Soil Bioremediation Strategies Based on the Use of Fungal Enzymes

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1.1 Introduction

The pollution of soils as a result of anthropogenic activities is the object of a growing interest since a few decades at most, compared to more than two centuries of industrial activities. Soil contamination requiring clean up is present at approximately 250000 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, near than 4 000 industrial contaminated sites have been inventoried, with more than 70% presenting a pollution of the sediments and subsoil and/or surface water resources (BASOL 2008). In addition, agricultural soils are also contaminated by 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 the 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 productions is generally to be excluded as they are of a high risk for human health, but is expected to provide suitable

soils for the 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 become available for less risky uses at an economically acceptable cost. Development of replanting programs at 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 a great interest in the valorisation of polluted soils.

Among available processes allowing the reuse of treated soils, bioremediation is of first interest. It consists in the use of the capacity of micro-organisms to transform pollutants, thus offering permanent solutions such as immobilisation or degradation of the contaminants. Because of their powerful capabilities, filamentous fungi, and especially ligninolitic (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 micro-organisms, however, is wrought with problems (Whiteley and Lee 2006). The accumulation in the environment of highly toxic pollutants only emphasises the fact that micro-organisms, by themselves, are insufficient to protect the biosphere from anthropogenic pollution. Furthermore, although micro-organisms may enhance the transformation of the pollutants making them more effective agents of biodegradation, it leads to the generation of a considerable amount of biomass. Any biostimulation approach has limited potential since individual bacteria able of remediating 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 that limits, further, the practicality of using micro-organisms during these processes.

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

1.2 Principles of soil bioremediation

1.2.1 Définitions

The nature and the origin of pollution due to human activities are very variable (industry, agriculture, transport...). Continuous uses of organic and metal pollutants due to the human activities deteriorate the agricultural productions, the ecosystems functions and the quality of soils and subsoil waters. According to their extent, we can distinguish between two types of pollution:

- Diffuse pollution which concerns significant soil surfaces and which originated primarily from use of liquid or solid products (e.g. pesticides) or of 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 pollutants. Heavy metals and mineral oil are identified as the main soil contaminants followed by organic contaminants including polycyclic aromatic hydrocarbons and aromatic hydrocarbons.

Two types of methods can be developed for soil treatment i) *in sit*u without excavation, ii) *ex situ* with excavation. The first method is useful in the presence of a deep contamination of the soil by pollutants, which are often volatile. It also adapted to large surfaces contamination. *Ex situ* processes begin by the excavation or scraping 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 micro-organisms by specific supply of nutriments or additives (biostimulation). Refinements to the process also include isolation and/or production of degradative organisms or enzymes, which are then re-introduced in the polluted material (bioaugmentation).

1.2.2 Bioremediation techniques

The bioremediation techniques include a set of biological systems using micro-organisms to cleanse various types of polluted media: air, water or soil. Bioremediation aims at decreasing pollutants amounts in soils by any natural process. Accelerated bioremediation consists in an

increase of the biodegradation or the biotransformation of contaminants by bioaugmentation or biostimulation. In case of biostimulation, soil properties such as pH, pedoclimate, redox potential can be altered by the presence of the additives. Biostimulation and bioaugmentation are often used in conjunction to supply the nutrients and to enhance the microbial growth and the other to enhance environmental hazard waste degradation (Whiteley and Lee 2006).

Bioslurry, biopile and landfarming are the main methods commonly used for bioremediation of polluted soils which are consistent with the use of fungal enzymes. In bioslurry, water is mixed with the sieved polluted soil to produce a slurry treated in a bioreactor. The use of reactors provides a 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, fixed or rotative, make it possible to treat high concentrations of pollutants in the sludge. Soils with high clay content are easily treated by bioslurry. Research concerns also the simultaneous or previous use of advanced oxidation processes 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 brings water, surfactants and nutrients. All the plants 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. Vapor generation during aeration can be controlled and treated. A closely 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. By 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).

1.2.3 Interest of bioremediation versus 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 the enhanced natural attenuation, consisting in 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 by contrast with physico-chemical treatments. The first parameter is related to the pollutant. When bioavailable, common chemical compounds are generally well degraded by microorganisms. On the contrary, ageing of the pollutant appears to limit biodegradation, as pollutants become less available for degradation 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 the target pollutant levels are reached. Their disadvantages are that they require a long time to start to work, associated with a pollutant concentration threshold which can be achieved through bioremediation.

1.2.4 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 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 only in 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.
- The last process, 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 give high-molecular weight compounds, which are stable and often incorporated into cellular components (cell walls…) 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.

1.2.5 Bioremediation of metallically polluted soils

Currently, the most used techniques for the stabilization soils contaminated by heavy metals are containment, the solidification/stabilisation or the setting in discharge.

Some plant species had 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 economically 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 root of photosynthetic plants and mycelia

of higher fungi, this last symbiotic partner play an evident role on the attenuation of metal mobility and toxicity (Smith and Read 1997).

Many research showed 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 their plant host not mycorrhized plants. The identified mechanisms combined the reduction of the absorption of metals in the cytoplasm and on the immobilization of metallic pollutants outside the cells by secretion of ligands in 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 that enables them to detoxify their cytoplasm against metallic stress (Courbot et al. 2004).

Great differences were 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 exhibited a higher tolerance to high heavy metal levels, compared to fungal isolates from non polluted sites. They are also able to transfer their tolerance to host plants when they are associated with (Adrianson et al. 2004, 2005; Colpaert and Van Aasche 1987, 1992).

Knowledge of the mechanisms implied in the tolerance of some symbiotic fungi to the metal pollutants make it possible their relevant use, 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 implies however the selection of adapted and effective fungal species and the optimisation of their use under site conditions.

1.3 Relevance of fungal enzymes for soil bioremediation

1.3.1 Filamentous fungi

The degradation of organic compounds (natural or xenobiotics) through microbial metabolic processes is considered to be the primary mechanism of biological transformation. The different groups of micro-organisms can mediate an almost infinite number of biochemical transformations. The most numerous 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 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, but a sexual stage is quite often discovered, in which case these organisms are reclassified into other classes. Fungi are more tolerant to acidic soils and low moisture than bacteria. They can be pathogenic for plants and animals, or associated with plants in forming mycorrhyzae.

1.3.2. 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 occurring in the extracellular environment. Many studies identified the role of that enzymatic machinery (e.g., laccase, lignin peroxidase and Mn-dependent peroxidase) in the transformation capacity of lignonolytic fungi towards a wide range of organic pollutants in contaminated soils (Pointing 2001, , Riva 2006, Anke 2006, Gianfreda et Rao 2004, Baldrian 2006 and further in the text).

1.3.2.1 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 (Martinez 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 catalytical 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 are able to oxidize and depolymerise their natural substrate, ie 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 and 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 namely for soils historically contaminated with aromatic hydrocarbons and detoxified by autochtonous fungi producing peroxidases (D'Annibale et al. 2006).

1.3.2.2. Laccases

Laccases belong to a large group of enzymes termed multicopper oxidases, which includes among others ascorbate oxidases and ceruloplasmin. Their name originates from plants lacquer, were first described in *Rhus vernicifera* by Yoshida in 1883, which ranks them among the oldest enzymes ever described. They are produced by plants, insects (*Bombyx sp*.), bacteria (*A. lipoferum*) and 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 by 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 (Figure 1).

Despite the amount of information on laccases as well as other related blue copper oxidases, details of dioxygen reduction in these enzymes are not fully understood (Garavaglia et al. 2004).

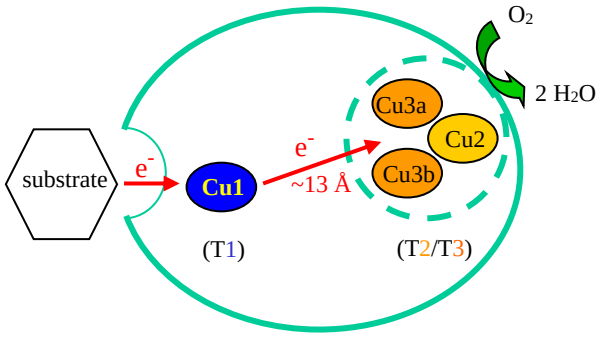

Figure 1: Principle of the oxidoreduction reaction catalyzed by laccases

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 600nm (Leontievsky et al. 1997). One interesting feature with these enzymes is that they seem to show a relatively high activity in the degradation of the 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 2006). Biological functions of laccases reflect their diversity and clearly depend on the producing micro-organism. 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 the fungus virulence.

Being able to oxidize various aromatic compounds, laccases have an excellent potential as industrial biocatalysts for many applications like wood fiber modification in paper pulp industry (Lignozym®-process, Call and Mücke 1997), or green organic chemistry and water/soil remediation, in order to protect 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 an intense research and development activity in recent years is that laccases cumulate 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 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 as other oxidases (like peroxidases).

Laccases are therefore involved in the transformation of a wide range of phenolic compounds including natural substrates as lignin and humic substances but also 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).

1.3.3. Examples of xenobiotic biotransformation mediated by fungal enzymes

Here, we would like to focus on tree families of these compounds, ie PAH, nitroaromatic compounds and phenolic estrogenic chemicals, because of their present or future importance in soil contamination.

1.3.3.1 Polycyclic aromatic hydrocarbons (PAH)

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants widely distributed in terrestrial and aquatic environments (Samanta et al. 2002) as diffuse pollutants (Johnsen and Karlson 2007) when 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 American Environmental Protection Agency (Mougin 2002) owing to their mutagenic and carcinogenic potential. Microbial bioremediation of PAHs has become popular since November 2002, when used to remove the pollution from the Prestige ship spill on the north

coast of Spain (Alcalde et al. 2006). Numerous micro-organisms, including bacteria, yeasts or fungi are known to be able to degrade PAHs (Mougin 2002; Aitken and Long 2004). Bacteria show the advantage of being able to use PAHs as a sole source of carbon but are unable to mineralise them entirely, contrary to soil fungi. In order to combine the advantages of both types of micro-organisms, 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 the degradation has been established by numerous studies. In 1996, Johannes et al. reported that a lacccase from *T. versicolor* oxidized about 35% of the acenaphtylene in solution after 72 hours of enzymatic treatment. Since this pioneer work, many results, some times 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 to its high ionisation potential, whereas Pickard et al. (1999) and Wu et al. (2008) work conclude to the opposite result. It is generally reported that the transformation of PAH is significantly enhanced in the presence of mediators, ie chemical compounds that are reducing 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 were demonstrated to be potential mediators in these reactions (Johannes and Majcherczyk 2000).
Several micro-organisms 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 the ligninolytic system 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 micro-organisms that limit laccase activity (Canet et al. 2001). However, a recent work from Wu and coworkers (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 86% and 60%, respectively.

1.3.3.2 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 estimated annual production of 10^8 tons (Ye at 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 nitro-reductase systems (Nishino et al. 2000). However, this enzymatic conversion of nitro groups lead 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 hazardness and/or toxicity (Sax and Lewis 1999). During the last few decades, extensive research has resulted in isolation of a number of micro-organisms with potential to degrade nitro-aromatic 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 imply mono or dioxygenases to eliminate the nitro groups from mono-nitrophenols, as exemplified more than fifty 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 the suitable oxidative enzymes 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 two-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 of nitro-aromatic removal mainly concern bench-scale assays and mostly use soil slurry technologies (for a recent review of bioremediation techniques, see 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 by 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 by SABRE (sequencial 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: (i) survival of inoculum gets difficult because of the chemicals toxicity, (ii) reduction of chemical load is limited, (iii) presence of heavy metals inhibits treatment. To overcome these limitations, immobilization of degradative micro-organisms or enzymes has been successfully used (Kulkarni and Chaudary 2007). As an example, immobilized laccase have been showed degrade nitrophenol (Alexander 1999).

1.3.3.3 Endocrine-disrupting phenolic compouds

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 disruptors 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 mainly focused on two families: (i) alkylphenols such as nonylphenol and octylphenol and (ii) 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 there 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 was reported that nonylphenol, octylphenol, bisphenol A and ethynylestradiol (synthetic estrogen) adsorbed on sea sand (2 pmol/g) 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 BPA 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.

1.3.4 Engineering of fungal oxidases

Numerous works cited above show that fungal laccases can be efficient tools for bioprocesses leading to polluted water cleanup (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: (i) a redox potential (so far, in the range 0.4–0.8 V) lower than that of the targeted organic compound for transformation, (ii) an acidic optimal pH for activity which is too low compared to pHs of effluents or soils and (iii) 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 tool to overcome such limitations and several attempts are reported to improve 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 neither the substrate cavity geometry nor 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 whose 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 laccases 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). Thet showed 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 Asn mutation led to a significant shift of the pH ($\Delta pH = 1.4$) 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 1 generation of directed evolution, one mutant displayed about 3.5-fold higher activities than 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 intends 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 eightfold to the highest yet reported for a laccase in yeast (18 mg/liter) 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 of enzyme, together with a 1.4-fold increase in k_{cat} whereas the wild type, the best mutant enzymatic properties (K_M for ABTS and guaiacol, thermo- and pH stability, optimal pH) are not changed (Hu et al. 2007).

1.3.5 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, ie their mineralization, relies on the action of additional intracellular enzymes present in their originating fungi or in others soil endogenous micro-organisms 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 allow overcoming a limiting drawback of bioremediation, ie, 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 reaction 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, thus reducing 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 micro-organisms undoubtedly presents some advantages, both from an environmental, engineering or economical points of view. Enzymes allow overcoming some limitations of the micro-organisms.

● Their 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 micro-organism.

● They cove 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 do 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 from an enzymatic point of view than using whole cells

● It is easy to control the ecological impact on field: to be efficient in the degradation of the targeted pollutant, cells must stay alive, thus likely to multiply and unbalance 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 micro-organisms after the treatment.

In addition to the advantages of using enzymes to overcome the drawbacks of micro-organisms, 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 micro-organisms (GEMs) with enhanced capabilities can also be produced according to 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 micro-organisms after their spreading (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 conclude 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 generalized to other enzymes and further applications, it would render the use of enzymes for remediation process even more attractive.

1.3.6 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 dynamic and activity of enzymes in soil. The diversity of physicochemical proprieties of soil and surface properties of enzymes make it difficult to understand the mechanisms implied in the interactions between these two interfaces. Soil medium is a physical environment organized in aggregates (Brewer 1964), theatre of the biological events and fluxes of water, air and matter, at various levels of structure which it is necessary to recognize and take into account to optimise bioremediation techniques of soils; especially by understanding and managing implied local processes is soils (fixation of pollutants, enzymes adsorption…).

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 enzymes based techniques. This modelling can not be possible without a precise characterisation of the soil structure and the thermodynamic state of the soil water interacting with this structure. New paradigm recently developed ad based on a fine characterisation of the pedostructure and pedoclimate, that make it possible the gap between pedology and soil physics will be helpful for the understanding of enzyme-soil interaction and for the optimizing the use of extracellular enzymes in bioremediation programs (Braudeau and Mohtar 2004, 2008).

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

1.3.6.1 Heterogeneity and availability of pollutants in the soil medium

Soil pollution with organic contaminants is often accompanied with high concentration of heavy metals like mercury, 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 the soil to the water phase. Numerous studies have been performed for many years in a great number of laboratories (Reid et al. 2000). Synthetic classical surfactants and biosurfactants have been extensively taken into account. Thus a number of hydrocarbon degrading micro-organisms produce extracellular emulsifying agents, which enhance contact between them and hydrocarbon. Nevertheless, inhibitions of pollutant transformation have 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).

1.3.6.2 Behaviour of enzymes in the soil medium

Soil is a porous medium characterised by a higher interfaces between solid, liquid and gaseous phases. The thermodynamic state of the soil water medium, which constitutes the local physical conditions, namely the pedoclimate, affect the biogeochemical and biological process in soil and by consequence the interactions between enzymes and soil medium (Braudeau and Mohtar 2008). In other hand, soil clay minerals had high adsorptive proprieties that affect directly the interaction of enzymes with physical surfaces (Gianfreda et al. 1991; Ramirez-Martinez and Mc Laren 1966). The strong affinity of enzymes for the solid-liquid and liquid-gas interfaces results on frequents interactions of these proteins with surfaces in soils. The great variety of physicochemical proprieties and monomers structures of the

enzymes makes their adsorption capacities rather higher compared to sugars or nucleic acids. The physicochemical proprieties of soil have direct effects on the adsorption intensity of enzymes and on the quantity of adsorbed proteins. Many studies highlighted the role of protein conformation on their adsorption properties. Indeed, structured conformation of protein on the soil surfaces has opposite effects as it increase the entropy of the system and by the same way leads to an increase of the specific interfacial area of the contact surface between enzyme and the soil interface (Quiquampois 1987; Sandwich and Schray 1988). Adsorption of enzymes on soil surfaces implied both electrostatic and hydrophobic interactions (Norde 1986; Staunton and Quiquampois 1994). This affect 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 soil nitrogen cycle and in consequence subjected to biodegradation (Quiquampois 2000; Quiquampois et al. 1995). Indeed, interactions of enzymes with solid surfaces had significant effects on their activity, but also on their degradation.

The interaction of enzymes with soil surfaces, especially clay minerals, can affect the 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 of the velocity of the enzymatic reaction and the catalytic activity (Gianfreda et al. 1991; Ramirez-Martinez and Mc Laren 1966). Irreversible negative effects of the adsorption of enzymes on their catalytic activity was observed and supposed to be reliable to the variation in the pH of the activity of the adsorbed protein (Quiquampois 1987). Mechanisms were supposed to be involved in the interaction of enzymes with mineral surfaces. This mechanisms my include variations of the microenvironment of the enzyme, like 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 had also a protective effect on the catalytic activity of enzymes by reducing the adsorption of enzymes on clay surfaces. Experimental studies showed that the destruction of organic matter increases

the quantities of adsorbed enzymes and reduced by consequence 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 the bioremediation programs. The hydrophobic/hydrophilic properties of soils contribute also to the interactions of proteins and soils and on the enzymes conformations. These properties vary according the mineral compositions of soils.

1.3.6.3 Production of fungal oxidases

Another important limitation to the use of enzymes in environmental treatment processes is the 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 laccases in rather high amount (about 20-50 mg/L), 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 inducer 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 increase significantly laccase production by a factor ten compared to the production without any inducer.

Another mean to enhance 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 the overexpression of these enzymes is still 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 bodies 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 micro-organism since glycosilation 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 shows no extensive hyperglycosilation and therefore have been used as an expression system to produce laccases but they generally show low production level in comparison to others proteins (Sigoillot et al. 2003; Valkonen 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 is recognized as GRAS by the US administration (USFDA 2003).

Yeasts, because of their ability to growth rapidly on simple media up to high cell densities at low cost, together with the ease of manipulation of unicellular organism and an eukaryotic organization enabling post-translational modifications (Jolivalt et al. 2005) are also favourable hosts. Historically, the bakers yeast *Saccharomyces cerevisiae* is the most popular and several laccases have been expressed in this host at low expression level (Kiiskinen and Saloheimo 2004; Klonowska et al. 2005). Another yeast, *Yarrowia lipolytica* was applied to the production of *T. versicolor* laccase (Jolivalt et al. 2005). Combined to the knowledge of substrate-enzyme interactions derived from *T. versicolor* laccase structure, it allowed engineering the enzyme by site-directed mutagenesis. However, the expression level of laccases remains low at 2 mg/L. The expression level in *Y. lipolytica* is expected to be increased ten fold by the use of multi-copy vectors and the strong hp4d promoter (Madzak et al., 2004).

1.4 Prospects for future research

1.4.1 Improving the ability of natural enzymes to transform pollutants

The development of the capabilities of given strains by genetic construction leading to GEMs or genetically modified organisms (GMOs) offers promising opportunities to obtain enzymes with improved catalytic capabilities. These strategies require the knowledge of structural and catalytic properties of 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 requires a continuous supply of H_2O_2.

Taking into account 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, that seems to regulate the redox potential of the enzyme, may offer exciting opportunities (Piontek et al. 2002). That way could allow suppressing the requirements for redox mediators/

1.4.2 Discovering enzymes with new or increased potential

The majority of enzymes utilized in biotechnology are still derived from well-characterized non-extremophilic micro-organisms. 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 the 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 new way. By contrast, novel cellulases have been produced and characterized from the extremophilic filamentous fungi *Penicillium citrinum* (Dutta et al. 2008).

1.5 Conclusions

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

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