New Insights into the Use of Filamentous Fungi and Their Degradative Enzymes as Tools for Assessing the Ecotoxicity of Contaminated Soils During Bioremediation Processes

Christian Mougin, Nathalie Cheviron, Marc Pinheiro, Jérémie D. Lebrun, Hassan Boukcim

New Insights into the Use of Filamentous Fungi and their Degradative Enzymes as Tools for assessing the Ecotoxicity of Contaminated Soils and Bioremediation Processes

Christian Mougin[1*], Nathalie Cheviron[1], Marc Pinheiro[1], Jérémie D. Lebrun[1,2], and Hassan Boukcim[3]

[1]UR 251 PESSAC, INRA, Route de St-Cyr, 78026, Versailles cedex, France
[2]Laboratoire Biosol, ESITPA, 3 rue du Tronquet, BP 40118, 76134 Mont-Saint-Aignan cedex, France
[3]Valorhiz, Bat 6, Parc Scientifique Agropolis II, 2196 Boulevard de la Lironde, 34980 Montferrier le Lez, France

1. Introduction

Among available processes allowing the reuse of polluted soils, bioremediation is of priority interest. It exploits the capability of microorganisms, mainly bacteria or fungi, to transform pollutants, thus offering permanent solutions such as immobilization or degradation of the contaminants. For more than 2 decades, powerful capabilities of filamentous fungi, and especially those of ligninolytic white-rot basidiomycetes, have been studied and used to target specific pollutant in waste and soils (Mougin et al. 2003; Asgher et al. 2008; Novotny et al. 2009). The use of bioremediation, however, is not lacking of problems. The possible accumulation in the environment of toxic pollutants or transformation products emphasizes the fact that microorganisms, by themselves, can be insufficient to protect the biosphere from adverse toxic effects.

Here, we would like to demonstrate that fungal enzymes appear to be promising tools either for remediating polluted soil or for the assessment of its possible ecotoxicity during the bioremediation process. We illustrate in that chapter the relationship between fungal enzymes involved in Polycyclic Aromatic Hydrocarbons (PAHs) and their ecotoxicological assessment when also considered as biomarkers. Researches on the development of these biomarkers are also presented considering model pollutants.

2. From soil bioremediation to ecotoxicological risk assessment, the example of Polycyclic Aromatic Hydrocarbons

PAHs constitute one of the most ubiquitous families of organic pollutants found in the environment. Extensive efforts of research have been developed in the field of soil bioremediation, since many PAHs and their transformation products are known to be toxic, mutagenic and carcinogenic (Penning et al. 1999; Bolton et al. 2000). Yet, remediation strategies have been reviewed by Gan et al. (2009). Fungal metabolism of PAHs has been and remains extensively studied because of the increasing development of remediation processes using filamentous fungi (Mougin 2002). Numerous fungi among zygomycetes, deuteromycetes, ascomycetes and basidiomycetes metabolize PAHs. Bacteria and fungi

exhibit distinct pathways for PAH transformation. Non-ligninolytic fungi produce metabolites including trans-dihydrodiols, phenols, quinones, tetralones and dihydrodiol epoxides. In addition, ligninolytic basidiomycetes are able to cleave the aromatic rings, mineralize them and also produce quinones. All of these fungal metabolites are produced during phase-1 reactions. During phase-2 metabolism, some of them are conjugated with hydrophilic moieties. Then, fungal metabolites can be retained within the cells, or released in their environment.

2.1. Fungal biotransformation of Polycyclic Aromatic Hydrocarbons and consequences upon their eco-toxicity

The biotransformation of PAHs, as well as other organic pollutants, can be due to direct metabolism or indirect effect of organisms on the environment. Three processes are typically involved in direct metabolism, namely biodegradation, cometabolism and synthesis (Mougin 2002).

During biodegradation, one or several interacting organisms metabolize a given PAH 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 PAH metabolism in the environment is cometabolism, in which organisms grow at the expense of a cosubstrate to transform the chemical 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 PAH, 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. In that case, 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. However, because of their chemical structure, PAHs can be conjugated, but they are poorly subjected to oligomerization.

Fungal metabolism of PAHs, as described below, has been extensively described in the past by Cerniglia and co-workers (ie Cerniglia and Sutherland 2001). Biodegradation aspects of PAHs have been reviewed by Haritash and Kaushik and Mougin et al. in 2009. Often, the first

step of PAH metabolism consists in ring epoxidation by a cytochrome P450 monooxygenase, leading to an unstable arene oxide in animals. Arene oxides are immediately hydrated to trans-dihydrodiols by epoxide hydrolase, or rearranged non-enzymatically to phenols. The carcinogenicity of trans-dihydrodiols can be lower than that of the parent compound (benzo[a]pyrene trans-9,10- and 4,5-dihydrodiols) or higher (benzo[a]pyrene trans-7,8-dihydrodiols). The non-enzymatic rearrangement of a PAH arene oxide in solution produces phenols.

When a monooxygenase catalyzes the second oxidation of a PAH trans-dihydrodiol, the result is a dihydrodiol-epoxide. Benzo[a]pyrene trans-7,8-dihydrodiol 9,10-oxide, produced by *Cunninghamella elegans*, is the ultimate carcinogenic and mutagenic metabolite of benzo[a]pyrene in mammals. The fungus also produces benzo[a]pyrene trans-9,10-dihydrodiol 7,8-oxide, which is less mutagenic. Dihydrodiol-epoxides can be metabolized further by epoxide hydrolase to tetrahydrotetraols.

Phenols and trans-dihydrodiols derived from PAHs are detoxified during phase 2 reactions by alkylation or conjugation with another molecule, including sulfate, glucosides, glucuronides and xylosides. Phenanthrene and pyrene have been shown to be converted into methoxylated compounds by *Aspergillus niger*. Sulfate conjugation, a common mammalian detoxification reaction, is also performed by fungi such as *C. elegans*. Glucuronic acid conjugates of PAHs are detoxification products in fungi. A soluble UDP-glucosyltransferase from *C. elegans* catalyses the conjugation of several PAHs. Fungi produce also glucose or xylose conjugates from PAHs, which are no more toxic.

Finally, several strains of fungi produce quinones. Non-ligninolytic fungi formed quinones from trans-dihydrodiols. By contrast, white-rot fungi produce extracellular enzymes (mainly lignin peroxidase - LIP, manganese-dependent peroxidase - MNP and laccase - LAC) that oxidize PAHs to form quinones. These fungi also partly metabolize PAHs to CO_2 and unidentified minor products.

In addition to various diseases and endocrine disorders, several PAHs induce mutagenic, teratogen and carcinogenic effects. Bioremediations processes generate compounds more polar than the parent molecules, PAHs hydroxides, ketones, and quinones, through atmospheric and metabolic reactions. Despite this fact, these polar compounds induce adverse effects on the health of environment, as well as the health of humans. Yet, in humans, lung cancer and bronchitis are possible consequences. Toxicity in animals is mainly associated to the binding of PAHs compounds on aryl hydrocarbon receptor (AhR) and thyroid hormone-related endpoints (Bekki et al. 2009). PAH quinones and ketones, which have functional groups with low polarity, have significant activities using AhR tests, thyroid receptor-based tests, and have estrogenic/antiestrogenic activity. Quinones also induce the generation of reactive oxygen species. In all living organisms, PAH metabolites can elicit several toxic and biochemical responses such as derivation of drug-metabolizing enzymes (cytochrome P450…). These results suggest that they might have various toxic activities in animals, more generally in the environment. In all cases, further studies on the toxicity and eco-toxicity mechanisms are necessary. Then, methyl-substituted PAHs are often in mixture with unsubstituted PAHs, and they are known to by mutagenic and carcinogenic.

2.2. Ecotoxicological assessment during PAH-contaminated soil bioremediation

According to the previous knowledge, the possible ecotoxicity of PAHs and related transformation products should be assessed in soils during bioremediation processes, either at the laboratory scale, or *in situ* at the field scale.

The performance of a biological treatment of a PAH-contaminated soil has been evaluated at the field scale (Lors et al. 2009). After six months of incubation, the biological treatment led to a significant reduction of 2- and 3-ring PAHs and to a lesser extent to 4-ring PAHs. As a consequence a significant decrease of the acute ecotoxicity was observed passing from highly ecotoxic before treatment to non-ecotoxic according to *Lactuca sativa* seedling and growth inhibition test and *Eisenia fetida* mortality test. This could be related to the bioavailability of PAHs. Indeed, tests performed on aqueous leachates of the soil showed a strong decrease of 2- and 3-ring PAHs correlated with a significant reduction of acute and chronic ecotoxicity responses. The biological treatment led to the mutagenicity reduction and the genotoxicity disappearance in the leachate. Thus, bioassays are complementary to chemical analyses to evaluate the efficiency of a bioremediation process and to evaluate the bioavailability of the organic pollutants as the total concentration of a contaminant is not the only criterion to consider. The comparison of the ecotoxic responses allowed underlining the best sensitivity of the earthworm, Microtox, Alga and Ames bioassays among the assays used.

In our laboratory, we performed some years ago an experiment intended to demonstrate the efficiency of the white-rot *Trametes versicolor* to decrease the amounts of PAHs in industrial soils (Rama et al., 2001). Solid lignocellulosic carriers have been developed to inoculate the fungus into a manufactured gas plant site soil. Pelleted wheat bran carriers were very efficient in stimulating the growth of fungi in the soil containing about 2800 mg kg^{-1} PAHs. Fungal biomass and activity of extracellular LACs produced by *T. versicolor* (as markers of metabolic activity in the contaminated soil), decreased after 2 weeks of incubation. Supplementing the soil with a mixture of carbon, nitrogen and phosphorus enhanced the fungal activity period.

In that experiment, LAC activity was measured during several weeks after inoculation of *T. versicolor*. The profile of laccase production was quite similar to that of fungal biomass production during the considered period. Laccase activity was the highest between 1 to 2 weeks after inoculation (450 nmol min^{-1} g^{-1}), and then gradually decreased. LAC identity was checked by SDS-PAGE and activity measurement. Its production seemed closely related to fungal growth. In addition, it has been shown that selected PAHs could be inducers of LACs. Yet, 9-florenone, as a metabolite of fluorene formed through LAC oxidation (Mougin et al. 2002a and b), increased 22-fold the activity of LAC produced by *T. versicolor*. That result demonstrated that enzymes involved in PAH metabolism can also be up or down-regulated by fungal exposure to their enzymatic substrate.

Very recently, we started an experiment intended to correlate PAH-spiked soil bioremediation by the fungus *T. versicolor*, and ecotoxicological assessment of the process. An agricultural soil was spiked with phenanthrene (90 mg kg^{-1} dry soil) and benzo[a]pyrene (60 mg kg^{-1} dry soil), supplemented with rice bran (7 % w/w on a dry basis) and mixed. Experiments were conducted in polyethylene jars refilled with 9.0-kg dry soil during 40 weeks, in the dark at room temperature. Inoculation devices have been developed in order to protect *T. versicolor*, provide nutrients, and ensure a significant fungal growth within the soil. Then, these devices

should allow an easy harvesting of fungal biomass exposed to contaminants for biomarker measurements. Three situations were considered using that inoculation device (control with PAHs and without fungus, control without PAHs and with the fungus, complete assay). In a fourth situation, the fungus was inoculated after growth and coating of lignocellulosic pellets (Rama et al. 2001). All fungal inoculations were performed at the beginning of the experiments, then after 21 weeks of incubation.

Whatever the inoculation process used, phenanthrene disappeared almost totally (> 98%) within the first 10 weeks in the 3 spiked-soils. By contrast, benzo[a]pyrene was more recalcitrant to fungal attack, with a 40% decrease in mean after 40 weeks.

As soil functioning biomarkers, global enzymatic activities have been monitored during the remediation process, namely dehydrogenase (ADH), β-galactosidase (GAL), urease (URE) and arylsulfatase (ARS)(Figure 1).

As concluding remarks of that part, we can underline that our results generally indicated a good relationship between global enzymatic activities in soils and fungal inoculation. An understanding of biochemical and physiological mechanisms involved in biological responses is necessary to understand particular situations. Because PAH degradation profiles were quite similar in the four jars, it remains difficult to link enzymatic activities to soil contamination, including residual PAHs and their new transformation products.

3. Fungal enzymes as candidates for biomarker development

Filamentous fungi such as white-rot basidiomycetes are among the major decomposers of biopolymers in the environment. They have developed non-specific and radical-based degradation mechanisms occurring in their extracellular vicinity. Numerous studies have identified the role of that enzymatic machinery (e.g. LIP and MNP, LAC…) in the transformation capacity of ligninolytic fungi towards a wide range of organic pollutants in contaminated soils (Gianfreda et Rao 2004; Baldrian 2006). In addition, these fungi possess also intracellular enzymes involved in transformation reactions, such as cytochromes P-450.

3.1. The extracellular oxidoreductases

Lignin peroxidase (LIP, EC 1.11.1.13) and manganese peroxidase (MNP, EC 1.11.1.14) were discovered in the strain *P. chrysosporium*. LIP and MNP catalyse the oxidation of lignin units by H_20_2. If LIP degrades non phenolic lignin units, MNP generates Mn^{3+} which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units. A versatile peroxidase (VP) has been also described in *Pleurotus sp.* and other fungi as a third type of ligninolytic peroxidase that combines the catalytic properties of LIP and MNP, being able to oxidize typical LIP and MNP substrates. In addition, a novel ligninolytic peroxidase gene (ACLnP) was cloned and characterized from the brown-rot fungus *Antrodia cinnamomea* (Huang et al. 2009). Peroxidases are glycosylated proteins with an iron protoporphyrin IX (heme) prosthetic group located at the active site. Therefore, MNP are able to oxidize and depolymerise their natural substrate, ie lignin, as well as recalcitrant xenobiotics such as nitroaminotoluenes and textile dyes (Knutson et al. 2005). 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). Recently, a novel group

of fungal peroxidases, known as the aromatic peroxygenases (APO), has been discovered (Pecyna et al. 2009). Members of these extracellular biocatalysts produced by agaric basidiomycetes such as *Agrocybe aegerita* or *Coprinellus radians* catalyze reactions (for example, the peroxygenation of naphthalene, toluene, dibenzothiophene, or pyridine) which are actually attributed to cytochrome P450 monooxygenases.

Laccases (LAC, EC 1.10.3.2) belong to a large group of multicopper oxidases, which includes among others ascorbate oxidases and ceruloplasmin. They occur widely in lignin degrading filamentous fungi, including white-rot basidiomycetes. 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. Their occurrence, characterization, functions and applications have been reviewed in recent years (Baldrian 2006; Mougin et al. 2003). 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, azo dyes and PAHs, the later chemicals being the major source of contamination in soil. The potential use of these oxidative enzymes for the detoxification of organic pollutants has been extensively reviewed (Couto and Herrera 2006; Gianfreda et al. 2004; Mougin et al. 2003).

3.2. Intracellular cytochromes P450

Cytochromes P450 constitute a large family of heme-thiolate proteins widely distributed among living organisms. In most cases, they function as monooxygenases by binding and activating molecular oxygen, incorporating one of its atoms into an organic substrate, and reducing the second atom to form water. The result of P450 catalysis, depending on the protein and its substrate, results in most cases in hydroxylation, but epoxidation, heteroatom dealkylation, deamination, isomerization, C-C or C=N cleavage, dimerization, ring formation or extension, dehydration, dehydrogenation or reduction have also been reported. For most eucaryotic P450s, a FAD/FMN-dependent NADPH-P450 reductase is needed to transfer the electrons used for oxygen activation from cytosolic NADPH. In filamentous fungi, P450s and reductases are usually microsomal membrane-bound proteins, exposed to the cytosol. Nevertheless, soluble forms of P450s, coupling P450 and reductase in a single fusion protein, have also been found in bacteria and fungi.

P450s are encoded by a superfamily of genes. The sequences of more than 500 of them have already been recorded in all living organisms. They are named, and classified in more than 150 families, according to the identity in amino acid sequences of the deduced proteins. With a few exceptions, based on phylogenetic considerations, proteins with 40% or less sequence identity are considered to define a new family. When two P450s are more than 55% identical, they are designated as members of a same subfamily. Families are designated by a number, subfamilies by a letter, following the prefix CYP. Through fungal genome sequencing projects, the discovery of P450s has advanced exponentially in recent years. More than 6000 fungal genes coding for putative P450s from 276 families have been identified.

Cytochrome P450 enzymes in the fungal kingdom have been reviewed by Cresnar and. Petric in 2011. Knowledge concerning regulation of fungal P450s is only starting to accumulate. P450 expression is can be regulated by fungal exposure to xenobiotics (agrochemicals, ethanol, or drugs like phenobarbital or aminopyrine). Fungal P450s can be also inhibited by

mechanism-based inactivators (ie 1-aminobenzotriazole). Numerous environmental pollutants act as P450 effectors.

3.3. Other enzymatic systems

Although not formerly involved in xenobiotic metabolism, fungi synthetize numerous enzymatic systems. Extracellular one, synthetized by wood decaying and phytopathogenic fungi, is the flavohemoprotein cellobiose dehydrogenase (CDH, EC 1.1.3.25. It can be used as a component of amperometric biosensors for detecting quinones (Karapetyan et al. 2006).

In addition, as many organisms, fungi produce hydrolases involved in carbon, nitrogen, phosphorus and sulfur cycles. These enzymes are commonly used for many years as sensitive indicators of soil functioning. Nevertheless, the relevant interpretation of their expression level, especially in a situation of contamination, requires a referential based on their natural spatiotemporal variability. Main hydrolases are β-glucosidase (GLU, EC 3.2.1.21), β-galactosidase (GAL, EC 3.2.1.23), N-acetyl-β-glucosaminidase (NAG, EC 3.2.1.30), urease (URE, EC 3.5.1.5), acid phosphatase (PAC, EC 3.1.3.2), alkaline phosphatase (PAL, EC, 3.1.3.1) and arylsulfatase (ARS, EC 3.1.6.1). Expression of these enzymes by fungi exposed to pollutants is not extensively studied.

All the previous paragraphs confirm that fungi possess and produce a wide range of enzymatic systems. Studying the specificity, the sensitivity and the dose-response relationships of these systems with fungal exposure to pollutants could ensure the identification of potential biomarkers.

4. Ways for research in the field of soil ecotoxicology associated to remediation processes

Mechanistic studies considering the regulation of fungal enzymatic systems are required in order to develop biomarkers and ecotoxicological tools. Targeted enzymes must belong to distinct families of fungal enzymes, either involved in pollutant transformation during remediation processes, or involved in nutrient cycles. *T. versicolor* is retained in that preliminary approach as a fungal model efficient in degrading pollutants. In addition, the panel of environmental pollutants has been extended to metals, because organisms are exposed to mixture of inorganic and organic pollutants in soils.

4.1. Laccase regulation as a starting point

Among fungal enzymes, laccases (LACs) have been detected and purified as constitutive or inducible isoenzymes from many strains. The stimulation of laccase production with respect to the culture medium composition has also been investigated. Metal ions and organic molecules have also been assayed for their ability to enhance the production of the inducible form of LACs. Gallic and ferulic acids were used, mainly because of their structural analogy with lignin model compounds. Moreover, 2,5-xylidine (2,5-dimethylaniline) has often been used to increase enzyme production in laboratory experiments by stimulating the expression of an inducible form of LAC. The substrate range of laccase includes some potential pollutants of the environment. Unfortunately, very little data are available concerning the

ability of these xenobiotics of environmental interest to interact with LAC production. We measured LAC activity in liquid cultures inoculates by *T. versicolor* (Mougin et al. 2002b). Agrochemicals, industrial compounds and their transformation products have been assayed for their ability to enhance LAC production in liquid cultures, when added at the concentration of 0.5 mM. After 3 days of treatment, enzymatic activity in the culture medium was increased 14-fold by 4-*n*-nonylphenol and 24-fold by the aniline. LAC activity was enhanced 10-fold by oxidized derivatives of the herbicide diquat, 17-fold by N,N'-dimethyl-N-(5-chloro,4-hydroxyphenyl)urea and 22-fold by 9-fluorenone, as presented above.

In a second time, we attempted to demonstrate LAC induction at the transcriptomic level. For that purpose, we followed the production of the gene AF414109 encoding the isoform of LAC A in *T. versicolor* cultures, and the gene U44430 encoding the isoform B. RNAs were quantified by real time PCR and their expression compared to enzymatic activity. The chemical retained as reference inducer was 2,5-dimethyaniline.

Our results showed that mRNA production and LAC activity were well correlated in mycelium of the fungus after 2 days of exposure to the inducer, with a good dose-response relationship (Figure 2A). The two markers were also linked regarding the time of exposure (Figure 2B).

By contrast, fungal exposure to 2,5-dimethylaniline failed to modify amounts of gene U44430, suggesting distinct regulation pathways for the two subfamilies of LACs; and a specificity of responses according to chemicals.

4.2. Towards the use of profiles of fungal enzymes as tools for ecotoxicological assessment?

In our laboratory, we developed a program of research intended to understand the physiological and physico-chemical mechanisms governing the biological response of filamentous fungi to environmental pollutants. One first objective is the development of fungal biomarkers for ecotoxicity assessment. Because some information was already available concerning organic pollutants (see above), recent experiments were developed with metals as pollutants. First experiments are performed in pure fungal liquid cultures, and extended to polluted soils in a second time. The conclusions are relevant in the case of soil PAH contamination, because they consider in all cases the chemical speciation of the pollutants, their bioavailability, and the exposure of organisms.

The relationship between the physiological state of fungi and the response of their functional enzymatic system has been clarified (Lebrun et al. 2010). Our study aims at establishing how the development phases modulate the secretion of enzymes in *T. versicolor* exposed to a single pollutant, copper (Cu). For that purpose, extracellular hydrolases (GLU, GAL, NAG, PAC, PAL, ARS) and oxidoreductases (LIP, MNP and LAC) were monitored in liquid cultures for two weeks. Cu was added either during the growing or stationary phases at 20 or 200 ppm. Our results showed that Cu at the highest concentration modifies the secretion of enzymes, whatever the development phase to which the fungus was exposed. In a general way, the production of hydrolases is decreased by Cu whereas that of oxidoreductases is highly increased. However, the sensitivity of enzyme responses to Cu depends on the phase development and the type of secreted enzyme. Furthermore, LIP, which was not measured in the control cultures, was specifically produced in the presence of Cu. Our results confirm that

oxidoreductases may be appropriate biomarkers for ecotoxicity assessment. It remains now necessary to investigate the response of other types of fungal enzymes after exposure to chemicals in mixtures.

Then, the sensitivity of biological responses must be addressed regarding the bioavailability of pollutants. Lebrun et al. (2011b) aimed at enhancing the secretion of lignin-modifying oxidoreductases in *T. versicolor* by favouring the bioavailability of essential metals. For this purpose, the fungus was exposed to Cu or zinc (Zn) in liquid culture media exhibiting different complexation levels. Metal speciation was determined experimentally or theoretically to quantify free metal species, supposed to be the most bioavailable, and species complexed to ligands. Although Zn^{2+} contents were high in media, Zn had no effect on oxidoreductase production. Conversely, Cu highly induced MNP and LAC productions until 40 and 310 times when compared to unexposed controls. This inductive potential was highly correlated to Cu^{2+} contents in media. Furthermore, in lowly complexing media, the response threshold of oxidoreductases to Cu greatly decreased and an unexpected production of LIP occurred, as a confirmation of the previous result.

Moreover, Lebrun et al (2011a) investigated the effect of Zn, Cu, lead (Pb) and cadmium (Cd), tested alone or in equimolar cocktail, on the secretion profiles at enzymatic and protein levels *in T. versicolor* cultures. They monitored extracellular hydrolases (GLU, GAL, NAG and PAC) and ligninolytic oxidases (LAC and MNP). Fungal secretome was analyzed by electrophoresis and LAC secretion was characterized by western-blot and mass spectrometry analyses. They showed that all hydrolase activities were inhibited by the metals tested alone or in cocktail, whereas oxidase activities were specifically stimulated by Cu, Cd and metal cocktail. At protein level, metal exposure modified the electrophoretic profiles of fungal secretome and affected the diversity of secreted proteins. The LAC isoenzymes, A and B were differentially glycosylated according to the metal exposure. The amount of secreted LAC A and LAC B was strongly correlated with the stimulation of LAC activity by Cu, Cd and metal cocktail. In conclusion, modification of enzyme activities can now be linked to modifications of protein secretion, in relation to transcriptomic probes.

Finally, the relation between degradation of PAHs and the ligninolytic enzymes remains difficult to be established in the soil matrix. Studies in liquid medium showed the ability of these enzymes to degrade the PAHs and principles governing the various reactions are globally established. However, the experiments of biodegradation of PAHs in soil have generally provided conflicting results. These results could be explained firstly by the difficulty of extracting these enzymes in the soil and measure of their activities; and secondly by the absence of the knowledge on the interactions between soil physical properties and the pollutant bioavailability and enzymatic activities. These are new challenges for the environmental sciences, especially by the study of physical coupling of processes in soils (Braudeau and Mohtar 2009).

5. Conclusions and perspectives

Through this chapter, we highlighted the following points:
- Considering the example of PAHs, we showed the complexity of the biochemical reactions catalyzed by fungi during remediation processes.

- These complex pathways resulted in the formation of numerous metabolites, exhibiting in most cases a real ecotoxicity.
- Fungal enzymatic systems either involved in pollutant transformation or not, behave as potent biomarkers for ecotoxicological assessment.
- It remains necessary to develop additional research to understand the biochemical mechanisms responsible of fungal biological responses.
- That knowledge allowed considering profiles of protein expression as biomarkers of soil ecotoxicity.

More research remains necessary to identify the most relevant protein profiles produced by fungi exposed to soil pollutants. It is also essential to understand all the mechanisms governing protein expression in polluted soils, as well as the selectivity and sensitivity of the responses. Proteomics and transcriptomics are actual approaches to study these topics. It is also fundamental to study the role of the soil physics properties in the enzymatic reactions measured *in situ* in order to allow the coupling between physical and biological processes in soils. That will make it possible, through a mechanistic modeling, the generalization of the results and the scale transfer for the use of the enzymes as biomarkers of soils ecotoxicity in the environmental diagnosis.

Acknowledgement

The authors thank VALORHIZ, the French Ministry for the Higher education and Research and French Public company OSEO for their financial contributions to this R&D project. The authors also thank Dr Claude Jolivalt (Ecole Nationale Supérieure de Chimie, Paris, France) for performing RNA extraction and real-time PCR experiments, as well as Dr Karine Laval and Dr Isabelle Trinsoutrot-Gattin (ESITPA, Mont St-Aignan, France) for for co-managing the Ph.D research of Jeremie D. Lebrun.

References

Asgher M, Bhatti HN, Ashraf M, Legge, R (2008) Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 19:771-783.

Baldrian P (2006) Fungal laccases - occurrence and properties. FEMS Microbiol Rev 30:215-242.

Bekki K, Takigami H, Suzuki G, Tang N and Hayakawa K (2009) Evaluation of toxic activities of Polycyclic Aromatic Hydrocarbon derivatives using *in* vitro bioassays. J Health Sci 55:601-610.

Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ (2000) Roles of quinones in toxicology. Chem Re Toxicol 13:135-160.

Braudeau E, Mohtar RH (2009) Modeling the soil system: bridging the gap between pedology and soil-water physics. Global Planet Change 67:51-61.

Cernilia CE and Sutherland JB (2001) Bioremediation of polycyclic aromatic hydrocarbons by ligninolytic and non-ligninolytic fungi. In: Gadd GM (ed), British Mycological Society Symposium Series, Fungi in Bioremediation. Cambridge University Press, Cambridge, pp 136–187.

Couto SR, Herrera JLT (2006) Industrial and biotechnological applications of laccases: a review. Biotechnol Adv 24:500-513.

Cresnar B and Petric S (2011) Cytochrome P450 enzymes in the fungal kingdom. Biochim Biophys Acta 1814:29-35.

D'Annibale A, Rosetto F, Leonardi V, Federici F, Petruccioli M (2006) Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. Appl Environ Microbiol 72:28-36.

Gan S, Lau EV, Ng HK (2009) Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). J Hazard Mat 172:532-549.

Gianfreda L, Rao MA (2004) Potential of extra cellular enzymes in remediation of polluted soils: a review. Enz Microb Technol 35:339–354.

Haritash AK, Kaushik CP (2009) Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. J Hazard Mat 169:1-15.

Huang ST, Tzean SS, Tsai BY, Hsieh HJ (2009) Cloning and heterologous expression of a novel ligninolytic peroxidase gene from poroid brown-rot fungus *Antrodia cinnamomea*. Microbiology-SGM 155: 424-433.

Karapetyan KN, Fedorova TV, Vasilchenko LG, Ludwig R, Haltrich D, Rabinovich ML (2006) Properties of neutral cellobiose dehydrogenase from the ascomycete *Chaetomium* sp. INBI 2-26(-) and comparison with basidiomycetous cellobiose dehydrogenases. J Biotechnol 121:34-48.

Knutson K, Kirzan S, Ragauskas A (2005) Enzymatic biobleaching of two recalcitrant paper dyes with horseradish and soybean peroxidase. Biotechnol Lett 27:753–758.

Lebrun JD, Trinsoutrot-Gattin I, Laval K and Mougin C (2010) Insights into the development of fungal biomarkers for metal ecotoxicity assessment: case of *Trametes versicolor* exposed to copper. Environ Toxicol Chem 29:902-908.

Lebrun JD, Demont-Caulet N, Cheviron N, Laval K, Trinsoutrot-Gattin I, Mougin C (2011a) Secretion profiles of fungi as potential tools for metal ecotoxicity assessment: a study of enzymatic system in *Trametes versicolor*. Chemosphere 82:340-345.

Lebrun JD, Lamy I, Mougin C (2011b) Favouring the bioavailability of Zn and Cu to enhance the production of lignin-modifying enzymes in *Trametes versicolor* cultures. Bioresource Technology 102:3103-3109.

Lors C, Perie F, Grand C, Damidot D (2009) Benefits of ecotoxicological bioassays in the evaluation of a field biotreatment of PAHs polluted soil. Global Nest J 11: 251-259.

Mougin C (2002) Bioremediation and phytoremediation of industrial PAH-polluted soils. Polycycl Aromat Comp 22:1011-1043.

Mougin C, Jolivalt C, Malosse C, Sigoillot JC, Asther M and Chaplain V (2002a) Interference of soil contaminants with laccase activity during the transformation of complex mixtures of polycyclic aromatic hydrocarbons (PAH) in liquid media. Polycycl Aromat Comp 22:673-688.

Mougin C, Kollmann A and Jolivalt C (2002b). Enhanced production of laccases by the fungus *Trametes versicolor* by the addition of xenobiotics. Biotechnol Letters 24:139-142.

Mougin C, Jolivalt C, Briozzo P, Madzak C (2003) Fungal laccases: from structure-activity studies to environmental applications. Environ Chem Letters 1:145-148.

Mougin C, Boukcim H, and Jolivalt C (2009) Bioremediation strategies based on fungal enzymes as tools. In: ASingh, A, Kuhad RC and Ward OP (eds), Advances in Applied Bioremediation. Springer, Soil Biology Series, Chapter 7, pp 123-149.

Novotny C, Cajthaml T, Svobodova K, Susla M, Sasek V (2009) *Irpex lacteus*, a white-rot fungus with biotechnological potential – review. Folia Microbiologica 54:375-390.

Pecyna MJ, Ullrich R, Bittner B, Clemens A, Scheibner K, Schubert R, Hofrichter M (2009) Molecular characterization of aromatic peroxygenase from *Agrocybe aegerita*. Appl Microbiol Biotechnol 84:885–897.

Penning TM, BurcZynski ME, Hung CF, McCoull KD, Palackal NT, Tsuruda LS (1999) Dihydrodiol dehydrogenases and polycyclic aromatic hydrocarbon activation: generation of reactive and redox active o-quinones. Chem Res Toxicol 12:1-18.

Figure 1. Dehydrogenase (A, ADH), β-galactosidase (B, GAL), urease (C, URE) and arylsulfatase (D, ARS) activities in the PAHs-spiked soils during the fungal remediation process. Symbols refer to: ●, control with PAHs and without *Trametes versicolor*; o, control without PAHs and with *Trametes versicolor*; ▼, complete assay with *Trametes versicolor* in the inoculation devices; ∆, complete assay with *Trametes versicolor* inoculated after growth on lignocellulosic pellets.

A Transient peak of ADH activity is noticed several weeks after fungal inoculation, as already reported in 2002. GAL activity was the highest in the soil inoculated with lignocellulosic pellets. There were no differences in URE activities until 25 weeks; then it decreased except in the jar inoculated with pellets. Finally, ARY activity was difficult to interpret.

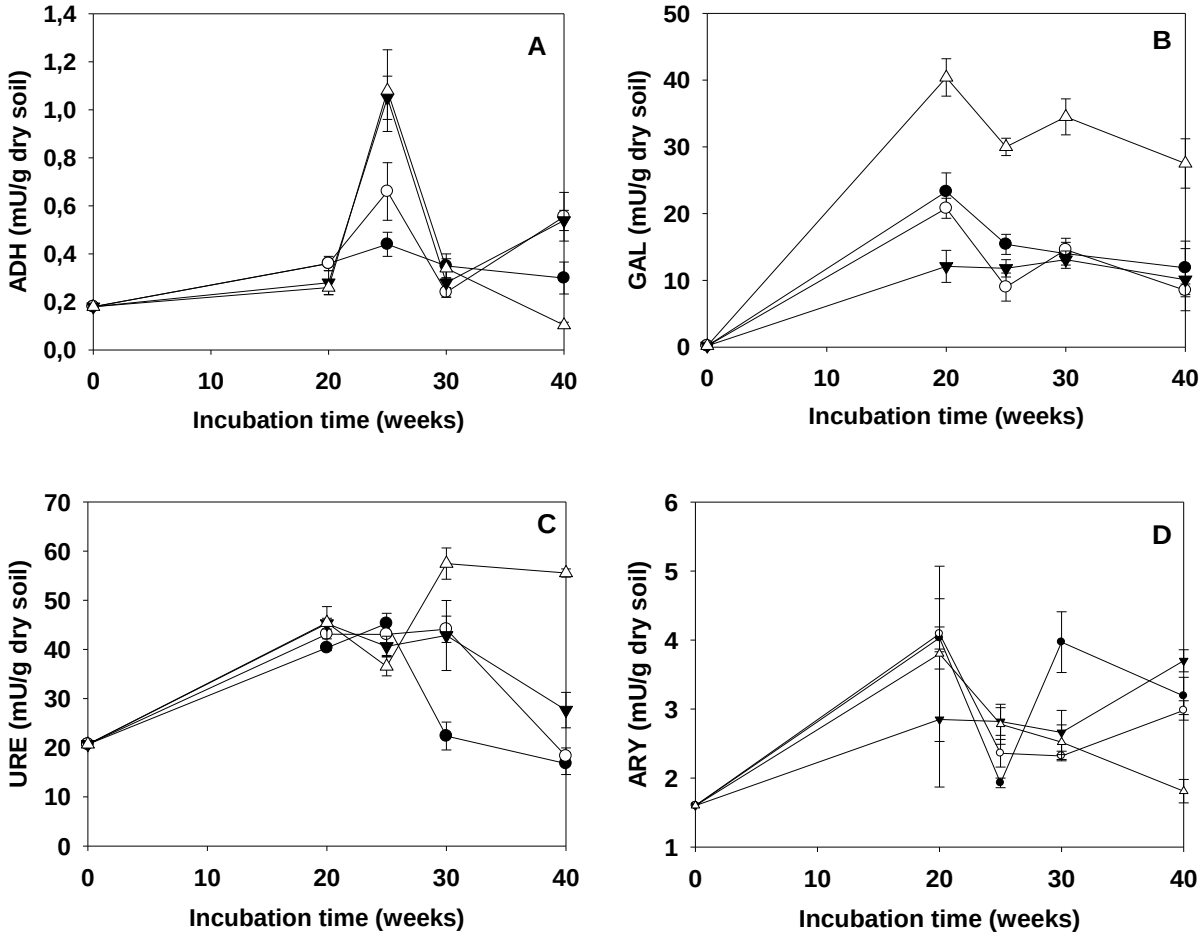

Figure 2. Laccase mRNA production (grey bars, gene AF414109) and laccase activity (black solid line) in liquid cultures of *T. versicolor* spiked with 2,5-dimethylaniline, with respect to inducer concentration (A, after 2 days of induction), and the length of exposure (B, inducer concentration of 100 µM).
Results exhibited good relationships of mRNA production and LAC activity with respect to inducer concentration and length of exposure.