewater	Submerged culture	*Trametes pubescens*	COD and TPP removal laccase production	Strong (2008)
	Combined Fenton process and yeasts	*Cryptococcus laurentii; Filobasidium* sp.	COD and TPP removal	Santos et al. (2014)

COD chemical oxygen demand, *TPP* total polyphenolic content, *SSF* solid-state fermentation

pomace and well in the mixtures of pruning and marc. Solid-state fermentation by *Pleurotus* of winery by-products produces food of good quality for human consumption, and, after the harvesting of mushrooms, the resulted fermented viticulture wastes may feed ruminants (Sanchez et al. 2002).

9.4.2 Production of Enzymes or Other Value-Added Compounds

Grape pomace, the main by-product from the wine industry, proved to be a good substrate to induce production of commercially valuable hydrolytic enzymes such as cellulases, pectinases, and xylanases, using *Aspergillus awamori* in a solid-state fermentation (SSF) process (Botella et al. 2005). Grape pomace and wine lees were an eco-friendly solid matrix for the growth of *Trichoderma viride* WEBL0703 (conidia yield around 6×10^9 colony forming units (CFU)/g initial dry substrate), a common biocontrol agent against phytopathogens, simultaneously producing important enzymes like chitinase, β-glucanase, and pectinase (Bai et al. 2008). More recently, a mixed culture of ascomycetous molds (*Trichoderma harzianum*,

Aspergillus niger, *Penicillium chrysogenum*, and *P. citrinum*) cultured both in submerged and SSF conditions, and in a medium with dried post-fermentation grape wastes as the only carbon source (Karpe et al. 2015a), produced commercially important metabolites during the process such as a cellulolytic enzyme complex. The enzyme activity yields varied with cultural conditions: cellulase activity was high in SSF, while β-glucosidase activity was superior in submerged fermentation. Contrary to the other tested molds, *Penicillium chrysogenum* was able to metabolize pentoses to alcohols (arabitol and xylitol), degrade tannins and lignin, and produce medicinal important metabolites, such as syringate (Karpe et al. 2015b).

Microbial production of organic acids is a promising approach to obtain extremely useful building blocks as starting materials for the chemical industry (Jin and Kelly 2009). Moreover, winery by-products have been described as low-cost substrates to produce pigments, namely, carotenoids. Buzzini and Martini (1999) report a maximum yield of carotenoids of 630 ug g^{-1} dry cell weight by cultures of the yeast *Rhodotorula glutinis* with grape must as the sole carbon source. Biological surfactants, which are more biodegradable, biocompatible, and less toxic than their counterparts, are another type of value-added products that can be obtained from SSF of grape wastes, using fungal species such as *Pleurotus djamor* (Velioglu and Ozturk Urek 2015).

9.5 Conclusion

Since composting, landfilling, or incineration add low value to wine residues and are likely to raise environmental concerns, other management strategies focused on the production of commercially valuable products (e.g., enzymes, biopolymers) and even fine chemicals (i.e., flavors, fragrances, and phytochemicals such as polyphenols) bring valorization of this kind of by-products to a higher economic level. Nonetheless, the possibility of using the winery and distillery wastes as raw materials, with the aim to obtain different products, depends also on the market value of these value-added products and the costs related to their management as wastes (Bustamante et al. 2008).

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Chapter 10
Biosorption of Dye and Heavy Metal Pollutants by Fungal Biomass: A Sustainable Approach

Himani Meena and Siddhardha Busi

10.1 Introduction

Dye and heavy metal pollutants present in the aquatic and terrestrial ecosystem are hazardous to the environment as well as the human health due to their toxicity even at the lower concentration. Industrial effluents produced from printing, food colorant, textile, dyes (Patel 2016), gold mining, cement production, and coal industry production (Rahman and Singh 2016) released directly into the environment at large scale magnify the pollution level and cause harmful effects on the human health as well as reduce the quality of ecosystem.

10.2 Dyes and Heavy Metals

Dyes are aromatic compound which can be divided into two major groups based on their synthesis, i.e., natural and synthetic dye (Tahir et al. 2016). Natural dyes are mainly used to dye leather and natural proteins. Synthetic dyes have advantage over the natural dyes as its color fastness and brightness. Synthetic dyes are produced by complex process of chemical synthesis, having aromatic structure with delocalized electron which possesses various functional groups (Tahir et al. 2016) contributes to its recalcitrant property (Kristanti et al. 2016).

Synthetic dyes are subdivided into auxochromic (–SO_3H, –OH, –NH_2, and –COOH) and chromophoric dyes (–N=N–, –NO_2, –C=O) including quinonoid rings.

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The broad range of dyes depends on two main parameters: (i) the presence of chromophore group in the aromatic ring and (ii) anionic (direct, acid, and reactive dyes), cationic (basic dyes), and nonionic (disperse dyes) based on dissociation while interacting with water molecules (Tahir et al. 2016).

10.2.1 Anionic Dyes

Textile industries and printing industries are the major source for the azo dye pollution and responsible for severe health issues, i.e., provoking allergic reactions, cancer, and mutations. Azo dyes are electron-deficient xenobiotic components with chromophore group –N=N–, which have been released in the environment (Patel 2016) by loading dangerous organic pollutants, potential toxicants (Corso and Almeida 2009), and hazardous pollutants (He et al. 2016). Azo dyes contain sulfonate functional group, with high molecular weight which restricts its entry into the cells (Solis et al. 2012).

Acid Magenta, Acid Navy Blue, Fast Red A and Acid Sulfone Blue, Orange-HF (diazo dye) (Kaushik and Malik 2010), Direct Blue 151 and Direct Red 31 (Lalnunhlimi and Krishnaswamy 2016), and Acid Orange 7 [*p*-(2-hydroxy-1-naphthylazo) benzene sulfonic acid] are azo dyes specially used in cosmetics as well as detergent, silk, and wool industries for dyeing. Their high toxicity leads to allergic reactions like upper respiratory tract, skin and eye irritation, dermatitis, and anemia in humans. Azo chromophore donates electron and reduced to generate toxic form, responsible for the carcinogenic nature. 1-Amino-2-naphthol, the reduced form, is well known for inducing bladder tumors. Enzymatic degradation causes aromatic amines production and responsible for mutagenic activity (Gupta et al. 2006). The microbial degradation initiates with cleavage of azo linkages which involves an anaerobic azoreductase and redox mediator to shuttle the electron, the intracellular reductase, and the extracellular dye which requires cofactors such as NADH, NADPH, and FADH (Solis et al. 2012).

Acidic dyes also known as anionic dyes with anionic group in their chemical structure include direct, mordant, reactive dyes. Acidic dyes are more problematic than basic or disperse dyes due to their water solubility and high reactivity (Laszlo 1994). Congo red is a well-known brownish red crystal, anionic dye with high water solubility and stability. The benzidine- and napthoic-derived Congo red are mainly used in clinical laboratories as pH indicator for diagnosis and histological studies for amyloidosis and to check the free HCl presence into gastric contents. It affects blood clotting factors and enhances allergic reactions (Gupta et al. 2006). Anionic dyes like Reactive Black 5 (Huanga et al. 2016), Reactive orange 16, Brilliant Red HE 3B (Chander et al. 2004) and Remazol Blue, Reactive textile dye (Tastan et al. 2009), and some direct dyes Blue 71 and Red 31 (He et al. 2016) which are widely used into textile industry are hazardous to human health. The anionic dyes are unable to degrade due to presence of complex aromatic structure (Huanga et al. 2016).

10.2.2 Cationic Dyes

Cationic dyes are water soluble and well known as basic dyes. Crystal Violet can cause bladder cancer in human and is harmful to fish if it is absorbed into the fish skin and metabolized into leucocrystal violet form. The leuco form can also induce hepatic and lung tumor (Smitha et al. 2017). The other cationic dye Rhodamine B also has carcinogenic effects (Maurya et al. 2006). Methylene blue (Maurya et al. 2006) is a cationic dye which is able to cause breathing problem and skin and eye irritation if inhaled or swallowed (Priac et al. 2014).

Other aromatic synthetic dyes are triphenylmethane which includes methyl violet, cotton blue, and malachite green that contribute 10–15% (Chen and Ting 2015) annual dye release into the environment through pharmaceuticals, acrylic (Asfaram et al. 2016), leather, and paper industries. Malachite green an N-methylated diaminotriphenylmethane dye is a highly recommended component for fish farming industry due to antifungal (Asfaram et al. 2016), antibacterial, and antiprotozoan nature. These toxic components are recalcitrant and can cause mutation and cancer (Chen and Ting 2015). It is harmful to aquatic animals as it blocks direct penetration of sunlight (Chowdhury et al. 2011) and enhances malignant cell formation (Jasinska et al. 2012).

10.2.3 Heavy Metals

Heavy metal pollutants generated through various industrial practices, i.e., combustion, extraction (Jarup 2003), mineral processing, and metallurgical process, and released liquid effluents, etc. are highly toxic to human health and atmosphere. Due to their nondegradable nature, it can accumulate into living system and cause potential irreversible health effects even at small quantity (Ahluwalia and Goyal 2007). Lead released through paint industry, photographic materials, battery manufacturing, and pigments poses a huge danger for the environment and humans. Lead poisoning causes severe health problem, i.e., hemolytic anemia (Faryal et al. 2007). Chromium highly present in industrial effluents is released from leather tanning and metal finishing. It is a carcinogenic metal, exists in oxidative states Cr III and Cr VI, and causes kidney failure, diarrhea, stomach ulcers, and allergic reactions (Ata et al. 2012). Mercury commonly found as methylmercury (MeHg) is able to enter the human blood and can cross placenta through the barriers. MeHg has toxic effects on neurological and cardiovascular system and gastrointestinal tract (Taylor and Williamson 2017). Cadmium and arsenic both present in natural form due to its anthropogenic activity. Cadmium and arsenic are found in aquatic environment having detrimental effect on the aquatic entity (Tan et al. 2016). The combination of zinc with acids or oxygen can cause extreme harm to biological systems. Copper affects human health by causing chronic diseases and leads to kidney and liver failure if consumed at high amount. Nickel metal mainly found in volatile form as $Ni(CO)_4$ causes death if inhaled (Siddiquee et al. 2015).

10.3 Traditional Methods

10.3.1 Adsorption

The mechanism involved the adsorption of soluble organic dyes from the waste water to highly porous, solid surface. It should be high surface capacity to adsorb the maximum amount of the pollutant and can be reusable (Veglio and Beolchini 1997).

10.3.2 Membrane Processes

Membrane process involves filtration of the polluted effluent to clarify the dye or metal and concentrate to recover the particular for reuse. The common membrane filtration processes are ultrafiltration, nano-filtration, and reverse osmosis (Gunatilake 2015). According to Gunatilake (2015), ultrafiltration requires membrane filter with pore size of 5–20 which allows the segregation of the molecules based on their weight ranging from 1000 to 100,000 Da. Reverse osmosis applies pressure to transfer the molecules through the membrane; it blocks the passage of solutes and separates solvent.

10.3.3 Oxidative Processes

Conversion of hazardous compounds into less toxic organic material using chemical agents, i.e., sodium hypochlorite, comes under the chemical oxidative processes. It involves ozonation process, photochemical oxidation process, and electrochemical oxidation process (Holkar et al. 2016).

10.3.4 Coagulation-Flocculation and Precipitation

The mechanism of coagulation-flocculation and precipitation requires electrostatic attraction between the oppositely charged soluble dye and polymer molecules to remove toxic dyes and heavy metals from the industrial effluents (Holkar et al. 2016).

10.3.5 Electrocoagulation

Electrocoagulation involves several mechanisms such as electrolytic reactions at electrodes, formation of coagulants in aqueous effluent and adsorption of soluble or colloidal pollutants on coagulants, and removal by sedimentation and flotation.

Electrocoagulation technique is easy to employ and does not require chemical treatment with production of less sludge amount (Gunatilake 2015).

10.3.6 Ionic Exchange

The ionic exchange is mainly based on the ionic interaction between functional group on ionic species and an adsorptive solid material (Veglio and Beolchini 1997).

10.4 Advantage Over Traditional Method

Rapid increase in industrialization and anthropogenic activity leads to increased production of toxic effluents accommodating various organic pollutants, toxic metals, and their metalloids. At industrial level effluent discharge demands increasing expenditure (Fomina and Gadd 2014). These physiochemical techniques are costly and may not always be practicable and non-ecofriendly, and also their metal-binding properties are nonspecific. Biosorption involves both physiological, biochemical, and metabolically independent processes based on surface complexation, ion exchange, precipitation, and absorption (Fomina and Gadd 2014). Fungal biosorption depends on various parameters, i.e., metal ion or dye concentration, biomass concentration, pH, temperature, and pretreatment of the biomass (Kapoor and Viraraghavan 1997). The ability to solubilize complex metal compounds, environmental adaption capability at various physiochemical conditions, and high surface area-volume ratio are important factor that increases fungal usage over other microorganisms (Leitao 2009).

10.5 Fungal Biosorbent

Biosorption is a versatile mechanism with combined adsorption and absorption process that mediate the adhesion of any substance on biological material. Adsorption mechanism involves the attachment of organic or inorganic matter onto biological subsistence surface or coupling of ions onto surface of biological entity in removal process from aqueous solution. Biosorption diverges the process into bioadsorption, bioabsorption, or bioaccumulation (Fomina and Gadd 2014).

Biosorbent manifests higher compatibility for metal ions as well as for the organic or inorganic pollutants and beneficiary for organic pollutant degradation, metal recovery, and recycling. Microbial origin, i.e., bacteria, cyanobacteria, algae, and fungi, can be utilized as biosorbent. Fungi cell composed of thick cell wall contains prominent structures like mannan, chitin, glucans, extracellular proteins, polysaccharides, and pigment which assist the metal binding at various peculiar binding sites (Gadd 2008). Fungi can familiarize with any bionomic condition and

survive in any habitat. Fungal species, i.e., *Trichoderma*, are easy to cultivate due to fast-growing nature and able to produce spore (Siddiquee et al. 2015).

Both live and dead fungal biomasses are applicable to eliminate dyes/heavy metals from the contaminated terrestrial and aquatic ecosystem. Madani et al. (2015) analyzed the biosorption capacity of the fungal biomass of *Pleurotus mutilus* for biosorption of Fe^{+3} and Mn^{+2}. FTIR data showed the presence of alcohol (–C–O), hydroxyl (–OH), amino ($–NH_2$), and carboxyl group (–C=O) on biosorbent which are responsible for biosorption of positively charged metal ions (Fe^{+3} and Mn^{+2}) with negatively charged functional group. *Trichoderma versicolor* fungal biomass analyzed for biosorption of chromium (VI) and Remazol Black B reactive dye in the study conducted by Aksu et al. (2007). Chromium (VI) biosorption was observed at initial concentration of 15–30 mg l^{-1}, and chromium uptake was found to be in the range of 2.34–4.04 mg/g^{-1}. The biosorption of Remazol Black B reactive dye also estimated with *Trichoderma versicolor*, but the biosorption process was slow compared to the chromium (VI). Dye biosorption was found to be 92.4% with the incubation time of 5–8 days with initial dye concentration of 58.4 mg l^{-1}.

Kuhar and Papinutti (2013) reported that due to overpressure during dye/heavy metal degradation, fungi do not outlast. They analyzed the effect of vanilloids on *Ganoderma lucidum* while removing the gentian violet (GV) and malachite green (MG) dyes, clotrimazole, and cadmium metal. They are added into culture medium and observed high adsorption capacity into fungi after vanilloids addition. Recently report shows that modification of fungal biomass increases the activity. *Aspergillus versicolor* modified by using cetyl trimethyl ammonium bromide (CTAB) enhances the fungal biosorption of Reactive Black 5 at various physiochemical conditions compared to the unmodified (Huanga et al. 2016). White rot fungi *Coriolopsis* sp. (1c3), known for decolorizing anthraquinone and heterocyclic and azo dyes, were analyzed for triphenylmethane (TPM) dye biosorption and found able to reduce TPM dyes. Akar and Divriklioglu (2010) reported the increased fungal biosorption of *Agaricus bisporus* with small modification using CTAB for Reactive Red dye biosorption. Fungal biomass can accumulate dyes into cell wall and internal space. *Candida tropicalis* proficiently bioaccumulate the dyes, i.e., Remazol Blue, Reactive Black, and Reactive Red. *Candida albicans* isolated from industrial discharge are found to degrade the azo dye Direct Violet 51 at lower pH conditions (Vitor and Corso 2008). Living fungal biomass equally degrades the heavy metal pollutant as dyes based on their interaction. Metal resistance developed by organisms involves particular mechanism, i.e., cell membrane metal efflux, intracellular chelation, and phytochelatins and metal compartmentalization (Errasqun and Vazquez 2003).

Mucor indicus avowed as fermenting agent which convert hexose and pentose into volatile solvent ethanol. The dimorphic fungus is also known for the metal-binding affinity due to the presence of chitin and chitosan. Javanbakht et al. (2011) conducted the study using *M. indicus* of different morphology and analyzed as biosorbent for lead removal from the contaminated solution. Fungal biomass has great prospective to produce extracellular enzymes during whole life cycle. Fungal enzyme mediated metal binding and acts as feasible biosorbent agents (Sharma and Malaviya 2016). Srivastava and Thakur (2006) performed detoxification of heavy metal chromium (Cr) based on the enzyme-mediated process using *Aspergillus niger* fungi isolated from tannery

effluents. Sewage sludge-isolated nonpathogenic *Trichoderma atroviride* was analyzed for metal tolerance and uptake capacity and effectively removed copper, zinc (Errasqun and Vazquez 2003), and cadmium (Mohsenzadeh and Shahrokhi 2014). Nongmaithem et al. (2016) assessed *Trichoderma* isolates MT-4, UBT-18, and IBT-I biosorption capacity to remove nickel and cadmium at higher metal concentration. *Rhizopus arrhizus* established with great potential to uptake 18.5% thorium among microorganisms, i.e., *Penicillium* spp., *Aspergillus* spp., *Mycobacterium smegmatis*, *Citrobacter* spp., and *Pseudomonas* spp. Bhainsa and D'Souza (2009) reported the *Aspergillus fumigatus* for metal uptake capacity, which was higher compared to the *Rhizopus arrhizus* with 37% (Errasqun and Vazquez 2003).

Dead cells execute more number of favors over the live cells such as no special media requirement for growth, easy to desorption and recovery, facile regeneration process, and no lethal effects of toxicants (Kabbout and Taha 2014; Kapoor and Viraraghavan 1997). Sanghi et al. (2009) found that dead white rot fungus *Coriolus versicolor* is able to remove chromium (Cr^{6+}) by forming complex with its polysaccharide component of the cell wall. Immobilization processes provide protection to the cell from physical damage, minimize the medium viscosity, procure protease activity, supply sufficient amount of oxygen, and increase mass transfer between media and cells (Daassi et al. 2013). Goyal et al. (2014) examined the bioreductant activity of immobilized white rot fungus *Coriolus versicolor* for decolorization at various parameters, i.e., pH, nutrient, and oxygen. Daassi et al. (2013) embedded the fungal biomass *Coriolopsis gallica*, *Bjerkandera adusta*, *Trametes versicolor*, and *Trametes trogii* onto Ca-alginate beads. The beads were further used for the removal of Lanaset Grey (LG), a metal textile dye comprising of organometal complexes (chromium III and cobalt).

Tannery dye Black Dycem TTO decolorization by white rot fungi *Trametes versicolor*, *Ganoderma lucidum*, and *Irpex lacteus* was evaluated and suggested adsorption and biodegradation as key mechanism (Baccar et al. 2011). Yang et al. (2011) compared the biosorption capacity of unmodified and CDAB-modified nonviable *Aspergillus oryzae* biomass for removal of Acid Blue 25 and Acid Red 337 using single and binary system and found that modified fungal biomass was more efficient than the unmodified. Maurya et al. (2006) formulated biosorbent from *Fomes fomentarius* and *Phellinus igniarius* for biosorption of methylene blue and Rhodamine B. Dead *Aspergillus niger* was pretreated with $NaHCO_3$ which improved the biosorption capacity and enhanced Congo red removal from the solution (Fu and Viraraghavan 2002).

10.6 Biosorption Mechanism

Heavy metal affects fungal growth, and metabolism results in uptake of metals through various processes (Kapoor and Viraraghavan 1997) including chemisorption, adsorption-coupled reduction process, ion exchange, precipitation (Pundir et al. 2016), and electrostatic interaction as shown in Fig. 10.1.

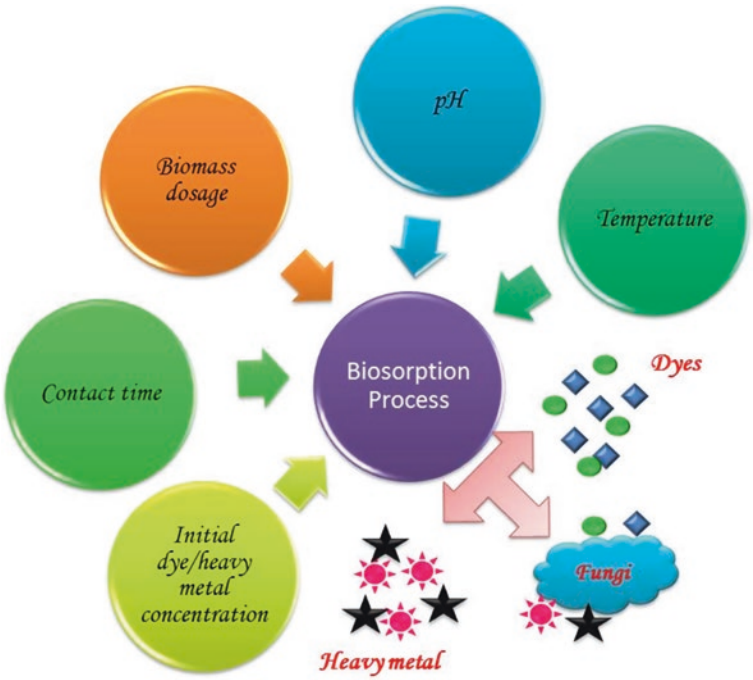

Fig. 10.1 Factors affecting fungal biosorption for dye/heavy metal

10.6.1 Molecules Translocation Through Cell Membrane

Transportation of dye/heavy metal ions into the cell from the extracellular environment using active/passive transport mechanism through the cell membrane is required for removal of dye/heavy metal ions from the environment. Two type of transportation are present in cell metabolism-dependent mechanism which require energy and allow dye/heavy removal from the aqueous solution (Veglio and Beolchini 1997).

10.6.2 Chemisorption/Electrostatic Interaction

Chemisorption involves bonding formation in chemical group (hydroxyl, amine, phosphoryl, thiol, etc.) present on fungal cell wall/surface and substrate to be adsorbed. Chemisorption excludes dependency on metabolic reaction within fungal cell (Bhainsa and D'Souza 2009) and forms strong bonding than ion exchange (Sheoran and Sheoran 2006). Bhainsa and D'Souza (2009) analyzed thorium (IV) uptake mechanism of *Aspergillus niger* using Temkin isotherm and first- and second-order Lagergren equations which clearly indicate that it involves chemisorption mechanism.

10.6.3 Ion Exchange

The functional groups present on cell surface interact with the metal ion known as ion exchange mechanism, for the uptake of metal ions from the surrounding environment and decontaminated (Kapoor and Viraraghavan 1997). Cationic ion exchange relied on environmental condition, metal concentration, and presence of other metals.

10.6.4 Precipitation

Condensation of dye/heavy metal concentration in the absence of water molecules known as precipitation that hepls in removal of contaminants in bulk amount. Gibert et al. (2005) removed Zn metal as merging of two chemical reaction metal (oxy) hydroxides and carbonates precipitation and sorption onto surface of municipal compost. This process depends on metal solubility, metal ion concentration, and presence of relevant anions and wetland pH (Sheoran and Sheoran 2006).

10.7 Factors Affecting Biosorption Mechanism

Biosorption process depends on various factors that influence the biosorption mechanism, i.e., pH, biosorption rate, initial dye/heavy metal concentration, metal speciation, solubility of metal ions, temperature, contact time, and binding site present on fungi for biosorption of dye/heavy metals (Veglio and Beolchini 1997).

10.7.1 pH

pH is one of the major factors that affect dye/heavy metals uptake from the environment. Change in pH enhances the biosorption rate as decreasing pH provides large number of free binding site and hence increases metal uptake (Dwivedi 2012). Maximum Cd removal capacity was higher at pH 9.0 compared to pH 5.0 and pH 7.0 by *Trichoderma* sp. (Mohsenzadeh and Shahrokhi 2014). Thorium removal by *Aspergillus fumigatus* was found to be at pH 4.0 with maximum metal ion removal capacity (Bhainsa and D'Souza 2009). Excess H^+ ions provide positive charge to *Coriolus versicolor*, and surface positive charges interact with $HCrO_4$– with higher affinity at pH 2.0 (Sanghi et al. 2009).

10.7.2 Initial Concentration of Dye/Heavy Metal

Initial dye concentration plays an important part that determine molecules mass transfer resistance between the medium and sorbent (Khataee et al. 2013). Methylene blue biosorption increased from 0.8 to 9 mg/g *Aspergillus fumigatus* fungal biomass with the initial dye concentration of 2–24 mg/g (Kabbout and Taha 2014). The effect of initial concentration on metal ion biosorption is analyzed by using initial concentration of chromium [Cr(III)/ Cr(VI)] ranging from 0 to 500 mg/L. Reduction of Cr (IV) to Cr (III) by microalga *Nannochloris oculata* was observed over 200 mg/L initial concentration which proves that initial concentration also matters along with fungal biomass and contact time (Kim et al. 2011).

10.7.3 Metal Speciation

Solubility and mobility of metal ions depend on metal speciation which refers to the quantification of metal ions into solution as hydroxides, organometallic compounds, and other biomolecule form.

10.7.4 Temperature

Temperature between 25 and 30 °C is the most favorable condition for the optimum fungal growth. Temperature also affects the enzymatic degradation of the dye by fungi (Kabbout and Taha 2014). Iqbal and Saeed (2007) studied the influence of physiochemical parameters on dye biosorption capacity, i.e., pH, contact time, initial dye concentration, and temperature on loofa sponge-immobilized fungal biomass and free fungal biomass *Phanerochaete chrysosporium* on Remazol Brilliant Blue R. The biosorption equilibrium was analyzed at different temperature ranging from 20 to 50 °C with initial concentration of 100 mg l^{-1}. The biosorption of Remazol Brilliant Blue R increased till 30 °C and decreased with increasing temperature.

10.7.5 Contact Time

Contact time also plays an effective role in fungal biosorption indicating the efficiency of biosorbent to remove pollutant from the environment. Yousefi et al. (2015) evaluated the metal adsorption efficacy of dead mass of *Aspergillus niger*. Quick removal of metal ions depends on the interchange between the functional groups present on fungal cell surface at initial reaction period and further reduction into

metal ions adsorption due to intracellular aggregation of the metal particles. Adsorption rate at initial 5 min for metal ions (zinc (II), cobalt (II), and cadmium (III)) was quick until it reaches equilibrium condition.

10.7.6 Biomass Dosage

Biomass dosage has its own importance in enhancing the decolorization by increasing dosage. The efficiency of decolorization rapidly increased in dose-dependent manner, as 0.1 mg/ml Congo red dye was found to be 90.58% at 3 g of white rot fungus, *Ceriporia lacerate*. Sometimes rapid increase in adsorption surface area and biosorption sites can hinder the adsorption rate of biomass dosage (Wang et al. 2017). Akar and Divriklioglu (2010) explained that later constant biosorption is possessed due to binding site superabundance of dyes particles which occupied the biosorbent surface. List of fungal biomass used as biosorbent for dye/heavy metal biosorption is given below in the (Table 10.1).

10.8 Biosorption Isotherms Models

The association that materialize between adsorbate concentration and adsorbed compound per biosorbent biomass designate as biosorption equilibrium isotherm. Indulgement of various parameters which explains the compatibility with adsorbent and the surface properties of the biosorbent and analyzes the biosorption capacity for individual toxic/hazardous compounds (Abdel-Ghani and El-Chaghaby 2014). The isotherm equilibrium generates an equation that is helpful to compare the diverse biosorbent and to optimize the process for future experiment (Ata et al. 2012). Gadd (2008) explains that the parameters, viz., pH, biomass dosage, presence of metal ions, and their cationic opposites, are applicable in single-batch system. Many scientists prefer breakthrough curves to calculate sorbent efficiency due to complexity of flow/continuous system. The equilibrium data can be explained by plotting the sorbent uptake value (q_e) against the end sorbent concentration (C).

$$q_e = \frac{V\left(C_i - C\right)}{S} \tag{10.1}$$

Solution volume contacted with the sorbent (V), sorbate initial and end concentration (C_i and C), and biosorbent amount (S) affirm as dry weight.

A wide range of models are present from simple to complex multidimensional models, i.e., *Langmuir*, *Freundlich*, *Temkin*, *Dubinin-Radushkevich*, *Flory-Huggins*, *Sip*, *Khan*, *Toth*, *Redlich-Peterson*, and *Radke-Prausnitz*, to explain the equilibrium isotherm for biosorption of dye/heavy metal.

Table 10.1 List of fungal biomass used as biosorbent for dye/heavy metals biosorption

S. No.	Live/dead fungal biomass	Biosorbed dye/heavy metal	References
1	*Trametes versicolor*, *Ganoderma lucidum*, *Irpex lacteus*	Black Dycem	Baccar et al. (2011)
2	*Coriolopsis gallica*, *Bjerkandera adusta, Trametes versicolor*, *Trametes trogii*	Lanaset Grey (LG)	Daassi et al. (2013)
3	*Aspergillus oryzae*	Acid Blue 25; Acid Red 337	Yang et al. (2011)
4	*Fomes fomentarius Phellinus igniarius*	Methylene Blue, Rhodamine B	Maurya et al. 2006)
5	*Aspergillus niger*	Congo red	Fu and Viraraghavan (2002)
6	*Agaricus bisporus*	Reactive Red dye	Akar and Divriklioglu (2010)
7	*Candida tropicalis, Candida albicans*	Remazol Blue, Reactive Black, Reactive Red, Direct Violet 51	Vitor and Corso (2008)
8	*Mucor indicus*	Lead (Pb^{+})	Javanbakht et al. (2011)
9	*Aspergillus niger*	Chromium (Cr)	Srivastava and Thakur (2006)
10	*Trichoderma atroviride*	Copper, zinc	Errasqun and Vazquez (2003)
11	*Aspergillus fumigatus*	Thorium	Bhainsa and D'Souza (2009)
12	*Coriolus versicolor*	Chromium (Cr^{6+})	Sanghi et al. (2009)
13	*Pleurotus mutilus*	Fe^{+3} and Mn^{+2}	Madani et al. (2015)
14	*Ganoderma lucidum*	Gentian violet, malachite green, clotrimazole cadmium	Kuhar et al. (2013)
15	*Trichoderma versicolor*	Chromium (VI) Remazol Black B	Aksu et al. (2007)

10.8.1 Langmuir Equilibrium Model

Hypothesis proposed by Langmuir model describes the consistency of energies adsorption without adsorbent renascence on the plane of the surface. Accuracy of the *Langmuir* model defines uniform distribution of adsorbent on the specific position situated on the biosorbent surface as monolayer, and the saturation of the sites blocks the excess adsorbate's adhesion. The isotherm can be represented as

$$q_e = \frac{Q_0 b C_e}{1 + b C_e} \tag{10.2}$$

C_e represents the equilibrium concentration (mg/L), qe represents the amount adsorbed per specified amount of adsorbent (mg/g), and Q_0 represents the amount of adsorbate required to form a monolayer.

Bhainsa and D'Souza (2009) observed maximum loading capacity of filamentous fungal biomass *Aspergillus fumigatus* for biosorption of thorium (Th) metal ions by *Langmuir* model. The statistical data showed that the maximum loading capacity (*Q0*) per gram biosorbent was 99, 143, and 455 mg thorium at initial pH 2, 3, and 4. The uptake of thorium was pH dependent which enhances with increase in pH range from 2.0 to 4.0. Maximum intake was noticed as 455 mg of thorium per gram biosorbent at pH 4.0 with estimated value of 370 mg Th/g fungal biomass. Increased pH allows binding of bivalent $Th(OH)_2^{2+}$ to the biomass due to thorium's lower solubility. According to Eq. (10.2), biosorption process is convenient when the *R*L value is less than 1, where 50 mg Th/L showed *R*L values of 0.59, 0.53, and 0.83 at the pH 2, 3, and 4, respectively. This demonstrates the suitability of the biosorption process for the thorium removal.

Tan et al. (2010) calculated biosorption capacity for biosorption of basic orange dye by dried fungal biomass *Azolla filiculoides. Langmuir* isotherm model determined the maximum biosorption capacity of *Azolla filiculoides* as 833.33 mg/g biosorbent and 3.67×10^{-3} L/mg of the K_L (consumed energy for biosorption). Correlation coefficient (R2) 0.996 also showed the favorability of the isotherm. This study indicates the basic orange dye biosorption by dried *Azolla filiculoides* which supports the formation of monolayer onto binding sites present on biomass surface.

10.8.2 Freundlich Equilibrium Model

Freundlich model suggests (Saha and Orvig 2010) the multilayer formation of adsorbate over a heterogeneous surface with different energies as a result of uneven dispersion of the adsorbate and is independent of biosorbent saturation. Mathematical representation for the *Freundlich* models is expressed as

$$q = kC_f^{(1/n)} \tag{10.3}$$

k and *n* (nondimensional) represents Freundlich constants, based on the estimation using the slope and the intercept of the plot drawn between 1n *q* and 1n C_f.Linear form of the *Freundlich* equation can be expressed as

$$\ln q = 1/n \ln C_f + \ln k$$

The model can be considerable to design biosorption process if the *n* follows the range between 1 and 10. This represents the higher affinity in between biosorbent and adsorbate which increases the biosorption rate.

Patel and Suresh (2008) analyzed the equilibrium data using *Freundlich* model for the biosorption of Reactive Black 5 dye by *Aspergillus foetidus.* Fungal biomass of

Aspergillus foetidus was pretreated with 0.1 M sodium hydroxide (NaOH) and compared the biosorption capacity with untreated fungal biomass. The K_f values for *Freundlich* isotherm increased with the elevated temperature (30–50 °C) signified the endoergic nature of the biosorption of Reactive Red 5 dye by pretreated *Aspergillus foetidus* biomass with 0.1 M NaOH. The studies showed that *Freundlich* isotherm model was fitted for anticipation of the isotherm for lesser dye concentration. Analytical data proposed the heterogenic nature of the *Aspergillus foetidus* mediated biosorption and pretreatment with 0.1 M NaOH enhanced the biosorption capacity.

10.8.3 Temkin Equilibrium Model

Temkin isotherm assumes the concomitant interaction of adsorbate-adsorbate on adsorption isotherm which produces heat energies that decrease with increasing coverage (Smitha et al. 2017).

$$q_e = \mathrm{RT}/b1\mathrm{n}\left(\mathrm{AC}_e\right) \tag{10.4}$$

The equation can be expressed in a linear form

$$q_e = \beta 1n\alpha + \beta 1n C_e$$

Constant β represents the adsorption heat generated by the interaction, where β is equal to (RT)/b.

Madani et al. (2015) observed biosorption of Fe^{+3} and Mn^{+2} by *Pleurotus mutilus* fungal biomass and used different isotherm to explain simple biosorption equilibrium data. The pH varied from range 1 to 8.5 and biosorption was executed for Mn^{2+} and Fe^{3+} ions. The biosorption of ions depends on the ionic form of the metal and electrical charge present on biomass mainly concerned with pH of the solution. The pH preferred for biosorption studies of Mn^{2+} and Fe^{3+} ions were 2.6 and 8, respectively. The Temkin isotherm found b constant as 684.383 and K_T as 34.982 with R2 value of 0.955 for Fe^{3+} and for Mn^{2+} b constant as 0.002 and K_T 1 and R2 value of 0.841.

10.8.4 Dubinin-Radushkevich Equilibrium Model

Dubinin and Radushkevich (1947) proposed an isotherm model which explains the effects of porous structure of biosorbent that indirectly affects biosorption capacity. The Dubinin-Radushkevich avoids the existence of constant sorption potential and heterogeneous surface of the biosorbent. The isotherm can be represented as

$$q = q_{\max} \exp\left(-B\varepsilon^2\right) \tag{10.5}$$

q_{max} stands for the monolayer saturation capacity (mol g^{-1}), constant B is correlated with biosorption energy, and ε represents Polanyi potential.

$$\varepsilon = \mathrm{RT}\ln\left(1+1/C_{\mathrm{f}}\right) \tag{10.6}$$

where R characterized as gas constant (8.314 J mol^{-1} K^{-1}) and T represents absolute temperature (K). The linearized form can be expressed as

$$\ln q = -B\varepsilon^2 + \ln q_{\max} \tag{10.7}$$

Dubinin-Radushkevich describes mean biosorption energy and determined chemical or physical nature of biosorption process. Biosorption energy (E) can be measured as

$$E = \frac{1}{\sqrt{2B}} \tag{10.8}$$

The biosorption energy ranges from 8 to 16 kJ mol^{-1} for chemical ion exchange, and biosorption energy less than 8 kJ mol^{-1} describes physical nature of the process.

Ahmad et al. (2013) studied the biosorption of zinc ions (Zn^{2+}) by *Candida utilis* and *Candida tropicalis*. The biosorption energy calculated from the *Dubinin-Radushkevich* isotherm describes the chemical ion exchange nature as the values fall under 9.81–11.16 kJ mol^{-1} for *Candida utilis* and 10.15–12.33 kJ mol^{-1} for *Candida tropicalis*. Akar et al. (2013) examined the biosorption potential of the filamentous fungal *Thamnidium elegans* for the removal of Reactive Red 198 dye. They studied the effect of various parameters such as contact time, temperature, initial dye concentration, and pH. The biosorption energy observed 18.39 kJ mol^{-1} for batch system and 16.09 kJ mol^{-1} for continuous column system. These energy values show the chemical ion exchange nature of the biosorption process.

10.9 Desorption and Reuse

Desorption process is the major part of the biosorption process which relieves the adsorbed molecule attached to the biosorbent using different kind of elution solution. It permits the recovery and reuse of the particular adsorbate. Biosorbent loses the biosorption capacity after elution, so the elution system should not harm the biosorbent properties while eluting the adsorbate. For the purpose organic solvents as desorbing agents, i.e., ethanol and surfactants chemical like NaOH and $CaCl_2$, are available for regeneration and elution of the adsorbate (Fomina and Gadd 2014).

Patel and Suresh (2008) performed the desorption process for the recovery of Reactive Black 5 dye by exposing the fungus *Aspergillus foetidus* with pH range

from 3 to 10 with 0.1 M and 1 M NaOH. At higher pH due to electrostatic repulsion between cationic fungal cell surface and anionic reactive dye, so desorption was observed at higher pH condition. The desorption percentage at 0.1 M NaOH was 90%, whereas in case of 1 M NaOH, 32% was observed.

Bhainsa and D'Souza (2009) desorbed thorium (Th) bound with *Aspergillus fumigatus* biomass. They compared different desorbing agents: ethylenediaminetetraacetic acid (EDTA), calcium carbonate ($CaCO_3$), sodium carbonate (Na_2CO_3), and sodium bicarbonate ($NaHCO_3$). The 97% and 99% desorption was observed in 1 M Na_2CO_3 and 1 M $NaHCO_3$ eluting agents, respectively. At 0.1 M EDTA concentration, 91% thorium desorption was found where as in 1 M $CaCO_3$ maximum desorption found was 93%.

Javanbakht et al. (2011) treated the *Mucor indicus* used for biosorption of lead with 0.05 M HNO_3 for 1 h to perform desorption of the ions. Acidic elution is one of the important approaches for desorption process. Nitric acid (HNO_3) and hydrochloric acid (HCl) are found to be the favorable desorbing agents due to their proton exchange properties, where HNO_3 eluted 80% lead from the fungal biomass of *M. indicus.*

10.10 Conclusion

Fungal biosorption can be considered as feasible biological approach that is ecofriendly as well as cost-effective. The biosorption capacity of the fungal biomass can be enhanced by pretreatment of the biomass or immobilization onto immobilizing agents that protects fungal biomass from the harsh conditions during biosorption process. Biosorption process can be enhanced by optimization of various physiochemical parameters, i.e., pH, incubation time, temperature, biomass dosage, and monitoring the initial dye/heavy metal concentration. Further we can use biosorption process for the recovery of organics removal, recovery of pharmaceuticals and precious metal and elements.

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Chapter 11
Application of Myconanotechnology in the Sustainable Management of Crop Production System

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11.1 Introduction

Nanomaterials, in general, are metal particles of less than 100 nm in at least one dimension and are the backbone of nanoscience and technology. Science of engineering nanomaterials or specifically nanotechnology is a novel scientific impend that involves the use of materials and equipment capable of manipulating physical as well as chemical properties of a substance at molecular levels. The combination of nanotechnology with biotechnology has significantly expanded the application domain of nanomaterials in diverse fields. A variety of single-walled and multiwalled carbon nanotubes (SWCNTs/MWCNTs), magnetized iron (Fe) nanoparticles, aluminium (Al), copper (Cu), gold (Au), silver (Ag), silica (Si), zinc (Zn) nanoparticles and zinc oxide (ZnO), titanium dioxide (TiO_2), cerium oxide (Ce_2O_3), etc. are being developed (Nair et al. 2010). The nanomaterials are used in medicine, water purification, wastewater treatment, environmental remediation and food processing and packaging, besides many industrial and household purposes and advancement of smart sensors (Byrappa et al. 2008; Gao et al. 2009; Lee et al. 2010; Zaragoza et al. 2011; Prasad et al. 2016). These materials are also being used in agriculture production and crop fortification (Bouwmeester et al. 2009; Nair et al. 2010; Sharon et al. 2010; Emamifar et al. 2010). The advancement of nano-devices and nanomaterials has been credited to shape agriculture and plant biotechnology in a modern way. Nanotechnology is one of the latest lines of technological innovations which occupies a prominent position in transforming agriculture and food production. The use of nanomaterials in agriculture, however, is relatively new and needs further investigation. Agriculture is the backbone of developing countries where more than 60% of the population depend on it for their survival (Brock et al. 2011). Nanotechnology has the capability to revolutionize the agricultural and food

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industry with novel implements for the control of diseases at molecular level, faster disease detection, enhancement in the ability of plants to absorb nutrients and water, etc. (Prasad et al. 2017). It can also upgrade the understanding of the biology of various crops and thus can potentially increase the yield and or nutritional qualities of plants. It can also be used to develop improved systems to detect environmental conditions (Tarafdar et al. 2013). The agriculture is facing new challenges like soil and plant health, increase in the disease intensity, emergence of new diseases and threats to agricultural production due to changing weather patterns (Biswal et al. 2012; Prasad et al. 2014). Nanobiotechnology can also be used for detection of viruses or disease-infecting particles and thus holds the potential for primordial detection and eradication. It has provided smart sensors and delivery systems which help the agricultural industry fight against viruses and other crop pathogens (Prasad et al. 2012, 2017). In the agricultural sector, nanotech research and development are likely to abet and frame the next level of development of genetically modified crops and precision farming techniques (Prasad et al. 2012, 2014). Research is going on to synthesize nanostructured catalysts which will be able to increase the efficiency of pesticides and herbicides, thus allowing lower doses to be used. Nanotechnology will also protect the environment indirectly through the use of renewable energy sources and filters or catalyse pollutants to reduce pollution and clean out existing pollutants in soil and water.

Thus, due to the beneficial usefulness of nanoparticles, rapid developments are going on to synthesize metallic and bimetallic nanomaterials. Various methodologies have been formulated to synthesize noble metal nanoparticles of specific shape and size depending on specific requirements. Biosynthesis of nanoparticles as an emerging highlight of the connection of nanotechnology and biotechnology has received enlarged interest due to a growing need to develop environmentally benevolent technologies in material syntheses. Biological entities have an exclusive potential to synthesize molecules with selective properties, thus becoming an impending tool for nanoparticles synthesis (Vinod et al. 2010). Biosynthesis of NPs has been carried out by exploiting microbes (Prasad et al. 2016). Surface complexation of nanogold with amino acids and proteins is a promising field of research (Mohanpuria et al. 2008). It has been shown that extracellularly produced silver or gold nanoparticles using fungi (e.g. *Fusarium oxysporum*) can be used in different fields. Biosynthesized nanoparticles have large number of applications such as in nonlinear optics, spectrally discriminating coating for solar energy absorption, biolabelling and intercalating materials for electrical batteries, as optical receptors, as catalyst in chemical reactions and as antibacterial capacities (Mohanpuria et al. 2008; Suman et al. 2010; Aziz et al. 2015, 2016).

Myconanotechnology can offer green and eco-friendly alternatives for plant disease management. As compared to other microbes, fungi are used as effortless biomanufacturing units for nanoparticles (NPs) synthesis (Prasad 2016, 2017). The non-pathogenic nature of some fungal species in combination with the ease of production and handling will increase the mass production of silver nanoparticles. Recently, a wide range of fungi have been screened for their ability to produce silver nanoparticles (Alghuthaymi et al. 2015). Different researchers have reported the

mycosynthesis of gold, silver, gold silver alloy, selenium, tellurium, platinum, palladium, silica, titania, zirconia, quantum dots, usnic acid, magnetite, cadmium telluride and uraninite nanoparticles (Alghuthaymi et al. 2015). Nanotechnology helps in inaccessible activation and monitoring of quick nano-delivery systems which can assist future agricultural growers to minimize fungicides and pesticides use. Nanoparticle-mediated gene transfer would be helpful for development of crops resistant to pathogens and pest. In this review we have tried to evaluate the works carried out as regard to the role of fungi in the synthesis of nanoparticles and application of those nanoparticles in different fields of agriculture like seed germination, plant growth, protection and production, pathogen detection, pesticide/herbicide residue detection, etc.

11.2 Synthesis of Nanoparticles by Microfungi

The use of microbial cells for the production of nanosized material has emerged as a new approach (Gericke and Pinches 2006). Several fungal species like *Fusarium*, *Aspergillus*, *Verticillium*, *Penicillium*, etc. have been used as promising resources for nanoparticle fabrication. Different fungal species are dexterous source for production of metal NPs both intra- and extracellularly. Fungi have a number of advantages for NPs synthesis over other microbes and plant materials. The use of fungi in the synthesis of NPs is potentially important since they produce large quantities of enzymes and are easy to handle in the laboratory (Mandal et al. 2006; Mohanpuria et al. 2008; Prasad et al. 2016). Since the NPs are produced extracellularly, they are easy to purify and can be directly used in different applications (Mukherjee et al. 2008; Gaikwad et al. 2013). Mesh of fungal mycelia can endure flow pressure and other conditions in bioreactors or other chambers as compared to plant materials or bacteria (Narayanan and Sakthivel 2010). Most fungi have a high tolerance towards metals and a high wall-binding potential, as well as intracellular metal uptake capabilities. Different fungi and metal NPs produced by them are listed in Table 11.1.
☺

11.2.1 Biosynthesis of Nanoparticles (NPs) by Fusarium, Penicillium, Aspergillus *and* Verticillium

Dias et al. (2002) screened different *Fusarium* spp. for their ability to produce nanoparticles, and they reported that *F. oxysporum* synthesized the smallest size of silver (Ag) NPs. *F. oxysporum* could reduce aqueous silver ions in water and extracellularly generated Ag-NPs (Ahmad et al. 2003; Birla et al. 2013; Bansal et al. 2004). TEM observations revealed that *F. oxysporum* produced spherical,

Table 11.1 List of fungi that synthesize metal NPs of various shapes and sizes under different reaction conditions and their applications in agriculture

Fungi	Method	Reaction condition	Reaction time (h)	Temp. (°C)	Shape	Size (nm)	Synthesis location	Nanoparticles produced	Applications	Reference
Alternaria alternata	NA	Cell-free filtrate	24	RT	S,T, H	20–60	Extracellular	Silver	Antifungal	Gajbhiye et al. (2009)
Aspergillus clavatus	Reduction	Active biomass	48–72	RT	S, T, H	10–25	Extracellular	Silver	Antibacterial	Verma et al. (2010)
A. flavus	Reduction	NA	NA	NA	NA	7–10	Extracellular	Silver	NA	Vigneshwaran et al. (2006)
A. fumigatus	Reduction	NA	NA	NA	NA	5–25	Extracellular	Silver	NA	Bhainsa and D'Souza (2006)
A. fumigatus	Reduction	NA	NA	NA	NA	50	Extracellular	Silver	NA	Navazi et al. (2010)
A. niger	NA	Cell-free filtrate	96	28 ± 2	S, E	NA	Extracellular	Silver	Antifungal	Fateixa et al. (2009)
A. niger	NA	NA	NA	NA	NA	15–20	Extracellular	Silver	Antibacterial	Kumar et al. (2008)
A. tamarii	NA	NA	NA	NA	NA	NA	Extracellular	Silver	NA	Kumar et al. (2012)
A. terreus	NA	NA	NA	NA	NA	1–20	Extracellular	Silver	Antifungal	Li et al. (2012)
Aspergillus sp.	NA	NA	NA	NA	NA	25	Extracellular	Zinc	NA	Raliya and Tarafdar (2014)
A. oryzae var. *viridis*	NA	Active and inactive biomass and cell-free extract	72–120	25	S, various shapes (cell-free filtrate)	10–60	Mycelial surface	Gold	NA	Binupriya et al. (2010)
A. sydowii	NA	Active biomass	NA	NA	S	8.7–15.6	Extracellular	Gold	NA	Vala (2014)

Cladosporium cladosporioides	NA	NA	NA	NA	NA	10–100	Extracellular	Silver	NA	Choudhury et al. (2010)
Colletotrichum sp.	NA	Active biomass	96	25–27	S,	60–80	Mycelial surface	Gold	NA	Shankar et al. (2003)
					LA	Undefined				
Coriolus versicolor	NA	NA	NA	NA	NA	20	Extracellular	Cadmium sulphide	NA	Sanghi et al. (2009)
Cylindrocladium floridanum	NA	Active biomass	168	30	S	5–35	Outer surface of the cell wall	Gold	NA	Narayanan and Sakthivel (2010)
Epicoccum nigrum	NA	Active biomass	72	27–29	NA	5–50	Intra- and extracellular	Gold	NA	Sheikhloo and Salouti (2011)
Fusarium acuminatum	NA	NA	NA	NA	NA	5–40	Extracellular	Silver	Antibacterial	Ingle et al. (2009)
F. semitectum	NA	Active biomass	24	RT	S	10–80	Extracellular	Gold	NA	Sawle et al. (2008)
F. solani	NA	NA	NA	NA	NA	5–35	Extracellular	Silver	NA	Ingle et al. (2009)
F. oxysporum	NA	NA	NA	NA	NA	5–15	Extracellular	Silver	NA	Ahmad et al. (2003)
F. oxysporum	NA	NA	NA	NA	NA	3–11	Extracellular	Zirconia	NA	Husseiny et al. (2015)
F. oxysporum	NA	NA	NA	NA	NA	5–15	Extracellular	Silica, titania	NA	Narayanan and Sakthivel (2010)
F. oxysporum	NA	NA	NA	NA	NA	20–50	Extracellular	Magnetite	NA	Bharde et al. (2006)
F. oxysporum	NA	Active biomass	72	NA	S, T	8–40	Extracellular	Gold	NA	Mukherjee et al. (2008)

(continued)

Table 11.1 (continued)

Fungi	Method	Reaction condition	Reaction time (h)	Temp. (°C)	Shape	Size (nm)	Synthesis location	Nanoparticles produced	Applications	Reference
Fusarium oxysporum	NA	NA	NA	NA	NA	10–25	Extracellular	Silver	NA	Kumar et al. (2007)
F. oxysporum	NA	NA	NA	NA	NA	2–5	Extracellular	Silver	Antibacterial	Khosravi and Shojaosadati (2009)
F. oxysporum	NA	NA	NA	NA	NA	5–60	Extracellular	Silver	NA	Mohammadian et al. (2007)
F. oxysporum	NA	NA	NA	NA	NA	30	Extracellular	Silver	NA	Khosravi and Shojaosadati (2009)
F. oxysporum f. sp. *lycopersici*	Reduction	NA	NA	NA	NA	10–50	Intracellular/ extracellular	Platinum	NA	Riddin et al. (2006)
F. oxysporum f. sp. *vasinfectum*	NA	Active biomass	24	30	S	3–30	Extracellular	Silver	Antibacterial	Joshi et al. (2013)
Helminthosporium solani	NA	Active biomass	72	37 ± 1	S, R, T, P, Py, St	2–70	Extracellular	Gold	NA	Kumar et al. (2008)
Hormoconis resinae	NA	Active biomass	24	30	S	3–20	Extracellular	Gold	NA	Mishra et al. (2010)
Neurospora crassa	Reduction	Active biomass	24	28	S	32	Intracellular	Gold	NA	Longoria et al. (2011)
Neurospora crassa	Reduction	NA	NA	NA	NA	60	Extracellular	Silver	NA	Longoria et al. (2011)
Penicillium brevicompactum	NA	Supernatant, cell-free filtrate, active biomass	12–72	30	S, T, H	10–60	Intracellular	Gold	NA	Mishra et al. (2011)

Penicillium fellutanum	Reduction	NA	NA	NA	NA	5–25	Extracellular	Silver	NA	Kathiresan et al. (2009)
P. nalgiovense	NA	NA	NA	NA	NA	15–25	Extracellular	Gold	NA	Maliszewska et al. (2013)
P. purpurogenum NPMF	NA	NA	NA	NA	NA	5–25	Extracellular	Silver	Antibacterial	Nayak et al. (2010)
Penicillium sp.	Reduction	NA	NA	NA	NA	16–40	Extracellular	Silver	NA	Bawaskar et al. (2010)
Penicillium sp.	NA	NA	NA	NA	NA	25–30	Extracellular	Silver	Antibacterial	Singh et al. (2014)
P. rugulosum	NA	Supernatant, cell-free filtrate, growth medium	8–24	30	S, T, H	2–80	NA	Gold	NA	Mishra et al. (2011)
Penicillium sp. 1–208	NA	Cell-free filtrate, active biomass	0.08–8	NA	S	30–50 40–60	Intracellular	Gold	NA	Du and He (2011)
Phanerochaete chrysosporium	NA	NA	NA	NA	NA	5–200	Extracellular	Silver	ND	Vigneshwaran et al. (2006)
Phoma glomerata	NA	NA	NA	NA	NA	60–80	Extracellular	Silver	ND	Birla et al. (2013)
Phoma sp.3.2883	NA	NA	NA	NA	NA	70–75	Extracellular	Silver	ND	Chen et al. (2003)
Pleurotus sajor-caju	NA	NA	NA	NA	NA	5–50	Extracellular	Silver	Antibacterial	Nithya and Ragunathan (2004)

(continued)

Table 11.1 (continued)

Fungi	Method	Reaction condition	Reaction time (h)	Temp. (°C)	Shape	Size (nm)	Synthesis location	Nanoparticles produced	Applications	Reference
Rhizopus oryzae	NA	Cell-free filtrate	24	30	S	16–25	NA	Gold	NA	Das et al. (2012)
Saccharomyces cerevisiae	NA	Active biomass	<24	30	S	15–20	Cell wall,	Gold	NA	Sen et al. (2011)
			>24			30	Cytoplasm			
Sclerotium rolfsii	NA	Cell-free filtrate	NA	RT	S	25.2 ± 6.8		Gold	NA	Narayanan and Sakthivel (2010)
Trichoderma asperellum	NA	NA	NA	NA	NA	13–18	Extracellular	Silver	NA	Mukherjee et al. (2008)
Trichoderma viride	NA	NA	NA	NA	NA	5–40	Extracellular	Silver	Vegetable and fruit preservation	Fayaz et al. (2009)
Trichothecium sp.	NA	NA	NA	NA	NA	5–200	Intracellular/ extracellular	Gold	NA	Ahmad et al. (2005)
Trichoderma sp.	NA	NA	NA	NA	NA	8–60	Extracellular	Silver	NA	Devi et al. (2013)
Usnea longissima	NA	NA	NA	NA	NA	50–200	Extracellular	Usnic acid	Antifungal	Shahi et al. (2003)
Verticillium luteoalbum	NA	Cell-free filtrate, active biomass	72	28	S	>10	Extracellular	Gold	NA	Ahmad et al. (2003)

Verticillium sp.	Reduction	NA	NA	NA	NA	2–20	Intracellular	Silver and gold	NA	Sastry et al. (2003)
Verticillium sp.	Reduction	NA	NA	NA	NA	20–50	Extracellular	Magnetite	NA	Bharde et al. (2006)
Volvariella volvacea	NA	Cell-free filtrate	NA	NA	NA	20–150	Extracellular	Silver	NA	Philip (2009)
Yarrowia lipolytica	NA	Active biomass	120	30	Various shapes depending on Au^{3+} concentration	NA	Intracellular	Gold	NA	Pimprikar et al. (2009)

NA not determined, *S* spherical, *E* elliptical, *H* hexagonal, *R* rods, *T* triangular, *P* pentagonal, *Py* pyramidal, *St* star, *LA* large aggregates, *RT* room temperature

well-dispersed Ag-NPs with size ranging between 5 and 13 nm. These Ag-NPs showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* besides good cytotoxic activity (Husseiny et al. 2015). Rapid and extracellular synthesis of silver nanoparticles using fungus *F. oxysporum* was also reported by Gopinath et al. (2015). When *F. oxysporum* was treated with aqueous $ZrF6^2$ anions, it could produce zirconia NPs by extracellular enzyme-mediated hydrolysis of the anionic complexes at room temperature (Bharde et al. 2006; Riddin et al. 2006). *F. oxysporum* and *Verticillium* sp. could also produce magnetite NPs in the presence of ferric and ferrous salts (Duran et al. 2010). Ishida et al. (2013) found monodispersed and spherical Ag-NPs with high antifungal activity against *Candida* sp. and *Cryptococcus* sp. produced by *Fusarium oxysporum*. Duran et al. (2010) screened strains of *F. oxysporum* f. sp. lycopersici and found that the strain successfully produced inter- and extracellular platinum NPs. Duran et al. (2010) and Khosravi and Shojaosadati (2009) also found that *F. oxysporum* could synthesize Ag-NPs and the biosynthesized Ag-NPs showed antibacterial properties when incorporated in textile fabrics. Rapid and extracellular synthesis of gold nanoparticles, using a plant pathogenic fungus *F. oxysporum* f. sp. *cubense* JT1 (FocJT1), was reported by Thakker et al. (2013). Korbekandi et al. (2013) through SEM analysis observed single almost spherical (25–50 nm) or aggregate (100 nm) Ag-NPs produced by *F. oxysporum*. TEM micrograph showed polydisperse spherical and ellipsoid silver nanoparticles of size ranging from 1 to 50 nm produced by *F. semitectum* with strong antibacterial activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Shelar and Chavan 2014). Numerous strains of the fungus *F. oxysporum* were studied by Ingle et al. (2008) for their ability to produce extracellular metal NPs and 5115 strains produced Ag-NPs. Nanoparticles of 10–100 nm size and different shapes, like hexagons, pentagons, circles, squares, rectangles and also irregularly shaped, were produced both intra- and extracellularly by *F. oxysporum* culture filtrate (Mohammadian et al. 2007; Deepa and Panda 2014). Ingle et al. (2009) and Deepa and Panda (2014) investigated the synthesis of Ag-NPs by *F. acuminatum* isolated from infected ginger and evaluated its antimicrobial activity against some bacterial human pathogens. Twelve fungal isolates of *F. oxysporum* could produce Ag-NPs in varied quantities (Rajput et al. 2016). The production of Ag-NPs by *F. oxysporum* was also investigated by Khan et al. (2014). They found that exposure of *F. oxysporum* to silver ion leads to the formation of silver nanoparticles. Ahmad et al. (2003) evaluated the biosynthesis of extracellular Ag-NPs of 5–15 nm dimensions by *F. oxysporum* through an enzymatic process. Extremely stable and crystalline Ag-NPs were produced by treating the filtrate of the fungus *F. semitectum* with a silver nitrate solution (Ingle et al. 2009; Bawaskar et al. 2010). Reduction of silver ions to Ag-NPs was confirmed when Ag^+ ions exposed to *Fusarium* isolates were characterized by them using UV_{Vis} spectroscopy and XRD. Sadowski et al. (2008) evaluated TEM images of the NPs and suggested that NPs were multi-dispersed and mostly spherical.

Penicillium sp. could successfully reduce $AuCl_4$ and carry out intracellular biosynthesis of gold NPs when exposed to $AuCl_4$ in aqueous condition (Honary et al. 2013). *P. citrinum* produced silver nanoparticles having a uniform spherical shape

with a size range of 90–120 nm which was determined by scanning electron microscopy (SEM). The gold nanoparticles were synthesized by reducing the aqueous gold ions using the culture supernatant of *P. chrysogenum* isolated from Ahar copper mine (Sheikhloo and Salouti 2011). Transmission electron microscopy showed intracellular formation of spherical-, triangular- and rod-shaped gold nanoparticles of 5–100 nm size (Shaligram et al. 2009). They achieved in vitro biosynthesis of Ag-NPs by *P. fellutanum* using $AgNO_3$ as a substrate isolated from coastal mangrove sediments. Silver NPs were also produced by *P. purpurogenum*, having antimicrobial activity against pathogenic gram-negative bacteria, viz. *Escherichia coli* and *Pseudomonas aeruginosa*, and gram-positive bacteria, viz. *Staphylococcus aureus* (Singh et al. 2014). Maliszewska et al. (2013) reported the production of Ag-NPs by a non-enzymatic and eco-friendly process. The cell-free filtrate of *P. nalgiovense* AJ15, containing cysteine proteins, played an important role in the production of silver NPs. Singh et al. (2014) reported good production of Ag-NPs by an endophytic *Penicillium* sp. isolated from healthy leaves of *Curcuma longa*, which showed good antibacterial activity against MDR *E. coli* and *S. aureus*.

Many species of filamentous fungus, *Aspergillus*, has been known for rapid extracellular synthesis of NPs. *A. fumigatus* fairly produced monodispersed 5–25 nm Ag-NPs (Mohanpuria et al. 2008). Soil fungi *A. niger* was reported to produce Ag-NPs by Kumar et al. (2008). *A. flavus* accumulated monodispersed silver nanoparticles on the surface of its cell wall when treated with silver nitrate solution (Jain et al. 2013). Jain et al. (2011) found *A. aeneus* NJP12 isolated from rhizospheric soil, which showed ability to biosynthesize extracellular spherical ZnO nanoparticles in the range of 100–140 nm. They also evaluated the synthesis of spherical metallic silver nanoparticles by means of the cell-free filtrate of *A. flavus* NJP08 supplemented with aqueous silver (Ag+) ions using transmission electron microscopy (TEM) and energy dispersive spectroscopy (EDS). Jain et al. (2011) calculated the average particle size using dynamic light scattering (DLS) measurements and was found it to be 17 ± 5.9 nm. *A. flavus* accumulated Ag-NPs on the surface of its cell wall after 72 h when treated with silver nitrate solution having a characteristic absorption peak at 420 nm and emission peak at 553 nm (Navazi et al. 2010). Bhainsa and D'Souza (2006) investigated extracellular biosynthesis of silver nanoparticles using *A. fumigates*. This process was quite rapid, and silver nanoparticles were formed within minutes of silver ion coming in contact with the cell filtrate. Saravanan and Nanda (2010) also investigated the extracellular biosynthesis of Ag-NPs using *A. fumigatus*. *A. clavatus* also could synthesize silver nanoparticles extracellularly (Verma et al. 2010). Li et al. (2012) evaluated the synthesis of Ag-NPs using a reduction of aqueous Ag+ ion, with the culture supernatants of *A. terreus*, occurred at ambient temperature and in a few hours. Mycosynthesized Ag-NPs were polydispersed spherical particles ranging from 1 to 20 nm diameter which could efficiently inhibit *Escherichia coli*, *Candida albicans* and *Pseudomonas fluorescence*. Ninganagouda et al. (2014) screened fungi for the extracellular production of silver nanoparticles by *Aspergillus* sp., *Rhizopus* sp., *Fusarium* sp. and *Penicillium* sp., and he found the maximum production of Ag-NPs by fungus *Aspergillus* sp. of spherical shape

and size ranged between 20 and 55 nm. Raliya and Tarafdar (2014) investigated an environmental-friendly process for the synthesis of Ag-NPs using *A. tamarii*, and their scanning electron microscope result showed the distribution of spherical Ag-NPs ranging from 25 to 50 nm. They also reported the synthesis of zinc, magnesium and titanium NPs by using six different *Aspergillius* species, viz. *A. flavus*, *A. terreus*, *A. tubingensis*, *A. niger*, *A. fumigatus* and *A. oryzae*, by employing various precursor salts of sulphates, nitrates, chlorides and oxides.

The use of eukaryotic organisms for nanoparticle synthesis was first established by the use of *Verticillium* sp. for the synthesis of gold nanoparticles (Au-NPs). As compared to other group of microorganism, fungi is known to synthesize a good quantity of nanoparticles, and Gericke and Pinches (2006) reported that *Verticillium luteoalbum* secreted an extracellular enzyme which was used for the synthesis of Au-NPs (Gericke and Pinches 2006). A green chemistry route, based on the bioreduction of $AuCl_4^-$ ions by the fungus *Verticillium* sp., for the formation of gold nanoparticles was demonstrated by Mukherjee et al. (2001). The TEM micrograph showed entrapment of gold nanoparticles on the cell wall and cytoplasmic membrane after reaction of a single *Verticillium* cell with gold ions (Mukherjee et al. 2001). Bharde et al. (2006) established that nanoparticulate magnetite was produced at room temperature extracellularly by *Verticillium* sp. , with mixtures of ferric and ferrous salts. In the reaction, extracellular hydrolysis of the anionic iron complexes by cationic proteins secreted by the fungi results in the production of crystalline magnetite particles at room temperature.

11.2.2 Biosynthesis of Nanoparticles by Other Microfungi

The structure of biogenic NPs depends on the biological species involved, for example, *Colletotrichum* sp. produced essentially spherical NPs under the same conditions (Shankar et al. 2003). Extracellular biosynthesis of silver nanoparticles (Ag-NPs) by *Trichoderma reesei* (also known as *Hypocrea jecorina*) when exposed to the silver nitrate solution was established by Vahabi et al. (2011). Silver nitrate solution prompted the fungus to produce enzymes and metabolites for its own survival resulting in the production of Ag-NPs. The white rot fungus, *Phanerochaete chrysosporium*, formed stable Ag-NPs when treated with aqueous silver nitrate medium (Vigneshwaran et al. 2006). Chen et al. (2003) evaluated the extracellular formation of Ag-NPs ranging from 60 to 80 nm using *Phoma* sp. 3.2883 when exposed to an aqueous silver nitrate solution at room temperature (Chen et al. 2003). Similar result was obtained by Birla et al. (2009) using another species of *Phoma*, viz. *P. glomerata*. An endophytic fungi *Colletotrichum* sp., growing in the leaves of *Geranium*, produced gold nanoparticles (Au-NPs) when exposed to chloroaurate ions (Shankar et al. 2003). These NPs were decahedral and icosahedral in shape and size ranged from 20 to 40 nm. Endophytic fungus *Colletotrichum* sp. ALF2-6 inhabiting *Andrographis paniculata* could synthesize well-dispersed silver nanoparticles with size ranging from 20 to 50 nm characterized using UV-visible

spectrometry with maximum absorption conferring at 420 nm (Azmath et al. 2016). *Trichoderma asperellum*, a biocontrol agent, could produce stable Ag-NPs with size ranging from 13 to 18 nm with well-defined morphology (Gaikwad et al. 2013). Five *Trichoderma* species, viz. *T. asperellum*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii* and *T. virens*, were screened for the production of Ag-NPs and could produce single or aggregated Ag-NPs with round and uniform shape having a size of 8–60 nm (Devi et al. 2013). Extracellular synthesis of Ag-NPs of 2–5 nm size by yeast strain MKY3 was reported by Kowshik et al. (2003). TEM analysis showed the presence of polydispersed and spherical-shaped particles produced by *Cladosporium cladosporioides* in $AgNO_3$ solution (Balaji et al. 2009). Out of 18 different *Phoma* sp., *P. sorghina* was found to be the potential species as a novel synthesizer of Ag-NPs by Gade et al. (2013). *P. sorghina* was reported to produce silver rods. *Hormoconis resinae* was proved to be an excellent fungal source among the various fungi screened for the extracellular synthesis of significantly stabile gold NPs (Mishra et al. 2010). Using yeast cells, Bao et al. (2003) evaluated a simple and efficient biosynthesis technique to prepare biocompatible cadmium telluride (CdTe) QDs with tunable fluorescence emission. The filamentous fungus *Neurospora crassa* was found to be an impending biological means for the production of mono- and bimetallic Au-/Ag-NPs (Longoria et al. 2011). The mycelia-free culture filtrate of the *Nigrospora oryzae* with gold chloride could produce Au-NPs of 6–18 nm diameters (Kar et al. 2014). *Schizophyllum commune* grown in the medium containing silver nitrate was tested for the extracellular synthesis of silver nanoparticles, and they could successfully produce Ag-NPs (Sastry et al. 2010).

11.3 Synthesis of Nanoparticles by Macrofungi

Pleurotus ostreatus was screened for the production of laccase enzyme which was in turn used in the synthesis of gold nanoparticles (Ahmed et al. 2015). The immobilized fungus *Coriolus versicolor* which helped in bioremediating cadmium could also synthesize stable CdS NPs in aqueous conditions (Sanghi et al. 2009). Owaid et al. (2015) found that the hot water extract of fresh basidiocarps of an edible mushroom, *Pleurotus cornucopiae* var. *citrinopileatus*, could produce silver nanoparticles (Ag-NPs) by bio-reduction of silver nitrate. The spherical-shaped morphology and <100 nm particle size of Ag-NPs produced by *P. cornucopiae* were confirmed by field-emission scanning electron microscopy (FESEM) and high-resolution transmission electron microscopy (HRTEM). These Ag-NPs inhibited the growth of all *Candida* species tested. Extract of another edible mushroom *Volvariella volvacea* was used for extracellular synthesis of Au, Ag and Au-Ag-NPs in water (Philip 2009). These Au-NPs were of different sizes (20–150 nm) and shapes from triangular nanoprisms to nearly spherical and hexagonal. The size and shape of gold nanoparticles were also found to depend on temperature of the extract. The silver nanoparticles are spherical with size of about 15 nm. The Ag-NPs synthesized by edible *Agaricus bisporus* showed a remarkable antimicrobial activity

against different clinically important pathogenic microorganisms (Sudhakar et al. 2014). Biosynthesis of silver nanoparticles (Ag-NPs) from dried mushrooms of Bandipora district (Jammu and Kashmir) and their effectiveness against methicillin-resistant *Staphylococcus aureus* (MRSA) strains were evaluated by Haq et al. (2015). A total of five different mushroom species, viz. *Agaricus bisporus*, *Helvella lacunosa*, *Ganoderma appalanatum*, *Pleurotus florida* and *Fomes fomenterieus*, were found as good producers of Ag-NPs. The biosynthesized Ag-NPs were characterized by UV-vis spectroscopy, FT-IR and TEM. The Ag-NPs produced by *A. bisporus* showed highest zone of inhibition against MRSA strains. The filtrate of *Agaricus bisporus*, *Calocybe indica*, *Pleurotus florida* and *P. platypus* was used as reducing and stabilizing agent for 1 mm of $AgNo_3$ aqueous solution. As a result they were found to produce stable Ag-NPs (Sujatha et al. 2013). The aqueous silver ions (Ag+) were reduced to silver metal nanoparticles (Ag m-NPs), when treated with the *Pleurotus ostreatus* fungal supernatant. SEM and TEM images showed formation of well-dispersed Ag-NPs of 50 nm, and the presence of silver was confirmed by EDX analysis. The Ag-NPs had great antimicrobial potential against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Vibrio cholera* (Devika et al. 2012). *Pleurotus* sp. was allowed for biosynthesis of iron nanoparticles in a culture media. TEM images revealed the depositions of NPs in both inside as well as outside of cells indicating the biosynthesis iron NPs (Mazumdar and Haloi 2011). Yehia and Sheikh (2014) evaluated the synthesis of silver nanoparticles (Ag-NPs) using a reduction of aqueous Ag^+ ions with culture supernatant from *Pleurotus ostreatus*. TEM studies showed the size of the Ag-NPs to be in the range of 4–15 nm, and these Ag-NPs possessed potent antifungal, antidimorphic and anticancer activities (Yehia and Sheikh 2014).

11.4 Application of Myconanoparticles in Agriculture

11.4.1 Plant Germination and Growth

During the last decades, many researchers have studied the effects of nanoparticles on plant germination and growth with the target to endorse its use for agricultural improvements. Zheng et al. (2005) evaluated the effects of nano- and non-nano-TiO_2 on the growth of naturally aged spinach seeds. They reported that nano-TiO_2-treated seeds, over a germination period of 30 days, produced plants with 73% more dry weight, three times higher photosynthetic rate, and 45% increase in chlorophyll-a formation compared to that of the control. It was found that the growth rate of spinach seeds was inversely proportional to the material size signifying that the smaller the nanomaterials the better the germination. According to them, the nanosized TiO_2 penetrated into the seed resulting into the increased seed germination and absorption of inorganic nutrients, fastened the breakdown of organic substances and also caused quenching of oxygen free radicals formed during the photosynthetic process, hence increasing the photosynthetic rate. Khodakovskaya et al. (2009)

reported that multiwall carbon nanotubes (MWCNTs) could penetrate into tomato seeds and increase the germination rate up to 90% (compared to 71% in control) and also the plant biomass by increasing the seed water uptake in 20 days. Studies on the influence of metal nanoparticles (Si, Pd, Au, Cu) on germination of lettuce seeds, by Shah and Belozerova (2009), indicated that nanoparticles (at different concentrations) had a positive influence on seed germination. The influence was measured in terms of shoot-to-root ratio and growth of the seedling. Ma et al. (2010) and Lin and Xing (2007) studied the effects of four oxide nanoparticles (cerium oxide, CeO_2; lanthanum (III) oxide, La_2O_3; gadolinium (III) oxide, Gd_2O_3; ytterbium oxide, Yb_2O_3) on the radish, rape, tomato, lettuce, wheat, cabbage and cucumber plant species. They found that the root growth depended on nanoparticles and its concentration. Ma et al. (2010) reported that the three types of nanoparticles (La_2O_3, Gd_2O_3, Yb_2O_3) greatly affected root growth at 2000 mg/L concentration. Oancea et al. (2009) hypothesized that controlled liberation of active plant growth stimulators and other chemicals encapsulated in nanocomposites made of layered double hydroxides (anionic clays) could be the feasible option for organic agriculture. However, according to Monica and Cremonini (2009), the consequences of nanoparticles on plants can be positive or negative. One of the important consequences for nanomaterials applications in seed germination is their phytotoxicity. The type and concentration of nanomaterial determines the level of phytotoxicity. For example, Nair et al. (2011) tested the applicability of fluorescein isothiocyanate (FTIC)-labelled silica nanoparticles and photostable cadmium-selenide (CdSe) quantum dots for their capability to be used as biolabels and for promoting seed germination. They found that FTIC-labelled silica nanoparticles induced seed germination in rice, while quantum dots arrested the germination. Root length of radish, rape canola, ryegrass, lettuce, corn and cucumber species was found to inhibit with the use of 200 mg/L nano-Zn and ZnO by Lin and Xing (2007). Ma et al. (2010) found that the nano-CeO_2 did not affect root elongation in plant species except for lettuce at 2000 mg/L concentration. Thus, the phytotoxic behaviour of the nanomaterials needs to be meticulously understood before utilizing under field conditions. A possible solution to avoid the phytotoxicity to ornamental and specialty crop plant species would be to grow the plant seedlings in a greenhouse and later transferring them to the field (Bergeson 2010). It has always been a debate the applicability and phytotoxicity of silver nanomaterials in agriculture by the EPA. Bergeson reported that there are more than 100 pesticides that contain Ag due to its antimicrobial properties. However, toxicity of Ag-NPs to ecosystem and human is a major apprehension. Lu et al. (2010) reported that the citrate-coated colloidal Ag-NPs were not genotoxic (genetic), cytotoxic (cell) and phototoxic (toxicity through photodegradation) to human; however, citrate-coated Ag nanoparticles in powder form were toxic. It was also found that the phototoxicity of the powdered Ag-NPs was interestingly repressed when they were coated with biocompatible polyvinylpyrrole. Exploring such biocompatible coatings to reduce or inhibit the toxicity of nanomaterials would increase the chances of applying nanomaterials in plant germination and growth. However, it is also needed to explore

the undesirable effect of such coatings on the desired seed/plant properties and the effectiveness of nanomaterials.

11.4.2 Plant Disease Management and Protection

In recent years, efforts have been made to develop harmless management methods to control food crop diseases that pose fewer hazards to humans and animals and have focused on overcoming the lack of synthetic fungicides (Falletta et al. 2008). There are some studies available dealing more specifically with the action of Ag-NPs against clinical isolates and American Type Culture Collection (ATCC) strains of *Candida* spp. and *Trichophyton mentagrophytes* (Panacek et al. 2006; Min et al. 2009; Li et al. 2012). The use of Ag-NPs as antimicrobial agents has become more extensive because of their easy and cost-effective production. Various modes of inhibitory action of Ag-NPs to plant pathogens attracts the scientists for controlling various plant pathogens in a moderately safer way compared to synthetic fungicides (Park et al. 2006; Oh et al. 2006), for example, Ag-SiO_2 NPs have a strong antifungal effect against *Botrytis cinerea* (Oh et al. 2006). Gajbhiye et al. (2009) evaluated the combined effect of fluconazole and Ag-NPs for their antifungal activity against *Phoma glomerata*, *Phoma herbarum*, *F. semitectum*, *Trichoderma* sp. and *C. albicans* by disc diffusion technique. Ag_2S nanocrystals on amorphous silica particles show antifungal activity against *A. niger* (Fateixa et al. 2009). The impending biocidal efficiency of ZnO and $ZnTiO_3$ nano-powders against the fungus *A. niger* was also assessed by Ruffolo et al. (2010) and Jo et al. (2009). Silver ions and NPs were evaluated to possess the antifungal activity on *Bipolaris sorokiniana*, *Magnaporthe grisea* and the unidentified ambrosia fungus *Raffaelea* sp. (responsible for the transience of a large number of oak trees in Korea) (Jo et al. 2009). Panacek et al. (2006) assayed the fungistatic and fungicidal property of the Ag-NPs against certain pathogenic yeasts such as *Candida albicans* (I and II), *C. tropicalis* and *C. parapsilosis*. The effect of Ag-NPs on plant pathogenic fungi *Fusarium culmorum* and *F. oxysporum* was studied by Kasprowicz et al. (2010) and Musarrat et al. (2010), respectively. The growth of phytopathogen *Colletotrichum gloeosporioides*, responsible for anthracnose in a wide range of fruit, significantly decreased in the presence of Ag-NPs in a dose-dependent manner (Ma et al. 2010). Zinc oxide nanoparticles (ZnONPs) repressed the growth of *B. cinerea* by upsetting cellular functions, which caused deformation in fungal hyphae. Moreover, ZnONPs inhibited the growth of conidiophores and conidia of *Penicillium expansum*, which finally led to the decease of mycelial mats (Krishnaraj et al. 2012). Silver nanoparticles may be less toxic to humans and animals than synthetic fungicides (Alghuthaymi et al. 2015). A comparative study of elemental and nano-sulphur against facultative fungal food pathogen, *A. niger*, was conducted by Choudhury et al. (2010), and they found that nano-sulphur was more efficient than its elemental form. Krishnaraj et al. (2012) studied different concentrations of Ag-NPs to determine the inhibitory activity on fungal plant pathogens, viz. *Alternaria alternata*, *Sclerotinia*

sclerotiorum, *Macrophomina phaseolina*, *Rhizoctonia solani*, *B. cinerea* and *Curvularia lunata*. Grippingly, 15 mg concentration of Ag-NPs showed tremendous inhibitory activity against all the tested pathogens. In in vitro studies, Saharan et al. (2013) mentioned in his findings that chitosan and Cu-chitosan NPs, due to their uniform size and stability, might contribute to their higher antifungal activity against *A. alternata*, *M. phaseolina* and *R. solani* (Krishnaraj et al. 2012). Cu-chitosan NPs also showed maximum inhibition rate of spore germination of *A. alternata*.

Plant pathologists are working to find a solution for protecting plant, food and agriculture products from bacteria, fungal and viral agents. A number of nanotechnologies have perked up existing crop control protocols in short to medium term (Abd-Elsalam 2012; Li et al. 2007). Nanomaterials are being produced that proffer the opportunity to administer pesticides, herbicides and fertilizers more efficiently and safely by controlling specifically when and where they are released (Rai and Ingle 2012). Many studies have revealed that metal NPs are effective against plant pathogens, insects and pests (Choudhury et al. 2010). For example, an eco-friendly fungicide is being developed that uses nanomaterials to release its pathogen-killing properties only when it is inside the embattled pathogen (Liu 2006). Alghuthaymi et al. (2015) reviewed the nanotechnological application in plant pathology with potential technological developments outlined in Fig. 11.1.

Khaydarov et al. (2011) reported a tremendous protective effect on the causal organisms of powdery mildew or downy mildew when aqueous silicate solution was used to treat diseased plants. Kanto et al. (2004) showed that this solution also promoted the physiological activity and growth of plants and induced disease and stress resistance in plants. It was found that 100% growth inhibition of *Pseudomonas syringae* and *Xanthomonas campestris* pv. *vesicatoria* occurred at 100 ppm concentration when different concentrations of nanosized silica-silver were evaluated for growth inhibition of phytopathogenic bacteria and fungi by Oh et al. (2006). *Magnaporthe grisea*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*,

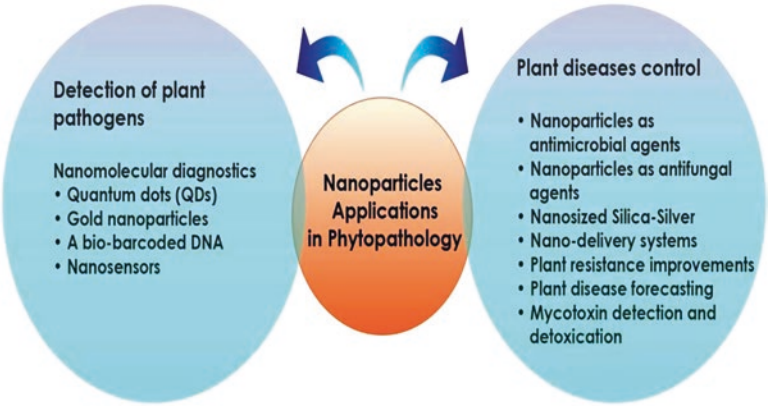

Fig. 11.1 Potential nanotechnology applications in plant pathology. (Adapted from Alghuthaymi et al. 2015)

Pythium ultimum and *Rhizoctonia solani* also showed 100% growth inhibition at 10 ppm concentration of the nanosized silica-silver. Nanosized silica-silver at 0.3 ppm concentration also efficiently inhibited *Erysiphe cichoracearum* causing powdery mildews of pumpkin in greenhouse and also in field assays (Kim et al. 2008). The antifungal efficacy of colloidal nanosilver (1.5 nm average diameter) solution was reported against rose powdery mildew caused by *Sphaerotheca pannosa* var. *rosae* by Kim et al. (2008). Nanocopper was reported to be highly effective against bacterial blight of rice (*Xanthomonas oryzae* pv. oryzae) and leaf spot of mung (*X. campestris* pv. phaseoli).

11.4.3 Improving Plant Resistance

Everyday plants are prone to many disease-causing agents like insect pest, nematodes and other pathogens and also different abiotic stresses, which lead to the tremendous economic loss. To evade these losses, the only substitute is to develop resistant varieties of plants. This, in turn, would help us in managing the above-mentioned agents to surmount the problem of economic loss. The advanced techniques of nanobiotechnology offer a splendid set of procedures using NPs, nanofibers and nanocapsules to multiply genes and, thus, develop plant resistance (Rai and Ingle 2012; Mcknight et al. 2003; Torney et al. 2007). The thriving incorporation and integration of plasmid DNA in the plant genome has been confirmed through gene expression (Filipenko et al. 2007). Nanoparticle-mediated plant transformations have the potential to improve plants and disease resistance through genetic amendments and alterations (Torney et al. 2007). Nanotechnology can explicitly aim specific plant microbe interactions and afford new methods to solve many phytopathological problems in agriculture for crop disease management (Filipenko et al. 2007). An example was mentioned by Bouwmeester et al. (2009) that introduction of resistance genes in plant cells using nanotechnological approaches may lead to the progress of resistant varieties which in turn lessen expenses on agrochemicals required for disease control.

11.4.4 Nanopesticides

According to Bergeson (2010), nanopesticides "involve either very small particles of pesticidal active ingredients or other small engineered structures with useful pesticidal properties". The rapid development in nanopesticide research over the last few years have stimulated a number of international organizations to consider potential issues relating the use of nanotechnology for crop protection (Kar et al. 2014). Microorganisms have caused marvellous environmental, ecological changes. This is the result of ingress of new diseases into environment leading to the disease and death of plants. Agricultural crops and forestry should therefore be protected

against the invasions of insect pests and pathogens. A sustainable mechanism for disease control is therefore essential, and the improvement of nanopesticides can facilitate the control plant diseases (Bouwmeester et al. 2009). Nanopesticides can enhance the diffusion and wettability of agricultural formulations which in turn help the reduction in organic solvent runoff and unwanted pesticide movement (Bergeson 2010). Nanomaterials and biocomposites possess valuable properties such as rigidity, permeability, crystallinity, thermal stability, solubility and biodegradability essential for formulation of nanopesticides (Bouwmeester et al. 2009; Bordes et al. 2009). Nanopesticides also possess large specific surface area and hence augmented affinity to the target (Jianhui et al. 2005). Bergeson (2010), Bouwmeester et al. (2009) and Lyons and Scrinis (2009) discussed about different delivery techniques of nanopesticide, like nanoemulsions, nanoencapsulates, nanocontainers, nanocages, etc., for plant protection. Some of the applications of nanomaterials in agricultural plant protection and production have been mentioned in Table 11.1. The basic criteria of the nano-formulations should mortify faster in the soil and slowly in plants with residue levels below the regulatory criteria in foodstuffs. Such type of NPs was developed by Jianhui et al. (2005). They reported the development of sodium dodecyl sulphate-modified photocatalytic TiO_2/Ag nanomaterial conjugated with dimethomorph. Silver has wide applications in metal or compound form. Recently, nanotechnology has increased the effectiveness of Ag-NPs because of its antimicrobial activity against pathogens and nontoxicity to humans (Kim et al. 2008; Elchiguerra et al. 2005; Yeo et al. 2003; Aziz et al. 2016). A mechanism for disease control is therefore required, and the development of nanopesticides can help control plant diseases (Bouwmeester et al. 2009). Woo et al. (2009) reported that Ag-NPs caused damage to fungal hyphae of *Raffaelea* sp. causing oak wilt, by reducing microbial absorption, and increased inhibition of fungal growth and conidial germination. Similarly, Min et al. (2009) also found that Ag-NPs surprisingly inhibited the hyphal growth of *R. solani*, *S. sclerotiorum* and *S. minor* in a dose-dependent manner in vitro. The antifungal activity of different forms of silver ions and NPs against *B. sorokiniana* and *M. grisea* was observed by Jo et al. (2009). They found that both silver ions and Ag-NPs could manipulate the colony formation of spores and disease progress of phytopathogenic fungi. These results advocate that Ag-NPs may have a huge impend of nanopesticides on control of phytopathogens.

11.4.5 Plant Pathogen Detection and Study of Plant Disease Mechanisms

Although researches on plant pathogen detection are in preliminary stage, instinctively, nanoparticles can be used as biomarkers or as a rapid diagnostic tool for revealing of bacterial, viral and fungal phytopathogens in agriculture (Boonham et al. 2008; Chartuprayoon et al. 2010). Nanoparticles-based sensors might offer

enhanced detection limits in detecting viral pathogens in plant (Baac et al. 2006). Nanoparticles can either be directly modified to be used for pathogen detection or used as a diagnostic tool to detect compounds which indicates the diseased condition (Khot et al. 2012). Nano-chips are types of microarrays that contain fluorescent oligo arrest probes through which the hybridization can be detected (Yao et al. 2009). They could detect *Xanthomonas axonopodis* pv. *vesicatoria* that causes bacterial spot disease in Solanaceae plants, using a fluorescence silica nanoparticle in combination with antibody, indicating a potential for nanoparticle application in disease detection. Singh et al. (2010) used nanogold-based immunosensors which could detect the Karnal bunt (*Tilletia indica*) disease in wheat using surface plasmon resonance. Mostly, research attempted to detect the disease using SPR sensor in wheat is used for seed certification and to establish plant quarantines. Plants respond to different stress conditions through physiological changes. It is thought that different plant hormones, viz. jasmonic acid, methyl jasmonate and salicylic acid, regulate the induction of systemic defence in plants leading to the physiological changes in plants as a result of the plant responses to stress condition (Khot et al. 2012). Wang et al. (2010) utilized this indirect incentive to develop a sensitive electrochemical sensor, using modified gold electrode with copper nanoparticles, to monitor the levels of salicylic acid in oil seeds for detection of the fungi *Sclerotinia sclerotiorum*. They lucratively and perfectly measured salicylic acid using this sensor. Research on similar sensors and sensing techniques should be broad and to be extended for detecting pathogens, their by-products, or for monitoring physiological changes in plants. Thus, it can be said that researches on pathogen detecting nanosensors for their in vivo application would be extremely valuable for faster diagnosis and disease management.

11.4.6 Soil Stabilization and Remediation

Nanoparticles are one of the latest materials that can be used in soil stabilization and remediation. Different particles contribute in the formation of soil. The sub-micrometric particles, i.e. clays, are one of them. Ghormade et al. (2011) mentioned that common clays are layered phyllosilicate materials, with a polymeric silicate base, which are nanodimensional in one plane. Transmission electron (TEM) and high-resolution transmission electron (HRTEM) microscopy revealed that clays are composed of stacked tetrahedral and octahedral sheets (Wilson et al. 2008). Natural weathering of bedrocks results in the formation of NPs such as iron and silica. Allard et al. (2004) reported that other naturally occurring NPs are iron oxides (2–5 nm length), as colloidal phases of ferrihydrite, coupled with organic matter in river-borne material. However, Yang and Watts (2005) showed that alumina NPs (aluminium oxide) had negative effect on plant root growth. The effect of Na^+-modified montmorillonite nanoclay on stabilization of clay properties was examined by Nohani and Alimakan (2015). They found that liquid and plastic limits rise with increasing nanoclay content and the highest soil resistivity was seen by adding 1.5%

of nanoclay using the uniaxial and CBR tests. However, low percentage of nanoclay generally improves the soil properties. Soil remediation is another facet of the role of NPs in soil ecology. Recent study, a nanotechnology-based soil binder which is a sort of quick-setting organic and biodegradable mulch, was developed by a US-based company (ETC Group 2004). This binder reacted with the silicates in the soil and resulted into self-assemblage of silicates into a kind of crust that remained up to a year in the soil. The crust was found to prevent soil runoff and allowed seeds blended into crust to germinate. Therefore, this soil binder was sprayed over 1400 acres of Encebado Mountain in New Mexico and Mendocino County, California, to prevent soil erosion and forest fires. Bioremediation of toxic metals present in the soil is an important aspect that affects plant growth. For example, copper, as a free ion, catalyses to produce damaging radicals, and in environment all life forms attempt to prevent copper toxicity for survival. The common wetland plants or the peats *Phragmites australis* and *Iris pseudacorus* convert copper into metallic NPs in and around their roots with aid from endomycorrhizal fungi when grown in Cu-contaminated soil (Manceau et al. 2008). Thus, peat cultivation can contribute to copper biorecycling and rhizosphere containment to avert copper biomagnification in soil, resulting into soil stabilization and remediation.

11.4.7 Pesticide Residue Detection

There are about 1045 chemicals in the soil reported by Food and Drug Administration (FDA) as pesticide residues (Sadrieh 2005). Earlier detection of such pesticide residues was done by traditional gas or liquid chromatography (GC/LC) mass spectroscopy (MS) techniques. In spite of accuracy and reliability, these traditional techniques are time-consuming due to the steps involved, such as field sample collection, solid-phase extraction in laboratory, analysing the sample and comparing the obtained spectral peaks with references to establish the pesticide residues. But after development of nanobiotechnology, nanomaterial-based nanosensors can be used to detect such pesticide residues as an alternate to traditional techniques (Stan and Linkerhagner 1996; Balinova et al. 2007). Nanosensors for pesticide residue detection offer "high sensitivity, low detection limits, super selectivity, fast responses, and small sizes" (Liu et al. 2008). Application province of nanomaterial-based sensing for pesticide residue detection is enormous; however, some issues such as (1) availability of the nanomaterials sensitive to common pesticide residues, (2) ease of sensor fabrication techniques and instrumentation, (3) desired reliability and repeatability in trace level detection, (4) cost and (5) issues related to nanomaterial exposure to the surrounding environment are to be considered for economical, sustainable application (Khot et al. 2012). According to Dyk and Pletschke (2011), it is not possible to test randomly for all pesticides in samples. At the starting point, the dogmatic institutions can use nanosensors to perceive major residuals that are enormously harmful to human health. Efficient nanomaterials can also be used as substitute to sensors for pesticide recognition.

11.4.8 Pesticide Degradation

Degradation of bio-recalcitrant pollutants using NPs is one of the good innovative approaches (Joo and Cheng 2006; Zhang 2003). NPs, in addition to its use for pesticide and herbicide detection, have also been applied for pesticide degradation in recent years. Yu et al. (2007) found that the attraction of peroxide or hydroxyl radical and electron transfer enables the photolytic degradation of organochlorine pesticides on the surface of nano-TiO_2 film. Zeng et al. (2010) also established that organophosphorus and carbamate pesticides in tomato leaves and soil can also be photocatalytically degraded at a rate of 15–30% using a rhenium (Re3þ)-doped nano-TiO_2. This nanomaterial was also proficient for degrading carbofuran at a rate of 55%, which is 30% higher than natural degradation. Recent studies revealed that pesticides such as atrazine, molinate and chlorpyrifos can be degraded with the help of nanosized zerovalent iron (ZVI, 1–100 nm). However, Joo and Cheng (2006) found that cyclodiene insecticides, such as endosulfan, were generally resistant, i.e. they could not be degraded using NPs. Applications of nanosized ZVI and iron oxide NPs may be used for removal of humic material and toxins from the soil (Giasuddin et al. 2007; Waychunas et al. 2005). However, very less work has been done on the performance duration of these nanoparticles/colloidal systems. A persistent organic pollutant, viz. lindane, found in drinking water as well as in food was scavenged and degraded by the application of NPs such as biopolymer-stabilized FeS (200 nm) (Paknikar et al. 2005). Other approaches such as photocatalytic putrefaction of pesticide residues by means of titania doped with Fe_2O_3 or other metals sprayed directly on crops or incorporated into the pesticide formulation are found to be promising (Sasson et al. 2007). For direct surface modification of colloidal pesticides, layer-by-layer surface (LbL) nano-engineering is a new strategy, which utilizes sequential adsorption of oppositely charged polyelectrolytes to form a complex congregation through electrostatic interactions (Yang and Watts 2005). Encapsulation of microcrystals of the insecticide imidacloprid (IMI) by LbL assembly using polysaccharides chitosan and sodium alginate followed by addition of photocatalytic NPs is an effective method for pesticide surface modification (Guan et al. 2008). They also reported about the photocatalytic degradation and mineralization of the IMI by TiO_2 NPs (~30 nm) and silver and sodium dodecyl sulphate-modified TiO_2 NPs.

11.4.9 Field Sensing Systems to Monitor the Environmental Stresses and Crop Condition

Nanomaterials also play an important role in promoting sustainable agriculture and provide better food worldwide (Gruere 2012). In developing countries, nanotechnology has received significant importance for enhancing agricultural productivity, along with other emerging technologies such as biotechnology including

genetics, plant breeding, disease control, fertilizer technology, precision agriculture and other associated fields (Sastry et al. 2010; Jha et al. 2011). Nanotechnology can be used for combating the plant diseases either by controlled delivery of functional molecules or as indicative tool for disease detection (Chen and Yada 2011). Signalling networks of wireless nanosensors placed across cultivated fields afford essential data leading to the best agronomic proficient processes resulting in minimizing resource utilization and maximizing the product output (Scott and Chen 2012). Such kind of signals can provide information about optimal timing for planting and harvesting crops and the level of water; time of application of agrochemicals like fertilizers, pesticides and herbicides; and other treatments that need to be administered for a specific plant physiological, pathological and environmental conditions. Scott and Chen (2003) demonstrated that the crop nutrient status, insects, pathogens, weeds, moisture level, soil fertility, soil temperature, etc. can be recognized and measured with the help of nanosensors and other field-sensing devices which in turn help in real-time monitoring of the crop growth and provide indispensable data for precision farming practices leading to diminish agricultural inputs and maximizing resource output and yield (Singha et al. 2015).

11.5 Conclusion and Future Perspectives

Nanotechnology is progressively being integrated into the agricultural sector. Nowadays the interest in the use of fungi in this field is mounting day by day as fungi are capable to possess relatively rapid and environmentally "clean" nanobiofactories for metallic NPs in comparison to other chemical and physical techniques. Uncontrolled overuse of fungicides in agricultural field has led to many adverse effects on human health and adverse effects on pollinating insects and domestic animals, causing soil as well as water pollution and directly or indirectly affecting the ecosystems. One of the suitable solutions for this problem is the intelligent use of chemicals on the nanoscale. The use of NPs in agriculture is one of the advantageous techniques as these nanomaterials are used onto the part of the plant that was attacked by disease or pest, and because these carriers are self-regulated, the required amount of medication can be delivered to the targeted tissue.

However, being in the very early stage of this field, there are several questions with remarkable scientific or practical importance need to be addressed, in lieu of the prompt advancement in the study of phytotoxicity, uptake and accumulation of NPs in the past few years. More nanophytopathological studies on physiology of host and pathogen, interaction, infection process and disease diagnosis will help in developing new disease management approach including nanopesticides that are less harmful to the environment than conventional formulations (Gericke and Pinches 2006).

Earlier research has evidently established the activities of NPs in plant species, but how and why different plant species exhibit different resistance to NPs is yet

unexplored. How plant species and environmental factors affect the uptake and accumulation of NPs is yet to be investigated (Singha et al. 2015). Different xylem structures of different plants, apoplastic or symplastic movement, movement through plasmodesmata of NPs, etc. all these factors are interrelated to each other. More studies are necessary in the field to confirm this hypothetical view. The concern on the toxicity of NPs in the environment has come into account during the last decade, with the increase of NPs industry and their manifold applications. However, the green syntheses of NPs using fungi are eco-friendly, safe and nontoxic approach with great advantages over physical and chemical methods as they are always cheap, reliable and nontoxic and as it does not use any toxic chemicals and specific higher temperature and pressure (Chaudhari et al. 2016). Myconanoparticles synthesis, and their characterization and application in agriculture, has been shown in Fig. 11.2. NPs are also used as nano-biosensors for detecting pathogens and soil quality and plant health monitoring, as antimicrobial agents, etc. The use of nanotechnology brings major benefits to farmers by food production and to the food industry through the improvement of novel products through food processing,

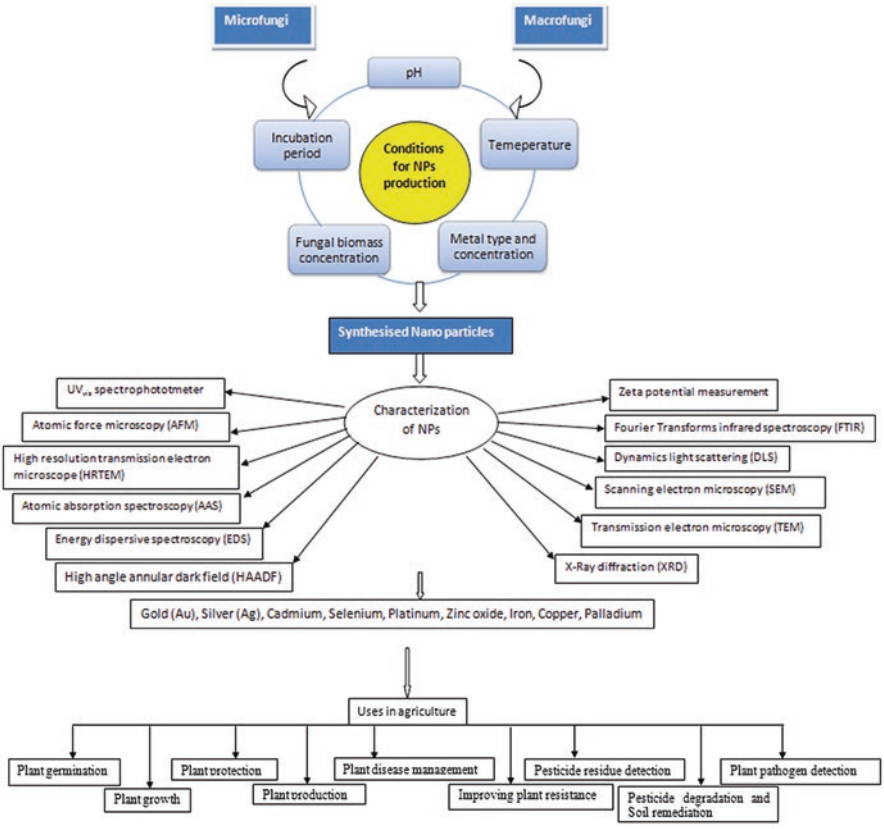

Fig. 11.2 Myconanoparticles: synthesis, characterization and their application in agriculture

preservation and packaging as NPs can prevent contamination. The use of NPs for plant disease conquest has not been adequately explored. The use of NPs for delivery of antimicrobial agents will be highly challenging in the near future for rehabilitation of all phytopathological problems. Although this review narrates the application of NPs for different agricultural practices, however, further expanded investigation and research are needed to widen their applications, possibilities and methodologies in agriculture.

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Chapter 12
Obligate Marine Fungi and Bioremediation

V. Venkateswara Sarma

12.1 Introduction

There are 1112 species of marine fungi belonging to 472 genera that have been formally reported. These include 805 species belonging to *Ascomycota*, 21 to *Basidiomycota*, and 26 to *Chytridiomycota* and related groups, 43 to asexual morphs of filamentous fungi in addition to 138 species belonging to ascomycetous yeasts and 75 to basidiomycetous yeasts all coming under 129 families and 65 orders (Jones et al. 2015). This shows that marine fungi are taxonomically diverse and hence they could be ecologically a united group but not taxonomically.

Several mycologists have attempted to provide a definition for what is a marine fungus. Kohlmeyer and Kohlmeyer (1979) proposed that "obligate marine fungi are those that grow and sporulate exclusively in a marine or estuarine habitat," while "facultative marine fungi are those from freshwater and terrestrial milieus able to grow and possibly also sporulate in the marine environment." Recently, Pang et al. (2016) provided a consensual definition for a marine fungus which is as follows: "any fungus that is recovered repeatedly from marine habitats because (1) it is able to grow and/or sporulate (on substrata) in marine environments; (2) it forms symbiotic relationships with other marine organisms; or (3) it is shown to adapt and evolve at the genetic level or be metabolically active in marine environments." Though this modified definition expands the scope of what is a marine fungus and includes marine-derived fungi also as marine fungi, one distinction that still rests with obligate marine fungi is that they don not occur in the terrestrial environments. Hence credence has been given to obligate marine fungi of the above fact, and further discussion is based on the recognition of this fact.

Marine fungi were studied following two main techniques: (1) direct examination method where the natural samples such as woody substrata, from coastal

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environments, are examined under dissection/stereomicroscopes to locate fungal reproductive structures and prepare slides to identify them under compound microscopes and (2) plating of water and soil samples from coastal environments on agar media. While in the former technique, mostly the obligate marine fungi are encountered when samples were collected from natural samples exposed to marine waters, the latter technique mostly yielded only aspergilli and penicilli and other fast-growing anamorphic states of filamentous fungi (Newell 1976; Kohlmeyer and Kohlmeyer 1979; Hyde et al. 2000).

Marine fungi grow on a variety of substrata including leaves, roots, pneumatophores, prop roots, seedlings, wood, soil, algae, corals, calcareous tubes of mollusks, guts of crustaceans, and living animals. While driftwood samples accommodate a large amount of obligate marine fungi, the mangroves have outnumbered them as per recent reports (Hyde et al. 2000).

Bioremediation includes two words, viz., "bio" and "remediation." While remediation is solving a problem, bioremediation is the involvement of living organisms or parts thereof to treat effluents and bring out mitigation. Bioremediation, according to EPA, is a "treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or non-toxic substances." Hence, it is a process where living organisms are used to remove or contain the contaminations or pollutants that are leaked into the environment. Living organisms used in bioremediation could include different groups of organisms, viz., algae, bacteria, fungi, plants, etc. Within a group also there could be various groups of organisms that could remediate a pollutant. For example, within filamentous fungi, there are different groups including soil fungi, airborne fungi, litter fungi, marine fungi, endophytic fungi, etc. that are involved in bioremediation. In this chapter, bioremediation by obligate marine fungi is discussed.

12.2 Lignocellulolytic Enzymes and Bioremediation

It is a well-known fact that the fungi are the major decomposers of woody substrata in terrestrial ecosystems. Marine ecosystems are no exception to this. What makes the difference is that while the main decomposers of lignin in the terrestrial environments are white rot fungi belonging to the *Basidiomycota*, it is marine ascomycetes in the marine environment. The number of basidiomycetes is very low among the obligate marine fungi, and they are tiny and microscopic in nature. One of the main reasons for lesser diversity and smaller size of marine basidiomycetes could be due to the fact that the tidal turbulence and exposure to water from wavy action of the seawater might hamper fruition of macroscopic fruit bodies. This has reflected on the diversity of marine mycota wherein around 80% of them belong to *Ascomycota*. The fact that the lignin is a rich carbohydrate available in the natural woody substrata from coastal environments has made many of the marine ascomycetes to evolve to develop the capability to produce ligninolytic enzymes. This has been

proved through many studies in in vitro (Rohrmann and Molitoris 1992; Raghukumar et al. 1994, 1996; Bucher et al. 2004; Luo et al. 2005).

Lignocellulosic complex of plant materials has cellulose, hemicellulose, and lignin in their cell walls. Depending on the degradation of these three components, the decay types are classified into three types according to Eaton and Hale (1993): (1) **white rot** where both enzymatic and nonenzymatic degradations of all wood components occur, (2) **soft rot type** where enzymic degradation of cellulose and hemicellulose occurs fully but lignin degradation is limited, and (3) **brown rot type** where a faster cellulose and hemicellulose degradation occurs but is mainly due to nonenzymic oxidation and here, once again, the lignin degradation is limited. After cellulose, lignin is considered to be the most abundantly available aromatic polymer (Boominathan and Reddy 1992).

The cellulolytic, xylanolytic, and ligninolytic enzymatic activities of different obligate marine fungi reported are provided in Table 12.1. Mouzouras (1989) has observed morphological decay features indicating soft and white rot decays in wood samples colonized by marine fungi. Endoglucanase activity on carboxymethylcellulose as indicator of utilization has been shown in more than 30 different strains of marine fungi (Rohrmann and Molitoris 1992; Raghukumar et al. 1994; Pointing et al. 1998). *Corollospora maritima* Werderm., *Pleospora pelagica* T.W. Johnson (=*Monodictys pelagica* T.W. Johnson) (Rohrmann and Molitoris 1992), *Halojulella avicenniae* (Borse) Suetrong, K.D. Hyde & E.B.G. Jones (=*Julella avicenniae* Borse), *Lignincola laevis* Höhnk, *Nia vibrissa*, and *Stagonospora* sp. (Pointing et al. 1998) have been shown to be rapidly utilizing cellulose (Hyde et al. 1998).

The pioneering work of Sutherland et al. (1982) has established the mineralization of lignin by marine fungi. In this work, they have employed ^{14}C-labeled maple and spruce lignin for mineralization to $^{14}CO_2$. They could establish only 5–7% mineralization after 30 days of treatment. Subsequently Bergbauer and Newell (1992) have shown that *Phaeosphaeria spartinicola* Leuchtm isolated from *Spartina alterniflora* could degrade lignocellulose and produce dissolved organic carbon. It was shown by them that this fungus could mineralize up to 3.3% of the lignin after 45 days of incubation. Raghukumar et al. (1996) have demonstrated the mineralization of ^{14}C-labeled lignin by one obligate marine fungus *Saagaromyces ratnagiriensis* (S.D. Patil & Borse) K.L. Pang & E.B.G. Jones (*Halosarpheia ratnagiriensis* S.D. Patil and Borse) along with two other marine-derived fungi.

Synthetic dyes are used as analogues of ligninolytic enzyme production. Some of these dyes are used as indicators of production of these enzymes. Various studies have been conducted to screen the lignocellulolytic enzymes by obligate marine fungi. For example, azure B was decolorized by *Bathyascus grandisporus* K.D. Hyde, *Cryptovalsa halosarceicola* K.D. Hyde, *Rhizophila marina* K.D. Hyde and E.B.G. Jones, and *Verruculina enalia* (Kohlm.) Kohlm. & Volkm.-Kohlm. Decolorization of azure B indicates production of ligninolytic peroxidase (Bucher et al. 2004). Poly-R could be degraded by *Periconia prolifica* Anastasiou; *Aigialus grandis* Kohlm. & S. Schatz; *Astrosphaeriella striatispora* (K.D. Hyde) K.D. Hyde; *Bathyascus grandisporus*; *Cryptovalsa halosarceicola*; *Dactylospora mangrovei* E.B.G. Jones, Alias, Abdel-Wahab & S.Y. Hsieh: *Oceanitis cincinnatula* (Shearer &

Table 12.1 Cellulolytic, hemicellulolytic, and ligninolytic activities of obligate marine fungi

S. no.	Name of the fungus	Cellulolytic activity	Hemicellulolytic activity	Ligninolytic activity	References
Ascomycetes					
1	*Acrocordiopsis patilii*	+	–	+	Bucher et al. (2004)
2	*Aigialus grandis*	+	+	+	Bucher et al. (2004) and Raghukumar et al. (1994)
3	*Aigialus mangrovei*	+	+	+	Raghukumar et al. (1994)
4	*Amylocarpus encephaloides*	+		+	Rohrmann and Molitoris (1992)
5	*Aniptodera salsuginosa*	+	+	+	Bucher et al. (2004)
6	*Anthostomella* sp.	+	+	+	Luo et al. (2005)
7	*Arenariomyces trifurcatus*			+	Rohrmann and Molitoris (1992)
8	*Ascocratera manglicola*	+	+	–	Bucher et al. (2004)
9	*Astrosphaeriella striatispora*	+	+	+	Bucher et al. (2004)
10	*Bathyascus grandisporus*	+	+	+	Bucher et al. (2004)
11	*Biconiosporella corniculata*	–		+	Rohrmann and Molitoris (1992)
12	*Botryosphaeria* sp.	+	+	–	Bucher et al. (2004)
13	*Buergenerula spartinae*	+		+	Gessner (1980)
14	*Ceriosporopsis halima*	–		+	Rohrmann and Molitoris (1992)
15	*Chaetomium ramipilosum*	+		+	Rohrmann and Molitoris (1992)
16	*Corollospora angusta*	+	+	–	Luo et al. (2005)
17	*Corollospora besarispora*	+	+	–	Luo et al. (2005)
18	*Corollospora filiformis*	+	+	–	Luo et al. (2005)
19	*Corollospora intermedia*	+		+	Rohrmann and Molitoris (1992)
20	*Corollospora lacera*	+		+	Rohrmann and Molitoris (1992)
21	*Corollospora maritima*	+	+	+	Bucher et al. (2004) and Rohrmann and Molitoris (1992)
22	*Cryptovalsa halosarceicola*	+	+	+	Bucher et al. (2004)
23	*Cucullosporella mangrovei*	+	+	+	Luo et al. (2005)
24	*Dactylospora mangrovei*	+	+	+	Bucher et al. (2004)

25	*Diaporthe salsuginosa*	+	+	+	Luo et al. (2005)
26	*Eutypa* sp.	+	+	−	Bucher et al. (2004) and Luo et al. (2005)
27	*Ascosalsum cincinnatula*	+	+	+	Bucher et al. (2004)
28	*Halorosellinia oceanica*	+	+	+	Luo et al. (2005), Pointing et al. (1998) and Raghukumar et al. (1994)
29	*Halosarpheia fibrosa*	+	−	+	Luo et al. (2005)
30	*Helicascus kanaloanus*	+		+	Pointing et al. (1998)
31	*Helicascus nypae*	+	+	−	Bucher et al. (2004)
32	*Hypoxylon* sp.	+	+	+	Luo et al. (2005)
33	*Julella avicenniae*	+		+	Luo et al. (2005), Pointing et al. (1999), and Vrijmoed et al. (1999)
34	*Kallichroma tethys*	+	+	+	Bucher et al. (2004) and Luo et al. (2005)
35	*Hypocrea* sp.	+	−	−	Bucher et al. (2004)
36	*Leptosphaeria avicenniae*	+	+	+	Luo et al. (2005)
37	*Leptosphaeria obiones*	−		+	Rohrmann and Molitoris (1992)
38	*Leptosphaeria* sp.	+	+	+	Bucher et al. (2004) and Gessner (1980)
39	*Lignincola laevis*	+	+	+	Bucher et al. (2004), Pointing et al. (1998, 1999), Rohrmann and Molitoris (1992), and Vrijmoed et al. (1999)
40	*Lineolata rhizophorae*	+	+	+	Luo et al. (2005)
41	*Linocarpon bipolaris*	+	+	+	Bucher et al. (2004)
42	*Lulworthia lignoarenaria*	+		+	Rohrmann and Molitoris (1992)
43	*Lulworthia grandispora*	+	+	+	Bucher et al. (2004)
44	*Lulworthia* sp.	+	+	+	Bucher et al. (2004)), Gessner (1980), Mouzouras (1986)), Rohrmann and Molitoris (1992)), and Vrijmoed et al. (1999)
45	*Marinosphaera mangrovei*	+	+	+	Bucher et al. (2004)
46	*Marinospora longissima*	−		+	Rohrmann and Molitoris (1992)

(continued)

Table 12.1 (continued)

S. no.	Name of the fungus	Cellulolytic activity	Hemicellulolytic activity	Ligninolytic activity	References
47	*Massarina acrostichi*	+	+	−	Bucher et al. (2004)
48	*Microascus senegalensis*	−		+	Rohrmann and Molitoris (1992)
49	*Morosphaeria velatospora*	+	+	+	Bucher et al. (2004)
50	*Nautosphaeria cristaminuta*	−		+	Rohrmann and Molitoris (1992)
51	*Neptunella longirostris*	+	+	+	Bucher et al. (2004)
52	*Patellaria* sp.	+	+	+	Luo et al. (2005)
53	*Passereniella obiones*	+		+	Gessner (1980)) and Rohrmann and Molitoris (1992)
54	*Phragmitensis marina*	+	+	−	Bucher et al. (2004)
55	*Phaeosphaeria typharum*	+			Gessner (1980)
56	*Phaeosphaeria* sp.	+			Gessner (1980)
57	*Pleospora pelagica*	+		+	Gessner (1980)
58	*Pleospora vagans*	+			Gessner (1980)
59	*Quintaria lignatilis*			−	Luo et al. (2005) and Raghukumar et al. (1994)
60	*Quintaria* sp.	+	+	+	Bucher et al. (2004)
61	*Remispora stellata*	−		+	Rohrmann and Molitoris (1992)
62	*Rhizophila marina*	+	+	+	Bucher et al. (2004)
63	*Rimora mangrovei*	+	+	+	Pointing et al. (1998), and Raghukumar et al. (1994)
64	*Saagaromyces ratnagiriensis*	+	+	+	Luo et al. (2005), Raghukumar et al. (1994), and Vrijmoed et al. (1999)
65	*Salsuginea ramicola*	−	+	+	Bucher et al. (2004)
66	*Savoryella lignicola*	+	+	+	Bucher et al. (2004), Leightley and Eaton (1977) Pointing et al. (1998, 1999)), and Mouzouras (1986)
67	*Savoryella longispora*	+	+	+	Luo et al. (2005)
68	*Sordaria fimicola*		+	+	Raghukumar et al. (1994)
69	*Swampomyces triseptatus*	+	+	+	Luo et al. (2005)

70	*Torpedospora radiata*	+		+	Pointing et al. (1998)
71	*Trematosphaeria mangrovei*	+		+	Pointing et al. (1998, 1999) and Mouzouras (1986)
72	*Trematosphaeria* sp.	+		+	Pointing et al. (1998)
73	*Verruculina enalia*	+	+	+	Bucher et al. (2004), Luo et al. (2005), and Raghukumar et al. (1994)
Mitosporic fungi					
74	*Alternaria alternata*	+			Gessner (1980)
75	*Alternaria maritima*	+	+		Leightley (1980)
76	*Asteromyces cruciatus*	+		+	Gessner (1980), Rohrmann and Molitoris (1992), and Sutherland et al. (1982)
77	*Camarosporium roumeguerii*	–		+	Rohrmann and Molitoris (1992)
78	*Cirrenalia macrocephala*	+	+	+	Leightley (1980), Leightley and Eaton (1977), and Rohrmann and Molitoris (1992)
79	*Cirrenalia pygmaea*	–		+	Mouzouras (1986), Raghukumar et al. (1994), and Rohrmann and Molitoris (1992)
80	*Cirrenalia tropicalis*	+		+	Leightley (1980) and Rohrmann and Molitoris (1992)
81	*Cytoplacosphaqera phragmiticola*	+	+	+	Bucher et al. (2004)
82	*Cytospora rhizophorae*	–	–	+	Bucher et al. (2004) and Rohrmann and Molitoris (1992)
83	*Dactylaria* sp.	+	+	+	Bucher et al. (2004)
84	*Dendryphiella arenaria*	+			MacDonald and Speedie (1982)
85	*Dendryphiella salina*	+	+	+	Bucher et al. (2004), Rohrmann and Molitoris (1992), and Sutherland et al. (1982)
86	*Diplodia oraemaris*			+	Sutherland et al. (1982)
87	*Epicoccum purpurascens*	+			Gessner (1980)
88	*Exserohilum rostratum*	+			Gessner (1980)
89	*Gliocladium* sp.		+	+	Raghukumar et al. (1994)
90	*Gongronella* sp.		+	+	Raghukumar et al. (1994)

(continued)

Table 12.1 (continued)

S. no.	Name of the fungus	Cellulolytic activity	Hemicellulolytic activity	Ligninolytic activity	References
91	*Monodictys pelagica*	−		+	Rohrmann and Molitoris (1992)), Pointing et al. (1998), and Sutherland et al. (1982)
92	*Papulaspora halima*			+	Sutherland et al. (1982)
93	*Periconia prolifica*	+	−	+	Bucher et al. (2004)
94	*Phoma* sp.	+	−	+	Bucher et al. (2004), Rohrmann and Molitoris (1992), and Gessner (1980)
95	*Phomopsis* sp.	+	+	−	Bucher et al. (2004)
96	*Stagonospora* sp.	+		+	Pointing et al. (1998) and Gessner (1980)
97	*Trichocladium achrasporum*	−	−	+	Bucher et al. (2004)), Mouzouras (1986), and Rohrmann and Molitoris (1992)
98	*Trichocladium alopallonellum*	+		+	Eaton and Jones (1971), Gessner (1980)Leightley (1980), Leightley and Eaton (1977), and Rohrmann and Molitoris (1992)
99	*Varicosporina rumulosa*	+		+	Gessner (1980) and Rohrmann and Molitoris (1992)
100	*Zalerion varium*	+	+	+	Bucher et al. (2004) and Raghukumar et al. (1994)
101	*Zalerion maritimum*	+		−	Rohrmann and Molitoris (1992) and Sutherland et al. (1982)
Basidiomycetes					
102	*Calathella mangrovei*	+	+	+	Luo et al. (2005)
103	*Digitatispora marina*	+	−	+	Rohrmann and Molitoris (1992)
104	*Flavodon flavus*	−	−	+	Raghukumar et al. (1999)
105	*Nia vibrissa*	+	+	+	Leightley and Eaton (1977), Pointing et al. (1998), Rohrmann and Molitoris (1992), and Sutherland et al. (1982)

+ indicates the presence of particular enzymatic activity; − indicates lack of particular enzymatic activity; empty box indicates either it is not determined or is unknown

J.L. Crane) J. Dupont & E.B.G. Jones (=*Ascosalsum cincinnatula* Shearer & J.L. Crane); *Kallichroma tethys* (Kohlm. & E. Kohlm.) Kohlm. & Volkm.-Kohlm.; *Leptosphaeria* sp.; *Lignincola laevis*; *Linocarpon bipolare* K.D. Hyde; *Lulworthia grandispora* Meyers; *Lulworthia* sp.; *Marinosphaera mangrovei* K.D. Hyde; *Halomassarina thalassiae* (Kohlm. & Volkm.-Kohlm.) Suetrong, Sakay., E.B.G. Jones, Kohlm., Volkm.-Kohlm. & Schoch (=*Massarina thalassiae* Kohlm. & Volkm.-Kohlm.); *Morosphaeria velatospora* (K.D. Hyde & Borse) Suetrong, Sakay., E.B.G. Jones & Schoch (=*Massarina velatospora*); *Neptunella longirostris* (Cribb & J.W. Cribb) K.L. Pang & E.B.G. Jones; *Quintaria* sp.*; Rhizophila marina*; *Salsuginea ramicola* K.D. Hyde; and *Verruculina enalia.* A clearance of the Poly-R dye in the agar medium indicates production of lignin-modifying enzymes (Bucher et al. 2004). Laccases are produced by the following marine fungi: *Acrocordiopsis patilii* Borse and K.D. Hyde; *Aigialus grandis*; *Cryptovalsa halosarceicola*; *Cucullosporella mangrovei* (K.D. Hyde and E.B.G. Jones) K.D. Hyde & E.B.G. Jones; *Diaporthe salsuginosa* Virjmoed, K.D. Hyde & E.B.G. Jones; *Digitatispora marina* Doguet; *Halenospora varia* (Anastasiou) E.B.G. Jones; *Halocyphina villosa* Kohlm. & E. Kohlm.; *Halorosellinia oceanica* (S. Schatz) Whalley, E.B.G. Jones, K.D. Hyde & Laessøe; *Halosarpheia fibrosa* Kohlm. & E. Kohlm.; *Kallichroma tethys*; *Leptosphaeria avicenniae* Kohlm. & E. Kohlm.; *Leptosphaeria* sp.; *Nia vibrissa*; *Quintaria lignatilis* (Kohlm.) Kohlm. & Volkm.-Kohlm.; *Rhizophila marina*; *Saagaromyces abonnis* (Kohlm.) K.L. Pang & E.B.G. Jones; *S. ratnagiriensis*; and *Verruculina enalia* (Rohrmann and Molitoris 1992; Raghukumar et al. 1994, 1996; Bucher et al. 2004; Luo et al. 2005). It could be seen from the above list that most of them are ascomycetes. Among the lignin-degrading enzymes, laccase (EC1.10.3.2, benzenediol:oxygen oxidoreductase) is a multicopper blue oxidase which can oxidize ortho- and para-diphenols and aromatic amines by removing an electron and proton from a hydroxyl group to form a free radical. Since these enzymes lack substrate specificity, they can degrade a wide array of xenobiotics including industrial colored wastewaters (D'Souza et al. 2006).

Xylan is a main component of hemicellulose, and its degradation indicates the ability of a strain to degrade hemicellulose. Xylan gives brownish tinge to the pulp and paper, and it is removed through chemical bleaching in the pulp and paper industries. Among the different terrestrial filamentous fungi, the species belonging to *Trichoderma* are widely used in biobleaching. Several obligate marine fungi have also been implicated in the xylanase activity. These include *Aigialus grandis*; *A. mangrovis* Borse; *Aniptodera salsuginosa* Nakagiri & Tad.; *Ascocratera manglicola* Kohlm.; *Astrosphaeriella striatispora*; *Bathyascus grandisporus*; *Corollospora maritima*; *Cryptovalsa halosarceicola*; *Cytoplacosphaeria phragmiticola* Petr.; *Dactylospora mangrovei*; *Eutypa* sp.; *Halocyphina villosa*; *Halomassarina thalassiae*; *Helicascus nypae* K.D. Hyde; *Kallichroma tethys*; *Lignincola laevis*; *Linocarpon bipolaris*; *Lulworthia grandispora*; *Marinosphaera mangrovei*; *Lophiostoma acrostichi* (K.D. Hyde) Aptroot & K.D. Hyde (=*Massarina acrostichi* K.D. Hyde); *Morosphaeria velatispora*; *Nia vibrissa*; *Neptunella longirostris*; *Oceanitis cincinnatula*; *Paradendryphiella salina* (G.K. Sutherl.) Woudenberg & Crous; *Phragmitensis marina* K.M. Wong, Poon & K.D. Hyde; *Rimora mangrovei*

(Kohlm. & Vittal) Kohlm., Volkm.-Kohlm., Suetrong, Sakay. & E.B.G Jones; *Salsuginea ramicola* K.D. Hyde; *Savoryella lignicola* E.B.G. Jones & R.A. Eaton; and *Verruculina enalia* (Rohrmann and Molitoris 1992; Raghukumar et al. 1994; Bucher et al. 2004; Luo et al. 2005). Though many obligate marine fungi have been shown to be having xylanolytic activity, they have not been studied in biobleaching. However, there is a huge potential in this area.

Ligninolytic and xylanolytic enzymes from terrestrial fungi have been used in various biotechnological applications including biobleaching, biopulping, and bioremediation technologies (Reddy 1995). A marine basidiomycete, *Flavodon flavus*, has been shown to be efficient in decolorization of paper mill bleach plant effluents (Raghukumar et al. 1996) and synthetic dye decolorization (Raghukumar et al. 1999) in addition to simultaneous detoxification and decolorization of molasses spent wash by immobilized cells (Raghukumar et al. 2004) and removal of colored pollutants in paper industry (Raghukumar 2002). There is a need for such concerted and intensive studies with the obligate marine fungi also taking into the account that many of them have ligninolytic and xylanolytic activity as shown above.

12.3 Dye Decolorization

Alcohol distilleries, dye making, leather, pulp and paper, and textile industries are among a few of the industries that discharge highly colored effluents (D'Souza et al. 2006). Among different kinds of synthetic dyes, the azo compounds are the most diverse and are used in different industries including cosmetics, food, paper printing, and textiles. Though they are xenobiotic in nature and hence generally are recalcitrant, certain microorganisms, particularly basidiomycetes, have developed enzyme systems for decolorization and mineralization of these dyes under certain environmental conditions. We have fairly good information about bacterial decolorization and degradation of azo dyes (Pandey et al. 2007). At present, the removal of dyes from effluents is done by physicochemical means which are expensive and cause disposal problems due to accumulation of concentrated sludge. Biological systems seem to be one of the best alternatives that could circumvent the above problems as has been contended in a review by Robinson et al. (2001).

Dye effluents could be treated with filamentous fungi, particularly those belonging to basidiomycetous fungi, as part of green technologies, because they produce all three main types of lignin-degrading enzymes, viz., lignin peroxidase, manganese-dependent peroxidase, and laccases which were implicated in the degradation of dyes and dye effluents (Blanquez et al. 2008). In addition to fungi belonging to *Basidiomycota*, several species belonging to *Ascomycota* and anamorphic fungi also have been shown to produce some of these enzymes particularly the laccases (Baldrian 2006) and interestingly many marine fungi also (Raghukumar et al. 1994; Pointing and Hyde 2000; Bucher et al. 2004; D'Souza et al. 2009).

Strains of *Phanerochaete chrysosporium* have been shown to degrade different azo dyes such as orange II, azure B, tropaeolin O, Congo red, amaranth, and orange

G (Cripps et al. 1990). The degradation ability is more efficient under nitrogen-limiting conditions and in the presence of peroxide against many of the above dyes, whether it be in cultures or crude lignin peroxidase treatments (Cripps et al. 1990). Nitrogen-limiting conditions also seem to be effective against other complex dyes such as crystal violet, cresol red, bromophenol blue, malachite green, and brilliant green when *P. chrysosporium*-grown cultures were treated (Bumpus and Brock 1988) or crude lignin peroxidase preparations from this fungus were used in the treatment (Paszczynski et al. 1992). While the above studies are with terrestrial fungi, there are very few reports available with marine fungi vis-à-vis dye decolorization. *Flavodon flavus*, a basidiomycete isolated from marine environment, has been shown to be decolorizing synthetic dyes such as Congo red, Poly-B, Poly-R, and Remazol brilliant blue R. This fungus also decolorized pigments in the molasses spent wash more efficiently under low nitrogen with glucose or sucrose as the carbon sources. In addition to the molasses spent wash, this fungus could also reduce the total phenolic and COD up to 50% and toxicity completely (Raghukumar 2004).

Pulp and paper industries release enormous amount of waste lignin to the order of 30–50 × 10^6 tons per year (Kuhad and Singh 1993). They release large volumes of wastewater effluents that contain dark-colored, high molecular weight, modified, and chlorinated lignins, phenols, dioxins, and furans. The rich information about the decolorizing capability of bleach plant effluents of pulp and paper industries by white rot fungi including *Phanerochaete chrysosporium* (Michel et al. 1991), *Tintoporia barbonica* (Fukuzumi et al. 1977), and *Trametes versicolor* (Bajpai et al. 1993) have been highlighted. However, there is a lack of data on the obligate marine fungi on these aspects (Raghukumar 2004). Three marine fungi were studied for decolorization of bleach plant effluents, of which *Flavodon flavus*, a basidiomycetous fungus, has been shown to be effective at both acidic and alkaline pH. Interestingly bleach plant effluent stimulates production of lignin-modifying enzymes, MNP, and laccases in these fungi thus indicating that modified lignin or degradation products of lignin, in the form of small molecular weight aromatic compounds, act as inducers (Raghukumar 2004). In this study, it has also been proved the capability of decolorization of paper mill effluent by a nonwhite rot obligate marine fungus, viz., *Saagaromyces ratnagiriensis* (an ascomycete).

As could be seen from the above account, there are only a few studies that have been conducted with the obligate marine fungi, and they are in the area of screening of their ligninolytic enzymes. More studies are required in the area of applications both at laboratory level and at field level particularly in the areas of biopulping, biobleaching, and decolorization of colored effluents from leather, sugar, textile, pulp, and paper industries. Isolation of obligate marine fungi is a tedious process as they are slow growing and have fastidious nutritional requirements. Though many studies have been conducted, they are mostly on the biodiversity and ecology of marine fungi (Hyde et al. 2000; Sarma and Hyde 2001). Only a few workers have attempted to isolate the obligate marine fungi. Due to this reason, the potential of obligate marine fungi, in the area of bioremediation, has largely remained a mystery.

12.4 Hydrocarbon Degradation

Hydrocarbon contamination from petrochemical industry is one of the major environmental problems faced by humanity. Leakage and accidental spillage of petroleum and petroleum products are a common feature during exploration, production, refining, transport, and storage, to the tune of 2,00,000–6,00,000 metric tons per year (Kvenvolden and Cooper 2003; Das and Chandran 2011). These components are known to be carcinogenic and neurotoxic organic pollutants causing death to plants, animals, and microbes. The disadvantages of present methods in remedial strategies are that (1) while incineration or burial in landfills is cost prohibitive when amount of contamination is large, (2) mechanical and chemical methods have limited effectiveness in addition to being expensive. Bioremediation employs microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities, and this method is noninvasive and relatively cheap. The efficacy and success of oil spill bioremediation are dependent on the ability to establish and bring in conditions that favor enhanced oil biodegradation rates. An important requisite to that end is the presence of microorganisms with appropriate metabolic capabilities followed by providing an efficient nutrient composition and physicochemical conditions for survival of the microorganisms for successful biodegradation. The oil spill bioremediation has two main methods, viz., (1) bioaugmentation that involves addition of known oil-degrading bacteria to increase the existing microbial population and (2) biostimulation that involves enhancing the growth of native oil degraders by adding nutrients or other growth-limiting co-substrates (Leahy and Colwell 1990; Das and Chandran 2011). Literature on hydrocarbon bioremediation shows that mostly bacteria are studied and used (Atlas 1981; Atlas and Bartha 1992). Species belonging to *Aeromicrobium*, *Brevibacterium*, *Burkholderia*, *Dietzia*, *Gordonia*, and *Mycobacterium* that are isolated from petroleum-contaminated soils have been proved to be potential organisms for hydrocarbon degradation (Chaillan et al. 2004), while degradation of polyaromatic hydrocarbons by *Sphingomonas* was reported by Daugulis and McCracken (2003). Interestingly, even though algae and protozoa are an important component of the microbial community in aquatic ecosystems, very scant information is available on these organisms in terms of hydrocarbon biodegradation. When compared to bacteria, we have less information on fungi as degraders of petroleum hydrocarbons. That could be due to the fact that fungi are not as efficient as bacteria or that less research has been conducted with fungi. The following fungal genera, viz., *Amorphotheca*, *Graphium*, *Neosartorya*, and *Talaromyces*, have been isolated from the petroleum-contaminated soils and proved to be having efficacy in hydrocarbon degradation (Chaillan et al. 2004). Terrestrial fungal genera such as *Aspergillus*, *Cephalosporium*, and *Penicillium* also have been found to be good candidates of crude oil hydrocarbon degradation (Singh 2006; Das and Chandran 2011).

In the case of obligate marine fungi, there is only one study that is available and that too mostly on the arenicolous marine fungi (Kirk and Gordon 1988). In their study, Krik and Gordon (1988) have shown that 14 strains belonging to *Corollospora*,

Dendryphiella, *Lulworthia*, and *Varicosporina* species grew using alkanes and alkenes as sole carbon sources and mineralized n[1-^{14}C] hexadecane. More specifically, all arenicolous strains belonging to *Corollospora maritima* Werdermann, *C. lacera* (Linder) Kohlm., *C. intermedia* I. Schmidt, *Lulworthia lignoarenaria* Koch et Jones, *Dendryphiella salina* (Sutherland) Pugh et Nicot, and *Varicosporina ramulosa* Meyers and Kohlm. could grow on hexadecane as the sole carbon source, whereas none of the strains of *Arenariomyces trifurcatus* Hohnk and Jones or *Nereiospora cristata* (Kohlm.) Jones, Johnson, and Moss could utilize this hydrocarbon as the sole carbon source. Only 3 out of 27 lignicolous marine fungi could utilize hexadecane as the sole carbon source including *Zalerion maritimum* (Linder) Anastasiou, *Verruculina enalia* (Kohlm.) Kohlm. and Volkm.-Kohlm., and *Halokirschsteiniothelia maritima* (Linder) S. Boonmee & K.D. Hyde. Others that could not utilize hexadecane as the sole carbon source were strains of *Leptosphaeria obiones* (Cruoan and Cruoan) Sacc., *L. albopunctata* (Westend.) Sacc., *L. oraemaris* Linder, *Buergenerula spartinae* Kohlm. and Gessner, *Lignincola laevis* Hohnk (all from *Spartina alterniflora* culms), *Lignincola laevis*, *Halosphaeriopsis mediosetigera* (Cribb and Cribb) T.W. Johnson, *Halosphaeria appendiculata* Linder, *Haligena elaterophora* Kohlm., *Ceriosporopsis halima* Linder, *Remispora maritima* Linder, *R. quadriremis* (Hohnk) Kohlm., *Lulworthia* sp., *Marinospora calyptrata* (Kohlm.) Cavaliere, *Monodictys pelagica* (Johnson) E.B.G. Jones, *M. putredinis* (Wallr.) Hughes, *Cirrenalia macrocephala* (Kohlm.) Meyers and Moore, *Periconia prolifica* Anastasiou, *Dictyosporium pelagicum* (Linder) G.C. Hughes ex Johnson and Sparrow, and *Trichocladium achrasporum* (Meyers and Moore) Dixon. However, all these species could mineralize the labeled hexadecane compound when amended with glucose (Kirk and Gordon 1988).

The study by Kirk and Gordon (1988) also has shown that the 14 arenicolous and 3 lignicolous strains also utilized 1-hexadecene, pristine, and to some degree the tetradecane as sole carbon sources. *Lulwoana uniseptata* (Nakagiri) Kohlm., Volkm.-Kohlm., J. Campb., Spatafora & Grafenhan (=*Zalerion maritimum*) was the only species that could grow appreciably in dodecane and decane. Interestingly none of these strains utilized octane, hexane, mineral oil, kerosene, or naphthalene as sole carbon sources. While hexadecane and mineral oil were non-toxic to all strains on glucose amended medium, hexane was toxic to all and kerosene to some extent to most strains even on glucose amended medium, has been found in the above study. Further, strains of *Periconia prolifica* and *Marinospora calyptrata* (Kohlm.) A.R. Caval grew much better on glucose with mineral oil or hexadecane than on glucose alone. Usually the marine-derived fungi grow much better and faster under laboratory conditions than obligate marine fungi which are more successful in the marine environment (Kohlmeyer and Kohlmeyer 1979). We need studies conducted on obligate marine fungi in the field conditions also. This is because the woody substrata immersed in the seawater always support obligate marine fungi. However, the same marine fungi are either slow growers on artificial media or would lose the viability upon repeated subculturing. Since it has been evidenced through examination of woody substrata that the obligate marine fungi grow well on such substrata in seawater, it could also be surmised that obligate

marine fungi would also degrade hydrocarbons well in their natural conditions in the seawater. However, this needs to be tested. But scaling up of their inocula in the artificial conditions, in the laboratories, and for field applications may pose a problem which needs to be studied thoroughly.

12.5 Factors for Bioremediation

Among various factors influencing degradation of petroleum hydrocarbons, the composition and inherent biodegradability of the hydrocarbons are important. Temperature seems to have an effect. For example, an increase in viscosity of the oil and a decrease in solubility of hydrocarbons occur at low temperatures, while converse is true where a better degradation occurs at higher temperatures has been found (Foght et al. 1996). Among different nutrients, nitrogen, phosphorus, and to some extent iron seem to aid in successful degradation of hydrocarbon pollutants (Cooney 1984). However, an excessive usage of NPK may give negative effects (Oudot et al. 1998; Chaineau et al. 2005; Carmichael and Pfaender 1997). A rapid and complete degradation of petroleum hydrocarbon pollutants occurs under aerobic conditions as the initial cleavage is an oxidative process involving oxygenases and peroxidases (Fritsche and Hofrichter 2000). Mechanisms involved in the (a) appropriate attachment of microbial cells to the substrates and (b) the production of biosurfactants by the hydrocarbon-degrading microorganisms should be studied well (Hommel 1990). Among different enzymes, cytochrome P450 alkane hydroxylases, a part of heme-thiolate monooxygenases, have been found to play an important role in the microbial degradation of oil, chlorinated hydrocarbons, fuel additives, etc. (Van Beilen and Funhoff 2007), and hence their screening should be carried out, as such enzymes seem to be contained by eukaryotes and have been proved in yeasts (Scheuer et al. 1998).

12.6 Future Prospects

The above account shows that there is very little information available regarding the obligate marine fungi in the area of bioremediation. The demand for usage of dyes is going to increase and not going to decrease. At the same time, the various kinds of pollutants including colored and hydrocarbon pollutants also are going to increase, and the challenge of decolorization and detoxification of these colored pollutants is also going be tougher. There seem to be very few attempts made to conduct studies on the utilization of the obligate marine fungi in the area of dye decolorization or hydrocarbon degradation. This is mainly because of very few experts working on obligate marine fungi and most of the reports available are on the taxonomy, biodiversity, and ecology of obligate marine fungi. There should be more attempts and efforts to isolate the obligate marine fungi, deposit them in the

recognized culture collection centers, and screen them for various bioremediation studies so that they could be utilized in the bioremediation applications. Further, studies on bioengineering aspects of obligate marine fungi including fermenter trials, media optimization, and growth parameters also need to be studied.

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Chapter 13
Fungal-Derived Chitosan-Based Nanocomposites: A Sustainable Approach for Heavy Metal Biosorption and Environmental Management

Subhaswaraj Pattnaik and Siddhardha Busi

13.1 Introduction

In the beginning of the twenty-first century, the world has come to a stage of technological advancement in day-to-day life. However, massive industrialization, unplanned urbanization, and indiscriminate use of chemicals and pesticides in agricultural sectors resulted in serious environmental concern in the form of water contamination. Water is the most vital resources in human society with a significant impact on economy, society, and environment. With the ever-increasing industrialization process, there is a concomitant discharge of toxic heavy metals, recalcitrant dyes, and other chemicals to the water bodies which resulted in severe toxicity to aquatic organisms especially to human populations (Reddy and Lee 2013). In addition, the presence of heavy metals and recalcitrant azo dyes in water also has a significant contribution toward human health disorders and more importantly toward environmental deterioration.

13.2 Heavy Metal Decontamination and Related Side Effects

Though the discharge of wastewater from industries contain both inorganic and organic contaminants, inorganic heavy metal contaminants possess serious threat to living organisms especially to humans owing to their tendency to accumulate in living organisms. In addition to that, accumulation of many heavy metal ions such as chromium, cadmium, lead, nickel, copper, zinc, mercury, and arsenic tends to be

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highly toxic or may be even carcinogenic in nature (Fu and Wang 2011). Lead is an important heavy metal, and its presence in the environment beyond the threshold level proves to be detrimental to serious health conditions such as dysphoria, cancer, brain disorders, kidney malfunction, and central nervous system impairment owing to its cumulative accumulation properties (Ngah and Fatinathan 2010). Meanwhile, accumulation of more fluoride ions in the living organisms leads to the development of fluorosis with severe consequences. As both the heavy metals have significant attributes toward health disorders individually, the co-occurrence of both fluoride and lead in water proves to have life-threatening effects to aquatic organisms as well as to human beings in particular (Cho et al. 2016). In between the two stable oxidation states of chromium (Cr) in the environment, Cr (VI) possesses remarkable toxicity to humans and has the characteristic property to act as carcinogenic agent. Cr (VI) is predominantly present in the wastewater discharged from leather, textile, dye, and canning industries and has a significant adverse effects on living organisms and particularly aid to environmental deterioration owing to its high solubility in water and ease in reduction process (Amuda et al. 2009; Sivakami et al. 2013).

Copper is one of the most essential trace elements required for various physiological activities in human beings. Besides, it has a special role to play in plant metabolism. Though copper is important to living organisms in a particular range of concentration, the presence of copper ions in various water resources beyond the threshold level has raised tremendous public concern with potential health risks such as vomiting, cramps, convulsions, severe undesirable fatal effect, and even death (Ngah and Fatinathan 2010). Zinc is an important trace element found in the earth's crust and is essential to regulate an array of physiological and biochemical processes in plants as well as humans. Though zinc proves to be elemental to various metabolic processes and human health, at higher doses, zinc can cause severe health problems such as skin irritations, vomiting, nausea, and even anemia. Nickel is one of the well-known heavy metals with potential carcinogenic property. Besides, it also exerts severe health consequences such as pulmonary fibrosis, kidney dysfunction, skin dermatitis, etc. on exceeding the determined critical level (Fu and Wang 2011). In addition to these heavy metals, mercury (Hg), cadmium (Cd), and arsenic (As) also have tremendous potential to cause severe environmental degradation owing to their high toxicity, prevalence, existence, and persistence in the environment (Wang and Chen 2014).

The presence of these heavy metals beyond their critical level in the environment resulted in severe toxicity to animals and humans. Besides, the potential of these heavy metals in altering the regular biological processes is one of the crucial issues to take care of. In this regard, there is an urgent need for the removal of such highly toxic metal ions from the environment especially from discharged wastewater of several industries (Liu et al. 2009). Several conventional methods are applied to eradicate the nuisance of the toxicity possessed by heavy metals. These traditional methods include chemical precipitation, chemical reduction, electrolysis, coagulation/coprecipitation, oxidation/precipitation, ion exchange, adsorption, nanofiltration, reverse osmosis, bioremediation, etc. (Wang and Chen 2014) (Fig. 13.1). However, each of the abovementioned conventional method has been found to be

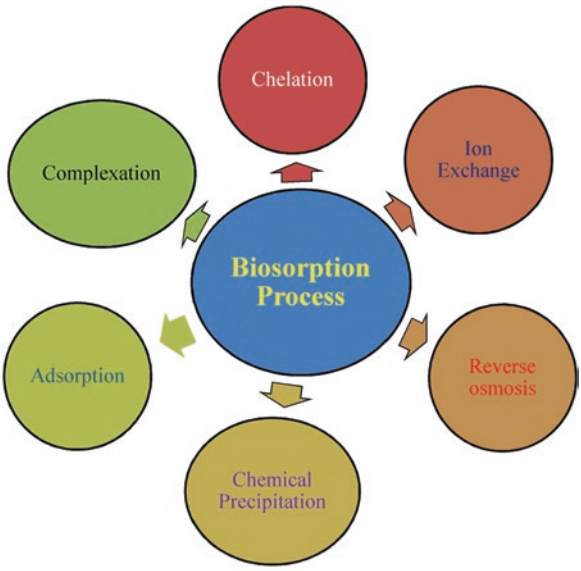

Fig. 13.1 Conventional biosorption process of heavy metals

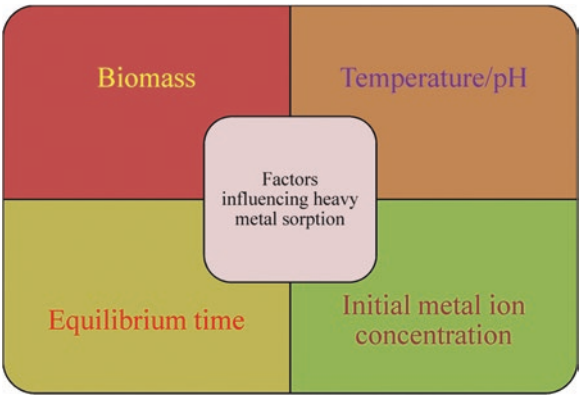

Fig. 13.2 Physical and biological factors influencing heavy metal bioremediation

limited by cost, complexity, and efficiency in remediating highly toxic heavy metals and dyes from the environment. In this context, highly effective and desirable techniques should be employed to remediate metals from soil and water (Kamari et al. 2011). The removal of heavy metals and dyes from wastewater always remains a daunting task for environmentalists to quest for effective strategy development to remediate contaminants and pollutants from the environment. Among the techniques employed for heavy metal remediation, adsorption process is recognized as the most effective strategy in terms of its high efficacy, operational simplicity, cost-effectiveness, regeneration capacity, easy recovery, and sludge-free operation (Sivashankar et al. 2014; Zhang et al. 2016). In this context, extensive research is in progress to develop novel adsorbents with unique sorption properties for the removal of heavy metals and dyes (Fig. 13.2).

Several natural and synthetic polymeric materials have been synthesized or chemically modified for the removal of heavy metal ions from aqueous solutions. Synthetic polymers such as ion exchange resins and chelating resins have been widely used as effective sorbents to collect radioactive nuclides and toxic metals from aqueous solutions. However, synthetic polymers possess toxicity and other health consequences. Therefore, the current research on heavy metal remediation is focused toward naturally occurring biopolymers to achieve effective sorption abilities for multivalent metal ions. Natural materials such as chitosan, cellulose, zeolites, clay, coal, and some microbial biomass products are classified as low-cost sorbents due to their cost effectivity. Naturally occurring biopolymers are competing with the conventional technologies and other similar materials of biological origins that have been intensively used as sorbents for heavy metal ion removal from water and industrial effluents (Khan et al. 2011).

However, to improve their absorption capacity and enhance the separation rate, the design of and exploration of novel adsorbents still need an insight into the matter. Recently, nanometer-sized hierarchically structured metal oxides such as magnetically functionalized iron oxides and its novel composites have been used for wastewater treatment and have shown remarkable potential due to their large surface areas (Liu et al. 2009).

13.3 Biosorption Process

The problems associated with conventional remediation strategies that lead to quest for novel, innovative, and cost-effective technologies for the removal of heavy metals from wastewater has been directed toward biosorption, i.e., adsorption of heavy metals through biological materials. The biological materials may be an algae, bacteria, fungi, or yeasts. All these microorganisms are well established for their metal biosorbent potential that can be correlated to their efficacy in metal sequestering (Das et al. 2008). The use of microorganisms and plants for remediation purposes is thus a possible solution for heavy metal pollution and subsequent environmental degradation (Dixit et al. 2015).

13.3.1 Bacteria as Biosorbent

Bacteria are most frequently used as efficient biosorbents on account of their small size, ubiquity, and capability to grow under controlled environmental conditions and most importantly their resistance against a wide range of varying environmental parameters (Ahemad and Kibret 2013). Considering the threat possessed by toxic heavy metals to microbes, plants, and human health, experiments were formulated to characterize the bacterial strain and to evaluate the metal biosorption ability of bacterial strains. *Bacillus thuringiensis* OSM29 (Acc. No. HM222647) isolated

from rhizospheric soils of cauliflower (*Brassica oleracea*) grown in metal-contaminated soil showed significant metal sorption efficacy (Oves et al. 2013). Besides, a number of bacterial species are well known to treat highly toxic polycyclic aromatic hydrocarbons (PAHs) and heavy metals present in wastewater in an efficient manner. Long-term waste discharge enables the microbes to tackle with the combined pollution of PAHs and heavy metals to a considerable extent. Bacterial species such as *Stenotropho monasmaltophilia* and *B. thuringiensis* FQ1 possess significant remediation efficiency of more than 90% in the removal of PAHs and heavy metals such as cadmium and copper (Chen et al. 2014; Jiang et al. 2015; Liu et al. 2017). The bioremediation efficiency of bacteria in removing heavy metal pollutants from environment depends on certain optimization parameters such as pH, biomass concentration, temperature, initial metal ion concentration, and equilibrium time (Aryal and Kyriakides 2015).

13.3.2 Algae as Biosorbent

The presence of heavy metal ions such as lead, copper, cadmium, zinc, and nickel as common contaminants in industrial wastewater leads to pollution of natural environment. Biosorption is considered as an innovative technology to remove heavy metal ions from wastewaters using predominantly inactive biomass and nonliving algae as absorption mechanisms in living algae are more complex than nonliving algae since absorption takes place during the growth phase, and intracellular uptake of heavy metal ions usually occur in this phase. In contrast, nonliving algae cells absorb metal ions (the process called biosorption) on the surface of the cell membrane, and it is considered an extracellular process. The toxic level of heavy metal ions in variant algal species can be highly strain specific, which consequently determines the potential remediation capacity using a specific algal strain (Zeraatkar et al. 2016).

Water pollution is one of the most serious problems because inorganic and organic wastes are discharged to the aquatic environment either in water-soluble or insoluble forms. Lead and cadmium are considered to be the most toxic and hazardous to the environment. Microalgae biosorbent seems to be more promising than microalgae because of easier cultivation, higher production yield, high efficacy, and more importantly higher-specific biosorption area. Blue-green algae (cyanobacteria) including *Dunaliella*, *Spirulina* (*Arthrospira*), *Nostoc*, *Anabaena*, and *Synechococcus* are the typical examples that exhibited the potential as biosorbents for efficient removal of heavy metals from wastewaters (El-Sheekh et al. 2005). Cyanobacteria have some advantages over other microorganisms due to their greater mucilage volume with high-binding affinity, large surface area, and simple nutrient requirements. In this context, the biosorption of Cd(II) and Pb(II) from aqueous solution onto the biomass of the blue-green alga *Anabaena sphaerica* as a function of pH, biosorbent dosage, contact time, and initial metal ion concentrations was investigated. The results suggested that the biomass of *A. sphaerica* is an extremely

efficient biosorbent for the removal of Cd(II) and Pb(II) from aqueous solutions (Abdel-Aty et al. 2013).

In aqueous systems, chromium exists in various oxidation states, but trivalent and hexavalent are the most common oxidation states. Meanwhile, Cr (III) is sometimes required in little amounts for biological metabolisms, Cr (VI) is known to be a toxic and carcinogenic substance. *Chlorella*, *Spirulina*, and *Dunaliella* are the microalgae species that have been most widely tested for the removal of Cr (VI) (Gokhale et al. 2008). However, the use of microalgae in wastewater treatment has limitations because of the necessity of separation of microalgae from the treated water. Industrial filtration and centrifugation are the techniques currently available for the harvesting of the microalgae, but unfortunately, these techniques are not cost-effective. In this regard, immobilization of microalgae into polymeric matrices, such as alginate, silica gel, and carrageenan, was proposed (Rangsayatorn et al. 2004). In this context, for enhanced efficacy in heavy metal biosorption, Kwak et al. (2015) reported that a bead-shaped biosorbent for removal of Cr (VI) from industrial wastewaters/aqueous media was fabricated using water-soluble *Spirulina platensis* extract (SPE).

13.3.3 Yeasts as Biosorbent

In the day-to-day life with advancement in technological expansion, heavy metal pollution has become a most serious environmental problem. Manganese is released into the environment by fertilizers, petrochemicals, electroplating, and tanneries industries. Exposure to manganese causes serious health disorders such as neurotoxicity, gastrointestinal problems, and decreased hemoglobin levels (Henrik et al. 2004). Microorganisms, as heavy metal bioadsorbents, offer a new alternative for removal of toxic or valuable metals in water. *Saccharomyces cerevisiae* has received considerable attention due to its unique nature and capacity for metal sorption. It is one of the most promising biosorbents capable of removing metal ions from aqueous solutions in an effective manner. Heavy metals can be competently removed from water and other aqueous environments by *S. cerevisiae*. *S. cerevisiae* as biosorbents in metal biosorption have many advantages such as easier cultivation at large scale, inexpensive growth media, and high biomass yield; biomass of *S. cerevisiae* can be obtained from various food and beverage industries as a by-product and easier to get from fermentation industry (Fadel et al. 2015).

13.3.4 Fungi as Biosorbent

Microorganisms including fungi and bacteria have been reported to extract heavy metals from wastewater through bioaccumulation and biosorption. An attempt was, therefore, made to isolate bacteria and fungi from sites contaminated with heavy

metals for higher tolerance and removal from wastewater. Fungi such as *Aspergillus nidulans*, *Rhizopus arrhizus*, and *Trichoderma viride* showed maximum uptake capacity of Pb, Cd, and Cr, respectively. This indicates the potential of these identified fungi as efficient biosorbent for removal of high-concentration metals from wastewater and industrial effluents (Kumar et al. 2014).

Microorganisms including fungi have been reported to exclude heavy metals from wastewater through bioaccumulation and biosorption at low-cost and in eco-friendly way. An attempt was, therefore, made to isolate fungi from sites contaminated with heavy metals for higher tolerance and removal of heavy metals from wastewater. Four fungi (*Phanerochaete chrysosporium*, *Aspegillus awamori*, *Aspergillus flavus*, *Trichoderma viride*) were included in this study. The majority of the fungal isolates were able to tolerate up to 400 ppm concentration of heavy metals such as Pb, Cd, Cr, and Ni. From the results, it was observed that a substantial amount of heavy metals was removed by some of the fungi. This indicated the potential of these fungi as efficient biosorbent for removal of heavy metals from wastewater and industrial effluents containing higher concentration of heavy metals (Joshi et al. 2011).

In the year 2015, an attempt was made to observe fungal absorption behavior toward heavy metal at optimum pH and temperature conditions. For heavy metal removal, highly tolerant isolates of *Aspergillus* spp. along with the initial metal concentration and contact time were utilized. The results showed that the biosorption capacity of *A. flavus* was 20.75–93.65 mg/g for Cu(II) with initial concentration of 200–1400 ppm. On the other hand, biosorption capacity of *A. niger* for Pb(II) ranged from 3.25 to 172.25 mg/g with the same range of initial metal concentration. The knowledge of the present study will be helpful for further research on the bioremediation of polluted soil by fungi (Iram et al. 2015).

13.4 Use of Chitosan in Biosorption Process

Chitosan is a biopolymer obtained naturally from chitin by deacetylation process. Being natural in origin, chitosan holds the unique properties such as biodegradability, biocompatibility, and inbuilt antimicrobial property. This novelty in chitosan makes it the most suitable candidate for use as an adsorbent in the removal of toxic heavy metals and environmental management. Among the various biopolymers used in sorption process, chitosan has the highest sorption capacity for several heavy metal ions, possibly due to the presence of primary amine at C-2 position of the glucosamine residues (Malathi et al. 2014).

Toxic metal species are mobilized from industrial activities and fossil fuel consumption and eventually are accumulated through the food chain, leading to both ecological and health problems. Recent researches in the field of heavy metal remediation from wastewaters and sediments have been focused on the development of materials with enhanced affinity, capacity, and selectivity to target heavy metals. In

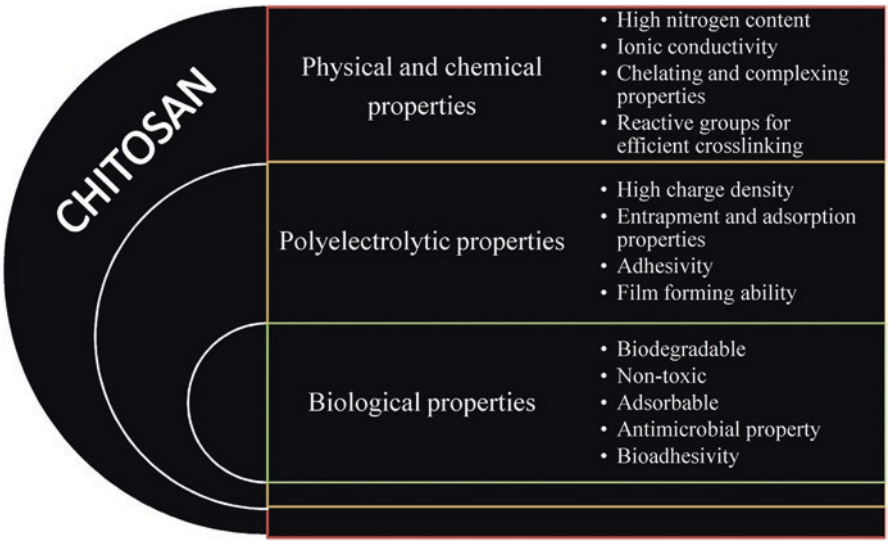

Fig. 13.3 Intrinsic properties of chitosan for biosorption of heavy metal ions and dyes

this context, the unique properties of chitin and its deacetylated derivative chitosan can be utilized for an array of applications (Franco et al. 2004).

Chitosan has been investigated by several researchers from the last couple of decades as a selective and novel biosorbent for the capture of dissolved dyes and heavy metals from aqueous solutions. This natural polymer possesses several intrinsic characteristics that make it an effective biosorbent for the removal of toxic chemicals and dyes (Fig. 13.3). Its use as a biosorbent is justified by two important advantages such as firstly, low cost compared to commercial-activated carbon and, secondly, its outstanding chelation behavior by its ability to tightly bind pollutants, in particular heavy metal ions (Crini and Badot 2008) .

13.5 Use of Chitosan-Based Composites as Biosorbents

Though chitosan remains one of the fundamental biopolymers used in heavy metal and toxic dye remediation from the last few decades due to the presence of amino and hydroxyl groups, it has certain drawbacks such as degree of softness, tendency to agglomerate, and nonavailability of reactive binding sites for biosorption of specific heavy metals as chitosan is very sensitive to change in pH values (Wan Ngah et al. 2011). Moreover, chitosan in its original form usually does not own specific selectivity for a certain kind of heavy metals or very high sorption capacity from complex polluted water or wastewater (Wang and Chen 2014).This has ultimately lead to decrease in the biosorption efficiency. To counteract the problems associated with chitosan and to utilize the efficacy of chitosan in biosorption process, the

research is focused toward the formulation of novel chitosan-based biocomposites that will increase the usability and efficacy of chitosan in bioremediation of heavy metals and environmental management (Malathi et al. 2014). To improve its performance as an adsorbent, cross-linking reagents such as glyoxal, formaldehyde, glutaraldehyde, epichlorohydrin, ethylene glycon diglycidyl ether, and isocyanates have been extensively used. Cross-linking agents not only stabilize chitosan in acid solutions so that it becomes insoluble but also enhance its mechanical properties (Wan Ngah et al. 2011).

In the quest for novelty in chitosan and chitosan-derived biocomposites in heavy metal removal, an attempt made to use chitosan-grafted cocoa husk char (CCH) as potential adsorbents for the removal of heavy metals such as Cr^{6+} and Pb^{2+} was examined. The study suggested the potential use of chitosan-grafted cocoa husk as an adsorbent to remove toxic heavy metal like chromium and lead from industrial wastewater. However, modification of this agricultural waste with chitosan characteristically enhances its performance in the removal of the heavy metals (Okoya et al. 2014). One of the most eminent drawbacks of chitosan in biosorption process is the post-adsorption separation technologies and regeneration of adsorbents. To overcome the problems related to the ease of separation and regeneration of adsorbents, recent research has been focused toward magnetic separation technology. In this context, magnetic chitosan composites are a novel material that exhibits a combinatorial property of good sorption behavior as that of chitosan and regeneration of adsorbents for reuse by using magnetic separation technologies. This resulted in an incredible enhancement in biosorption of various toxic pollutants in aqueous solution. These magnetic composites have a fast adsorption rate and high adsorption efficiency and are efficient to remove various pollutants, and they are easy to recover and reuse (Reddy and Lee 2013).

In an attempt to increase the biosorption efficiency of chitosan, silica gel was functionalized for the first time with chitosan to form silica/chitosan composite using bifunctional cross-linking agent, glutaraldehyde. The silica/chitosan composite showed a tremendous potential to remove heavy metals such as copper and lead by adsorption and complexation process. The sorption capacity of the composite was observed to be higher than unmodified sorbents (Gandhi and Meenakshi 2012). From the last few years, there is a considerable attention toward the fungal-derived polysaccharides in heavy metal removal from contaminated water. In this context, fungal-derived chitosan-glucan complex was assessed for their inherent property of efficient biosorption capacity of heavy metals such as copper and chromium and radioactive isotopes such as cobalt and uranium (Muzzarelli 2011). Though chitosan served as the most prominent biosorbent, it suffers certain limitations such as low thermal stability, inadequate mechanical properties, low porosity, etc. (Zhang et al. 2016). In this context, cross-linked chitosan-clay composite beads with epichlorohydrin were investigated systematically for biosorption efficacy. The results suggested the enhanced adsorption potential of the cross-linked chitosan composite in influencing the adsorptions of heavy metals such as Ni(II) and Cd(II) ions (Tirtom et al. 2012). In 2014, two novel chitosan (CS) adsorbents were developed, and the study suggested that the adsorption capacities of heavy metal ions like Cd(II) and

Pb(II) were enhanced after grafting and cross-linking (Kyzas et al. 2014). Arsenic poisoning is one of the most important concerns with widespread problems such as cancer, hypertension, and respiratory dysfunction (Guan et al. 2012). In addition to that, arsenic is the prime source of water contamination, and development of treatment technologies for the removal of arsenic is one of the biggest challenges in wastewater treatment. In this context, the adsorption properties of alumina have been widely recognized along with chitosan via cross-linking with oxalic acid to form an effective composite. The results emphasized the enhanced sorption capacity of the composite and suggested the application of this composite in wastewater treatment (Rahim and Haris 2015).

In an attempt to enhance the biosorption efficacy of chitosan, chitosan modification by introducing the desired properties in terms of physical, chemical, and mechanic properties is detrimental to enhance its metal sorption capacity, to improve its affinity for the metal, and to change the selectivity series for metal sorption. Chitosan modifications including various physical or chemical methods or hybrids have been reported and summarized. In this context, carboxymethyl chitosan (CM-chitosan) is one of the most fully explored chitosan derivatives due to its good sorption capacity for heavy metals. By grafting procedure, various functional groups or other polymers or molecules are introduced to enhance metal sorption capacity. Chemical grafting introduces some new specific functional ligands onto chitosan, which will enhance metal sorption performance by increasing the density of sorption sites, changing sorption sites to increase sorption selectivity, and altering pH range for metal sorption (Guibal 2004; Wang and Chen 2014).

In the last few years, research and development are focused toward the new and innovative biosorbents with increased efficiency toward an array of heavy metals. In this context, Sargin et al. (2015) exploited the preparation of three novel chitosan microcapsules from pollens of three common wind-pollinated plants (*Acer negundo*, *Cupressus sempervirens*, and *Populus nigra*). The sorption capacities of these synthesized microcapsules were compared with that of cross-linked chitosan beads without pollen grains, and the results suggested the tremendous biosorption potential of chitosan-based microcapsules in remediating heavy metals such as cadmium, copper, zinc, and chromium. This work has suggested the use of these microcapsules as advanced biosorption agent in heavy metal remediation (Sargin et al. 2015). In an era of massive industrialization and technological advancement, there is a frequent incidence of drinking water contamination by the release of heavy metal ions and toxic dyes leading to life-threatening health issues and serious environmental concern. In this context, to develop highly cost-effective, highly operation-convenient, and highly efficient natural polymer-based adsorbents with a new kind of efficient recyclable magnetic chitosan hybrid microspheres seems to be a quite interesting strategy (Luo et al. 2015). The results suggested the efficacy of the hybrid microspheres in bioremediation of heavy metal ions as compared to the traditional adsorbents.

Chitosan is a functional biopolymer which is desired in heavy metal removal due to its metal ion interacting groups. Meanwhile, algal biomass is one of the preferred candidates in heavy metal removal studies for its cost-effectiveness, and their cell

wall composition provides unique binding sites for heavy metal ions (He and Chen 2014). In this context, production of adsorbents by exploiting biological materials grafted with natural polymers like chitosan proves to be advantageous over the conventional synthetic adsorbents. The microbeads with bleached algal biomass and chitosan exhibited higher sorption capacity for cadmium and zinc ions than the plain glutaraldehyde cross-linked chitosan microbeads, suggesting the adequate contribution of the algal biomass to the sorption efficacy of chitosan (Sargin et al. 2016).

The presence and high concentration of Pb(II) in various water resources lead to severe adversity to the environment and public health. An attempt to remove Pb(II) ions from water in an efficient manner using graphite-doped chitosan composite (GDCC) was evaluated for the first time. The adsorption efficiency of GDCC was found to be pH dependent, and the maximum biosorption efficiency was observed to be approximately 98% at an optimum pH of 6. The results illustrated that GDCC has the tremendous potential to remove toxic Pb(II) ions from aqueous solution in an efficient manner (Gedam et al. 2015). The result suggested the use of GDCC in biosorption of other heavy metal ions in the future to maintain equilibrium in the environment.

Biochar is the carbonaceous product of the pyrolysis of organic matter and recently under limelight for its tremendous potential as an efficient environmental sorbent to remove various contaminants from soil and water systems. The reason behind this biosorption characteristic is the strong affiliation of biochar to heavy metal ions. In this context, to enhance the efficacy of unmodified biochar, Zhou et al. (2013) formulated batch sorption experiment with chitosan-modified biochars in heavy metal ion remediation. The results exhibited that, compared to the unmodified biochars, almost all the chitosan-modified biochars showed enhanced removal of three heavy metals such as lead, cadmium, and copper ions from aqueous solution (Zhou et al. 2013).

Uranium (VI) and thorium (III) are naturally occurring radioactive elements with tremendous nuclear significance as important resources of energy. However, the recovery of uranium (VI) from natural seawater and industry wastewater and the toxicity related to thorium remains an uphill task to do (Liu et al. 2013). Anirudhan and Rijith (2012) prepared a novel adsorbent, poly(methacrylic acid)-grafted chitosan/bentonite composite, through graft copolymerization reaction of methacrylic acid and chitosan in the presence of bentonite and N,N-methylenebisacrylamide as cross-linking agent and observed a tremendous increase in the adsorption of toxic heavy metals as compared to chitosan alone. Anirudhan et al. (2010) prepared a novel composite matrix, poly(methacrylic acid)-grafted composite/bentonite, through graft copolymerization reaction of methacrylic acid and chitosan in the presence of bentonite and N,N-methylenebisacrylamide and observed a similar trend in adsorption of toxic radionuclides.

13.6 Chitosan-Based Nanocomposites in Biosorption of Heavy Metals

In recent years, nanotechnology research is emerging as cutting-edge technology with interdisciplinary amalgamation of physical science, chemical science, biological sciences, material science, and therapeutics (Narayanan and Sakthivel 2010). Nanomaterials owing to their specific surface plasmon resonance properties and pronounced physical and chemical attributes have a multitude of applications in the field of medicine and agriculture and in remediation of toxic heavy metals.

Arsenic pollution is increasing throughout the world due to natural and heavy anthropogenic activities. The existing technologies for arsenic removal include oxidation/precipitation, coagulation/coprecipitation, nanofiltration, reverse osmosis, adsorption, ion exchange, solvent extraction, bioremediation, etc. Among these processes, adsorption is an effective and remarkable alternative due to its simplicity, ease of operation and handling, regeneration capacity, and sludge-free operation (Saha and Sarkar 2012). For the removal of arsenic from water, several adsorbents such as activated alumina, activated carbon, kaolinite clay, titanium oxide, cerium oxide, and silicium oxide and many natural and synthetic media have been reported (Sarkar et al. 2010). Alumina is the most promising adsorbent as it possesses excellent physical and textural properties compared to other transitional inorganic oxides. However, the main disadvantages of these materials are clogging of bed during scale-up operations and fouling problems caused by suspended solids due to the formation of metal hydroxides. Recently, application of nanomaterials for the removal of toxic pollutants has come up as an interesting and innovative area of research as nanoparticles exhibit tremendous adsorption efficiency especially due to higher surface area and greater active sites for interaction with metallic species (Hristovski et al. 2007). However, only a few reports are available in literature dealing with the applications of nanoscale alumina for the removal of arsenic from water. In this context, a reverse microemulsion method was applied to prepare the alumina nanoparticles. The main objective of this work was to develop a novel biosorbent by dispersing alumina nanoparticles in chitosan-grafted polyacrylamide (CTS-g-PA) and to determine the arsenic removal efficiency of the biosorbent (Saha and Sarkar 2012).

Heavy metals are essential to the human body in trace amount for a variety of physiological processes. However, rapid industrialization process leads to exposure and inhalation of high concentrations of heavy metals to the body which ultimately proves to be dangerous leading to serious damage to human health, due to their non-biodegradability, and can be accumulated in living tissues. In this context, determination of trace levels of heavy metals is immensely critical for human health, monitoring environmental deterioration and environmental protection (Ahmed and Fekry 2013). Chitosan is one of the most promising elements for efficient biosorption of heavy metal ions. Besides, metal oxide nanoparticles such as Fe_3O_4, ZnO, CeO_2, etc. have also been suggested as promising matrices with efficient adsorption capacity owing to their large surface-to-volume ratio, high surface reaction activity,

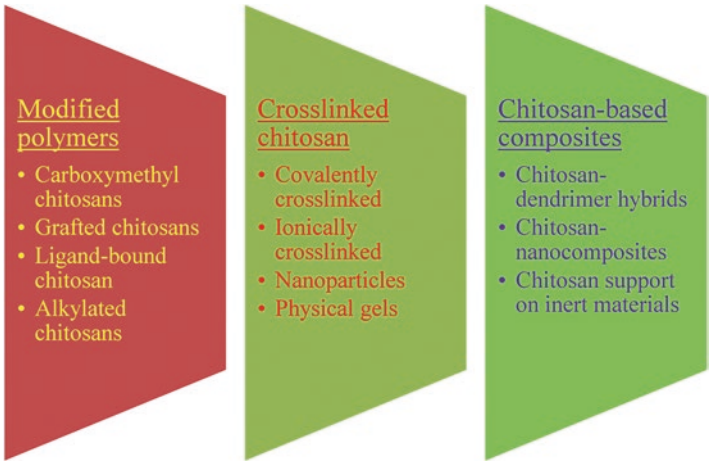

Fig. 13.4 Classification of chitosan and chitosan derivatives toward heavy metal biosorption

and high catalytic efficiency that can be helpful to obtain improved stability and sensitivity of a biosensor (Singh et al. 2007; Ansari et al. 2008; Wei and Wang 2008). However, the existing problem of aggregation and rapid biodegradation of Fe_3O_4 nanoparticles remains a limitation. This problem can be sorted out by modifying these nanoparticles using chitosan by preparing novel nanocomposites with advanced adsorption efficacy (Kaushik et al. 2008). In this context, an attempt was made with the Fe_3O_4/chitosan nanocomposite for the determination and successful removal of heavy metals such as arsenic, lead, and nickel ions from aqueous solution in a very efficient manner. The nanocomposite possesses excellent film-forming ability, good adhesion, biocompatibility, high mechanical strength and susceptibility to chemical modification of chitosan, and most importantly the enhanced adsorption capacity as compared to chitosan or Fe_3O_4 nanoparticles (Ahmed and Fekry 2013).

The phenomenon of adsorption is well recognized as an efficient, cost-effective, and eco-friendly approach using chitosan and chitosan-derived biopolymers for heavy metal bioremediation (Fig. 13.4). Chitosan modification is one of the significant interests due to exceptional properties and widespread applications. Among the materials used for such modification, carbon nanotubes (CNTs) have gained a considerable attention. Many research studies have shown the capability of CNTs in the adsorption and removal of different pollutants, heavy metals from the aqueous environment in an efficient manner (Hu et al. 2009). An attempt was made to formulate a multifunctional nanocomposite of chitosan, silver nanoparticles (Ag NPs), copper nanoparticles (Cu NPs), and CNTs. The adsorption efficiency of chitosan for Cu(II), Cd(II), and Pb(II) reached 60% using 5 g/L adsorbent dose, whereas the novel multifunctional composite reached staggering 100% adsorption efficiency with a reduction of the amount of adsorbent to 1 g/L (Alsabagh et al. 2015). Though laponite, a synthetic clay, acts as an excellent cationic adsorption agent due to its enhanced

surface area, its sensitivity to ions remains a problem causing aggregation and gelation in an ionic environment (Pinto et al. 2016). In this regard, chitosan might be a fruitful alternative to formulate unique nanocomposite in support for adsorption purposes. In this context, a novel chitosan and Laponite-based nanoparticle-polymer hybrid adsorbent was formulated. The novel adsorbent contains a lot of functional groups such as $–COO^–$, $–NH_2$, –OH, $–CONH_2$, and $–SO_3^–$, which aid to the enhanced capability of adsorption. Besides, the nanocomposite structure also significantly improves the adsorption rate and efficiency with an ample capacity to regenerate post-adsorption (Cao et al. 2017).

The heavy metal contamination and its bioremediation in an environment-friendly manner are of paramount importance in developing environmental management program. In this regard, from the last few years, nanostructured materials have received significant interest due to their characteristic ability to exhibit different physical and chemical properties as compared to their bulk materials in heavy metal remediation. In this context, chitosan composites have repeatedly been reported for enhanced adsorption capacity and resistance to acidic environment. Choo et al. (2016) made an attempt on the development of a novel biopolymer based on chitosan and halloysite nanotubes (HNT) and evaluated the copper removal intake as a potential application of this biocomposite. The results suggested the efficacy of the prepared biocomposite in bioremediation of copper in an efficient manner (Choo et al. 2016) (Table 13.1). Over the years continuous research is being carried out on finding a cost-effective and environment-friendly biosorbent owing to the toxicological aspect and bioaccumulation tendency of heavy metals. Chitosan from natural sources remains an excellent alternative to synthetic bioremediating agent. Besides, chitosan nanoparticles prove to be nontoxic and ready to use as more efficient for pollutant removal (Esmaeili and Asgari 2015). Apart from chitosan, sodium alginate, a natural polysaccharide, also serves as an efficient biosorbent for removal of toxic metal ions from wastewater (Lakouraj et al. 2014). Owing to the biosorption properties of chitosan and sodium alginate, a successful attempt was formulated to evaluate the bioremediation potential of alginate-coated chitosan nanoparticles (Esmaeili and Khoshnevisan 2016).

From the last few years, numerous works have been reported aiming on removal of various metals especially Hg(II) with adsorption onto chitosan materials. To enhance the adsorption selectivity, modification of chitosan grafted with carboxyl, amino, amido, imino, sulfonate, etc. is of paramount importance. Apart from traditional functionalization, modification with magnetic nanoparticles in order to form a magnetic chitosan derivative is an advanced and novel approach to deal with nuisance of heavy metal contamination. Magnetically modified chitosans were already exhibited for the removal of a number of heavy metals (Li et al. 2013). However, there is lack of extensive studies for the use of magnetic chitosan as novel adsorbent for Hg(II) removal. In this context, two modified chitosan derivatives and one chitosan adsorbent (CS) only cross-linked with glutaraldehyde, while the other (CSm), which is magnetically cross-linked with glutaraldehyde and functionalized with magnetic nanoparticles (Fe_3O_4), were prepared in order to compare their adsorption properties for Hg(II) removal from aqueous solutions. The results suggested the

Table 13.1 Chitosan composites' heavy metal remediation

S. no.	Composite	Heavy metal	Biosorption mechanism	References
1.	Chitosan–magnetite nanocomposites	Fe(III)	Adsorption	Namdeo and Bajpai (2008)
2.	Chitosan/poly(acrylic acid) magnetic composite	Cu(II)	Adsorption	Yan et al. (2012)
3.	Magnetic chitosan nanoparticles	Cu(II)	Adsorption	Yuwei and Jianlong (2011)
4.	Magnetic chitosan modified with diethylenetriamine	Cu(II), Zn(II), Cr(VI)	Adsorption	Li et al. (2011)
5.	Chitosan-bound Fe_3O_4	Cu(II), As(III), Pb(II)	Adsorption	Chang and Chen (2005) and Ahmed and Fekry (2013)
6.	Magnetically modified chitosan	Zn(II), Cr(III)	Adsorption	Fan et al. (2011) and Geng et al. (2009)
7.	Fe–chitosan	Cr(VI)	Adsorption	Zimmermann et al. (2010)
8.	Multiwalled carbon nanotubes/ chitosan nanocomposite	Ni(II),Cu(II), Cd(II), Zn(II)	Adsorption	Salem et al. (2011)
9.	Thiourea-modified magnetic ion-imprinted chitosan/TiO_2 composite	Cd(II)	Adsorption	Chen et al. (2012)
10.	Chitosan-capped gold nanocomposite	Zn(II), Cu(II), Pb(II)	Adsorption	Sugunan et al. (2005)
11.	AgNPs/CT membrane	As(III)	Adsorption	Prakash et al. (2012)
12.	Hydrogels of chitosan, itaconic, and methacrylic acid	Cu(II)	Adsorption	Milosavljevic et al. (2011)
13.	Chitosan entrapped in polyacrylamide hydrogel	Pb(II), UO2(II), Th(IV)	Adsorption	Akkaya and Ulusoy (2008)
14.	Chitosan-based hydrogels	Pb(II), Cd(II), Cu(II)	Adsorption	Paulino et al. (2008)
15.	Chitosan coated onto polyvinyl chloride (PVC) beads	Ni(II)	Adsorption	Krishnapriya and Kandaswamy (2009)
16.	Chitosan/poly(vinyl alcohol)	Pb(II), Cu(II)	Adsorption	Fajardo et al. (2012) and Salehi et al. (2012)
17.	Chitosan/montmorillonite	Cr(VI)	Adsorption	Fan et al. (2006)
18.	Dispersion of alumina NPs in chitosan-grafted polyacrylamide	As(III)	Adsorption	Saha and Sarkar (2012)

(continued)

Table 13.1 (continued)

S. no.	Composite	Heavy metal	Biosorption mechanism	References
19.	Multifunctional nanocomposite of chitosan, AgNPs, CuNPs, and carbon nanotubes (CNTs)	Cu(II), Cd(II), Pb(II)	Adsorption	Alsabagh et al. (2015)
20.	Chitosan and halloysite nanotubes (HNT)	Cu(II)	Adsorption	Choo et al. (2016)
21.	Alginate-coated chitosan NPs	Cu(II)	Adsorption	Esmaeili and Khoshnevisan (2016)
22.	Chitosan cross-linked glutaraldehyde functionalized with magnetic NPs	Hg(II)	Adsorption	Kyzas and Deliyanni (2013)
23.	Electrospun chitosan/GO nanofibers	Cu(II), Pb(II), Cr(VI)	Adsorption	Najafabadi et al. (2015)
24.	Chitosan/TiO_2 nanofibers	Cu(II), Pb(II)	Adsorption	Razzaz et al. (2015)

efficacy of magnetically functionalized chitosan in Hg(II) removal (Kyzas and Deliyanni 2013). It is already an established fact that the removal efficiency of metal ions increases by functionalization of magnetic nanoparticles into the chitosan matrix. In this regard, magnetic adsorbent, graphene oxide (GO) due to its large specific area and oxygenous functional groups including hydroxyl, carboxyl, and epoxy groups at the edges confirms their high adsorption performance on metal ions (Ma et al. 2012). In this context, GO was synthesized and functionalized with chitosan solution. The application of electrospun chitosan/GO nanofibers was successfully investigated for the removal of Cu^{2+}, Pb^{2+}, and Cr^{6+} ions from aqueous solutions (Najafabadi et al. 2015). In an attempt to modify chitosan with metallic nanoparticles, chitosan/TiO_2 nanofibrous adsorbents were developed by two methods containing TiO_2 nanoparticle-coated chitosan nanofibers and electrospinning of chitosan/TiO_2 solutions. The potential of nanofibrous adsorbents was evaluated for the removal of Pb(II) and Cu(II) ions in a batch mode (Razzaz et al. 2015). It has been reported earlier that chitosan beads cross-linked with glutaraldehyde immensely increase their sorption capacity for Cr(VI). However, the mechanical strength of chitosan beads needs to be improved. In this regard, several methods have been used to enhance the mechanical strength of chitosan beads through either physical or chemical modifications, and chemical modification by epichlorohydrin (ECH) is one of the most common strategies. The Fe^0 nanoparticles were successfully immobilized on ECH–chitosan beads for the removal of hexavalent chromium from aqueous solution. The study demonstrates that ECH–CS–Fe^0 beads could become an effective and promising technology for in situ remediation of Cr(VI) as well as other toxic heavy metals (Liu et al. 2012).

13.7 Chitosan-Based Nanocomposites in Biosorption of Dyes

The dark side of the advancement in the technological attire and massive industrialization programs is the pollution caused by the uncontrolled deposition of colored effluents having massive impact on environment as well as human health (Akar et al. 2016). Most of the dyes are used in the textile industry and resulted in an approximated 20% of water pollution worldwide (Gul et al. 2016). Dyes are one of the most hazardous materials found in industrial effluents having mutagenic and carcinogenic impact on human beings (Fan et al. 2013).

Reactive Black 5 (RB5) dye is one of the most commonly used synthetic reactive dyes in the dyeing industry to dye nylon, cotton, etc. RB5 possesses a highly stability profile against traditional physical, chemical, and biological treatment like that of other synthetic dyes (El Bouraie and El Din 2016). In this regard, hydrophilic and biocompatible chitosan–graphene mesostructures with emphatic surface area were prepared to investigate its role in the removal of RB5. The results of the experiment showed a significant potentiality in removing highly reactive RB5 from aqueous solution with a staggering removal efficiency of 97.5%. These results suggested the efficacy of chitosan–graphene mesostructures in treatment of other industrial effluent (Cheng et al. 2012). Acid orange 7 is a very popular water-soluble azo dye used for dyeing a variety of materials such as nylon, detergents, and cosmetics (Gupta et al. 2006). Being water soluble, acid orange 7 tends to be critical in maintaining dissolved oxygen level leading to severe waterborne health disorders. Sheshmani et al. (2014) explored the unique adsorption properties of magnetic graphene and chitosan in a novel nano-formulation to remove acid orange 7 from aqueous solution. The study suggests that the novel fabrication of magnetic grapheme with chitosan proves to be a promising nanoadsorbent for removal of anionic azo dyes from aqueous solution in an efficient manner (Sheshmani et al. 2014).

In the quest for removal of highly reactive dyes from water bodies, an attempt was made by Akar et al. (2016). A novel and environment friendly composite consisting of chitosan-alunite is used for the removal of highly reactive acid red 1 and reactive red 2. The results showed the potential of chitosan–alunite composite as effective biosorbent in removing these reactive anionic dyes from the aqueous systems (Akar et al. 2016). Gul et al. (2016) evaluated the selective adsorption of cationic and anionic dyes using chitosan-decorated Fe_3O_4 nanoparticles with cross-linked graphene oxide composite. Based on well-defined electrochemistry, easy protonation reversibility, and significant redox recyclability, polyaniline is considered to be one of the most promising classes of organic conducting polymers for effective dye removal in combination with starch, chitosan, and even bacterial extracellular polysaccharides. In this context, nanocomposite prepared from chitosan, polyaniline, and ZnO was formulated for the first time for effective and ecofriendly removal of highly reactive orange 16 (Pandiselvi and Thambidurai 2013). Silica-based materials are one of the prime choices for removal of environmental pollutants owing to their functional groups and selectivity toward the pollutants (Soltani et al. 2013). Biosilica is a silica-based material mainly consisting of silicon

dioxide which can be used as an alternative adsorbent in removal of organic dyes due to its high surface area along with functional groups (Dang et al. 2013). However, biosilica-based components possess some sort of drawback related to the revival of the fine adsorbents from the aqueous solution's post-adsorption process. In this regard, chitosan would be a suitable natural biopolymer for the immobilization of biosilica-based material process because of its hydrophilicity and biodegradability. In this context, a novel hybrid nanocomposite was formulated for biosilica grafted with chitosan for increased adsorption of acid red 88, textile dyes with respect to reaction time, temperature, pH, and concentration of adsorbate (Soltani et al. 2013).

With the ever-increasing population, there is an increase in the demand for textile products which eventually resulted in more release of dyes in the form of effluents leading to serious health consequences. With adsorption being the most suitable alternative for biosorption process, there is always a tendency to quest for more cost-effective adsorbents with enhanced biosorption efficacy (Zhou et al. 2016). In this context, chitosan-decorated lignocellulose fiber was formulated for removal of harmful acidic azo dyes in an efficient manner, and the results emphasized the use of novel nanocomposites as a sustainable approach for dye removal. Metal oxides possess tremendous potential applications on wastewater treatment due to their high surface area and low production and regeneration costs. Among the metal oxides, the role of magnesium oxide (MgO) is well established as novel wastewater treatment agent. The advanced metal sorption properties of both MgO and chitosan were designed in such a way to achieve significant biosorption of heavy metal ions and dye with the enhanced property of self-regeneration posttreatment (Haldorai and Shim 2014). The reusability and cost-efficient production efficacy of multifunctional chitosan–MgO composite suggested its multivariate application in environmental management of toxic heavy metal ions and dyes from wastewater in an eco-friendly manner. In wastewater treatment, removal of azo dyes is of paramount importance, and from the last few years, there is a tendency to formulate chitosan-based nanocomposites for the removal of the same. With the passing years, novel and innovative chitosan-based nanocomposites are gaining interest, and Kamal et al. (2017) explored the multifunctional properties of both chitosan and graphene oxide for the formulation of nanocomposite using tetraethyl orthosilicate as cross-linking agent for dye uptake and removal from aqueous environment in an eco-friendly approach.

In biosorption process, the cost-effectivity and regeneration of biosorption agent remain an age-old problem. Therefore, the research on biosorption has now considered the formulation of novel chitosan-based nanocomposites for enhanced biosorption of dyes and regeneration of the biosorbents after treatment. In this context, unique chitosan–polyvinyl alcohol–TiO_2 nanocomposites were designed in such a way that the biosorption efficacy is increased with a significant impact on environmental sustainability program (Rasoulifard et al. 2017).

13.8 Future Perspectives

With the ever-increasing population and their demand, there is a concomitant increase in the technological expansion in the industrialization which ultimately leads to the discharge of toxic heavy metal ions and synthetic dyes into the water bodies. The presence of these heavy metals and dye materials not only possesses harmful effects to the human health but also contributes in the dramatic environmental deterioration (Sivashankar et al. 2014). To attenuate the nuisance of the presence of highly toxic heavy metal ions and dyes, a number of traditional strategies such as chemical oxidation, coagulation–flocculation, adsorption, filtration, and photodegradation are regularly used. Among the available traditional biosorption strategies, adsorption remains the ultimate choice in terms of effectiveness, simplicity, and most importantly its impact on environmental management (Kadam and Lee 2015). In addition to that, adsorption of heavy metals and dyes using biological organisms and naturally derived biopolymers remains the most attractive alternative for targeting biosorption of heavy metals and toxic dyes. Chitosan, a naturally derived biopolymer, is extensively used in biosorption process (Obeid et al. 2013). However, with the drawbacks associated with chitosan as biosorption agent, the tendency of research on biosorption is focused toward the development of novel formulation of chitosan nanocomposites using the field of multidisciplinary nanotechnology which centered on its feasibility and environmental impact (Crini and Badot 2008; Travlou et al. 2013).

The inherent search for an effective biosorbent with a considerable attention toward efficient removal of highly toxic heavy metal ions and dyes leads to environmental sustainability. The use of chitosan and chitosan-based nanocomposites in biosorption process remains the limelight of the current research on heavy metal remediation owing to their biodegradability, cost-effectiveness, and enhanced capacity to bind to the metal ions, and most importantly it remains environmentally safe (Nithya et al. 2016). The tendency to work on naturally derived chitosan and chitosan-based nanocomposites has been increased day by day in the quest for finding environmentally reliable nanocomposites in heavy metal and dye remediation. The efficacy of chitosan-based nanocomposites in heavy metal bioremediation and dye sequestration and their widespread environmental implications have suggested the research community a wide array of attention for a healthy environment.

13.9 Conclusion

The bioavailability of natural polymers and their widespread applications in the field of pharmaceuticals, healthcare, agriculture, and environmental sustainability remain the backbone of drug discovery programs. The inherent use of natural biopolymers such as chitosan and chitosan-based nanocomposites in maintaining environmental sustainability will provide a ray of scope in heavy metal and dye remediation in an efficient manner to achieve a stable and healthy environment in the future.

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Chapter 14
Mycoremediation Mechanisms for Heavy Metal Resistance/Tolerance in Plants

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14.1 Introduction

Pollution is the commonest environmental problem faced by the world in the present era of industrialization and anthropogenic activities (Yu et al. 2011; Hamba and Tamiru 2016). It includes incidents of land, air and water resources being contaminated with toxic materials like pesticides, aromatic hydrocarbons, polythenes, heavy metals, e-wastes and other pollutants. The continuous burdening of these tenacious nondegradable, hazardous contaminants leads to a negative impact on plant, animal and human health (Yuan et al. 2004; Liu et al. 2013). In India, approximately 7.2 million tons of hazardous waste is generated, out of which 5.2 million tons is destined for disposal on land leading to soil turbulence, restricted crop production and inadequacy of clean water (Kamaludeen et al. 2003). Among all the pollutants, heavy metals, due to their wide distribution in the environment, are raising concern over their adverse effects on human health and the environment.

Heavy metals are elements with a specific density >5 g/cm^3, which include lead (Pb), cadmium (Cd), nickel (Ni), cobalt (Co), iron (Fe), zinc (Zn), chromium (Cr), arsenic (As), silver (Ag) and the platinum group of metallic elements (Banfalvi 2011). Their involvement in various biochemical and physiological functions in living organisms is known; however, their presence beyond threshold concentrations adversely affects the environment and living organisms (Jarup 2003). Heavy metal exposure is increasing in many parts of the world due to its tenacious nature. Presence of elevated concentrations of these toxic metals above the permissible

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limit in soil leads to their accumulation in plants and their subsequent transfer and biomagnification in the food chain. Prolonged exposure of heavy metals to animals or human beings causes liver and kidney malfunctioning, increases risk of cancer and leads to problem in the bone marrow and central nervous system (Lloyd and Cackette 2001; Rajendran et al. 2003; Abioye 2011). Therefore, their toxicity is an area of concern for ecological, evolutionary, nutritional and environmental reasons (Jaishankar et al. 2014; Nagajyoti et al. 2010).

Different conventional methods (mechanical and chemical) such as chemical precipitation, coagulation-flocculation, ion exchange and membrane filtration are generally used to mitigate heavy metal from contaminated sites. These approaches are not economical and eco-friendly as it produces a huge amount of toxic by-products, thereby creating issues for their disposal. To overcome these problems, bioremediation is a promising means of heavy metal treatment via different processes of intracellular accumulation, redox reactions, bioleaching, biosorption and biomineralization (Lloyd 2002). Since this technology uses microorganisms like bacteria, yeast and fungi for the treatment of heavy metal contaminants, it is an economic, feasible and sustainable approach. Bioremediation using fungi, termed as mycoremediation, gains a special attention for its remediation potential due to their ability of producing a large amount of biomasses and variety of extracellular enzymes, organic acids and different metabolites and their spatial distribution in different environmental conditions (Siddiquee et al. 2015). They also play an important role in remediation of contaminated sites by intensifying the phytoremediation ability of plants through enhancement of their nutrient acquisition and phytoextraction ability (Karlinski et al. 2010; Hou et al. 2017). Many microorganisms have evolved traits that allow them to survive in extreme conditions which can potentially be acquainted into higher plants for abiotic stress adaptation (Bohnert et al. 2006). A focused discussion on the role of fungi in heavy metal mitigation from contaminated sites and their role in enhancing heavy metal tolerance to the plants is presented herewith.

14.2 Heavy Metal Toxicity

Industrialization, urbanization and several other natural and anthropogenic activities have resulted in excess release and leaching of heavy metals into the soil, a cause of concern because of the congenital traits of heavy metals. Different heavy metals have different mechanisms of toxicity such as (i) lead causes oxidative stress due to free radical imbalance; (ii) mercury, arsenic and chromium are responsible for formation of harmful thiol or methyl derivative; (iii) aluminium and cadmium causes metal ion replacement; (iv) chromium(VI) disturbs the cell membrane permeability, causes imbalances in ion channels and damages DNA and protein; and (v) iron leads to corrosion, saturation, organ penetration and lipid peroxidation (Gupta and Diwan 2017). Excess availability of these heavy metals severely affects the soil and water quality along with limited biodegradation of organic contaminants in the environment (Atagana 2011; Siddiquee et al. 2015). Their addition in

soil has been known to inhibit soil respiration, nitrogen mineralization and nitrification (Sobolev and Begonia 2008), resulting in reduction of cultivable land (Oves et al. 2016). Crop cultivated in contaminated soil faces the problem of phytotoxicity and nutrient deficiency (Prasad et al. 2010; Hall 2002; Adams et al. 2014). There is also the risk to human health due to heavy metal contamination transferred to the food chain via irrigation of urban and industrial wastewater (Mahmood and Malik 2014). Toxicity of different heavy metals has been discussed below.

14.2.1 Arsenic

Arsenic (As) is an ubiquitous metalloid which occurs both naturally and anthropogenically in nature. The main source of arsenic toxicity is human activities such as mining, use of pesticide, burning of fossil fuels, use of arsenic-laden irrigation water, etc. Arsenic toxicity in plants results in reduction in biomass, grain yield, plant height, seed germination, leaf area and photosynthesis (Srivastava and Sharma 2013). The main environmental exposure to As for humans is through contaminated drinking water, crops and fodder (Nordstrom 2002).

14.2.2 Lead

Depending on the frequency of occurrence, toxicity and human exposure, lead was reported as the second major hazardous metal after arsenic (Jennrich 2013). Mining and smelting of lead ores, automobile exhausts, lead-containing paints, gasoline and explosives and effluents from storage battery industries result in lead contamination of the environment (Verma and Dubey 2003; Sharma and Dubey 2005). Lead contamination causes severe toxicity in plants resulting in stunted growth, blackening of root system and chlorosis, inhibits photosynthesis and mineral uptake and affects the membrane structure and permeability (Emamverdian et al. 2015). Young children are more vulnerable as lead adversely affects their health especially the development of the brain and nervous system (WHO, Fact Sheet 2017).

14.2.3 Cadmium

Cadmium (Cd) is a non-essential heavy metal pollutant due to its toxicity, long elimination half-life and high solubility in water. It is released in the environment mainly due to use of phosphatic fertilizers and sewage sludge in agriculture, mining and industrial activities (Satarug et al. 2003; Perfus-Barbeoch et al. 2002). In plants cadmium enters the root system via the xylem by different transporters and reaches aerial parts of the plant (Tian et al. 2017; Zhang et al. 2016). In plants its toxicity

causes retardation in photosynthesis, chlorosis and rolling of leaves, decreases leaf conductance and affects the carbon dioxide uptake in plants (Perfus-Barbeoch et al. 2002). Humans get exposed to Cd often via plant-derived food. It is a carcinogen and produces a number of negative effects to all systems in the body, including the reproductive, cardiovascular, eyes, kidneys, and even the brain (Bernard 2008). Airborne exposure of cadmium ion causes chronic toxicity leading to proteinuria and lung emphysema; in addition, its higher doses are known to cause skin ulcers and cancer too.

14.2.4 Chromium

Chromium (Cr), which prevails in a different oxidation state, is a non-essential, toxic metal for microorganisms and plants. Among all the oxidation states, the most stable form of chromium is Cr(III) and Cr(VI) in which Cr(VI) is considered as more toxic in comparison with less mobile Cr(III) (Cervantes et al. 2001). Cr(VI) associates with oxygen to form chromate and dichromate ions, while Cr(III) forms oxides, hydroxides and sulphates which remain to bind to organic matter in soil. The main source for chromium contamination in the environment is its wide use in industries for metal plating, wood preservation, etc. (Babula et al. 2008; Shtiza et al. 2008). The leather industries are a major route of chromium contamination, and they account for 40% of total industrial use (Shanker et al. 2005). Chromium toxicity results in inhibition of seed germination, enzymatic activities and other physiological processes of plants (Singh and Sharma 2017). They are known to get transported actively via specific essential element carriers such as iron and sulphur due to lack of specific transporters for Cr (Oliveira 2012). Chromium induces phytotoxicity by interfering plant growth, nutrient uptake and photosynthesis, elevating ROS production, causing lipid peroxidation and altering the antioxidant enzyme activities (Shahid et al. 2017).

14.2.5 Nickel

Nickel (Ni) is transported in plants via passive diffusion and active transport. Nickel is a constituent of several metalloenzymes such as urease, superoxide dismutase, Ni-Fe hydrogenases, methyl coenzyme M reductase, carbon monoxide dehydrogenase, acetyl coenzyme-A synthase, hydrogenases and RNase A (Ahmad and Ashraf 2012). Therefore, it is essential for redox reactions which are necessary for different cellular functions in plant. Although it is important for plant metabolism, it is toxic to plants at higher levels. Above permissible limit it alters normal metabolism of plants causing cellular injuries and at extreme level causes death of plant (Yusuf et al. 2011). Nickel toxicity symptoms in plants include chlorosis, necrosis, shoot-root growth retardation and decrease in leaf area (Singh et al. 2010).

14.3 Heavy Metal Toxicity and Detoxification Mechanisms in Plants

14.3.1 Toxicity

Several heavy metals are essential for normal plant growth; however, their elevated concentration results in growth inhibition and toxicity symptoms. Insufficiency of Cd detoxification is known to disturb redox control of cell and growth inhibition and stimulation of secondary metabolism which finally leads to cell death in plant (Schutzendubel and Polle 2002). Increased availability of Zn affects the plant by altering morphology of root and leaf, disintegration of cell organelles, membrane disruption, condensation of chromatin material and increase in number of nucleoli (Sresty and Rao 1999). Heavy metal toxicity in plant results in binding of metals to sulphhydryl groups of proteins leading to disruption of structure and their activity and displacement of essential metal ions from biomolecules, thereby causing deficiency symptoms, production of reactive oxygen species by autoxidation and Fenton reaction typically for iron or copper resulting in oxidative stress to plant and obstruction of the essential functional group mainly reported for mercury and cadmium (Fig. 14.1) (Schutzendubel and Polle 2002; Hall 2002). They also result in inhibition of cytoplasmic enzymes which eventually damage the cellular structures (Chibuike and Obiora 2014). Inhibition of important enzymes like δ-aminolevulinic acid dehydratase and protochlorophyllide reductase due to cadmium and lead toxicity is associated with impairment in chlorophyll biosynthesis and supply of Mg, Fe

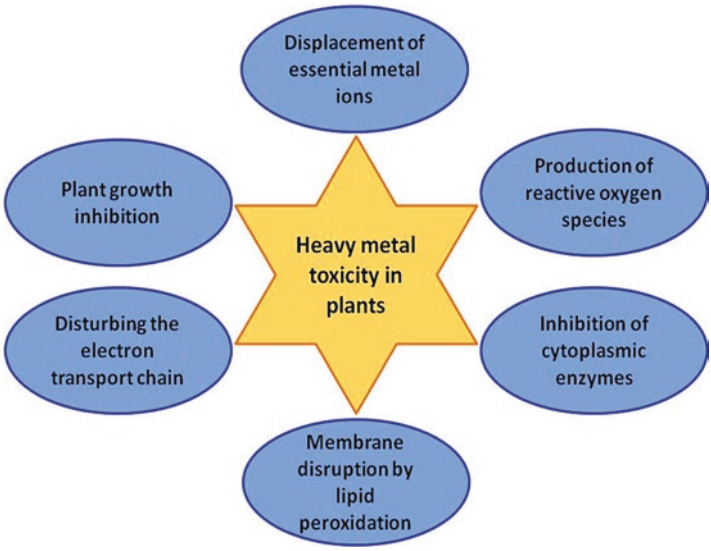

Fig. 14.1 Consequences of heavy metal toxicity in plants

and Zn (John et al. 2012). Cadmium and other metals resulted in depletion of glutathione which prevents damage to important cellular components during oxidative burst which is apparently an important step for Cd detoxification (Chao et al. 2011).

14.3.2 Detoxification of Mechanism

Being sessile plants are more susceptible to heavy metal toxicity. Metal resistance mechanism in plants is polygenic and occurs via two different ways, i.e. avoidance and tolerance (Nedkovska and Atanassov 1998). Heavy metal exposure initiates the first line of defence by involving plant structures like the cell wall, cuticles and trichomes which act as a barrier and restricts their entry in plants (Emamverdian et al. 2015). Once the heavy metals enter in plants, different cellular mechanisms such as chelation and compartmentalization, interaction with proteins, displacement of essential metal ions from their specific binding sites and ROS production are initiated in order to dissipate their detrimental effect on plants (Fig. 14.1) (Yadav 2010; Domínguez-Solís et al. 2004; Choppala et al. 2014).

Plants are known to resist the chromium-induced phytotoxicity through various mechanisms such as complexation of organic ligands, compartmentation into the vacuole and scavenging ROS (Shahid et al. 2017). Heavy metal uptake, internal sequestration and translocation in plant depend on different factors such as interaction of genotype with environmental conditions and plant's nutritional requirement (Hong-Bo et al. 2010; Chao et al. 2011). Besides, plants also have a general stress response involving the induction of stress molecules (such as metallothionein, phytochelatins, glutathione, organic acids), cellular exudates (flavonoid and phenolic compounds), heat-shock proteins, amino acids (proline, histidine) and hormones (salicylic acid, jasmonic acid and ethylene) (Emamverdian et al. 2015; Viehweger 2014). Plants also protect themselves against metal-induced oxidative stress by superoxide dismutase (SOD) induction and activation and synthesis of antioxidant catalase (Oves et al. 2016). Rout et al. (2000) identified the Cr-tolerant moong bean and rice cultivar using peroxidase and catalase activity as a marker. Metal detoxification mechanisms in plants depend on different factors such as type of metal, plant species and their tolerance level and environmental conditions. Some of the tolerance mechanisms of plants are discussed below.

14.3.2.1 Phytochelatins

Phytochelatins are reported as a biomarker for detection of heavy metal stress in plants. They play a pivotal role in heavy metal detoxification and maintenance of intracellular levels of essential metal ions. Phytochelatins are produced in response to varied metals such as Cd, As, Cu, Zn, Hg, etc. However, their biosynthesis is preferentially controlled by Cd (metal) and As (metalloid) stress. The amount of

phytochelatin synthesis when exposed to heavy metal depends on both the type of heavy metal and plant species. In a comparative study among three plant species *Onobrychis viciifolia*, *Lathyrus sativus* and *Medicago sativa*, *Onobrychis* was found to synthesize more phytochelatins as compared to the other two plant species under lead and copper toxicity. In addition, between the two heavy metals, lead was more effective in phytochelatin synthesis in all the three plants as compared to copper (Beladi et al. 2011). Similarly, the production of phytochelatin was enhanced in root when *Solanum nigrum* L. plant was exposed to 200 μmol/L of Cu resulting in its accumulation in root, thereby reducing its translocation from root to shoot (Fidalgo et al. 2013). Li et al. (2015) found the overexpression of phytochelatins in *Escherichia coli* for hyperaccumulation of cadmium. Phytochelatins are synthesized in cytosol from sulphur-rich, low-molecular-weight thiol glutathione (GSH) in presence of enzyme phytochelatin synthase (PCS) and are actively transported in vacuole in the form of metal-phytochelatin complex (Bricker et al. 2001; Freeman et al. 2004; Yadav 2010). Besides synthesis of phytochelatins, availability of GSH is another key factor to overcome and detoxify the oxidative stress caused by metal (Mishra et al. 2006).

14.3.2.2 Glutathione

Glutathione is a widely distributed sulphur compound actively synthesized from three amino acids (Noctor et al. 2011). Synthesis of glutathione involves the formation of a peptide bond between γ-glutamate and cysteine in the presence of γ-glutamylcysteine synthetase 1 (GSH1) followed by addition of glycine by glutathione synthetase (GSH2). The availability of sulphur greatly influences the synthesis of glutathione. High requirement of glutathione under heavy metal stress condition stimulates the sulphate uptake, reduction and its assimilation in order to fulfil the need of cysteine. The presence of cysteine residue contributes to the antioxidant property of GSH and is also known as a substrate for regeneration of other antioxidants (Jozefczak et al. 2012). Upregulation of high-affinity sulphate transporter gene ZmST1;1 expression in maize exposed to Cd, Zn and Cu also favours the role of glutathione (Nocito et al. 2006). Therefore, GSH is known to alleviate heavy metal stress by different mechanisms involving metal homeostasis, antioxidative defence and signal transduction (Jozefczak et al. 2012).

14.3.2.3 Metallothioneins

Metallothioneins (MT), gene-encoded polypeptides, are low-molecular-weight cysteine-rich metal-binding protein molecules (Cobbett and Goldsbrough 2002). They are widely present in animals, fungi and plant. Metallothionein biosynthesis is regulated at the transcriptional level and is influenced by different factors such as cytotoxic agents, hormones, nutrient deprivation, heat shock and heavy metals

such as Cd, Zn, Hg, Cu, Au, Ag, Co, Pb, Ni and Bi (Hossain et al. 2012; Cai and Ma 2003). Expression of metallothionein genes under different unfavourable conditions is a part of general stress response and may be indirectly connected to heavy metal status (Cobbett and Goldsbrough 2002). Metallothionein genes are differentially expressed in tissue-specific manner in plants in relation to their developmental stage in response to a number of stimuli. Higher expression of MT1 in roots and MT2 and MT3 in seeds has been reported by Guo et al. (2003). MT are also involved in a number of physiological and pathophysiological processes, viz. homeostasis of essential metals (Zn and Cu), detoxification of heavy metals and protection against oxidative stress (Ruttkay-Nedecky et al. 2013), protecting cells against UV or ionic radiation and cytotoxic alkylating agents and modulating oxygen free radicals, thereby preventing cells from apoptosis (Gumulec et al. 2014). Like phytochelatins, MT are localized in cytosol but are not transferred to vacuoles (Zhigang et al. 2006). Heavy metals affect plant indirectly by altering the growth and metabolism of microbes associated to the rhizospheric region of plants which leads to decrease in organic matter decomposition resulting in decline in soil nutrient, and this affects the growth of plants which sometimes results in death of plants (Fig. 14.2).

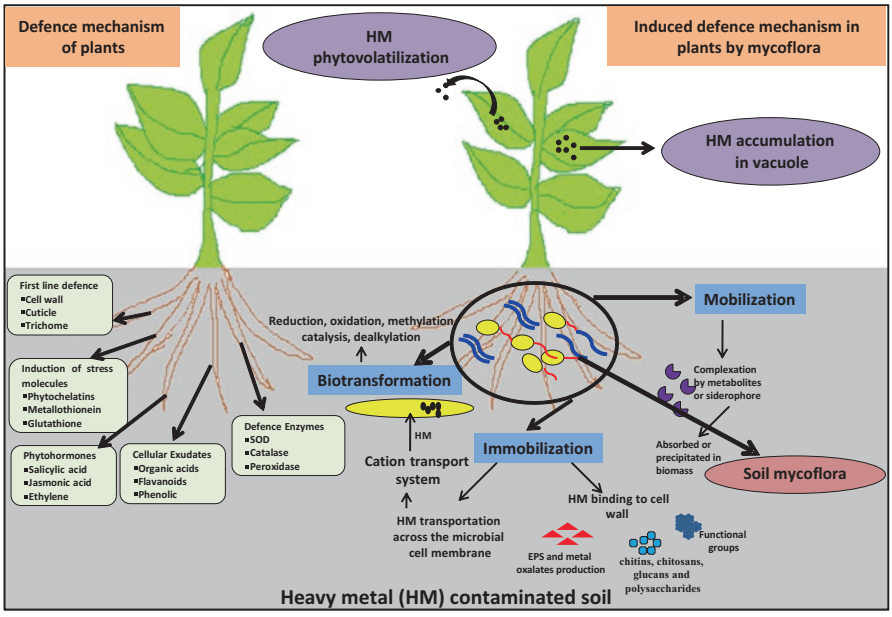

Fig. 14.2 Role of beneficial microbial intervention in inducing defence mechanisms of plant in heavy metal-contaminated soil

14.4 Role of Fungi in Remediation of Heavy Metal Contamination

Different physical, chemical and biological approaches are employed for remediation of heavy metal-contaminated sites such as immobilization, toxicity reduction, physical separation, extraction, precipitation, evaporation, electroplating, ion exchange, etc. (Singh and Prasad 2015; Abbas et al. 2014). These conventional methods have their own merits and demerits. These physical and chemical methods require high amount of energy and are usually uneconomical and non-eco-friendly. In recent years, biological agents mainly microorganisms, i.e. yeast, fungi or bacteria, are employed for removal of toxic wastes from the environment by utilizing their enzymatic activity (Strong and Burgess 2008). On exposure of toxic metals, several microbes are known to evolve with many metal-resistant genes as a means of their adaptation. These operon-clustered metal-resistant genes offer good potential for bioremediation of heavy metals (Das et al. 2016). These microbe-based technologies are known for complete mineralization of the pollutants through biostimulation, bio-augmentation, bioaccumulation, biosorption, phytoremediation and rhizoremediation (Busetti et al. 2005; Zolgharnein et al. 2010; Akpoveta et al. 2010; Perelo 2010). Therefore, use of such microbial technologies for removal of heavy metals in an economical and eco-friendly manner would be an ultimate solution for pollution abatement (Abioye 2011).

Application of fungi for remission of heavy metal-contaminated site is a pioneering technology and is often termed as mycoremediation (Hamba and Tamiru 2016). Mycoremediation is a promising approach over conventional methods due to the ubiquitous and dominant nature of fungi along with high surface area. These fungi are dominating in soil because of their diversity and survival in extreme conditions. They are known to extract and accumulate the contaminants in their mycelia or fruiting bodies. The key to mycoremediation is determining the right fungal species to target specific pollutants such as arsenic (As), lead (Pb) and cadmium (Cd). A number of fungal species are known for their heavy metal remediation potential like *Aspergillus niger*, *Aureobasidium pullulans*, *Cladosporium resinae*, *Funalia trogii*, *Pleurotus tuberregium*, *Ganoderma lucidum* and *Penicillium* spp. (Table 14.1) (Loukidou et al. 2003; Say et al. 2003; Akpaja et al. 2012). Different groups of fungi such as *Zygomycotina*, *Ascomycotina*, *Deuteromycotina* and *Basidiomycotina* have the ability to mitigate heavy metal contamination from soil and wastewater by a different type of mechanism with involvement of several enzymes as shown in Table 14.2. Different genera of fungi belonging to *Aspergillus*, *Penicillium* and *Cladosporium* isolated from the oxidation pond of sewage treatment plants were reported to have high level of tolerance for different metals and offered them as attractive potential candidates for further investigation (Table 14.2) (Siokwu and Anyanwn 2012).

The process of mycoremediation of heavy metals or other environmental pollutants mainly depends on fungi which enzymatically convert the pollutants into harmless products. As mycoremediation can be effective only where environmental

Table 14.1 Fungi involved in mycoremediation of heavy metals

Fungi	Heavy metal	Mechanism	Reference
Circinella sp.	Ni	Biosorption of Ni	Alpat et al. (2010)
Cunninghamella sp.	Pb	Biosorption	El-Morsy (2004)
Mucor sp.	Pb, Cd, Ni, Zn, Cu	Bioaccumulation	Zhang et al. (2017)
Rhizopus sp.	Pb, Cd, Cr, Cu, Ni, Zn	Biosorption	Pal et al. (2010), Luo and Xiao (2010), Shoaib et al. (2012), and Fourest and Roux (1992)
Trichoderma sp.	Cu, Pb ,Cd, As, Zn	Bioaccumulation	Fazli et al. (2015)
Penicillium sp.	Zn, Ni, Pb	Biosorption, bioaccumulation	Mohsenzadeh and Shahrokhi (2014)
Paxillus involutus	Cd	Accumulation of Cd in vacuole	Blaudez et al. (2000)
Phanerochaete chrysosporium	Pb, Cd	Bioaccumulation	Xu et al. (2014)
Aspergillus lentulus	Pb, Cu, Cr, Ni	Bioadsorption	Mishra and Malik (2014)
Pleurotus ostreatus	Pb, Cd	Bioaccumulation	Kapahi and Sachdeva (2017)
Pisolithus sp.	Pb, Cu, Cr, Ni, Zn	Bioadsorption	Tam (1995)
Hymenoscyphus ericae	Zn, Cd and Fe	Absorption	Binsadiq (2015)

conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow fungal growth and degradation to proceed at a faster rate. There are several *Aspergillus* spp. known to accumulate different heavy metals such as Cr, Ni, Cd, Pb, Cu and Zn (Table 14.1). The bioaccumulation potential of *Aspergillus* spp. depends on different physical factors such as pH, temperature, initial concentration of metal and contact time (Congeevaram et al. 2007; Thippeswamy et al. 2012; Joshi et al. 2011; Chandrakar et al. 2012; Siokwu and Anyanwn 2012). Similarly, bioaccumulation of Cu, Fe and Zn by *Candida* spp. and *Penicillium* spp. also depends on temperature, time and biomass (Anaemene 2012; Akhtar et al. 2008; Ahmad et al. 2005; Dugal and Gangawane 2012). Besides this different *Aspergillus* spp. with heavy metal and salt tolerance have been studied for their potential to accumulate different heavy metals from saline medium (Adeyemi 2009; Siokwu and Anyanwn 2012). A detailed heavy metal detoxification mechanism studied in one of the fungi *Phanerochaete chrysosporium* showed oxalic acid-mediated metal complex (oxalate metal) formation as one of the mechanisms of detoxification affecting its bioavailability (Xu et al. 2015). Other species of *Phanerochaete* are also known for degradation of polyaromatic hydrocarbons (Bisht et al. 2015). Phosphate-mediated metal tolerance has also been described in some bacterial species showing its relationship with polyphosphate complex formation (Grillo-Puertas et al. 2014).

Table 14.2 Different class of fungi producing different heavy metal metabolizing enzymes

Enzymes occurrence in nature	Mode of action	Taxon order	References
Laccases – extracellular	Oxidation of organic compounds(O_2-dependent)	*Ascomycota* and *Basidiomycota*	Baldrian (2006) and Majeau et al. (2010)
Tyrosinases – mainly intracellular sometime extracellular	Cresolase activity and catechols	*Ascomycota*, *Basidiomycota* and *Mucoromycotina*	Halaouli et al. (2006) and Ullrich and Hofrichter (2007)
Peroxidases – extracellular	Oxidation of organic compounds(H_2O_2-dependent); oxidation of Mn^{2+} to Mn^{3+}	*Basidiomycota*	Ruiz-Duenas and Martinez (2009) and Hofrichter et al. (2010)
Peroxidases – extracellular	Oxidation of aromatic compounds (H_2O_2-dependent)	*Basidiomycota*	Ikehata et al. (2005) and Hofrichter et al. (2010)
Manganese peroxidase – extracellular	Oxidation of Mn2+ to Mn^{3+} pounds (H_2O_2-dependent)	*Basidiomycota*	Hofrichter (2002), and Hofrichter et al. (2010)
Dye-decolorizing peroxidase – extracellular	Oxidation of organic compounds (H_2O_2-dependent) hydrolysing activity	*Basidiomycota*	Hofrichter et al. (2010)
Caldariomyces fumago haem-thiolate chloroperoxidase – extracellular	Halogenation of organic compounds in the presence of halides (H_2O_2-dependent); oxidation of phenols and anilines in the absence of halides (H_2O_2-dependent); peroxigination of alkenes and anilines and sulphoxidation in the absence of halides (H_2O_2- dependent)	*Ascomycota*	Hofrichter et al. (2010)
Reductive dehalogenase – membrane bound	Membrane-bound glutathione S-transferase and glutathione conjugate reductase activity	*Ascomycota* and *Basidiomycota*	Aust et al. (1990), and Reddy and Gold (2000)
Cytochrome P450 – membrane bound	Reduction reaction	*Ascomycota*, *Basidiomycota*, *Mucoromycotina* and *Chytridomycota*	Kasai et al. (2010) and Ullrich and Hofrichter (2007)
Haem thiolate peroxygenases – extracellular	Peroxigination of aromatic, aliphatic and alicyclic compounds (H_2O_2-dependent); bromination (H_2O_2-dependent); sulphoxidation	*Basidiomycota*	Hofrichter et al. (2010)
Lignin peroxidizes – extracellular	Oxidation of aromatic compounds (H_2O_2-dependent)	*Basidiomycota*	Ruiz-Dueñas and Martínez (2009) and Hofrichter et al. (2010)
Nitroreductases – cell bound	Reduction of nitroaromatic compounds (NADPH dependent)	*Ascomycota*, *Basidiomycota* and *Mucoromycotina*	Scheibner et al. (1997), Rieble et al. (1994), and Fournier et al. (2004)

14.4.1 Mode of Detoxification in Fungi

The ability of microorganisms to affect metal availability depends on the biosorption ability of the microbe. Biosorption, defined as removal of materials (compounds, metal ions, etc.) by the action of biological agents, occurs via ion exchange, chelation adsorption on cell surface and entrapment in inter- and intrafibrillar capillaries due to concentration gradient (Wierzba 2017). Moulds, yeasts and mushrooms are three groups of fungi which possess major practical importance for metal biosorption (Abbas et al. 2014; Wierzba 2017).

Biosorption is influenced by several factors such as living and nonliving state of biomass, types of biomaterials, properties of metal and other environmental conditions such as pH, temperature, etc. (Wang and Chen 2006). Enhanced sorption efficiency with increase in pH has also been shown for binding of heavy metal such as Pb and Cu using *P. canescens* and *P. purpurogenum* (Say et al. 2003; Ianis et al. 2006). *Saccharomyces cerevisiae* has good potential of accumulating Pb and Cd which gets affected by the factors such as carbon source, aeration and metal and biomass concentration. Damodaran et al. (2011) observed the increase in biosorption of Cd and Pb with increase in aeration and availability of glucose as carbon source which facilitates the increase in biomass of *S. cerevisiae*. And Thippeswamy et al. (2012) found increase in metal removal capability in *Saccharomyces* spp. with decrease in their biomass and proposed that low biomass results in high surface to volume which holds maximum heavy metals in both soluble and particular form by increasing metal interaction with active binding sites of the cell surface.

There are different mechanisms based on cell complexity by which metal is taken up by the microbes. Although the mechanism is not well understood, yet two modes of biosorption of heavy metals are known (Fig. 14.3). The first mode (passive mode) involves the uptake of metal, independent of the metabolic activity, where the precipitation, physical adsorption, ion exchange and complexation cause the binding of metal ions to the surface of cell wall and extracellular material (Veglio and Beolchini 1997). The second is efficient, active and energy-dependent mode, which involves the transportation of metals across the cell membrane (Abbas et al. 2014). Excellent metal-binding property of fungi is more advantageous due to the presence

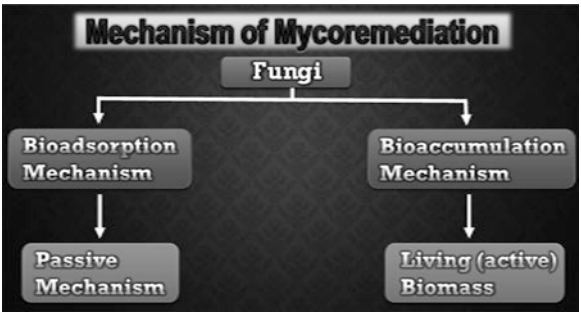

Fig. 14.3 Different mechanisms of heavy metal detoxification in fungi

of high percentage of cell wall materials (Abbas et al. 2014). Different types of ligands including carboxyl, amine, hydroxyl, phosphate and sulphhydryl groups are present on fungal cell surface through which metal ions are adsorbed by complexing with negatively charged reaction sites (Gupta et al. 2000). Other than the above-mentioned ligands, microbial cell wall is also rich in polysaccharides and glycoproteins which offer numbers of active site for metal chelation (Ahluwalia and Goyal 2007). The type of metal chelation differs in individual organisms (bacteria, algae, fungus and yeast) due to difference in cell wall composition. Fungal cell wall composed of chitin and chitosan is known for metal ion sequestration (Das et al. 2008). Mushrooms possess a very effective mechanism of heavy metal uptake through biosorption mechanism via mycelia, and they contain considerably higher concentration of heavy metal than fruits, vegetables and crop plants (Turkekul et al. 2004; Zhu et al. 2011). Mushrooms remove the heavy metals from contaminated sites by channelling them to their fruiting bodies, and they accumulate heavy metals like arsenic, cadmium, copper, lead, mercury and radioactive caesium and selenium.

Bioaccumulation is another process of metal removal using living cells, which is an active process that employs an energy-dependent metal influx mechanism (Errasquın and Vazquez 2003; Ting and Choong 2009). It is a slower process than biosorption and depends on favourable conditions and toxicity effects of the metal (Fig. 14.3) (Abbas et al. 2014). Microbes have progressed diverse approaches to overcome the toxic effects of metals and metalloids, by utilizing accumulation and resistance or, more interestingly, by reducing their bioavailability or toxicity through biomethylation and transformation. Several *Trichoderma* spp. are reported to accumulate a variety of heavy metals such as copper, zinc, cadmium and arsenic under in vitro conditions (Errasquın and Vazquez 2003; Harman et al. 2004; Zeng et al. 2010; Tripathi et al. 2017). Biomethylation is a conversion of organic and inorganic compounds of metals into their volatile cognate based on enzymatic processes (Mukhopadhyay et al. 2002). *T. asperellum* and *T. viride* are known to volatilize arsenic in liquid medium (Fig. 14.2) (Urík et al. 2007; Zeng et al. 2010; Srivastava et al. 2011).

14.4.2 Environmental Factors Affecting Detoxification

Different physical factors greatly influence the metal-removing ability of fungus. Effect of temperature, time, pH and biomass concentration is studied for biosorbent efficiency of the *Circinella* spp. for removal of Ni from aqueous solutions (Alpat et al. 2010; El-Morsy 2004). An alginate immobilized biomass-mediated formulation has also been shown to have ~25 to 30% increase for all tested metals after 15 min. *Mucor rouxii* has been found to uptake heavy metals from aqueous solution in order of Zn>Pb>Ni>Cd which was affected by pH (Yan and Viraraghavan 2003). Fungi like *M. hiemalis* and *Rhizopus* have been also shown to have metal sorption efficiency which varied based on temperature, pH, contact time and initial concentration of metals (Ahmad et al. 2005; Zafar et al. 2007). Congeevaram et al. (2007)

found the maximum removal of Cr(VI) (92%) and nickel, Ni(II) (90%), at pH 5.0 by *Aspergillus* sp. and Cr(VI) (90%) and Ni(II) (55%) at pH 7.0 by *Micrococcus* sp. Luo and Xiao (2010) found the higher uptake of Cd under weak acid condition as compared to strong acid condition, whereas almost no sorption occurred when the pH was lowered to 2.0. Other than Cr and Cd, another lead-tolerant fungal strain *R. arrhizus* was found which showed higher lead accumulation in the mycelia indicating that the cell surface functional groups of the fungus might act as ligands for metal sequestration resulting in removal of the metals from the aqueous culture media (Pal et al. 2010).

14.5 Plant: Microbe Coexistence as a Boon for Heavy Metal Stress Amelioration

Plant roots are surrounded by a narrow zone of soil termed as rhizosphere which serves as an active site for microbial activity. These microbes play a pivotal role in regulating plant health and development. Microorganism helps in plant growth promotion via different mechanisms such as biological nitrogen fixation, phosphate solubilization, ACC deaminase activity and production of siderophores and phytohormones (Souza et al. 2015; Prasad et al. 2015). Besides plant growth promotion, microorganisms also protect plants against a range of abiotic and biotic stresses involving different mechanisms due to their enormous metabolic and genetic capabilities (Meena et al. 2017). Different biochemical, physiological and molecular studies reveal that association of microbes with plants largely directs plants towards stresses (Farrar et al. 2014). Interaction of microbes with plants under stress conditions induces different local and systemic responses which help plants to combat these stresses by improving their metabolic capabilities (Van Nguyen et al. 2008). The role of several microorganisms belonging to genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Azotobacter*, *Trichoderma, Piriformospora indica*, etc. is well documented for mitigation of abiotic and biotic stresses in plant (Srivastava et al. 2016; Dixit et al. 2016; Tripathi et al. 2015; Gill et al. 2016). Phytoremediation strategies with appropriate heavy metal-adapted rhizobacteria or mycorrhizas have received more and more attention due to their ability to affect heavy metal mobility and availability to the plant through release of chelating agents, acidification, phosphate solubilization and redox changes (Hong-Bo et al. 2010). A number of rhizospheric and endophytic bacteria known through microbiome study, belonging to different taxa of *Flavobacteriales*, *Burkholderiales* and *Pseudomonadales*, have potential for heavy metal stress mitigation (Mesa et al. 2017). Bacterial movement from rhizoplane to rhizosphere has been reported as a possible mechanism of microbe-mediated heavy metal remediation (Pishchik et al. 2016). Sequential treatment with microbes is known to detoxify the heavy metal in the phosphate tailing contaminated soil influencing plant growth and stress amelioration in plants by improving nutrition, enhancing antioxidant system, modification of root architecture, enzyme

production and water use efficiency (Kamal et al. 2010; Nadeem et al. 2014; Huang et al. 2017). Mycorrhizal association of *Piriformospora indica* with plant are known to modulate growth, yields, secondary metabolites and adaptation to abiotic and biotic stresses (Prasad et al. 2008a, b, 2013; Gill et al. 2016).

Besides these plant growth-promoting rhizobacteria, arbuscular mycorrhizal fungi (AMF) and *Trichoderma* strains are also known to affect the heavy metal uptake mechanism of the plant and minimize the deleterious effects of heavy metals taken up from the environment on plants. These microbes are known to help in phytoremediation of heavy metal-contaminated soils through various processes. Siderophore-producing microbes sequester iron and nickel, thereby playing a dual role of sourcing iron for plant use and protecting against nickel toxicity (Glick 2003; Dimkpa et al. 2008). *Trichoderma*, a well-known biological control agent, contains multiple metal-detoxifying properties which are able to degrade organic contaminants; its beneficial association with plant is known to be effective against heavy metal (Cao et al. 2008) and metalloids (Tripathi et al. 2015, 2017). *T. atroviride* is reported to influence uptake and translocation of Ni, Zn and Cd in *Brassica juncea*, while *T. harzianum* was reported to promote growth of crack willow (*Salix fragilis*) in metal-contaminated soil (Adams et al. 2007). It is reported that *T. harzianum* strains can detoxify potassium cyanide and promote root growth of arsenic hyperaccumulating fern *Pteris vittata* (Lynch and Moffat 2005). Combined application of AM fungi and *T. harzianum* increased the tolerance and accumulation of *Eucalyptus globulus* to high concentrations of aluminium and arsenic in soil (Arriagada et al. 2007, 2009). The potential of *Trichoderma* to stimulate the growth of trees in heavy metal-contaminated soil has been investigated, and its role in revegetation and stabilization has also been demonstrated (Adams et al. 2007; Arriagada et al. 2007, 2009).

Association of arbuscular mycorrhizal fungi with plants has been known to alleviate heavy metal-induced stress in plants. Symbiosis with AM also enables metallophytes to grow under heavy metal stress by reducing heavy metal uptake in plant (Berreck and Haselwandter 2001). These metallophytes belong to the families *Brassicaceae* and *Caryophyllaceae*, known non-mycorrhizal plants (Demars and Boerner 1996). Metallophytes show their symbiosis (higher root colonization) with AM at the stage of high nutrient demand, i.e. at reproductive stage. Schutzendubel and Polle (2002) found the stimulation of phenolic defence system in *Paxillus–Pinus* mycorrhizal symbiosis under cadmium stress conditions, and they also showed possibility for providing protection by mycorrhizal association via GSH under heavy metal stress conditions as evidenced by higher thiol level. However, heavy metal tolerance in plant by AMF depends on the type of heavy metal contamination. The stress response of AMF is regulated by different factors such as plant species, the fungal species, the metal and its availability, soil fertility and plant growth conditions. They also reduce metal toxicity to the plants either by reducing their effects or by accumulating them into their biomass. Rhizospheric microbes result in complexation of heavy metals with dissolved organic matter (Li et al. 2013). The metal-resistant rhizomicrobes can serve as an effective metal sequestering and growth-promoting bioinoculant for plants in metal-stressed soil.

14.6 Fungal Mechanisms in Reducing Heavy Metal Toxicity in Plants

Plant growth-promoting microbes are widely known for their ability to improve plant growth and alleviate metal toxicity by either mobilizing/immobilizing or transforming metals in soil which can help to new strategies regarding microbe-mediated restoration strategies. Microbes are very closely involved in biogeochemistry of metals with a variety of processes determining their mobility and bioavailability. The processes including mobilization and immobilization of metals depend on the organisms involved as well as their environment and physicochemical conditions. Mobilization of metals occurs via different processes involving heterotrophic and autotrophic leaching, complexation/chelation by metabolites and siderophores and their volatilization due to methylation, whereas immobilization occurs due to metal sorption to biomass or exopolymers, transport and intracellular sequestration or precipitation as organic and inorganic compounds, e.g. oxalates (fungi) and sulphides.

14.6.1 Mobilization

Microbial mobilization or solubilization of insoluble metal compounds depends on different factors such as relative concentrations of anions and metals in solution, pH and the stability constants of the various complexes by using different processes. Microbes acidify their environment by release of protons through plasma membrane, maintenance of charge balance, release of organic acids and respiratory carbon dioxide accumulation for metal complex formation. Production of low-molecular-weight iron-chelating siderophores is the most common mode for iron acquisition by bacteria and fungus. Although siderophores are known for enhancing iron acquisition, they also have the ability to bind trace and toxic heavy metals; however, their affinity for them is slightly low (Gadd 2004; Rajkumar et al. 2010). Metal solubilized due to siderophore gets adsorbed or precipitated in the biomass which led to decrease in bioavailability of Cd, Zn and Pb (Diels et al. 1999). Siderophore has been also reported for reducing the heavy metal-induced degradation of IAA, thereby enhancing the ability of plants to combat against heavy metal stress (Neubauer et al. 2000).

14.6.2 Immobilization

Immobilization of metals by fungus through precipitation, sequestration or transformation is both a metabolism-dependent and metabolism-independent process (Gadd and Sayer 2000). Immobilization has also been reported to be of extracellular, cell surface and intracellular accumulation type (Veglio and Beolchini 1997). The first

site of interaction between metal and fungus is the fungal cell wall. Fungal cell wall is mainly composed of chitins, chitosans, glucans and other polysaccharides such as proteins and lipids which offer a variety of functional groups (carboxylate, hydroxyl, sulphate, phosphate and amino groups) which binds to different metal ions depending upon their availability. Binding of metal ions to ligands depends on several factors such as electrostatic interactions between them, electronegativity, metal accessibility, ionic radius and metal speciation (Rahman and Sathasivam 2015). Microbes also release extracellular polymeric substances (EPS), and a complex mixture of polysaccharides, mucopolysaccharides and proteins as a self-defence mechanism also has a capability to bind with potentially toxic metals (Gupta and Diwan 2017). Ionizable functional groups and noncarbohydrate substituents in EPS impart an overall negative charge to the polymer which enables it to sequester positively charged heavy metal ions (Gupta and Diwan 2017). Similarly, *Herminiimonas* arsenic oxydans induces biofilm formation on exposure of arsenic and its scavenging through EPS (Marchal et al. 2010). Oxalic acids released by fungi form complexes with soluble metal ions and immobilize them in the form of oxalates. Calcium oxalate is one of the common forms of oxalates in the environment associated with the precipitation of solubilized calcium (Gadd 2004). Other than calcium oxalates, fungi can also produce a number of metal oxalates such as Co, Cd, Mn, Sr and Zn (Franceschi and Nakata 2005). Jarosz-Wilkolazka and Gadd (2003) found the formation of oxalate crystals by white rot fungi (*Bjerkandera fumosa*, *Phlebia radiata* and *Trametes versicolor*) and the brown rot fungus, *Fomitopsis pinicola*, when grown in the presence of 0.5% $CaCO_3$-, $Co_3(PO4)_2$- or $Zn_3(PO_4)_2$-amended plates. However, crystals were not formed in $Zn_3(PO_4)_2$-amended plates. Metal transportation across the cell membrane is also one of the mechanisms involved by microbes which results in metal immobilization. Transportation of heavy metals across cell membrane may occur with same mechanism involved for important ions such as potassium, magnesium and sodium. Presence of heavy metal ions of same charge and ionic radius as that of essential metal ions might be transferred by cation transport system across the cell membrane (Javanbakht et al. 2014).

14.6.3 Biotransformation

Several species of fungi are known to exhibit a unique property of metal biotransformation which also contributes in removal of the heavy metals. They are capable of transformation of metals, metalloids and organometallic compounds through reduction, oxidation, methylation, catalysis and dealkylation which modify its mobility and toxicity. Methylation of metals and metalloids occurs by using one of the three pathways, i.e. S-adenosylmethionine, methylcobalamin or N-methyltetrahydrofolate (Mason 2012). Methylation of Hg, As, Se, Sn, Te and Pb is mediated by a number of bacteria and fungi under aerobic and anaerobic conditions (Gadd 2004). Methylation includes the transfer of methyl groups to the metals to form the compounds which vary in their solubility, volatility and toxicity. In secondary metabolic processes, carbon, oxygen, nitrogen and sulphur act as methyl

group acceptors. One of the possible mechanisms for the detoxification of arsenate is its conversion into methylarsonic acid or to dimethylarsinic acid. Conversion of water-soluble arsenate into volatile trimethylarsine is a multistep process which includes the initial reduction of arsenate into arsenite in the presence of arsenate reductase. The process continues with a sequence of methylation and reoxidation which is further followed by reduction of the organo-arsenical intermediates with S-adenosylmethionine as the usual methyl donor (Mukhopadhyay et al. 2002). Selenium methylation shares the same pathway, whereas, other metal ions such as Sn and Hg utilize the cobalamin pathway which involves the methylation of metal ions in their most oxidized form (Mason 2012). However, some evidences for methylation of arsenic and other metalloids by cobalamin pathway has also been reported (Wuerfel et al. 2012). Methylation of heavy metals by microbes, resulting in volatilization, has also been used successfully for in situ bioremediation of contaminated sites. With large surface area to volume ratio, fungi have been reported as an efficient agent for bioremediation of As, exploiting the mechanisms such as biomethylation, bioaccumulation, biosorption, biovolatilization, extra- and intracellular precipitation and active uptake (Srivastava et al. 2011; Singh et al. 2015; Zeng et al. 2015). Many fungal spp. are reported to methylate As(V) or As(III) to their organic forms such as monomethyl arsenate (MMA), dimethyl arsenate (DMA) or trimethyl arsenate TMA(V) or trimethyl arsine TMA(III) (Srivastava et al. 2011; Singh et al. 2015; Zeng et al. 2015). Efficient As removal by different fungal strains, viz. *Trichoderma* spp., *Neocosmospora* spp. and *Rhizopus* spp., from the growth medium has been reported earlier (Srivastava et al. 2011). Zeng et al. (2015) showed oxidation and methylation of As(III) inside the cells of *Trichoderma*, *Fusarium and Penicillium* into organic form of arsenic (MMA and DMA) through biotransformation. Despite As resistance, accumulation and volatilization, some fungal species showed substantial growth promotion in the presence of As(III) than their respective controls without As(III) (Srivastava et al. 2011; Feng et al. 2015). Fungi, such as *Scopulariopsis brevicaulis* and *Candida humicola*, methylate inorganic arsenic to produce volatile derivatives (Cullen and Reimer 1989). Some microorganisms gain energy by the reduction/oxidation of As(V)/As(III) for their growth (Oremland and Stolz 2003). Su et al. (2012) reported mechanism of As(V) reduction in three fungal species, viz. *T. asperellum* SM-12F1, *Penicillium janthinellum* SM-12F4 and *Fusarium oxysporum* CZ-8F1. Fungi mediates As(V) reduction, methylation and subsequent formation of As(III), MMAs and DMAs as the biotransformation products. Recently, a novel full-length arsenic methyltransferase (*WaarsM*) gene from a soil fungal strain *Westerdykella aurantiaca* with conserved SAM-binding motifs has been reported (Verma et al. 2016). Chen et al. (2017) also reported existence of *ArsM* enzyme with three conserved cysteines in the genome of *Aspergillus fumigates*, a common soilborne fungus. *AfarsM1* methylates MMA(III), but not trivalent As(III). It was proven by the heterologous expression of *AfarsM1* in an *E. coli* which conferred resistance to MMA(III) but not As(III).

14.7 Gene Modulation During Fungal Plant Interaction in Heavy Metal Stress

In order to avoid intoxication and retention of minimum value required for normal metabolism, organisms developed homeostatic mechanisms through modulation of expression of different genes. The increasing concern about environmental pollution due to heavy metals, whether of natural or anthropogenic origin, justifies the numerous studies on the mechanisms of metal tolerance. Heavy metal tolerance in mycorrhizal fungi through omics approaches and heterologous expression in model organisms *Oidiodendron maius* has been demonstrated. Functional complementation in *Saccharomyces cerevisiae* has allowed the identification of several ericoid mycorrhizal genes (i.e. antioxidant enzymes, metal transporters and DNA damage repair proteins) that may contribute to metal tolerance (Daghino et al. 2016). Mycorrhizal symbiosis, recognized as a crucial determinant for plant growth and productivity, has been shown to affect the expression of plant genes under heavy metal stress conditions (Repetto et al. 2003; Rivera-Becerril et al. 2005). Lanfranco et al. (2002) found the upregulation of metallothionein gene of *Gigaspora margarita* on exposure of Cu. ABC transporter gene (GintABC1) of G. intraradices has been reported to be upregulated on exposure to cadmium and copper (González-Guerrero et al. 2010). In an experiment, Hildebrandt et al. (2006) found the expression of several genes encoding protein involved in heavy metal stress tolerance, viz. Zn transporter, a metallothionein, heat-shock protein and a glutathione S-transferase in *Glomus intraradices* Sy167 in the presence of different heavy metals. They also found the upregulation of these genes in roots of *Medicago truncatula* grown in Zn-rich soil or in a non-polluted soil supplemented with 100 μM $ZnSO_4$. Foliar metallothionein and polyamine biosynthetic genes (*PaMT1, PaMT2, PaMT3, PaSPDS1, PaSPDS2 and PaADC*) were found to be induced in white poplar plants grown in heavy metal-polluted soil in the presence of AMF (Cicatelli et al. 2010). Almeida-Rodríguez et al. (2015) demonstrated symbiotic association between *Rhizophagus irregularis* and *Salix purpurea* L. in modulating plant responses under Cu stress as broad spectrum metabolic and physiological responses by the regulation of *aquaporin* genes *PIP1*;2 and *TIP2*;2 in *S. purpurea*. Metallothionein-mediated detoxification has been proposed under Cu stress through yeast complementation assays (Kohler et al. 2004). Polyamines are known for normal growth and development of eukaryotic organisms. In addition, there is abundant evidence for a stress-protective role of polyamines. Increased tolerance to multiple types of abiotic stress (Cu and Zn stress) in plants overexpressing polyamine biosynthetic genes has been reported (Franchin et al. 2007; Prabhavathi and Rajam 2007). Next-generation sequencing was used to study the whole genomes and transcriptomes of several heavy metal-tolerant organisms (He et al. 2011; Peña-Montenegro and Dussán 2013). Several studies on gene modulation due to arsenic toxicity and the role of different microbes for arsenic stress amelioration have been published earlier (Gao et al. 2013; Kintlová et al. 2017). Tripathi et al. (2015, 2017) have suggested the role of different *Trichoderma* species in reducing the grain

arsenic content through modulation of gene expression, anatomy and nutrient and mineral uptake. Genetic engineering of crop plants was suggested by several workers for phytoremediation of As to reduce As loading for safer food production due to plants' inability to methylate or volatilize As; hence, expression of *arsM* gene in plants would be a potential mitigation strategy to reduce uptake of total as well as toxic species of As in edible plant parts. Transgenic plants (viz. *Arabidopsis thaliana, Oryza sativa*) with induced As methylation property with the expression of *arsM* gene have been demonstrated under in vitro conditions (Meng et al. 2011; Verma et al. 2016). Meng et al. (2011) expressed *arsM* gene from the soil bacterium *R. palustris* in rice and reported that the transgenic rice significantly produced tenfold more volatile As than the non-transgenic control. In rice, overexpression of bacterial *arsM* gene was found to methylate As leading to reduced toxicity in rice. Furthermore, these transgenic lines expressing arsM exhibit less toxic organic As forms, MMA(V) and DMA(V), in the root and shoot of transgenic rice along with low As levels in rice grain (Meng et al. 2011). Use of a novel *arsM* gene (*WaarsM*) from a soil fungus *Westerdykella aurantiaca* can be suggested as a biotechnological solution to decrease As accumulation in plants (Verma et al. 2016).

14.8 Conclusion and Future Perspective

Mycoremediation is an eco-friendly 'green-clean' technology that has tremendous potential to be utilized in the cleaning up of heavy metals and organic pollutants. Association of plant and fungi can detoxify toxic metals, translocate and accumulate them in the above-ground biomass, which has to be then harvested for metal recovery. Despite tremendous potential for the application of mycoremediation in the cleaning up of contaminated soil, sediment and water, it has not been commercialized and used extensively on a large scale. There are many reports of heavy metal/metalloid uptake, detoxification and accumulation, but most of these are described at the laboratory scale in model plants (Hossain et al. 2012; Ovečka and Takáč 2014). Progress towards commercializing the phytobial remediation of heavy metals and metalloids has been hampered due to a lack of complete understanding of the metal uptake process from soil to roots, translocation from roots to shoots and accumulation in the biomass tissues. Several recent studies have attempted to unravel the mechanism of heavy metal and metalloid transport and accumulation in plants using transcriptomic and proteomics approaches (Cvjetko et al. 2014). Additionally, metabolomic analysis can help to identify the metabolites associated with heavy metal and metalloid stresses, which can be further mapped to its metabolic pathways to identify the related candidate genes (Mosa et al. 2016). However, more efforts are needed to enable imaging visualization and determination of metal and metalloid localization and distribution in plant tissues. Despite recent progresses in biotechnological applications and the availability of complete genome sequences of several plant species, the potential of mycoremediation and plant

involvement has still not been fully exploited for the successful application of this technology on a commercial scale for the cleaning of contaminated soil and water.

In the future, efforts should be made to develop strategies to improve the tolerance, uptake and hyperaccumulation of heavy metals/metalloids using genomic and metabolic engineering approaches. Pathways that control the uptake, detoxification, transport from root to shoot tissues and translocation and hyperaccumulation in the above-ground storage tissues can be engineered using gene-stacking approaches. Additionally, genome editing strategies can be designed using TALENs (transcription activator-like effector nucleases) technology or the powerful CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats) system to produce microbes/plants for bio/phytoremediation purposes. Recently, an efficient and successful CRISPR/Cas9-mediated targeted mutagenesis has been reported in *Populus* plants (Fan et al. 2015). This is a particularly interesting finding since *Populus* plants are known to be ideal plants for the phytoremediation of several toxic pollutants. Additionally, efforts should be made to develop breeding programmes to improve the biomass and growth habits of natural hyperaccumulators and breed those traits into non-food, high biomass and fast-growing plants for commercial phytoremediation of heavy metals and metalloids. Furthermore, efforts should be made to combine the phytoremediation approach with bioenergy through the dual use of plants for phytoremediation and biofuel production on contaminated lands. This approach would be useful to phytoremediate contaminated sites and simultaneously produce renewable energy that can offset the costs of applying these types of methodologies.

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