Chapter 7
Soil Bioremediation Strategies Based on the Use of Fungal Enzymes

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7.1 Introduction

The pollution of soils as a result of anthropogenic activities has received substantial attention in the past few decades, as compared with the previous two centuries of industrial activities. Soil contamination requiring clean-up exists at approximately 250,000 sites in the EEA member countries, according to recent estimates. And this number is expected to grow. The number of sites needing remediation will increase by 50% by 2025. In France, nearly 4,000 industrial contaminated sites have been listed, with more than 70% presenting pollution of the sediments and subsoil and/or surface water resources (BASOL 2008). In addition, agricultural soils are also contaminated with numerous chemicals resulting from atmospheric deposition (metals), direct contamination (e.g., use of pesticides) or amendments with contaminated residual organic products (wastewater, sludge and compost land filling). Because of pollution impacts on the environment (ecological diversity, ecosystem functioning) and human health (air quality and water resources), it is a great challenge to develop processes for soil rehabilitation.

In addition, the recent development of crops for green chemistry purposes, including the production of biomaterials and biofuels, limits worldwide the availability of soils for feed and food production. The reuse of decontaminated soils for agricultural production is generally to be excluded, as they are of a high risk for human health, but is expected to provide suitable soils for industrial crops. In the case of diffused pollution, in situ bioremediation techniques are better adapted for treatment of large surfaces of contaminated soils. Such treated land becomes available

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for less risky uses at an economically acceptable cost. Development of replanting programs on a large scale, using symbiotic fungi coupled with the bioremediation techniques based on the use of filamentous fungi and/or extra-cellular enzymes, is of great interest in the valorisation of polluted soils.

Among available processes allowing the reuse of treated soils, bioremediation is of priority interest. It exploits the capability of microorganisms to transform pollutants, thus offering permanent solutions such as immobilisation or degradation of the contaminants. Because of their powerful capabilities, filamentous fungi, and especially ligninolytic (white-rot) strains have been studied and used for at least 2 decades to target specific pollutants in wastes and soils (Aust 1989; Aitken 1993; Barr and Aust 1994).

The use of microorganisms, however, is fraught with problems (Whiteley and Lee 2006). The accumulation in the environment of highly toxic pollutants only emphasises the fact that microorganisms, by themselves, are insufficient to protect the biosphere from anthropogenic pollution. Furthermore, although microorganisms may enhance the transformation of the pollutants, making them more effective agents of biodegradation, they imply the use of a considerable amount of biomass. Any biostimulation approach has limited potential, since individual bacteria capable of remediation of a given pollutant may be inhibited by the presence of other pollutants. Another limiting factor in the bioremediation of polluted contaminated sites is the very slow rate of degradation, which further limits the practicality of using microorganisms in these processes.

Here, we would like to demonstrate that fungal enzymes appear to be promising tools for remediating moderately polluted soils, and that enzyme-based technology can be used even in the case of large-scale contaminations. We will describe in this chapter the main principles of soil bioremediation, discuss the relevance of fungal enzymes for soil bioremediation, and present some prospects for future research intended to improve the efficiency of these tools.

7.2 Principles of Soil Bioremediation

7.2.1 Definitions

The nature and the origin of pollution due to human activities are variable (industry, agriculture, transport, etc.). Continuous growth of organic and metal pollutants due to human activities causes deterioration in agricultural production, ecosystem functions and the quality of soils and subsoil waters. According to their extent, we can distinguish between two types of pollution:

- Diffused pollution which concerns significant soil surfaces, and which originated primarily from use of liquid or solid products (e.g., pesticides) or from atmospheric deposition,
- Punctual pollution, concerning limited surfaces and whose origin is generally accidental or chronic, generally due to industrial activity.

Contaminants can be mineral or organic compounds. Heavy metals and mineral oil are identified as the main soil contaminants, followed by organic contaminants including polycyclic aromatic hydrocarbons and aromatic hydrocarbons.

Two general methods can be developed for soil treatment: (1) in situ without excavation, and (2) ex situ with excavation. The first method is useful in the presence of deep contamination of the soil by pollutants, which are often volatile. It can also be adapted to large-surface contamination. Ex situ processes begin by the excavation or scraping off of the polluted soil, which can be moved into a treatment plant (off-site treatment) or treated on site. Only ex situ processes allow an efficient optimization of incubation parameters, including pH, aeration, agitation, moistening and addition of suitable electron acceptors, nutrients, solvents or surfactants. Ex situ processes enhance the rate of pollutant desorption, and increase the activity of native microorganisms by specific supply of nutrients or additives (biostimulation). Refinements to the process also include isolation and/or production of degradative organisms or enzymes, which are then introduced into the polluted material (bioaugmentation).

7.2.2 Bioremediation Techniques

Bioremediation techniques include a set of biological systems using microorganisms to clean various types of polluted media: air, water or soil. Bioremediation aims at decreasing pollutants amounts in soils by any natural process. Accelerated bioremediation involves increasing the rate of biodegradation or biotransformation of contaminants by bioaugmentation or biostimulation. In the case of biostimulation, soil properties such as pH, pedoclimate, and redox potential can be altered by the presence of the additives. Biostimulation and bioaugmentation are often used in conjunction with supply of nutrients, to enhance microbial growth and to improve environmental hazard waste degradation (Whiteley and Lee 2006).

Bioslurry reactor, biopile and landfarming are the main methods commonly used for bioremediation of polluted soils which are consistent with the use of fungal enzymes. In a bioslurry reactor, water is mixed with the sieved polluted soil to produce slurry treated in a bioreactor. The use of reactors provides rapid degradation of pollutants, due to enhanced mass transfer rates and increased contaminant-to-microorganism contact. The system can be supplemented with nutrients, electron acceptors, surfactants and degrading organisms (native or exogenous). The treatment units, static or mixed, make it possible to treat high concentrations of pollutants in the sludge. Soils with high clay content are easily treated by bioslurry. Other approaches involve combining advanced oxidation processes (used sequentially or in simultaneously) with biotransformation (e.g., addition of Fenton's reagent) (Mougin 2002).

Biopiles involve soil excavation, sifting and heaping into piles. The soil is packed on a protective layer formed by a bottom inert liner. Slotted or perforated piping placed throughout the pile collects leachates and forces air to move by

injection or extraction (static biopiles). The soil is periodically reversed in the dynamic biopile to ensure aeration. Nevertheless, the soil needs to be turned or tilled at certain times during the operational life of all biopiles to promote continued biodegradation. In addition, the watering system at the top of the pile distributes water, surfactants and nutrients throughout the soil. All of the material may be covered with a greenhouse or a gore-tex cover to regulate temperature and limit water evaporation. Volatile constituents tend to evaporate rather than biodegrade during treatment. Vapour generation during aeration can be controlled and treated. A related method is composting with addition of fertilizers such as manure (EPA 2008).

Landfarms are similar to biopiles in that they are aboveground, engineered systems that use oxygen from air to degrade pollutants. In contrast to biopiles, excavated soil is spread on the ground, and landfarms are periodically aerated by tilling or plowing to encourage microorganism growth. In some cases, polluted soil is incorporated in the top layer of an agricultural soil. Nutrients and moisture may be added, and collection of leachates may be necessary. Landfarming concerns all types of soil polluted by organics and heavy metals (Mougin 2002).

7.2.3 Interest of Bioremediation Vs. Physico-chemical Processes

Compared to physical or chemical remediation techniques, bioremediation is of major interest for a sustainable rehabilitation of contaminated sites, without strong modifications of soil properties. The bioremediation techniques are intended in priority for sites where there is no urgency for rehabilitation, and where the traditional methods of depollution are not adapted and/or ineffective. They could also be coupled with enhanced natural attenuation, in which involves the stimulation of faculties of the ecosystems to evolve and to regenerate. They allow also in most cases the subsequent reuse of cleaned soils.

Among the remediation methods available, several parameters indicate that bioremediation is an interesting technology in contrast to physico-chemical treatments. The first parameter is related to the pollutant. When bioavailable, common chemical compounds are generally well-degraded by microorganisms. On the other hand, ageing of the pollutant appears to limit biodegradation, as pollutants become less available for degradative enzymes. Bioremediation technologies can be applied to all types of soils, whatever their texture or permeability. They are partially governed by local constraints, such as space, noise, smell and dust. In other terms, off-site methods are useful in the case of urban areas. The advantages of bioremediation processes are that they are economically and environmentally acceptable solutions. They induce low costs, and the treated soil can be re-used if acceptable target pollutant levels are reached. Their disadvantages are that they require a long duration to achieve the required pollutant concentration thresholds.

7.2.4 Biotransformation Pathways of Organic Pollutants

The biotransformation of organic pollutants can be due to direct metabolism or to an indirect effect of organisms on the environment (Mueller et al. 1996). Three processes are involved in direct metabolism, namely biodegradation, cometabolism and synthesis.

During biodegradation, one or several interacting organisms metabolize a given xenobiotic into carbon dioxide and other inorganic components. In this way, the organisms obtain their requirements for growth and energy from the molecule. From an environmental point of view, biodegradation is the most interesting and valuable process, because it leads to the complete breakdown of a molecule without the generation of accumulating intermediates.

The prevalent form of xenobiotic metabolism in the environment is cometabolism, in which organisms grow at the expense of a cosubstrate to transform the xenobiotic without deriving any nutrient or energy for growth from the process. Cometabolism is a partial and fortuitous metabolism, and enzymes involved in the initial reaction lack substrate specificity. Generally, cometabolism results in only minor modifications of the structure of the xenobiotic, but different organisms can transform a molecule by sequential cometabolic attacks, or another can use cometabolic products of one organism as a growth substrate. Intermediate products with their own bio- and physico-chemical properties can accumulate, thus causing some adverse effects on the environment.

Synthesis includes conjugation and oligomerization. Xenobiotics are transformed into compounds with chemical structures more complex than those of the parent compounds. During conjugation, a xenobiotic (or one of its transformation products) is linked to hydrophilic endogenous substrates, resulting in the formation of methylated, acetylated, or alkylated compounds, glycosides, or amino acid conjugates. These compounds can be excreted from the living cells, or stored. During oligomerization (or oxidative coupling), a xenobiotic combines with itself, or with other xenobiotic residues (proteins, soil organic residues). Consequently, they produce high molecular weight compounds, which are stable and often incorporated into cellular components (cell wall) or soil constituents (soil organic matter). This biochemical process not only affects the activity and the biodegradability of a compound in limiting its bioavailability, but also raises concern about the environmental impact of the bound residues.

7.2.5 Bioremediation of Metal-polluted Soils

Currently, the techniques most used for the stabilization of soils contaminated by heavy metals are containment and solidification/stabilisation, or settling and discharge. Some plant species have a natural capacity to fix, degrade or eliminate the toxic chemicals and the pollutants from soils. The establishment of a vegetable cover on contaminated soils constitutes a bioremediation solution viable economi-

cally and complementary to the already existing techniques of depollution. As more than 90% of plant species are concerned with the mycorrhizal symbiosis which is established between roots of photosynthetic plants and mycelia of higher fungi, this symbiotic partnership plays an evident role in the attenuation of metal mobility and toxicity (Smith and Read 1997).

Much research has shown that the ectomycorrhizal fungi have extracellular and intracellular mechanisms which confer to them a tolerance to the presence of metal pollutants higher than that of the non-mycorrhized host plant. The identified mechanisms combine reduction of the absorption of metals in the cytoplasm and immobilization of metallic pollutants outside the cells by secretion of ligands into the medium or by their retention on the fungal cell wall (Bellion et al. 2006). Some fungal species from the basidiomycota phylum are able to produce metalothionein in great quantity, which enables them to detoxify their cytoplasm against metallic stress (Courbot et al. 2004).

Great differences have been observed between fungal species and isolates in their capacity to fix pollutants and to confer to the host plant a tolerance to toxicity. Isolated fungi from industrial contaminated sites exhibit a higher tolerance to high heavy metal levels, when compared to fungal isolates from non-polluted sites. They are also able to transfer their tolerance to host plants with which they have an association (Adrianson et al. 2004, 2005; Colpaert and Van Aasche 1987, 1992).

Knowledge of the mechanisms involved in the tolerance of some symbiotic fungi to metal pollutants makes possible their potential to contribute to the remediation of soils by supporting the accumulation and the immobilization of the pollutants in the roots of selected plants and the associated symbiotic fungi. This objective requires selection of adapted and effective fungal species and the optimisation of their use under site conditions.

7.3 Relevance of Fungal Enzymes for Soil Bioremediation

7.3.1 Filamentous Fungi

The degradation of organic compounds (natural or xenobiotic) through microbial metabolic processes is considered to be the primary mechanism of biological transformation. The different groups of microorganisms can mediate an almost infinite number of biochemical transformations. The most abundant organisms in soil are bacteria, whereas fungi form the largest biomass. They are involved in numerous functions, such as mineralization and humification of soil organic matter, biogeochemical cycles, production of toxins and compounds of industrial interest (antibiotics), and degradation of pollutants. The eukaryotic fungi comprise molds, mildews, rusts and mushrooms (all aerobic), as well as yeasts (fermenting organisms). Filamentous fungi are characterized by extensive branching and mycelial growth, as well as by the production of sexual (for asco- and basidiomycetes) and asexual spores. Deuteromycetes (*fungi imperfectii*) lack sexual reproduction capabilities,

but a sexual stage is quite often discovered, in which case these organisms are reclassified into other groups. Fungi are more tolerant to acidic soils and low moisture than bacteria. They can be pathogenic to plants and animals, or associated with plants in forming mycorrhyzae.

7.3.2 Fungal Oxidases

Filamentous fungi such as white-rot basidiomycetes, which are among the major decomposers of biopolymers, have developed non-specific and radical-based degradation mechanisms in their extracellular environment. Many studies have identified the role of this enzymatic machinery (e.g., laccase, lignin peroxidase and Mn-dependent peroxidase) in the transformation capacity of ligninolytic fungi towards a wide range of organic pollutants in contaminated soils (Pointing 2001; Riva 2006; Anke 2006; Gianfreda and Rao 2004; Baldrian 2006).

7.3.2.1 Peroxidases

Lignin peroxidase (LiP) and manganese peroxidase (MnP) were discovered in the mid-1980s in *P. chrysosporium*, and described as true ligninases because of their high redox potential (Martínez 2002). LiP and MnP catalyse the oxidation of lignin units by H_20_2. LiP degrades non-phenolic lignin units (up to 90% of the polymer), whereas MnP generates Mn^{3+}, which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units via lipid peroxidation reactions (Jensen et al. 1996). More recently, versatile peroxidase (VP) has been described in *Pleurotus* (Camarero et al. 1999) and other fungi (Pogni et al. 2005) as a third type of ligninolytic peroxidase that combines the catalytic properties of LiP and MnP (Heinfling et al. 1998), being able to oxidize typical LiP and MnP substrates.

Peroxidases share common structural and catalytic features: they are glycosylated proteins with an iron protoporphyrin IX (heme) prosthetic group located at the active site. Their catalytic mechanism involves a two-electron oxidation of the heme moiety to a high redox potential oxo-ferryl intermediate known as compound I. This two-electron reaction allows the activated enzyme to oxidize two substrate units. Two successive one-electron reductions return the enzyme to its resting state using a second intermediate, compound II (one-electron oxidized form) (Veitch 2004). The primary reducing substrate in the MnP catalytic cycle is Mn^{2+}, which efficiently reduces compound I and II, generating Mn^{3+}, which is stabilized by chelators such as oxalic acid, itself also excreted by the fungi. Chelated Mn^{3+} acts as a highly reactive, low molecular weight, diffusible redox-mediator. Its redox potential, up to 1.5 V, in turn oxidizes the organic substrate. Therefore, MnP enzymes are able to oxidize and depolymerise their natural substrate, i.e., lignin, as well as recalcitrant xenobiotics such as nitroaminotoluenes and texile dyes (Knutson et al. 2005; Wesenberg et al. 2003). Phenol cleanup by commercial horse radish

peroxidase (Wanger and Nicell 2002), or alternatively soybean peroxidase (Ryan et al. 2006) has been reported, but several drawbacks limit its widespread application, including intolerance of high concentrations of the primary substrate H_2O_2, low enzymatic reusability and financial costs (Nicell and Wright 1997). Bodalo et al. (2005) noted that the choice of peroxidase for wastewater treatment also depends on effluent characteristics, operational requirements and costs. The use of peroxidases for soil cleaning has been studied, specifically for soils historically contaminated with aromatic hydrocarbons and detoxified by autochtonous fungi producing peroxidases (D'Annibale et al. 2006).

7.3.2.2 Laccases

Laccases belong to a large group of enzymes termed multicopper oxidases, which includes among others ascorbate oxidases and ceruloplasmin. Their name originates from plant lacquer; they were first described in *Rhus vernicifera* by Yoshida in 1883, where they were ranked among the oldest enzymes ever described. They are produced by plants, insects (*Bombyx sp*.), bacteria (*A. lipoferum*) and they also occur widely in lignin degrading filamentous fungi, including the white-rot basidiomycete *Trametes versicolor*. They perform the reduction of dioxygen to water while oxidizing organic substrates by a one-electron redox process. Laccases can oxidize a wide range of aromatic substrates, mainly phenolic and anilines.

Laccases contain four copper ions distributed into three sites, defined according to spectroscopic properties. The different copper centres can be identified on the basis of their spectroscopic properties. The T1 copper is characterized as having a strong absorption around 600 nm, whereas the T2 copper exhibits only weak absorption in the visible region. The T2 site is electron paramagnetic resonance (EPR)-active, whereas the two copper ions of the T3 site are EPR-silent, due to an antiferromagnetic coupling mediated by a bridging ligand (Messerschmidt 1997). The substrates are oxidized by the T1 copper and the extracted electrons are transferred, probably through a strongly conserved His-Cys-His tripeptide motif, to the T2/T3 site, where reduction of molecular oxygen to water takes place (Fig. 7.1). Despite the substantial amount of information available for laccases as well as other related blue copper oxidases, mechanistic details of dioxygen reduction in these enzymes are not fully understood (Garavaglia et al. 2004).

Recently it was shown that white-rot fungi cultivated on natural solid lignin-containing substrates produce another form of laccases, lacking the T1 copper and named "yellow" laccases because these enzymes do not show the characteristic absorption band around 600 nm (Leontievsky et al. 1997). One interesting feature regarding these enzymes is that they seem to show a relatively high activity in the degradation of some polycyclic aromatic hydrocarbons (Pozdnyakova et al. 2004).

So far, more than 100 laccases have been purified from fungal cultures and characterized in terms of biochemical and catalytic functions (Xu et al. 1996). Their occurrence, characterization, functions and applications have been reviewed in recent years (Mayer and Staples 2002; Mougin et al. 2003; Baldrian 2006, Riva

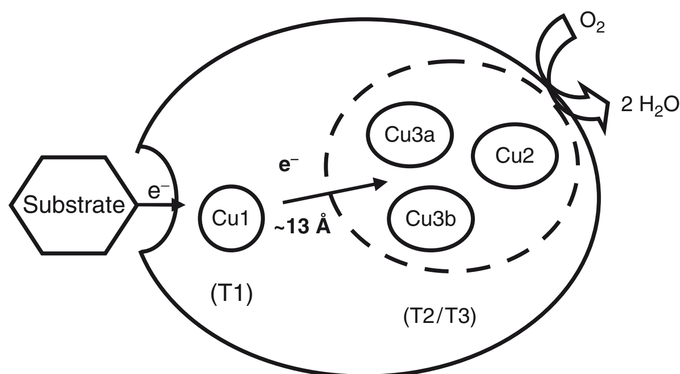

Fig. 7.1 Principles of the oxido-reduction reaction catalyzed by laccases

2006). Biological functions of laccases reflect their diversity, and clearly depend on the producing microorganisms. They are involved in many in vivo processes such as pigmentation, plant cell wall biosynthesis (which proceeds via oxidative polymerisation of monolignols in the cell wall matrix), phytopathogenesis or insect sclerotisation. Fungal laccases are involved in the process of wood delignification but also play a role in fungal morphogenesis, and could influence fungal virulence.

Being able to oxidize various aromatic compounds, laccases have excellent potential as industrial biocatalysts for many applications such as wood fibre modification in the paper pulp industry (Lignozym®-process, Call and Mücke 1997), or green organic chemistry and water/soil remediation, in order to protect the environment from damage caused by industrial or urban effluents (Xu 1999; Torres et al. 2003; Wesenberg et al. 2003; Dubroca et al. 2005). Commercially, laccases have been used to bleach textiles (Denilite® from Novozyme) or as a biosensor to distinguish between morphine and codeine.

One crucial point explaining such intense research and development activity in recent years is that laccases exhibit interesting properties: in addition to their broad specificity (which allows them to transform a wide range of substrates) and to their wide diversity, most fungal laccases are very stable, especially at pH values near neutrality, their organic substrate oxidation site exhibits a high redox potential [around 0.78V/normal hydrogen electrode (NHE)], and finally they use dioxygen, a harmless and abundant compound, as a co-substrate instead of oxygen peroxide which other oxidases (like peroxidases) use.

Laccases are therefore involved in the transformation of a wide range of phenolic compounds, including natural substrates such as lignin and humic substances. They can also transform xenobiotics such as trichlorophenols, pesticides, polynitrated aromatic compounds (Ramos et al. 2005), azo dyes and PAHs, which are a major source of contamination in soil; therefore, their degradation is of great importance for the environment. The potential use of oxidative enzymes from white-rot fungi for detoxification of these organic pollutants has been extensively reviewed (Torres et al. 2003; Pointing 2001; Gianfreda et al. 2004; Mougin et al. 2003; Couto and Herrera 2006).

7.3.3 *Examples of Xenobiotic Biotransformation Mediated by Fungal Enzymes*

Here, we would like to focus on three families of these compounds, i.e., polycyclic aromatic hydrocarbons (PAHs), nitroaromatic compounds and phenolic estrogenic chemicals, because of their present or future importance in soil contamination.

7.3.3.1 Polycyclic Aromatic Hydrocarbons (PAH)

PAHs are persistent organic pollutants widely distributed in terrestrial and aquatic environments (Samanta et al. 2002) as diffuse pollutants (Johnsen and Karlson 2007), where they result from fuel combustion of engines or as localized contaminants of old gas plants for example. They are composed of two or more fused benzene rings, and are classified as highly toxic (16 HAP have been listed as priority pollutants by the US Environmental Protection Agency (Mougin 2002) owing to their mutagenic and carcinogenic potential. Microbial bioremediation of PAHs has become popular since November 2002, when bioremediation was used to remove the pollution from the Prestige ship spill on the north coast of Spain (Alcalde et al. 2006). Numerous microorganisms, including bacteria, yeasts or fungi are known to be able to degrade PAHs (Mougin 2002; Aitken and Long 2004). Bacteria exhibit the advantage of being able to use PAHs as a sole source of carbon but are unable to mineralise them entirely, in contrast to soil fungi. In order to combine the advantages of both types of microorganisms, the use of consortia emerges as a promising method (Canet et al. 2001; Johnsen et al. 2005). White-rot fungi (Anastasi et al. 2008; Mollea et al. 2005; Cohen et al. 2002) were reported to be efficient in PAH degradation and the role of laccases in their degradation has been established through numerous studies. In 1996, Johannes et al. reported that a laccase from *T. versicolor* oxidized about 35% of acenaphtylene in solution after 72 h of enzymatic treatment. Since this pioneering work, many results, sometimes controversial, have been published. According to Collins et al. (1996) or more recently to Han et al. (2004), *T. versicolor* laccase does not oxidize phenanthrene in accordance with its high ionisation potential, whereas Pickard et al. (1999) and Wu et al. (2008) concluded the opposite result. It is generally reported that the transformation of PAH is significantly enhanced in the presence of mediators, i.e., chemical compounds that reduce substrates for laccases and are enzymatically transformed into radicals. These radicals in turn oxidize PAH: 80% of oxidation was reported for anthracene (Mougin et al. 2002), phenanthrene (Han et al. 2004) or benzo[a]pyrene (Mougin 2002). Some “natural” compounds such as tyrosine or cysteine have been demonstrated as potential mediators in these reactions (Johannes and Majcherczyk 2000).

Several microorganisms producing laccases, including *P. ostreatus* (Bogan et al. 1999) and *T. versicolor* (Rama et al. 2001) have been shown to efficiently transform PAH in soils. A positive correlation was found between PAH degradation

and ligninolytic enzymatic activity (Novotny et al. 1999). However, remedial strategies based on inoculation of PAH-degrading fungi seem to be difficult to apply under field conditions, due to sub-optimal growth conditions, high toxicity of PAH or potential interactions between microorganisms that limit laccase activity (Canet et al. 2001). However, recent research by Wu et al. (2008) on the direct application of free laccase in PAH-contaminated soil reported very promising results: a mixture of 15 PAHs was significantly degraded, anthracene and benzo[a]pyrene being the most efficiently transformed, to 8% and 60% respectively.

7.3.3.2 Nitro-Aromatic Compounds

Nitro-aromatic compounds are produced by incomplete combustion of fossil fuel or nitration reactions, and are used as chemical intermediates for synthesis of explosives (2,4,6-trinitrotoluene-TNT), pesticides (parathion), dyes or pharmaceuticals, with an estimated annual production of 10^8 tons (Ye et al. 2004). Therefore, large areas of soil and ground water have been highly contaminated by these xenobiotics. Nitro-aromatics are readily reduced by mammalian non-specific nitroreductase systems (Nishino et al. 2000). However, this enzymatic conversion of nitro groups leads to reactive carcinogenic derivatives such as nitroso and hydroxylamino groups. Nitro-aromatics are therefore now recognized as recalcitrant and given Hazardous Rating-3, where 3 denotes the worst level of hazard and/or toxicity (Sax and Lewis 1999). During the last few decades, extensive research has resulted in isolation of a number of microorganisms with the potential to degrade nitroaromatic compounds (see the review from Kulkarni and Chaudhari 2007) following aerobic or anaerobic pathways. Anaerobic processes generally lead to the formation of aromatic amines through a six-electron transfer mechanism, while aerobic pathways exploit mono- or dioxygenases to eliminate the nitro groups from mono-nitrophenols, as exemplified more than 50 years ago in *Pseudomonas sp.*, which is capable of converting 4-nitrophenol to hydroquinone, with the release of nitrite (Simpson and Evans 1953). However, bacteria utilizing nitro-aromatics as a sole source of C and/or N are very rare (Bennet et al. 1995). Because they possess suitable oxidative enzyme systems, white-rot fungi are capable of TNT degradation and mineralization to CO_2 (Pointing 2001). *Phanaerochaete chrysosporium* has been the organism of choice in such studies (Hodgson et al. 2000; Jackson et al. 1999; Bayman and Radkar 1997), and the involvement of the ligninolytic enzymatic system has been confirmed by studies using purified MnP (Van Acken et al. 1999). Addition of the surfactant Tween-80 to cultures of *P. chrysosporium* enhanced TNT mineralization 2-fold (up to 29.3% over 24 days) and reduced mutagenicity of aqueous TNT wastes by up to 94%, as measured using the *Salmonella*/microsome bioassay (Donnelly et al. 1997). Mineralization by *P. chrysosporium* has been demonstrated also for nitroglycerin in mixed culture with bacteria; however, anaerobic mineralization by bacteria was shown to occur at a faster rate (Bhaumik et al. 1997). Laccases have been shown to be involved in the degradation of TNT by catalysing the coupling of reduced TNT metabolites to the organic soil

matrix, which resulted in detoxification of the munition residue (reviewed by Duran et al. 2000). Recently, Nyanhongo et al. (2006) showed that a laccase from *Trametes modesta* was involved in immobilisation of TNT degradation products.

Soil remediation attempts for nitro-aromatic removal has mainly concerned bench-scale assays and has mostly used soil slurry technologies (Kulkarni and Chaudary 2007). Slurry processes consist of reactors filled with a mixture of soil and water to which co-substrates and nutrients can be added as necessary. There are two different approaches to TNT bioremediation in slurry reactors: mineralization of the explosive as the main target, and irreversible binding of TNT metabolites to the soil matrix (Esteve-Numez et al. 2001). A process designated as SABRE (sequential anaerobic biological remediation ex situ), developed and patented at the University of Idaho, consists of a consortium of facultative anaerobic organisms including strains of the genus *Clostridium* that transform explosives such as TNT to nontoxic, nonaromatic, and aerobically mineralizable products (Funk et al. 1995). *P. chrysosporium* was also reported to mineralize TNT present in soil at levels of up to 10,000 ppm (Fernando et al. 1990). However, the accumulation of starchy material in treated soil produces a high oxygen demand, which may be detrimental in agricultural soils because of the rapid development of anaerobic conditions when the soil is wetted, such as after irrigation or rainfall. Moreover, whole bacteria/fungi or their consortia used for degradation suffer from several drawbacks: (1) survival of inoculum gets difficult because of the chemicals toxicity, (2) reduction of chemical load is limited, and (3) presence of heavy metals inhibits treatment. To overcome these limitations, immobilization of degradative microorganisms or enzymes has been successfully used (Alexander 1999; Kulkarni and Chaudary 2007).

7.3.3.3 Endocrine-Disrupting Phenolic Compounds

Scientific and public attention has recently focused on the potential effects of certain environmental hormone-like chemicals on wildlife and human health. These chemicals, for the most part of anthropogenic origin, are known as endocrine disruptor chemicals (EDCs) because they modulate the endocrine system producing various pathologies, particularly during reproduction and development. In 2001, the Stockholm Convention under the auspices of United Nation Environmental Program specified a list of potential endocrine-disrupting chemicals in the environment, including certain pesticides, phthalates, phytoestrogenes, and several phenolic compounds (UNEP 2001).

Such concerns have heightened the need for novel and advanced bioremediation techniques to effectively remove these compounds from a variety of contaminated environmental media including water, sediments, sludge used to fertilize agricultural soils and soils (Duran and Esposito 2000; Romantschuk et al. 2000).

Enzymatic transformation of EDCs by the oxidative enzymes of ligninolytic fungi has mainly focused on two families: (1) alkylphenols such as nonylphenol and octylphenol, and (2) biphenyls such as biphenyl methane, known also as Bisphenol

A, usually used as a model compound for endocrine disruptors. Bisphenol A is a ubiquitous substance used mainly in the production of epoxy resins and polycarbonate plastics. The latter are used in food and drink packaging applications, while the former are commonly used as lacquers coating metal products such as food cans, or in water supply pipes. Because they are phenolic derivatives, these compounds are readily transformed by fungal laccases, as reported in several papers (Tanaka et al. 2001; Tsustumi et al. 2001; Saito et al. 2004). As an example, it has been reported that nonylphenol, octylphenol, bisphenol A and ethynylestradiol (synthetic estrogen) adsorbed on sea sand (2 pmol g^{-1}) was transformed by a laccase from *T. versicolor* at an optimum pH of 5. The authors suggest that the phenolic EDCs might have polymerized via enzymatic conversion to their phenoxy radicals. Our group (Dubroca et al. 2005) recently showed that the ligninolytic basidiomycete *T. versicolor* was able to catalyze partly the conversion of nonylphenol into carbon dioxide, and that laccases purified from *T. versicolor* cultures are involved in nonylphenol oxidative coupling, leading to oligomerization of nonyphenol via C-C bounds formation.

Very recently, Diano et al. (2007) showed that a laccase from *T. versicolor* immobilized on nylon membranes is able to transform efficiently Bisphenol A, and that the values of the percentage activity increases of immobilized enzymes proved to be higher at low substrate concentrations, i.e., at concentrations that really exist in polluted waters, considering the low aqueous solubility of these compounds.

7.3.4 Engineering of Fungal Oxidases

Numerous works cited above show that fungal laccases can be efficient tools for bioprocesses, leading to the cleanup of polluted water (Jolivalt et al. 2000) and soil bioremediation (Rama et al. 2001). Nevertheless, laccase-mediated biotransformation of xenobiotics in natural media suffers from several limitations of the enzyme: (1) a redox potential (so far, in the range 0.4–0.8 V) lower than that of the targeted organic compound for transformation, (2) an acidic optimal pH for activity which is too low compared to pHs of effluents or soils, and (3) the need for a redox mediator when the reducing substrate is too large to be accommodated into the active T1 site.

Engineering of laccases appears as a promising approach to overcome such limitations, and several attempts are reported to have improved laccase (or other enzymes potentially used in bioremediation processes) properties using biomolecular technologies (Lui et al. 2005). Two different and complementary approaches have been reported: a rationale approach based on structural knowledge of the protein, leading to targeted site-directed mutagenesis experiments, or more random-based directed evolution techniques.

Based on sequence alignments, without precise information concerning either the substrate cavity geometry or the interactions between amino acids and substrate, the pioneering work of Xu et al. (1996) and Xu (1999) suggested that a non-ligating

tripeptide in the vicinity of the active site was involved in the redox potential value. Although the redox potentials were not significantly altered, the triple mutants had a phenoloxidase activity, the pH optimum shifted 1 unit lower or higher while the kinetic parameters were greatly changed. The results were interpreted as possible mutation-induced structural perturbations of the molecular recognition between the reducing substrate and the enzyme. In 2002 for the first time, the three-dimensional structures of laccases from *T. versicolor* (Bertrand et al. 2002; Piontek et al. 2002), *P. cinnabarinus* (Antorini et al. 2002), *M. albomyces* (Hakulinen et al. 2002) with a full complement of copper ions was elucidated. In addition, two of these laccase structures have been obtained in the presence of a reducing substrate (Bertrand et al. 2002; Enguita et al. 2003). Our group showed that the presence of an arylamine (2,5-dimethylbenzeneamine or 2,5-xylidine) at the T1 active site of the enzyme revealed two important residues for the interaction between the amino group of the reducing substrate and the enzyme. In particular, aspartate 206 is hydrogen-bonded via the terminal oxygen of its side chain to the amino group of 2,5-xylidine. Moreover, the analysis of the dependence of kinetic parameters on pH suggests that an acidic residue may be involved in the binding of phenolic compounds. Site-directed mutagenesis experiments were performed towards Asp206 using the yeast *Yarrowia lipolytica* (Madzak et al. 2006). It was shown that the transformation rates remain within the same range whatever the mutation of the Asp206 and the type of substrate: at most a 3-fold factor increase was obtained for k_{cat} between the wild-type and the most efficient mutant Asp206Ala with ABTS as a substrate. Nevertheless, the Asp mutation led to a significant shift of the pH (pH = 1.4) for optimal activity against 2,6-dimethoxyphenol.

Engineering of laccase by laboratory evolution also showed interesting results as reported by the group of Ballesteros involved in the functional expression of a thermophilic laccase in *Saccharomyces cerevisiae* (Bulter et al. 2003). As the low aqueous solubility of some xenobiotics such as PAHs may require the addition of organic solvents to minimize mass transfer limitations, whereas laccases are known to be fairly unstable in such conditions, a thermophilic laccase was engineered by in vitro evolution to be highly active and stable in the presence of increasing concentrations of acetonitrile and ethanol. After only one generation of directed evolution, one mutant displayed about 3.5-fold higher activity than the parent type in the presence of 20% acetonitrile or 30% ethanol (Alcade et al. 2005). Mutant laccases were also tested for the oxidation of anthracene in the presence of 20% (v/v) acetonitrile (Zumarraga et al. 2007).

Another interesting attempt in laccase engineering aims at enhancing the expression of the enzyme in recombinant systems, which is an important bottleneck to overcome when aiming to use "optimized" enzymes in bioremediation processes. Directed evolution of a laccase from *Myceliophthora thermophila* (MtL) expressed in functional form in *Saccharomyces cerevisiae* improved expression 8-fold, to the highest level reported for a laccase in yeast (18 mg l^{-1}) at that time. Specific activities of MtL mutants toward ABTS and syringaldazine indicate that substrate specificity was not changed by the introduced mutations (Bulter et al. 2003).

Recently, random mutagenesis was performed, leading to an improved expression of a *T. versicolor* laccase in *Pichia pastoris* by 3.7-fold to 144 mg l^{-1} of enzyme, together with a 1.4-fold increase in k_{cat}. In comparison with the wild type, the best mutant enzymatic properties (K_M for ABTS and guaiacol, thermal- and pH-stability, optimal pH) were not changed (Hu et al. 2007).

7.3.5 Advantages of the use of Enzymes for Soil Bioremediation

The above examples show the potential of extracellular oxidases from white-rot fungi for the bioremediation of some aromatic pollutants. Even if the complete removal of these compounds, i.e., their mineralization, relies on the action of additional intracellular enzymes present in their originating fungi or in other soil-endogenous microorganisms and requires the presence of whole cells and their metabolic pathways, the biodegradation of the pollutant is efficiently started by these oxidases. The use of cell-free enzymes could therefore facilitate overcoming a drawback of bioremediation (the low degradation rate) by accelerating the initial degradation phase. The pollutant, being transformed into a very reactive radical by the enzymatic reaction, is then likely to react with other nucleophilic species in soil, leading to the formation of bound residues via coupling reactions to soil humic substances, a process analogous to humic acid synthesis in soils. In this case, the degradation of the pollutant is incomplete because no mineralization occurs, but the immobilized product is less bioavailable and thus has reduced its toxicity (Bollag 1992). As pointed out by several authors (Gianfreda and Rao 2004; Pointing 2001; Alcade et al. 2005; Ruggaber and Talley 2006; Kulkarni and Chaudary 2007), the use of enzymes instead of microorganisms undoubtedly presents some advantages, from environmental, engineering or economic points of view. Enzyme use overcomes some limitations of microorganisms.

- Enzyme sensitivity to the pollutant concentration changes is low: high pollutant concentration may be toxic for the cell, thus reducing the degradation efficiency, but low concentration may have a negative impact on the expression of the enzymatic system, especially when it is related to secondary metabolism of the microorganisms.
- Enzymes are active over a rather wide range of physicochemical (temperature, pH and salinity) gradients in the environmental matrix, often unfavourable to active microbial cells, as well as the presence of toxic substances or inhibitors of microbial metabolism.
- The biotransformation reaction can be selected not to generate toxic products, as is often the case with chemical and some microbiological processes.
- The requirement to enhance bio-availability by the introduction of organic co-solvents or surfactants is much more feasible for enzymes than for whole cells.
- It is easy to control the ecological impact in the field: to be efficient in the degradation of the targeted pollutant, cells must stay alive, which may cause an imbalance of the ecological equilibrium of the ecosystem, preventing any sustainable further

use of the soil for agricultural purpose for example. By comparison, the future of free enzymatic systems is more under control, since the enzymes are digested, in situ, by the indigenous microorganisms after the treatment.

In addition to the advantages of using enzymes to overcome the drawbacks of microorganisms, enzymes offer a series of intrinsic advantages, mainly focused on the capability of biomolecular engineering to improve the efficiency of the enzymes in bioremediation systems. The use of recombinant-DNA technology is likely to produce optimized biocatalysts, with high reaction activity towards recalcitrant pollutants, enhanced specificity and stability, at a higher scale and at a lower cost. Of course, genetically engineered microorganisms (GEMs) with enhanced capabilities can also be produced using the same technology, but the use of GEMs still faces significant constraints regarding their application. Release of GEMs into the environment is strictly regulated to avoid the spreading of undesirable mobile genetic elements such as recombinant plasmid containing antibiotic resistance markers. By comparison, pure enzymatic systems have a low ecological impact in soil because of their low life time: the enzymes are rapidly digested in situ by the indigenous microorganisms after their application (Ahn et al. 2002).

However, the number of published reports dealing with enzymatic remediation of soil is limited, owing to difficulties in the purification and cost of enzymes. A rare example is the remediation of 2,4-dichlorophenol-contaminated soil by laccase (Ahn et al. 2002). The authors compared the performances of both free and immobilized laccase, and concluded that taking into account the cost of immobilization and the activity loss during the immobilization procedure, the advantage of immobilized enzyme is minimal. Using free *T. villosa* laccase for soil remediation thus appears to be the more practical option. If such a conclusion could be extended to other enzymes and further applications, it would render the use of enzymes for the remediation process even more attractive.

7.3.6 Limitations of the Use of Enzymes for Soil Bioremediation

The use of fungal enzymes in the bioremediation of contaminated soils necessitates an accurate assessment of the activity of enzymes in soil. The diversity of physicochemical proprieties of soil and surface properties of enzymes make it difficult to understand the mechanisms involved in the interactions between these two interfaces. Soil medium is a physical environment organized in aggregates (Brewer 1964), which hosts the biological events and fluxes of water, air and matter, at various levels of structure. It is necessary to recognize and take into account these complexities in order to optimise bioremediation techniques of soils, in particular by understanding and managing the various local processes occurring in soils (fixation of pollutants, enzymes adsorption, etc.).

Regarding the complexity and the heterogeneity of soils, modelling of the thermodynamic status of the soil medium is a relevant tool for the monitoring of the

efficiency of bioremediation enzyme-based techniques. This modelling cannot be possible without a precise characterisation of the soil structure and the thermodynamic state of the soil–water interactions within this structure. A new paradigm has recently been developed, based on a fine characterisation of the pedostructure and pedoclimate, which makes it possible to bridge the gap between pedology and soil physics with a view to understanding of enzyme–soil interaction and for optimizing the use of extracellular enzymes in bioremediation programs (Braudeau and Mohtar 2004, 2009).

Thus, the use of enzymes for the remediation of polluted soils necessitates more knowledge about the effects of environmental conditions on the fungal survival and dissemination and on enzyme behaviour and activity. Many problems are identified as limiting factors to performing bioremediation of contaminated soils by organic pollutants.

7.3.6.1 Heterogeneity and Availability of Pollutants in the Soil Medium

Soil pollution with organic contaminants is often accompanied by high concentrations of heavy metals like lead or mercury. This pollutant mixture has multiple negative effects on the survival of fungi and soil microflora (Baldrian and Gabriel 1997; Baldrian et al. 1996; Bogan and Lamar 1996), on the catalytic activities of the enzymes, and by consequence on the effectiveness of bioremediation of soils.

A large variety of ionic and nonionic surfactants or emulsifiers may facilitate the partitioning of pollutants from the solid phase of soil to the water phase. Numerous studies on this topic have been performed for many years (Reid et al. 2000). Synthetic classical surfactants and biosurfactants have been extensively investigated. Thus, a number of hydrocarbon-degrading microorganisms produce extracellular emulsifying agents, which enhance contact between them and hydrocarbons. However, inhibition of pollutant transformation has often been reported in the presence of surfactants. Proposed mechanisms for inhibition of microbial degradation, mostly at supra-cmc levels, include surfactant toxicity, or preferential use of the surfactant as a growth substrate (Mougin 2002).

7.3.6.2 Behaviour of Enzymes in the Soil Medium

Soil is a porous medium which is characterised by high interfacial areas between solid, liquid and gaseous phases. The thermodynamic state of the soil water medium, which constitutes the local physical conditions, namely the pedoclimate, affects the bio-geochemical processes in soil and by consequence the interactions between enzymes and soil medium (Braudeau and Mohtar 2009). On the other hand, soil clay minerals have high adsorptive proprieties that affect directly the interaction of enzymes with physical surfaces (Gianfreda et al. 1991; Ramirez-Martinez and McLaren 1966). The strong affinity of enzymes for the solid–liquid and liquid–gas interfaces results in frequent interactions of these proteins with surfaces in

soils. The great variety of enzyme physicochemical properties and monomeric structures makes their adsorption capacities rather higher than those of sugars or nucleic acids. The physicochemical proprieties of soil have a direct effect on the adsorption intensity of enzymes and on the quantity of adsorbed proteins. Many studies have highlighted the role of protein conformation on their adsorption properties. Indeed, the structural conformation of proteins at soil surfaces has the opposite effect, as they increase the entropy of the system and at the same time lead to an increase of the specific interfacial area in the contact surface between enzymes and the soil interface (Quiquampois 1987; Sandwich and Schray 1988). Adsorption of enzymes on soil surfaces involves both electrostatic and hydrophobic interactions (Norde 1986; Staunton and Quiquampois 1994). This affects directly the activities of enzymes, and by consequence the degradation of xenobiotics, and the biogeochemical cycles of major elements like carbon, nitrogen or phosphate, in soils. Extracellular ligninolytic enzymes are both catalysts and important modules/elements (N source) in the soil nitrogen cycle, and consequently are subjected to biodegradation (Quiquampois 2000; Quiquampois et al. 1995). Indeed, interactions of enzymes with solid surfaces have not only had a significant effect on their activity, but also on their degradation.

The interaction of enzymes with soil surfaces, especially clay minerals, can affect enzyme activity. The main consequence of this interaction is a pH shift of the optimum catalytic activity of the enzymes adsorbed on electrically charged surfaces (Mc Laren 1954; Mc Laren et al. 1958). On the other hand, the adsorption of enzymes often induces a decrease in the velocity of the enzymatic reactions and catalytic activity (Gianfreda et al. 1991; Ramirez-Martinez and McLaren 1966). Irreversible negative effects of the adsorption of enzymes on their catalytic activity has been observed and supposed to be related to the variation in the pH of the activity of the adsorbed protein (Quiquampois 1987). Mechanisms were supposed to involve the interaction of enzymes with mineral surfaces. This mechanism may include variations of the microenvironment of the enzyme, such as local pH or ion concentration, and the modification of the conformation of the protein (Quiquampois 2000).

The effects of pH of the soil on the interaction of proteins with soil surfaces had been widely studied. Many observations indicate that the maximum adsorption of a protein on an electrically charged surface occurs often around the pI of the protein (Haynes and Norde 1994; Mc Laren et al. 1958; Norde 1986). The presence of organic matter on soils also has a protective effect on the catalytic activity of enzymes, by reducing the adsorption of enzymes on clay surfaces. Experimental studies have shown that the destruction of organic matter increases the quantities of adsorbed enzymes, and as a result reduces the activity of the protein (Quiquampois et al. 1995). Thus, the organic composition of polluted soils will have to be evaluated and managed to ensure the optimum activities of fungal enzymes used in bioremediation programs. The hydrophobic/hydrophilic properties of soils also contribute to the interactions of proteins and soils and on enzyme conformation. These properties vary according to the mineral composition of soils.

7.3.6.3 Production of Fungal Oxidases

Another important limitation to the use of enzymes in environmental treatment processes is enzyme availability, which depends on the quantity and cost of the enzyme. Numerous studies have been done to determine favourable conditions for laccase production by fungi (Tavares et al. 2005; Ikehata et al. 2004; Kahraman and Gurdal 2002; Pointing et al. 2000).

Filamentous fungi are able to produce laccase levels of about 20–50 mg l^{-1}; however, an efficient production system at bioreactor scale is still lacking, and several limitations must be overcome, such as uncontrolled fungal growth, the formation of polysaccharides around mycelia, and secretion of proteases that inactivate laccases (Rodriguez-Couto and Toca-Herrera 2007). The addition of inducers such as xylidine (Minussi et al. 2007) or its metabolites (Couto et al. 2002; Mougin et al. 2002; Kollmann et al. 2005), guaiacol (Ryan et al. 2007) for *T. versicolor* and copper for the white-rot fungi, *Pleurotus ostreatus* (Palmieri et al. 2000), *Trametes trogii* (Lenin et al. 2002) and *T. versicolor* (Tavares et al. 2005) have been found to significantly increase laccase production (by a factor of ten) compared to production without any inducer.

Another means of enhancing the enzyme availability is to overproduce it by recombinant organisms in which high production yields are achieved, making their production processes economically attractive. Unfortunately, the expression of oxidases from filamentous fungi is rather difficult in heterologous systems (Jolivalt et al. 2005) and over-expression of these enzymes has yet to be achieved. However, some work is in progress in this field.

Peroxidases suffer from multiple post-translational modifications, including disulfide bonds, *O*- and *N*-glycosilations as well as signal-peptide removal (Conesa et al. 2002), so that their expression in *E. coli* proceeds through inclusion body formation (Ryan et al. 2006). So far, efficient expression of peroxidases in heterologous systems has not been achieved, and they still have to be obtained from natural sources.

Fungal laccases undergo post-translational modification similar to those of peroxidases; their expression in a heterologous organism requires the use of a eukaryotic microbe, since glycosylation seems to be implicated in the stability of fungal laccases, impairing their production in *E. coli* (Yoshitake et al. 1993). *Aspergillus species* are capable of performing posttranslational modifications and show no extensive hyperglycosylation, and therefore have been used as an expression system to produce laccases. However, they generally show low production levels in comparison to other proteins (Sigoillot et al. 2003; Valkonen et al. 2003). Nevertheless, the commercial production of a laccase from *Myceliophthora thermophilia* expressed in *Aspergillus oryzae* has been undertaken by Novozymes. The manufacturing process for the enzyme production is done by submerged, fed-batch pure culture fermentation, and the laccase was generally recognized as safe (GRAS) by the US administration (USFDA 2003).

Yeasts are also favourable hosts, because of their ability to grow rapidly on simple media with high cell densities at low cost, together with the ease of manipulation of

this eukaryotic organism, enabling post-translational modifications. Historically, baker's yeast (*Saccharomyces cerevisiae)* is the most popular host, and several laccases have been produced at low expression level (Kiiskinen and Saloheimo 2004; Klonowska et al. 2005). Another yeast, *Yarrowia lipolytica*, has been used for production of *T. versicolor* laccase (Jolivalt et al. 2005). Combined with the knowledge of substrate–enzyme interactions derived from *T. versicolor* laccase structure, it made it possible to engineer the enzyme by site-directed mutagenesis. However, the expression level of laccases remains low at 2 mg l^{-1}. The expression level in *Y. lipolytica* is expected to be increased 10-fold by the use of multi-copy vectors and the strong hp4d promoter (Madzak et al. 2004).

7.4 Prospects for Future Research

7.4.1 Improving the Ability of Natural Enzymes to Transform Pollutants

The development of the capabilities of given strains by genetic construction, leading to genetically modified organisms (GMOs), offers promising opportunities for obtaining enzymes with improved catalytic capabilities. These strategies require knowledge of the structural and catalytic properties of the key enzymes involved in pollutant metabolism, as a basis of their directed evolution, to obtain the most effective isoforms. The fact that laccase can use atmospheric oxygen as a final electron acceptor represents a considerable advantage for industrial and environmental applications compared with peroxidases, which require a continuous supply of H_2O_2.

Taking into account the fact that the advantage of peroxidases is their high redox potential, engineering the active site of laccases to obtain high redox potential variants would be of considerable biotechnological interest. A complete knowledge of the molecular environment of laccase type 1 copper, which seems to regulate the redox potential of the enzyme, may offer exciting opportunities (Piontek et al. 2002). That approach could allow suppression of the requirements for redox mediators.

7.4.2 Discovering Enzymes with New or Increased Potential

The majority of enzymes utilized in biotechnology are still derived from well-characterized non-extremophilic microorganisms. Because extremophilic microorganisms are adapted to survive to adverse environmental conditions, they are expected to express enzyme activities, even under harsh conditions. Both terrestrial and aquatic environments may be extreme with respect to pH, temperature, salinity, or water activity. Utilizing natural biodiversity can speed up the process to find

performing enzymes, as compared to more sophisticated and more expensive engineering approaches. To our knowledge, no fungal peroxidases or laccases have been identified by this approach. By contrast, novel cellulases have been produced and characterized from the extremophilic filamentous fungi *Penicillium citrinum* (Dutta et al. 2008).

7.5 Conclusion

Biological processes remain of great interest for the remediation of contaminated soils. In that context, enzymes appear as potent tools, because they provide solutions to some limitations encountered with whole organisms. Fungal oxidases, such as peroxidases and laccases, have been extensively studied. They exhibit great potential for the transformation (degradation or coupling) of numerous types of pollutants. Nevertheless, their capabilities have been demonstrated in liquid axenic cultures and remain, in most cases, to be demonstrated in soils. Approaches to research, including the screening of enzymatic systems produced by extremophilic microorganisms, appear to provide a good opportunity to discover other powerful bio-catalysers.

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