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and
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Institute of Microbiology

PhD Course in Biochemical and Biological Evolution
(XXII cycle)

***In vivo and in vitro* degradation of aromatic contaminants by
white rot fungi. A case study: *Panus tigrinus* CBS 577.79**

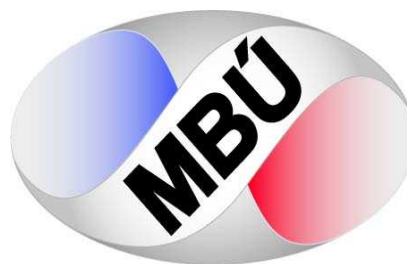
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Dipartimento Agrobiologia e Agrochimica

and

ACCADEMIA DELLE SCIENZE DELLA REPUBBLICA CECA

Istituto di Microbiologia

Corso di dottorato in Evoluzione Biologica e Biochimica
(XXII ciclo)

**Degradazione di contaminanti aromatici *in vivo* e *in vitro* ad opera
di funghi white rot. Il caso di *Panus tigrinus* CBS 577.79**

Settore Scientifico disciplinare: (Bio/19)

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1. INTRODUCTION

Since the industrial revolution, the great development of chemical, military and farming activities has led to the production and use of various chemicals of industrial synthesis. Plastics, plasticizers, fertilizers, herbicides, pesticides, as well as fuels and solvents turned out to be essential for the socio-economic development of industrialized countries. Due to the lack of environmental laws regulation, an enormous amount of these hazardous chemicals has been released into the environment. The fate of these compounds, alien to bioprocesses and thus to biodegradation (xenobiotics), has been the accumulation in the natural systems (air, water and soil) and in living organisms. Thus far, the protection of the natural equilibrium by developing environmentally sustainable industrial technologies on one side, and the remediation of historically polluted sites on the other side, have become major issues of concern.

1.1 Soil remediation

Focusing the attention on land contamination, a polluted site can be defined as an area where, due to past or present anthropic activities, a punctual alteration of the natural soil characteristics caused by polluting agents has been ascertained and therefore remedial activities are needed. In this meaning active or dismissed industrial sites, mining sites, scrap yards, waste treatment plants and dump sites are meant to be potential polluted sites. The number of these sites has been growing exponentially over the last years, especially in the industrialized countries: data about the year 2000 stated that in Italy there were around 10.000 potentially polluted sites in need for remedy, while the number reached almost 13.000 at the end of 2004.

Generally, environmental remediation deals with interventions pointing to the removal of the pollutants present in a given environmental matrix or, at least, to the reduction of their concentration. The conventional, rather antiquate remedial strategies consisted in *i*) excavation of the polluted layer of the soils which were successively buried in dump sites, and *ii*) coating of the polluted area with insulating barriers. The former resulted risky, especially during excavation procedures and movement to the dump site, while the latter just a temporary effective solution, causing expensive monitoring and maintenance.

Modern remedial approaches take aim at either the complete removal of the contaminants present in the soil, or at their transformation into lesser harmful substances, and can be divided in:

- *ex situ* treatments: the soil is dug out and transported to the treatment plant, which can be portable in loco (*on site*) or settled elsewhere (*off site*). Afterwards the soil is placed back.
- *in situ* treatments: the soil is treated directly in its natural state, with a variety of advantages with respect to *ex situ* treatments. Wider range of technologies available, better control of the remedial process and its evolution, shorter period of treatment and more homogeneous removal of the pollutants make *in situ* treatment preferable in many cases.

In addition to the former classification, mainly based on the site where the remedial actions take place, the diverse technologies currently available could be grouped into different kind of treatment.

1.1.1 Thermal treatments:

Generally make use of heat to separate, destroy, or immobilize contaminants. Thermal desorption and hot gas decontamination are separation technologies. Pyrolysis and conventional incineration destroy the contaminants. Vitrification destroys or separates organics and immobilizes some inorganics. The following technologies are considered the most effective:

- *Soil vapor extraction* (SVE) is an *in situ* unsaturated (vadose) zone soil remediation technology in which a vacuum is applied to the soil to induce the controlled flow of air and remove volatile (VOCs) and some semivolatile (SVOCs) contaminants from the soil. The gas leaving the soil may be treated to recover or destroy the contaminants, depending on local and state air-discharge regulations.
- *Hot Air/Steam Injection (in situ)* is often coupled with SVE; hot air or steam is injected below the contaminated zone to heat up the contaminated soil. The heating enhances the release of contaminants from the soil matrix. Some VOCs and SVOCs are stripped from the contaminated zone and brought to the surface through soil vapor extraction.
- *Radio Frequency/Electromagnetic Heating* Radio frequency heating (RFH) is an *in situ* process that uses electromagnetic energy to heat soil and enhance soil vapor extraction (SVE). RFH technique heats a discrete volume of soil using rows of vertical electrodes embedded in soil. The technique can heat the soils to over 300 °C.
- *Thermal desorption* is an *ex situ* treatment which removes harmful chemicals from the soil and other materials (like sludge and sediment) by using heat (90-650°C) to transform the chemicals into gases. These gases are collected with special equipment. The clean soil is

returned to the site. Thermal desorption is not the same as incineration, which uses heat to *destroy* the chemicals.

- *Vitrification* technology can be conducted *in situ* or *ex situ* and uses an electric current to melt the contaminated soil at high temperatures (1.600 to 2.000 °C). Upon cooling, the vitrification product is a chemically stable, leach-resistant, glass and crystalline material similar to obsidian or basalt rock. The high temperature component of the process destroys or removes organic materials. Radionuclides and most heavy metals are retained within the vitrified product.
- *Pyrolysis* is defined as chemical decomposition induced in organic materials by heat in the absence of oxygen. Pyrolysis typically occurs under pressure and at operating temperatures above 430°C. The pyrolysis gases require further treatment. The target contaminant groups for pyrolysis are SVOCs and pesticides. The process is applicable for the separation of organics from refinery wastes, coal tar wastes, wood-treating wastes, creosote-contaminated soils, hydrocarbon-contaminated soils, mixed (radioactive and hazardous) wastes, synthetic rubber processing wastes, and paint waste.
- *Incineration* is the process of burning (in presence of oxygen) hazardous materials to destroy harmful chemicals. Although it destroys a range of chemicals, such as PCBs, solvents and pesticides, incineration does not destroy metals. Generally incinerated soils cannot be placed back on site.

1.1.2 Physico-chemical treatments:

Physico-chemical soil treatment processes are mainly extraction processes. The extracting agent can be either air or aqueous solutions containing agents enhancing the performance (e.g. surfactants). The principle of *ex situ* treatments is soil excavation and subsequent concentration of the contaminants in a small residual fraction after separation, while *in situ* treatments aim at the leach out of the contaminants with a system of wells and pumps on the polluted area.

- *Soil venting* is an *in situ* technique relatively effective for VOCs in the vadose zone of the soil. Several injecting wells pump air into the polluted layer mobilizing and volatilizing pollutants; the air is then collected by other extracting wells and, often, by coupling soil vapour extraction systems. Air and gases are collected and treated by active carbon adsorption or incineration.
- *Soil washing* is an *ex situ* treatment mostly considered as a pre-treatment. Contaminants sorbed onto fine soil particles are separated from bulk soil in an aqueous-based system on

the basis of particle size. The wash water may be augmented with a basic leaching agent, surfactant, pH adjustment, or chelating agent to help remove organics and heavy metals. This approach is often coupled with other treatments like bioremediation or incineration.

- *Soil flushing* is the *in situ* extraction of contaminants from the soil with water or other suitable aqueous solutions (solvents, surfactants, acids and bases). Soil flushing is accomplished by passing the extraction fluid through in-place soils using an injection or infiltration process (wells). Extraction fluids must be recovered from the underlying aquifer and, when possible, recycled. As well as soil washing, this approach is often coupled with other treatments like bioremediation or active carbon sorption.
- *Dehalogenation* is a chemical process to remove halogens (usually chlorine) from a chemical contaminant (mainly PCBs, PCDDs, PCDFs), rendering it less hazardous. Glycolate dehalogenation makes use of a chemical reagent called APEG. APEG consists of two parts: an alkali metal hydroxide (the “A” in APEG) and polyethylene glycol (PEG), a substance similar to antifreeze. Sodium hydroxide and potassium hydroxide are two common alkali metal hydroxides. The process consists of mixing and heating the contaminated soils with the APEG reagent. During heating, the alkali metal hydroxide reacts with the halogen from the contaminant to form a non-toxic salt; and the PEG takes the location in the PCB molecule formerly occupied by the halogen making it less hazardous. At the end of the process the APEG reagent is extracted and recycled, the water phase is separated from the soil and treated appropriately and the remediated soil is brought back to its original location.
- *Solidification/Stabilization (S/S)* is a treatment technology for contaminated soils, either for clean up/remediation alone or as part of a brownfield redevelopment. Portland cement, often augmented with other materials, such as fly ash, lime kiln dust, cement kiln dust, and lime, is used as a binding reagent in S/S because of its ability to both solidify (change the physical properties) and stabilize (change the chemical properties) of a wide range of hazardous materials. Solidification increases the compressive strength, decreases the permeability, and encapsulates toxic elements. Stabilization converts hazardous elements into less soluble, mobile or toxic forms. Mixing the right combination of binding reagents into contaminated soils allows them to be either excavated and disposed of in a landfill, or re-used on site to support redevelopment.

1.1.3 Biological treatments (bioremediation)

Bioremediation is a grouping of technologies that use microbiota (typically, heterotrophic bacteria and fungi) to degrade or transform hazardous contaminants to materials such as carbon dioxide, water, inorganic salts, microbial biomass, and other byproducts that may be less hazardous than the parent materials. Biological treatment has been a major component for many years in the treatment of municipal and industrial wastewaters, but in recent years, biological mechanisms have been exploited to remediate contaminated ground water and soils (US-EPA, 1998; 2000).

During the late 1970s, 1980s, and early 1990s, bioremediation of any hazardous constituents or waste was considered innovative. Numerous applications of bioremediation are now widely accepted as a remedial alternative and are in wide use at sites contaminated with petroleum products and/or hazardous wastes. Some bioremediation technologies are still in development and should be considered innovative, while some others are still current topics of research. Since bioremediation technologies that fall into the innovative or research category have limited field implementation and effectiveness data, additional site assessment and treatability studies may be needed to confirm that a selected technology will be effective at a specific site.

As well as thermal or physico-chemical technologies, bioremediation techniques can be grouped in *in situ* or *ex situ* treatments, depending on the approach. The former aim at achieving an enhanced microbial activity towards pollutants without the need of excavating or handling the soil matter, the latter present higher costs and environmental risks for the digging out and moving processes, but generally are more effective with regard to highly persistent pollutants.

When *in situ* bioremediation is selected as a treatment, site-monitoring activities should demonstrate that biologically mediated removal is the primary route of contaminant removal. Sampling strategies should consider appropriate analyses and tests, as well as site heterogeneity. In some cases, extensive sampling may be required to distinguish bioremediation from other removal mechanisms or statistical variation. Small-scale treatability studies using samples from the contaminated site may also be useful in demonstrating the role that the biological activity plays in contaminant removal (US-EPA, 1998; 2000). The most common *in situ* treatment are:

- *Intrinsic in situ bioremediation* (or *Natural Attenuation, NA*) relies on natural processes to degrade contaminants without altering current conditions or adding amendments. Dilution, dispersion, sorption, volatilization, chemical reactions such as oxidation and reduction, biological reactions, and stabilization are the processes involved in NA of polluted sites.

This technique is considered suitable and most appropriate in case of subsurface plumes which are considered stable.

- In *Aerobic Bioventing*, contaminated unsaturated soils with low oxygen concentrations are treated by supplying oxygen to facilitate aerobic microbial biodegradation. Oxygen is typically introduced by air injection wells that push air into the subsurface; vacuum extraction wells, which draw air through the subsurface, may also be used. Extracted gases may require treatment since volatile compounds may be removed from the ground. Compared with soil vapor extraction (SVE), bioventing employs lower air flow rates that provide only the amount of oxygen required to enhance removal. Operated properly, the injection of air does not result in the release of the contaminants to the atmosphere through volatilization because of the low flow rates. Bioventing is designed primarily to treat aerobically biodegradable contaminants, such as non-chlorinated VOCs and SVOCs (e.g., petroleum hydrocarbons), that are located in the vadose zone or capillary fringe.
- *Cometabolic Bioventing* is a rather new technique which was successfully used to treat sites polluted with chlorinated solvents (trichloroethane and dichloroethene); similar to aerobic bioventing, it exploits competitive reactions mediated by monooxygenase enzymes (US-EPA, 2000). Bacterial monooxygenases catalyze the oxidation of hydrocarbons, often through epoxide intermediates, but these enzymes can also catalyze the dechlorination of chlorinated hydrocarbons. Thus, by supplying an appropriate organic substrate (propane) and air, cometabolic bioventing can elicit the production of monooxygenases, which consume the organic substrate and facilitate contaminant degradation (AFCEE, 1996; US-EPA, 1998).
- *Anaerobic Bioventing* has been found to be effective towards some chlorinated species which are not effectively treated aerobically. Microbes may degrade these contaminants directly via anaerobic reductive dechlorination or through anaerobic cometabolic pathways. Anaerobic reductive dechlorination is a biological mechanism typically marked by sequential removal of chlorine from a molecule. Microbes possessing this pathway do not gain energy from this process. Anaerobic cometabolism is similar to aerobic cometabolism in that microbes fortuitously degrade contaminants while reducing other compounds (cometabolites). Anaerobic bioventing uses the same type of gas delivery system as the other bioventing technologies, but injects nitrogen and an electron donor, instead of air, to establish reductive anaerobic conditions. The nitrogen displaces the soil oxygen, and small amounts of an electron donor gas (such as hydrogen and carbon dioxide) produce reducing conditions in the subsurface, thereby facilitating microbial dechlorination. This process may

be useful in treating highly chlorinated compounds such as tetrachloroethene (TCE), pentachlorophenol (PCP), and pesticides such as lindane and dichlorodiphenyl-trichloroethane (DDT).

- *Biosparging* involves the injection of gas (mainly air or oxygen and occasionally gas-phase nutrients), under pressure, into the saturated zone to promote aerobic degradation. The injection of gases below the water table distinguishes biosparging from bioventing. Typically, biosparging is achieved by injecting air into a contaminated subsurface formation through a specially designed series of injection wells. The air creates an inverted cone of partially aerated soils surrounding the injection point. The air displaces pore water, volatilizes contaminants, and exits the saturated zone into the unsaturated one. While in contact with ground water, oxygen dissolution from the air into the ground water is facilitated and supports aerobic biodegradation. The method is often coupled with soil vapor extraction to avoid losses. A number of contaminants have been successfully addressed with biosparging technology, including gasoline components such as benzene, toluene, ethylbenzene, and xylenes (BTEX) and SVOCs (US-EPA 2004).
- *Bioslurping* (also known as multi-phase extraction) is effective in removing free product that is floating on the water table (Battelle, 1997). Bioslurping combines the two remedial approaches of bioventing and vacuum-enhanced free-product recovery. Bioventing stimulates aerobic bioremediation of contaminated soils *in situ*, while vacuum-enhanced free-product recovery extracts light, nonaqueous-phase liquids (LNAPLs) from the capillary fringe and the water table. Bioslurping is limited to 7-8 meters below ground surface as contaminants cannot be lifted more than that by this method. A bioslurping tube with adjustable height is lowered into a ground water well and installed within a screened portion at the water table. A vacuum is applied to the bioslurping tube and free product is “slurped” up the tube into a trap or oil water separator for further treatment. Removal of the LNAPL results in a decline in the LNAPL elevation, which in turn promotes LNAPL flow from outlying areas toward the bioslurping well. As the fluid level in the bioslurping well declines in response to vacuum extraction of LNAPL, the bioslurping tube also begins to extract vapors from the unsaturated zone. This vapor extraction promotes soil gas movement, which in turn increases aeration and enhances aerobic biodegradation (Miller, 1996).

Processes consisting of removing contaminated materials for treatment are common to the *ex situ* remediation technologies. Contaminated media are excavated or extracted (*e.g.*, ground water removal by pumping) and moved to the process location, which may be within (*on site*) or adjacent

to the contamination zone (*off site*). The following techniques represent the most effective ex situ treatment currently available:

- *Landfarming* is useful in treating aerobically degradable contaminants. This process is suitable for non-volatile contaminants at sites where large areas for treatment cells are available. Land treatment of site-contaminated soil usually entails the tilling of 30-40 cm layer of the soil to promote aerobic biodegradation of organic contaminants. The soils are periodically tilled to be aerated, and moisture is added when needed. In some cases, amendments may be added to improve the tilth of the soil, supply nutrients, moderate pH, or facilitate bioremediation. Typically, full-scale land treatment would be conducted in a prepared-bed land treatment unit, an open, shallow reactor with an impermeable lining on the bottom and sides to contain leachate, control runoff, and minimize erosion and with a leachate collection system under the soil layer (US-EPA, 1993). The performance of land treatment varies with the contaminants to be treated. For easily biodegradable contaminants, such as fuels, land treatment is inexpensive and effective. Contaminants that are difficult to degrade, such as PAHs, pesticides, or chlorinated organic compounds, are topics of research and would require site-specific treatability testing.
- *Composting* is a controlled biological process that treats organic contaminants using microorganisms under thermophilic conditions (40°–50°C). For some practitioners, the creation of thermophilic conditions is the primary distinction between composting and biopiles (which operate at less than 40°C). In composting, soils are excavated and mixed with bulking agents and organic amendments, such as wood chips and vegetative wastes, to enhance the porosity of the mixture to be decomposed. Degradation of the bulking agent heats up the compost, creating thermophilic conditions. Oxygen content, moisture levels, and temperatures are monitored and manipulated to optimize degradation. Oxygen content usually is maintained by frequent mixing, such as daily or weekly turning of windrows. Surface irrigation often is used to maintain moisture content. Temperatures are controlled, to a degree, by mixing, irrigation, and air flow, but are also dependent on the degradability of the bulk material and ambient conditions. Composting has been successfully applied to soils and biosolids contaminated with petroleum hydrocarbons (*e.g.*, fuels, oil, grease), solvents, chlorophenols, pesticides, herbicides, PAHs, and nitro-aromatic explosives (US-EPA, 1998; 1997; 2004).
- *Biopiles* involve the mixing of excavated soils with soil amendments, with the mixture placed in a treatment area that typically includes an impermeable liner, a leachate collection system, and an aeration system. Biopiles are typically 2–3 meters high, and contaminated

soil is often placed on top of treated soil. Moisture, nutrients, heat, pH, and oxygen are controlled to enhance biodegradation. This technology is most often applied to readily degradable species, such as petroleum contaminants. Surface drainage and moisture from the leachate collection system are accumulated, and they may be treated and then recycled to the contaminated soil. Nutrients (*e.g.*, nitrogen and phosphorus) are often added to the recycled water. Alkaline or acidic substances may also be added to the recycled water to modify or stabilize pH to optimize the growth of select microbes capable of degrading the contaminants of concern. The air distribution system is buried in the soil as the biopile is constructed. Oxygen exchange can be achieved utilizing vacuum, forced air, or even natural draft air flow. Low air flow rates are desirable to minimize contaminant volatilization. If volatile constituents are present in significant concentrations, the biopile may require a cover and treatment of the offgas.

- *Slurry bioreactors* are utilized for soil, sediments, sludge, and other solid or semi-solid wastes. Slurry bioreactors are costly and, thus, are likely to be used for more difficult treatment efforts.

Typically, wastes are screened to remove debris and other large objects, then mixed with water in a tank or other vessel until solids are suspended in the liquid phase. Suspension and mixing of the solids may increase mass transfer rates and may increase contact between contaminants and microbes capable of degrading those contaminants (US-EPA, 1990). Mixing occurs in tanks or lined lagoons. Mechanical mixing is generally conducted in tanks. Typical slurries are 10–30% solids by weight. Aeration, with submerged aerators or spargers, is frequently used in lagoons and may be combined with mechanical mixing to achieve the desired results. Nutrients and other additives, such as neutralizing agents, surfactants, dispersants, and co-metabolites (*e.g.*, phenol, pyrene) may be supplied to improve handling characteristics and microbial degradation rates. Indigenous microbes may be used or microorganisms may be added initially to seed the bioreactor or may be added continuously to maintain proper biomass levels. Residence time in the bioreactor varies with the matrix as well as the type and concentration of contaminant (US-EPA, 1990).

Once contaminant concentrations reach desired levels on a dry-weight basis, the slurry is dewatered. Typically, a clarifier is utilized to dewater the slurry by gravity and the dry matter is separated.

In conclusion, *in situ* and *ex situ* biodegradation technologies are increasingly selected to remediate contaminated sites, either alone or in combination with other source control measures.

Bioremediation technologies have proven effective in remediating fuels and VOCs and are often able to address diverse organic contaminants including SVOCs, PAHs, chlorinated aromatic hydrocarbons (CAHs), pesticides, herbicides, and nitro-aromatic compounds (such as explosives), potentially at lower cost than other remediation options. Some bioremediation techniques are also able to address heavy metal contamination. A unique feature of biological treatment is the diversity of its application to solids, liquids, and liquid–solid mixtures, involving both *in situ* and *ex situ* environments. Amendments may be necessary to support or to enhance the biodegradation processes to improve the timeframe involved to achieve cleanup goals. Site characterization and long-term monitoring are necessary to support system design and sizing as well as to verify continued performance. There are also regulatory requirements to be addressed regarding system design, implementation, operation, and performance, including the disposition of liquid effluents and other wastes resulting from the treatment process. Bioremediation continues to be an active area of research, development, and demonstration for its applications to diverse contaminated environments.

1.2. Mycoremediation

A specific branch of bioremediation, called “mycoremediation”, has been gaining increasing interest in recent years: its name is due to the use of fungi for the remediation of polluted soils or other solid-liquid matrices.

With this respect, particularly promising appears to be the use of “white rot” fungi, an ecologically distinct group specialised in lignin breakdown. In addition to their natural substrate these fungi have been shown to degrade and, to some extent mineralize, a wide range of organic and xenobiotic pollutants such as petroleum hydrocarbons, chlorophenols, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and furans, pesticides, herbicides and nitroaromatic explosives (Alexander, 1994; Juasz and Naidu, 2000; Rabinovich *et al.*, 2004). An important characteristic, which distinguishes filamentous fungi from bacteria and makes them excellent candidates for soil bioremediation strategies, is the penetration of the fungal hyphae into the polluted matrix and the excretion of oxidative enzymes. These oxidases, mainly laccase, lignin peroxidase and manganese peroxidase, present very low substrate specificity and, being active in the extracellular environment, are able to attack scarcely bioavailable contaminants.

Over the past years it has been documented that the degradation mechanisms of white rot fungi are co-metabolic. In spite of their extraordinary degrading capabilities, fungi cannot use pollutants

to produce energy as well as bacteria do: they need an alternative carbon source. For this reason lignocellulosic residues are used as amendants to improve mycoremediation extents.

Furthermore, it is well known that fungi are involved in soil humification process: with this respect, the use of these organisms in soil remediation could lead, not only to the decontamination, but also to the re-use of the soil for agricultural purposes.

1.2.1. White rot fungi

“White rot” fungi are considered to be the most efficient lignin degraders in nature. Their name derives from the appearance of wood attacked by these organisms, in which lignin removal results in a bleached form.

Lignin is a complex polyphenolic polymer presenting highly heterogeneous structures and macromolecular features. These characteristics make this polymer particularly resistant to degradation by intracellular enzymatic systems. White rot fungi have been developing an extracellular, radical based degradation machinery with very low substrate specificity able to breakdown the lignin polymer. Ligninolysis *per se* does not support fungal growth, but is rather intended to open up the structure of woody materials so that polysaccharide-degrading agents can penetrate (Hammel, 1995). Indeed, this extracellular system is generally triggered by nutritional stresses, like N- or C-deficiency, even though there are exception to this observations (Hammel, 1995; Mester *et al.*, 1996).

Due to the non-specificity of their ligninolytic enzymes, white rot fungi have been shown to degrade a range of persistent aromatic pollutants with structural similarities to lignin, such as polycyclic aromatic hydrocarbons, chlorophenols, chloroanilines, polychlorinated biphenyls, dioxins, furans, nitroaromatic explosives, herbicides and pesticides (Pointing, 2001; Rabinovich *et al.*, 2004). In some cases significant mineralization of the contaminants was obtained (Hammel, 1995).

In addition to the extracellular degradative system, white rot fungi possess also an endocellular one involving cytochrome P-450 monooxygenase-epoxide hydrolase. This intracellular pathway is present in all eukaryotic organisms, in which regulates mainly the bioconversion of hormones and the detoxification of drugs and xenobiotics (Bernhardt, 2006). In wood rotting fungi cytochrome P-450 is supposed to cooperate with the ligninolytic system in the general mechanism of xenobiotic degradation (Bezalel *et al.*, 1996; Van den Brink *et al.*, 1998).

1.2.2. Ligninolytic enzymes produced by white rot fungi

White rot fungi variously secrete one or more of three main extracellular lignin degrading enzymes, lignin peroxidase (LiP, E.C. 1.11.1.14), Mn-dependent peroxidase (MnP, E.C. 1.11.1.13), and laccase (Lac, E.C. 1.10.3.2) (Tuor *et al.*, 1995). Some authors also report a novel Mn-independent MnP activity and other versatile peroxidase activities in some white rot fungi (Heinfling *et al.*, 1998; Camarero *et al.*, 1999; Ruiz-Duenas *et al.*, 2001).

In addition, the following enzymes are associated with lignin degrading enzymes in lignin breakdown, but are unable to degrade lignin alone: glyoxal oxidase (E.C. 1.2.3.5), superoxide dismutase (E.C. 1.15.1.1), glucose oxidase (E.C. 1.1.3.4), aryl alcohol oxidase (E.C. 1.1.3.7), and cellobiose dehydrogenase (E.C. 1.1.99.18). They produce H₂O₂ required by peroxidases (LiP and MnP) or serve to link lignocellulose degradation pathways (Leonowicz *et al.*, 2001).

Ligninolytic enzyme production by white rot fungi occurs during the secondary metabolism. All three major ligninolytic enzymes are encoded by gene families that allow production of multiple enzyme isoforms (Thurston, 1994; Tuor *et al.*, 1995; Martinez, 2002). In *Phanerochaete chrysosporium* cultures the lignin degrading system appears in response to nitrogen, carbohydrate, and sulfur starvation and the balance of trace metals, Mg²⁺ and Ca²⁺, is also important for the ligninolytic activity of the fungal cultures (Jeffries *et al.*, 1981). In contrast, in *Bjerkandera* sp. strain BOS55 the production of lignin degrading peroxidases was significantly improved in N-sufficient conditions (Mester *et al.*, 1996). Also growth conditions such as temperature and agitation of fungal cultures significantly affect the appearance and levels of activity of ligninolytic enzymes in white rot fungi (Vyas *et al.*, 1994; Darah and Ibrahim, 1996; Podgornik *et al.*, 2001). Additionally, recent research has shown that ligninolytic enzyme production by white rot fungi is also affected by mediator compounds, various other chemicals and required-metal (Mn²⁺, Cu²⁺) concentrations (Dittmer *et al.*, 1997; Scheel *et al.*, 2000; Galhaup *et al.*, 2002).

1.2.2.1. Lignin peroxidase

Lignin peroxidase (LiP, E.C. 1.11.1.14) is a glycosylated heme-containing peroxidase with a molecular mass of 40-45 kDa. In the presence of endogenously generated hydrogen peroxide LiP catalyzes oxidation of non-phenolic aromatic structures in lignin and generates aryl cation radicals.

LiP is secreted by the white rot fungi extracellularly, mostly at the onset of secondary metabolism, triggered by nitrogen limitation, *e.g.* in *P. chrysosporium* (Tien and Kirk, 1988). However, it can also be produced in nitrogen-rich conditions (Collins and Dobson, 1995). LiP

production in fungal cultures requires high oxygen levels but is suppressed by agitation of fungal cultures (Faison and Kirk, 1985). The production of LiP in agitated cultures of *P. chrysosporium* can be improved by the combination of a shift of agitation rate from a higher to a lower speed after the onset of secondary metabolism, a simultaneous decrease of the incubation temperature and a supply of dimethoxybenzylamine (Liebeskind *et al.*, 1990). Additionally, it was shown that LiP production is regulated by manganese and that manganese regulation is independent of nitrogen regulation in LiP and MnP production (Reddy and Dsouza, 1994; Hamman *et al.*, 1999). LiP production is completely suppressed in the presence of high Mn levels (Reddy and Dsouza, 1994). In contrast, Mn deficiency can replace high oxygen levels needed for LiP formation in *P. chrysosporium* cultures (Rothschild *et al.*, 1999).

During its catalytic cycle (Figure 1) LiP is oxidized by H_2O_2 to form a two-electron intermediate (compound I) which oxidizes substrates by removing one electron and produces a more reduced enzyme intermediate (compound II). This intermediate can then oxidize substrates by one electron, returning enzyme to its initial state. However, compound II has a very high reactivity with H_2O_2 , therefore in the presence of a poor substrate and excess H_2O_2 , it is instead converted to an inactive form of the enzyme (compound III) (Wariishi and Gold, 1989).

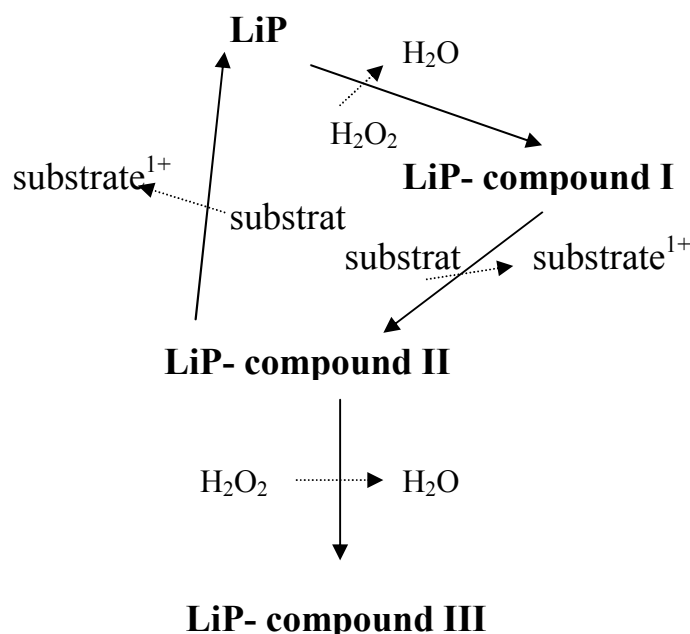


Figure 1: The catalytic cycle of fungal LiP

Compounds such as veratryl alcohol and tryptophane have been shown to have a protective effect against the enzyme inactivation by excesses of H_2O_2 (Collins *et al.*, 1997). When present, they are more favorable substrates for compound II and convert it into the resting enzyme, completing the catalytic cycle. An additional role for veratryl alcohol and tryptophane as diffusible mediators in the LiP-catalyzed oxidation of environmental contaminants has been proposed (Paszczyński and Crawford, 1991; Collins *et al.*, 1997).

1.2.2.2. Manganese peroxidase

Mn-dependent peroxidase (MnP, E.C. 1.11.1.13) is an extracellular heme-containing peroxidase that catalyzes an H_2O_2 -dependent oxidation of Mn^{2+} to highly reactive Mn^{3+} , which is stabilized by fungal chelators such as oxalic acid, and oxidizes phenolic components of lignin resulting in the formation of free radicals.

MnP is often produced in multiple isoforms exhibiting a molecular mass of 45-55 kDa (Lobos *et al.*, 1994; de la Rubia *et al.*, 2002; Martinez, 2002). These isoforms differ mostly in their isoelectric points, which are usually rather acidic (pH 3-4) (Palma *et al.*, 2000; Ha *et al.*, 2001). The expression of MnP in fungal cultures is regulated at the level of gene transcription by hydrogen peroxide and various chemicals including ethanol, 2,4-dichlorophenol as well as by the Mn^{2+} concentration and heat shock (Li *et al.*, 1995; Gettemy *et al.*, 1998; Scheel *et al.*, 2000).

The catalytic cycle of MnP (Figure 2) is similar to that of other heme peroxidases like LiP. It includes the native ferric enzyme as well as the reactive intermediates (compound I and compound II) (Hofrichter, 2002). In contrast to other peroxidases, MnP uses Mn^{2+} as the preferred substrate. The cycle is initiated by binding of H_2O_2 to the native ferric enzyme and a formation of an iron-peroxide complex (Hofrichter, 2002). The subsequent cleavage of the peroxide oxygen-oxygen bond requires a two-electron transfer from the heme resulting in a formation of MnP compound I. Afterwards one water molecule is expelled. A subsequent reduction proceeds through MnP compound II. A monochelated Mn^{2+} ion acts as the one-electron donor for this enzyme intermediate and is oxidized to Mn^{3+} . The reduction of MnP compound II proceeds in a similar way and another Mn^{3+} is formed from Mn^{2+} leading to generation of the native enzyme and release of the second molecule of water (Hofrichter, 2002).

Whereas MnP compound I resembles that of LiP and can, besides Mn^{2+} , be reduced by other electron donors (ferrocyanide, phenolics), MnP compound II is reduced by other substrates only very slowly and requires Mn^{2+} to complete the catalytic cycle. The Mn^{3+} formed during the cycle is stabilized by carboxylic acids (oxalate, malonate, malate, tartrate or lactate). The chelates of Mn^{3+}

cause one-electron oxidations of various substrates. MnP is, similarly to LiP, sensitive to high concentrations of hydrogen peroxide that cause reversible inactivation of the enzyme by forming MnP compound III.

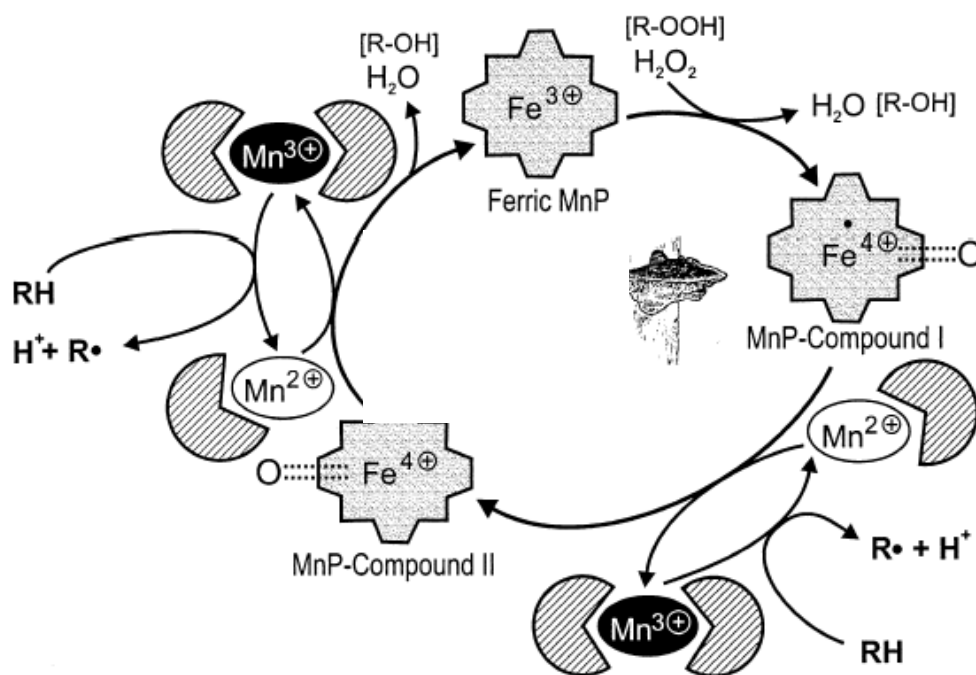


Figure 2: The catalytic cycle of fungal MnP (Hofrichter, 2002)

1.2.2.3. Laccase

Fungal laccases (Lac, E.C. 1.10.3.2; benzendiol:oxygen oxidoreductases) are copper-containing phenoloxidases. They belong to the group of blue copper oxidases which catalyze a four electron reduction of oxygen to water. Fungal laccases contain four copper atoms (all in 2+ oxidation state) in the molecule distributed among three different binding sites. These copper ions play an important role in the catalytic mechanism of the enzyme. In addition, the copper binding sites constituted of one cysteine and ten histidine residues are well conserved in fungal laccase sequences (Thurston, 1994).

Laccases are remarkably non-specific. They oxidize different compounds as a reducing substrate, e.g. phenols, polyphenols, and aromatic amines as well as non-phenolic organic substrates, by one-

electron abstractions resulting in the formation of reactive radicals undergoing further depolymerization, repolymerization, demethylation, or quinone formation. A rather broad substrate spectrum of laccases can additionally be extended by the addition of small molecular redox mediators (Bourbonnais *et al.*, 1995; Johannes and Majcherczyk, 2000).

Additionally to lignin degradation, fungal laccases are also involved in several other processes such as the development of fungal fruit bodies, pigmentation, pathogenicity, and sexual differentiation (Thurston, 1994; Leonowicz *et al.*, 2001).

Laccases are known to be produced by many fungi in multiple isoforms with a typical molecular mass of 60-80 kDa (Thurston, 1994). Laccases from white rot fungi can be intracellular or extracellular and are secreted mostly into the culture media. The research of laccases in other fungi suggested also the presence of laccases associated with fungal cell wall (Zhu *et al.*, 2001).

The production of laccase activity by white rot fungi can be improved by the addition of Cu^{2+} which regulates laccase production at the level of gene transcription (Palmieri *et al.*, 2000; Galhaup and Haltrich, 2001; Saparrat *et al.*, 2002). Several other chemicals such as 2,5-xylidine, veratryl alcohol, and guaiacol have also an inducing effect on laccase production (Eggert *et al.*, 1996; Quarantino *et al.*, 2007).

1.2.2.4. Versatile peroxidase

Versatile peroxidase (VP) has been discovered in *Pleurotus* and *Bjerkandera* species (Martinez *et al.*, 1996; Mester and Field 1998). VP is able to oxidize both LiP and MnP substrates and therefore can be considered an hybrid between the two enzymes. It has high affinity for Mn^{+2} , hydroquinone and dyes, and oxidizes also veratryl alcohol, dimethoxybenzene and lignin dimers. However, its catalytic efficiency is much higher in presence of Mn^{+2} than in presence of other aromatic substrates (Heinfling *et al.*, 1998). Its optimal pH for oxidation of Mn^{+2} (pH 5) and aromatic compounds or dyes (pH 3) differ, being similar to those of optimal MnP and LiP activity (Ruiz-Duenas *et al.*, 2001). A non-competitive inhibition was proposed for both substrates, which means that VP has, at least, two binding sites (Heinfling *et al.*, 1998; Martinez, 2002).

1.2.3. Production patterns and cooperation of ligninolytic enzymes

White rot fungi produce extracellular lignin degrading enzymes, the best characterized of which are lignin peroxidase, MnP, and laccase. According to the production patterns of the above three

enzymes, white rot fungi could be divided into three main groups, although overlaps and exceptions occur (Hatakka 1994).

The first group, LiP-MnP group, is represented by the fungi producing mainly LiP and MnP such as *P. chrysosporium*. The two other groups are: white rot fungi producing MnP and laccase (e.g., *Dichomitus squalens*, *Ceriporiopsis subvermispora*, *Pleurotus ostreatus*, *Lentinus edodes* and *Panus tigrinus*) and white rot fungi producing LiP and laccase (e.g., *Phlebia ochraceofulva*).

LiP, MnP, and laccase are highly non-specific with regard to their substrate range. In lignin degradation they act synergistically. LiP catalyzes oxidations in the alkyl side chains of lignin subunits to give benzoic aldehydes. Lignin degraded by LiP thus provides substrates for laccases (Leonowicz *et al.*, 2001). MnP cooperation with laccase has been shown to be important for the primary attack on lignin by the fungus *Rigidoporus lignosus* (Galliano *et al.*, 1991). Generally, fungi which do not express LiP produce MnP with features similar to LiP, the so called “hybrid MnP” (Mester and Field, 1998). Recently, laccase from *Stropharia rugosoannulata* (litter decomposing fungus) has been shown to oxidize Mn^{2+} to Mn^{3+} in the presence of Mn chelators leading to the production of hydrogen peroxide. The results demonstrated a role of laccase in providing H_2O_2 for MnP reactions (Schlosser and Hofer, 2002).

1.3 The white rot fungus *Panus tigrinus*

First studies of biochemistry, enzymology, and biodegradation capacity of white rot fungi were strongly concentrated on the fungus *P. chrysosporium* (Reddy, 1995; Tien and Kirk, 1988). However, other white rot fungi were found capable of xenobiotic degradation although differing in their ligninolytic enzyme pattern and enzyme regulation (Hatakka, 1994; Palma *et al.*, 2000).

P. tigrinus is a white rot fungus able to perform selective degradation of lignin in both woody and non-woody plants (Costa *et al.*, 2002). This interesting capability has suggested the use of this organism in biopulping techniques (Goncalves *et al.*, 2002). Moreover, *P. tigrinus* showed to be highly efficient in chlorophenol degradation: both fungal cultures, in which endogenous laccase mediators are produced, and its extracellular laccases were able to eliminate up to 0.4 g l^{-1} of di- and tri-chlorophenols from liquid medium or wastewaters (Rabinovic *et al.*, 2004). In addition, Nazareth and Sampy (2003) showed the ability of this basidiomycete to decolourize textile dyes.

The most of the informations available on *P. tigrinus* ligninolytic system belong to the strain 8/18 (Quaratino *et al.*, 2006). It has been shown that this strain, in both submerged and solid-state fermentation, produces high amount of laccase and manganese-dependent peroxidase (MnP), the latter being the main lignin-degrading enzyme (Leontievsky and Golovleva, 1990; Leontievsky *et*

al., 1994; Pozdnyakova *et al.*, 1999). In contrast to an earlier report indicating the presence of LiP (Kirk and Farrell, 1987), Maltseva and co-workers (1991) reported that the ligninolytic system of *P. tigrinus* is composed of MnP and laccase, but no LiP. Moreover, in an early report the MnP of *P. tigrinus* 8/18 was described as a common MnP, the molecular features of the enzyme and Mn-dependent reactions were described, and its ability to oxidize NADH following the oxidase pathway (without intermediate hydrogen peroxide) was demonstrated (Leontievsky *et al.*, 1990). In a successive work, (Lisov *et al.*, 2003) comprehensive study of the enzyme showed that the MnP of *P. tigrinus* 8/18 should be assigned to hybrid-MnPs.

The strain CBS 577.79 of *P. tigrinus* was shown to be an active producer of both laccase and MnP (Fenice *et al.*, 2003). Moreover, when grown on an agro-industrial effluent (olive-mill wastewaters, OMW), the same strain was able to both tolerate organic loads as high as 60 g l⁻¹ and to perform significant dephenolization and decolorization of the waste (D'Annibale *et al.* 2004). Differently from the strain 8/18, laccase is the predominant ligninolytic enzyme produced by the strain CBS 577.79 in both solid-state and liquid cultures (Quaratino *et al.*, 2007). With this respect, the major laccase isoenzyme produced by the fungus was purified and its main physicochemical and biochemical properties were characterized (Quaratino *et al.*, 2007). On the contrary, high MnP titres were produced in a low-N medium (1 mM of N as ammonium sulphate) whose composition was optimized for MnP production (Quaratino *et al.*, 2006). The same authors observed that, regardless of the cultural conditions, the onset of MnP activity always occurred in concomitance with nitrogen depletion.



Figure 1.3. Picture of *Panus tigrinus* fruit body.

1.4. Aim of the thesis work

Due to the above mentioned promising characteristics of the strain CBS 577.79 of *P. tigrinus*, the main aim of the present Ph.D project was to investigate its degradation capabilities in defined liquid media towards different classes of ubiquitous aromatic contaminants: polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorobenzoic acids (CBAs) and endocrine disrupting compounds (EDCs). Furthermore, in order to gain more information on its degradation machinery, the kinetic and redox properties of its major MnP isoenzyme (MnP II) were characterized. The isoenzyme in question along with a purified laccase from the same strain were exploited for *in vitro* degradation experiments of the above-cited classes of pollutants both in the presence and in the absence of redox mediators.

Additional goal of this Ph.D thesis was to assess the effectiveness of allochthonous white rot fungi in lab-scale mycoremediation trials of PAH-polluted environmental matrices (soils and lignocellulosic materials). All experiments were carried out under non-sterile conditions in order to assess the ability of the tested fungi to compete with the indigenous microbiota and colonize the matrix under study. The attention was focused on the effect of the inoculum lignocellulosic carrier on both growth and PAH-degradation performances due to the expected protective/trophic effects of these carriers and to the known co-metabolic nature of the degradation process in the specialized ecological group of white-rot fungi. An additional focus was an assessment of the impact of the current bioavailability of contaminants which is known to regulate the mass transfer rate of contaminants from the solid phase to the aqueous one. To this aim, two matrices derived from a wood treatment facility (*i.e.*, a historically contaminated soil and creosote-impregnated shavings) were selected and degradation data referred to the amount of the respective bioavailable fractions. The bioavailability issue was also faced by evaluating the impact of different surfactants (*i.e.*, Tweens and plant oils) on the PAH degradation ability of fungal strains that had been previously isolated from a historically contaminated site. Such assessment also regarded the impact of different fungus/surfactants application on the resident microbiota by using both cultivation-dependent (*i.e.* microbial counts) and molecular approaches (Denaturing gradient gel electrophoresis analysis of 16S DNA).

In the majority of cases, the outcomes of either fungal or enzymatic treatments were assessed from a toxicological viewpoint by using either bioluminescence (*i.e.*, *Vibrio fischeri*) or contact tests (*e.g.*, germinability of higher plants, *Collembola* mortality) or, in the case of EDCs, by determining their residual estrogenic activities.

2. IN VIVO AND IN VITRO PAH DEGRADATION BY *Panus tigrinus* CBS

577.79

In this chapter, the attention will be focused on polycyclic aromatic hydrocarbons (PAHs), a class of more than 200 compounds considered of great environmental relevance. Due to their frequent occurrence in the environment and their toxicological properties, 16 non-substituted PAHs have been included in the list of “priority pollutants” by the *Environmental Protection Agency of the United States* (US-EPA) and *World Health Organization* (WHO).

2.1 Polycyclic aromatic hydrocarbons (PAHs)

Recalcitrant compounds are a major hazard for the environment and, in many cases, they constitute risk to human and animal health. Special attention has been focused on contaminants, which, in virtue of their low aqueous solubility, are highly persistent. Among them, PAHs have aroused significant concern due to their toxicity and, for some members, due to carcinogenicity. Because of the increased consumption of fossil fuels, their occurrence in the environment has steadily increased since last 100 to 150 years (Cerniglia, 1992).

2.1.1 Sources and contamination:

The main sources of PAHs are both natural and anthropogenic. In the natural environment, one of their origin is related to pyrolysis of organic matter (*i.e.*, wood and biomasses) at high temperature. Another natural process causing the formation of PAHs is diagenesis, namely the heating of organic materials occurring at relatively low temperatures (100-150 °C) over an extended period of time. In this respect, fossil fuels arising from this process, like crude oil and coal, exhibit high content of PAHs.

The anthropogenic source is becoming more significant with increasing industrialization. The most important sources ascribable to human activities are associated with industrial processes like petroleum and carbon manufactures, wood treatment plants, power plants, municipal incinerators and many others activities in which combustion of fuels or organic materials is included. PAH contaminated sites are commonly associated with dismissed or active industrial plants, where accidental spills or leaks from storage tanks occurred. However, domestic sources of PAHs, like domestic heating, automobile exhaust fumes, and smoked food are considered relevant.

Soils can be polluted by PAHs at concentrations widely ranging from 1 µg/kg to 300 g/kg depending on the contamination source. The highest PAH levels have been so far detected in coal gasification sites (Bamfort and Singleton, 2005). Background levels of PAHs in the air are reported to be 0.02-1.2 mg/m³ in rural areas and 0.15-19.2 mg/m³ in urban areas. Background levels of PAHs in drinking water range from 4 to 24 ng/l (ATDSR 1995).

2.1.2 Physical and chemical properties:

PAHs are chemical compounds consisting of two or more benzene rings, fused together in different arrangements. In case an alkyl group or another radical is linked to the aromatic ring they are called “PAH derivatives”, while “heterocyclic aromatic compounds” are PAHs in which a carbon atom is replaced by nitrogen, oxygen or sulphur (*e.g.*, dibenzothiophene).

The main physico-chemical properties of PAHs are reported in Table 2.1.

Table 2.1. Physical properties of representative PAHs.

Compound	(*)	Molecular weight	log K _{ow} [†]	Water solubility (mg l ⁻¹)	Melting point (°C)	IP‡ (eV) ** [range]
Fluorene	1	166	4.18	1.98	116.5	7.88
Phenanthrene	1	178.24	4.57	1.29	101	8.03
Anthracene	1	178.24	4.54	0.07	216	7.43
Pyrene	1	202.26	5.18	0.135	156	7.53
Fluoranthene	1	202.26	5.22	0.26	111	7.90
Benzo[a]anthracene	1	228.28	5.61	0.011	162	7.56
Chrysene	1,2	228.28	-	2·10 ⁻³	254	7.59
Benzo[a]pyrene	1,2	252.32	5.91	3.8·10 ⁻³	179	7.12
Benzo[b]fluoranthene	2	252.32	5.80	1.5·10 ⁻³	168	7.65
Benzo[k]fluoranthene	2	252.32	6.06	0.8·10 ⁻³	217	7.48
Indeno(1,2,3-cd)pyrene	2	276	6.50	1.9·10 ⁻⁴	164	8.02

(*) 1, compounds addressed in the assessment of environmental effects; 2, compounds addressed in the assessment of human health effects.

(**) Ref. Bogan and Lamar, (1995) and Zheng and Obbard, 2002)

[†] Octanol/water partition coefficient; [‡] Ionization potential

PAHs are hydrophobic compounds, with high melting point; their persistence in the environment is mainly due to their low solubility in water (Cerniglia 1992). In general, volatility and solubility decrease with the increasing number of fused benzene rings (Wilson and Jones 1993).

2.1.3 Toxicity:

Many PAHs display acute carcinogenic, mutagenic and teratogenic properties and may induce tumours in some organisms at even single doses. Other non-cancer causing effects include adverse effects on reproduction, development and immunity (Eisler, 1987). Their effects have been found in many organisms, including humans and other mammals, birds, invertebrates, plants, amphibians and fishes. Mammals can absorb PAHs by inhalation, dermal contact or ingestion (Eisler, 1992).

Among the sixteen PAHs identified as “priority pollutants”, benzo[a]pyrene is known to be one of the most powerful carcinogens (Jusz and Naidu, 2000); carcinogenetic potential of other PAHs is expressed in relation to that of benzo[a]pyrene, as reported in Table 2.2.

Moreover, it has been recently shown that some PAHs have an anti-estrogenic effect, due to their ability to compete for the estrogenic receptor (ER). For this reason, PAHs are also included in the list of Endocrine disrupting chemicals (EDCs) (Arcaro *et al.*, 1999).

Table 2.2. Carcinogenetic factors related to benzo[a]pyrene of 16 individual PAHs recognized as “priority pollutants” by US-EPA (Nisbet and LaGoy, 1992)

PAH	Carcinogenetic factor	PAH	Carcinogenetic factor
Naphtalene	0.001	Benzo[a]anthracene	0.1
Acenaphthylene	0.001	Chrysene	0.01
Acenaphtene	0.001	Benzo[b]fluoranthene	0.1
Fluorene	0.001	Benzo[k]fluoranthene	0.1
Phenanthrene	0.001	Benzo[a]pyrene	1
Anthracene	0.01	Indeno[1,2,3-cd]pyrene	0.1
Fluoranthene	0.001	Dibenzo[a,h]anthracene	5
Pyrene	0.001	Benzo[g,h,i]perylene	0.01

2.1.4. PAH biodegradation by white rot fungi

Although several bacterial and fungal species are potentially involved in PAH oxidation, degradation of these contaminants is limited by their low water solubility (Baldrian *et al.*, 2000; Li *et al.*, 2009). Whereas soil bacteria were found to effectively degrade low molecular-weight PAHs (Cerniglia and Heitkamp, 1989), fungi belonging to the ecological group of white-rot basidiomycetes can also bring about the oxidation of more condensed PAH molecules with up to six aromatic rings and limited water solubility (Baldrian *et al.*, 2000). High molecular weight PAHs, in fact, in soil are mostly adsorbed or covalently bound onto solids (clays, organic matter and humic acids). Due to the necessity of intracellular uptake, bacterial degradation is strongly affected by the bioavailability of PAHs. On the contrary, fungi display several advantages such as penetration of the hyphae and excretion of oxidative enzymes in the polluted matrices, as already discussed in the section 1.2.

There have been many experiments in the last few years carried out to evaluate the PAH-degrading capability of white rot fungi (Pointing, 2001; Verdin *et al.*, 2004). In 1985, Bumpus and co-workers showed the ability of *Phanerochaete chrysosporium* to degrade recalcitrant compounds. In subsequent years, research was focused on the ability of other white rot species to degrade light and heavy PAHs and on the assessment of possible correlation between ligninolytic enzymes production and degradation extents. To date, the biodegradation potential of white rot basidiomycetes has been tested on both artificially contaminated soil (Pointing 2001; Sasek *et al.*, 2003) and real soil samples from polluted sites (D'Annibale *et al.*, 2005; Leonardi *et al.*, 2008). Moreover, several environmental factors such as interaction with soil microbiota, presence of other pollutants and bioavailability of the substrate have been investigated (Radtke *et al.*, 1994; Pointing, 2001; Federici *et al.*, 2007; Leonardi *et al.*, 2007; 2008).

2.1.5. Strategies to enhance bioavailability of PAHs

Although the works carried out in PAHs degradation by white rot fungi have proved the removal of organopollutants from the soil in laboratory conditions, a common feature in the reported studies has been the unpredictable level of transformation and mineralization compared to submerged liquid cultures (Boyle *et al.*, 1998). The low bioavailability of PAHs is often considered the major rate-limiting factor in the biodegradation of these compounds. Therefore, special attention requires the enhancement of PAHs availability by means of either surfactants or organic solvents.

2.1.5.1. Surfactants

A possible way to enhance bioavailability of hydrophobic organic compounds is the application of surfactants, the molecules of which comprises a hydrophilic head and a hydrophobic tail. Due to this amphiphilic structure, an important characteristic of surfactants is the fact that aggregates of 10 to 200 molecules, called micelles, are formed in solution above the critical micelle concentration (CMC).

Two mechanisms explain the increased bioavailability of hydrophobic compounds in the presence of surfactants: *i*) the solubility of the pollutant is increased because of the lipophilic organic fraction in micelles (Edwards *et al.*, 1991), and *ii*) the transport of the contaminant from the solid to the aqueous phase is favoured, probably due to reduction of surface tension of pore water in soil particles, interactions of the surfactant with solid interfaces, or interaction of the pollutant with single surfactant molecules (Volkerling *et al.*, 1998).

In many works it has been shown that non-ionic surfactants stimulate PAH degradation by increased bioavailability (Tiehm, 1994; Volkerling, 1995; Zheng and Obbard, 2001). For example, surfactants such as Tween 80 and polyoxyethylene 10 lauryl ether (PLE) increased anthracene, pyrene and benzo[a]pyrene degradation rate by 2 to 5-fold (Kotterman *et al.*, 1998). Marquez-Rocha *et al.* (2000) detected increased degradation of PAHs in artificially contaminated soil by *Pleurotus ostreatus* when Tween 40, Tween 80 and Triton X-100 were added. However, contradictory results are present in literature, since some authors revealed a certain inhibitory effect of surfactant on biodegradation (Laha and Luthy 1991, 1992; Grimberg *et al.*, 1995). In this respect, one hypothesis is that the microorganisms do not have access to the PAHs in the micellar phase. Another proposal is that surfactants may be toxic to the organism or used by microorganisms as carbon source. For the reasons mentioned above, careful study is needed before using surfactants for biological soil treatment.

2.1.5.2. Solvents

The use of water/organic solvent mixtures is an alternative to enhance the bioavailability of hydrophobic contaminants. Solubility of these compounds in organic solvents is usually several orders of magnitude higher than in water. Their use may be interesting for soil treatment because of the possible regeneration after the extraction process. However, the use of solvents presents several potential disadvantages such as high costs, solvent recycling problems and potential toxicity. Many organic solvents are toxic to living organisms because of their devastating effect on biological

membranes (Heipieper *et al.*, 1994). This factor correlates inversely with the hydrophobic character of the solvent, expressed by the logarithm of the partition coefficient between octanol and water (log K_{ow} value (Inoue and Horikoshi, 1989)).

The use of water-miscible co-solvents give rise to monophasic systems which allow the solubilisation of high amounts of poorly soluble substrates. These mixtures considerably reduce the mass-transfer limitations and, therefore, have been used for PAH degradation by both bacteria and white rot fungi. Morris and co-workers demonstrated that arithmetic increments of solvent in water increase PAH solubility in a logarithmic mode (Morris *et al.*, 1988). However, the ratio of solvent to be used is limited by its toxicity on the microorganism. As an example, acetone or ethanol in concentration higher than 20% had an inhibitory effect on the growth and the performances of the white rot fungus *Bjerkandera* sp. BOS55 (Field *et al.*, 1995). In that work, addition of acetone or ethanol in the ratio 11-21% (v/v) increased anthracene degradation rate by a factor of 2-3 compared to fungal cultures receiving 1 to 3% solvent. The degradation of 10 mg l⁻¹ was completed after 4 days of incubation. Water miscible solvents such as acetone, ethanol, acetonitrile, dimethyl sulfoxide and dimethylformamide have been successfully utilized in different proportion for the enzymatic conversion of PAHs (Pickard *et al.*, 1999; Gunther *et al.*, 1998; Eibes *et al.*, 2005; 2007)

2.1.6. *In vitro* degradation of PAHs by ligninolytic enzymes

Ligninolytic enzymes have been traditionally used for the degradation of organopollutants, xenobiotics and industrial contaminants as well as for biopulping and biobleaching in the paper industry (Pointing 2001; Cohen *et al.*, 2002; Rabinovich *et al.*, 2004). In the specific case of poorly soluble compounds, such as PAHs, the *in vitro* degradation requires a solubility-enhancing system to overcome mass transfer limitations. Low concentration of surfactants (*e.g.*, Tween and Triton series) have been successfully used for *in vitro* degradation studies (Camarero *et al.*, 2008; Canas *et al.*, 2007). In addition to the solubilising action, surfactants containing unsaturated fatty acids, such as Tween 80 and 85, could enhance the extent of degradation due to lipid peroxidation *via* the formation of peroxy radicals (Steffen *et al.*, 2003).

From a classical point of view, organic solvents have been used for the precipitation or denaturation of enzymes. However, it is not surprising that enzymes can be catalytically active in organic solvent systems since various enzymes, including lipases, esterases and dehydrogenases function in natural hydrophobic environments (Dordick, 1989). The most important criterion in selecting a miscible solvent is its compatibility with enzymatic activity retention. Hydrophilic solvents have a greater tendency to strip bound water from enzyme molecules (Klibanov, 2001),

therefore preliminary test on the stability of the enzymatic preparation in the selected reaction mixture is strongly recommended.

2.2 Anthracene and benzo[a]pyrene degradation by *Panus tigrinus* CBS 577.79 in direct micellar systems

2.2.1. Introduction

Aromatic contaminants with very low solubility in water, such as PAHs, have been found to be oxidized by both fungal cultures and fungal oxidases (Pointing, 2001; Hammel, 1995). Therefore, in order to increase their degradation in aqueous media, it is necessary to devise biocompatible liquid systems able to greatly increase the solubility of PAHs without negatively affecting the degradation performances of both living mycelia and their extracellular lignin-modifying enzymes.

In the present study direct micellar systems have been utilized to achieve solubilization of PAHs. It is well known that surfactants in solution undergo spontaneous self-association in globular aggregates above a threshold concentration often referred to as critical micelle concentration (CMC). In micelles, the hydrophobic portion of each surfactant molecule is confined in the apolar interior of the micelle which possesses fluidity and dielectric properties similar to bulk alkanes. The polar head groups are located in the external hydrophilic layer that represents the interface between the bulk oil and the aqueous medium.

Direct micelles are able to solubilise certain amounts of oil in their apolar interior (Christian and Scamehorn, 1995). Hence, the addition of an oil, in which PAHs were previously dissolved, results in the formation of chemical “microreactors” where the aromatic substrates are stored in the apolar core and can diffuse to hydrophilic layer to participate in chemical reactions taking place in the aqueous medium (Berti *et al.*, 2000). The ideal oil has to be non-aggressive to cellular membranes and capable of solubilizing high quantities of aromatic substrate. Suitable oils, in this respect, are oleic acid, glyceryl trioleate, isopropyl palmitate, methyloleate and δ -tetradecan-lactone (Berti *et al.*, 2000; Randazzo *et al.*, 2001). Nevertheless, it was shown that changing the oil consisted in a different phase behaviour and in a diverse micelles radius swelling, but the aromatic substrate bioconversions resulted to be optimal under all the conditions tested (Berti *et al.*, 2000).

Whereas the degradation of PAHs by different white rot fungi, such as those belonging to some species of *Phanerochaete*, *Pleurotus*, *Trametes* etc., is well documented (Bezalel *et al.*, 1996; Pointing, 2001), the PAH-degrading capabilities of the strain CBS 577.79 of *P. tigrinus* have not

been investigated yet. Thus far, the aim of the present work was to assess whether *P. tigrinus* cultures were capable of degrading two representative PAHs, the 3-ringed anthracene and the 5-ringed benzo[a]pyrene, in direct micellar systems. Such solubilising systems were prepared in two liquid growth media, each one leading to a preferential production of either laccase or manganese-dependent peroxidase (MnP) (Quaratino *et al.*, 2006; 2007). Moreover, the impact of such PAH-solubilizing system on ligninolytic enzymes and biomass production was monitored during the whole incubation period.

2.2.2. Materials and methods

2.2.2.1. Organism and inoculum preparation

P. tigrinus (strain 577.79) was obtained from the CBS culture collection (Baarn, The Netherlands). During the study, the strain was maintained on potato dextrose agar slants at 4° C and sub-cultured every month. Inocula were prepared by growing the fungus for 96 h at 28° C in 1 litre shaken flasks (150 rpm) filled with 200 ml of a medium containing 50 g l⁻¹ glucose and 2 g l⁻¹ yeast extract. At the end of incubation, pre-cultures were centrifuged (4,000 g, 10 min) and washed with deionized water. The mycelium was homogenized by Ultra-Turrax (IKA Labortechnik, Staufen, Germany) (two subsequent steps of 30 s each, at ca. 7,000 rpm) and diluted with deionized water to yield a biomass concentration of approximately 10 g l⁻¹.

2.2.2.2. Media composition and culture conditions

The low-nitrogen (LN) medium optimized for the production of MnP by *P. tigrinus* CBS 577.79 (Quaratino *et al.*, 2006) was modified as follows: 7 g l⁻¹ sucrose, 1 mM nitrogen (as (NH₄)₂SO₄), 5% (v/v) olive mill wastewaters (OMW), 0.001% (w/v) yeast extract and 1% of the surfactant Triton-X-100 (pH 5.0). The high-nitrogen (HN) medium had the following composition: 12.5 g l⁻¹ fructose, 20 mM nitrogen (as ammonium tartrate), 0.01% (w/v) yeast extract and 1% (v/v) of Triton-X-100. The pH of this medium was adjusted to 5.0 by adding few drops of 1 N NaOH.

All the fermentations were carried out under shaken conditions (120 rpm, 28° C) in Erlenmeyer flasks (250 ml), containing 50 ml of either LN or HN medium. Sterilization was performed in autoclave (120° C, 20 min) and successively the flasks were supplemented with 1% (v/v) of isopropylpalmitate (IPP), where anthracene or benzo[a]pyrene had been previously dissolved. The final concentration of the aromatic contaminants in the liquid media was 0.5 mM. After an

equilibration time of 24 h to allow the formation of microemulsions (Berti *et al.*, 2000), 3 ml inoculum were added to each flask. Cultures grown in micellar systems but in the absence of PAHs (mere sterile IPP added) and fresh non-inoculated medium containing micelles and PAHs were incubated in the same way and referred to as biotic controls (BC) and incubation controls (IC), respectively.

2.2.2.3. Enzyme and biochemical assays

Laccase activity was routinely assayed spectrophotometrically at 35 °C using 2,6-dimethoxyphenol (DMP) as a substrate and monitoring the formation of 3,3',5,5'-tetramethoxy-*p*-diphenoquinone (coerulignone) at 477 nm ($\epsilon = 14600 \text{ M}^{-1} \text{ cm}^{-1}$) (Slomczynsky *et al.*, 1995).

Manganese-dependent peroxidase activity was determined by the method of Waarishi and coworkers (1992). The assay mixture (1 ml) contained 0.5 mM MnSO₄ and 75 μM H₂O₂ in 50 mM tartrate buffer at pH 4.5. The formation of Mn(III)–tartrate complex was followed at 290nm ($\epsilon = 2860 \text{ M}^{-1} \text{ cm}^{-1}$). One unit of enzyme activity (U) is defined as the amount of enzyme which produces 1 μmol of product per minute under the assay conditions. Extracellular protein was determined by the dye-binding method using bovine serum albumin as a standard (Bradford, 1976). Microbial biomass concentration was measured by dry weight estimation: broth samples were filtered on pre-weighed Whatman GF/C discs ($\varnothing$ 47 mm), the harvested biomass was washed twice with distilled water, then with n-hexane and the filter was dried at 105 °C for 24 h, cooled in a desiccator and weighed. Total sugars were determined by the phenol–sulphuric acid method (Dubois *et al.*, 1956). Ammonium was determined spectrophotometrically at 670 nm by its reaction with hypochlorite ions and salicylate in the presence of sodium nitroprusside to yield a blue-green reaction product (Seo and Fritz, 2000).

2.2.2.4. Analytical methods

Samples (1 ml) from fungal cultures and respective IC were collected on a daily basis during the whole incubation period. Aliquots of the supernatant after centrifugation (14.000 rpm at 4 °C, for 5 min) were diluted with acetonitrile (1:10, v/v). In order to assess the possible occurrence of adsorption phenomena, the fungal biomass was extracted with 20 ml aliquots of acetonitrile for three times and the resulting extracts dried under vacuum by Rotavapor (Buchi, Basel, Switzerland) and resuspended in 2 ml of acetonitrile. Both diluted surnatants and biomass extracts were analyzed by reversed-phase HPLC. The pumping system (1525 Waters, Milford, MA) was equipped with an

Inertsil-PAH C 18 column (4.6×250 , particle size $\varnothing 5 \mu\text{m}$, GL Science Inc., Japan) and a Dual λ UV detector (2487, Waters, Milford, MA). An isocratic elution was applied using acetonitrile : water (70 : 30) and the elution of anthracene and benzo[a]pyrene was monitored at 254 nm.

2.2.3. Results and discussion

To the best of our knowledge, the present study is the first report on PAH degradation by a white rot fungus in direct micellar systems. The solubility-enhancing (micro-emulsion) system we employed was composed of the non-ionic surfactant Triton-X-100 (1% v/v) and isopropyl palmitate (IPP, 1% v/v). The former was selected on the basis of its chemical nature as previously discussed by other authors (Randazzo *et al.*, 2001): the phenyl ring on the polar head of the molecule could play an important role in both PAH solubilization and diffusion of the aromatic substrate from the reservoir located in the micellar core to the liquid medium. As for the latter, IPP proved to be a suitable apolar liquid, ensuring a high PAH solubilization without affecting bacterial cell growth and bioconversion (Berti *et al.*, 2000). Furthermore, a previous work (Kadimaliev *et al.*, 2006) revealed that the palmitic acid is one of the most abundant (>30%) fatty acids produced by the strain VKM F-3616D of *P. tigrinus*.

2.2.3.1. Enzymatic activities and fungal growth in direct micellar systems

The selected direct micellar system (DMS) was diluted in two different liquid media where the production of extracellular oxidases by the strain CBS 577.79 of *P. tigrinus* is differently regulated (Petruccioli *et al.*, 2009). The low-N medium was optimized for the production of MnP (Quaratino *et al.*, 2007), while the high-N medium led to the production of high yields of laccase and the suppression of MnP activity (Quaratino *et al.*, 2006).

Time courses of laccase and MnP activities in LN and HN liquid media are reported in Figures 2.2.1 and 2.2.2, respectively. Regardless of the presence or the absence of PAHs, the onset of MnP activity in the LN medium (Figure 2.2.1) added with DMS components occurred after 9-10 days of cultivation, when the N-source (1 mM ammonium sulfate) had almost been depleted from the medium (data not shown). This finding is in agreement with previous studies reporting that MnP production is a secondary metabolic event triggered by the N-limitation in *P. tigrinus* (Leontievsky *et al.*, 1994; Quaratino *et al.*, 2006) and other white rot fungi (Buswell *et al.*, 1995; Moilanen *et al.*, 1996).

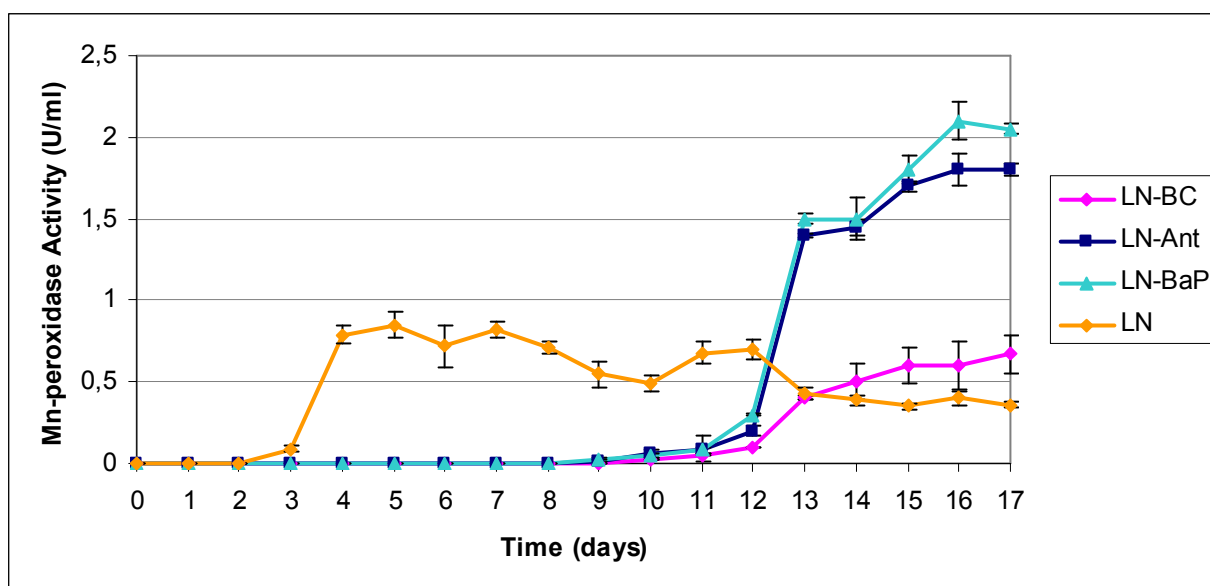


Figure 2.2.1. Time course of MnP activity in LN medium (LN, orange line) and in the same medium containing: DMS (BC, pink line), DMS + 0.5 mM anthracene (blue line) and DMS + 0.5 mM benzo[a]pyrene (light blue line). Data are the mean $\pm$ the standard deviation of triplicate experiments.

In addition, the presence of DMS strongly delayed the occurrence of the MnP activity peaks with respect to the medium in the absence of DMS (16 and 17 days in PAH-supplemented cultures and in the BC, respectively, vs. day 5). It is worth noting that maximal MnP activity values in the presence of anthracene and benzo[a]pyrene (1.89 and 2.12 U ml⁻¹, respectively), the highest among those reported for the strain CBS 577.79 in shaken cultures, resulted to be approximately threefold higher than those of the relative BC (0.67 U ml⁻¹). With this regard, a similar stimulating effect of either PAHs or PAH-degradation products on ligninolytic enzymes activities has already been reported by Cajthaml and co-workers (2008). No laccase activity was detected in *P. tigrinus* cultures grown under nitrogen-limiting condition, as already observed by Quarantino and colleagues (2006). In PAH-supplemented cultures, moreover, the MnP activity peaks in the presence of DMS were significantly higher than that in the control medium albeit with the above mentioned delay.

Table 2.2.1 shows that the biomass production in the presence and in the absence of DMS components did not significantly differ, thus suggesting the mildness of the PAH solubilising system towards the fungus. In addition, the residual concentration of the C-source at the end of the fermentation was similar to that observed for the control medium while the presence of PAHs depressed the sucrose consumption. This was somehow expected since the DMS allowed to solubilise both PAHs at an initial concentration (0.5 mM) which might have exerted inhibitory effects on the fungus.

Table 2.2.1. Dry biomass estimation and residual sucrose concentration at the end of the incubation (day 17) in *P. tigrinus* shaken cultures grown on LN medium. Data are mean $\pm$ standard deviation of three replicates.

Culture conditions	Dry Biomass (g l ⁻¹)	Residual C-source (% of original concentration)
LN medium	1.25 ^a $\pm$ 0.19	31.6 ^a $\pm$ 4.2
LN-BC	1.11 ^a $\pm$ 0.24	31.8 ^a $\pm$ 6.1
LN-Ant	1.45 ^a $\pm$ 0.29	46.1 ^b $\pm$ 2.7
LN-BaP	1.43 ^a $\pm$ 0.21	48.5 ^b $\pm$ 4.7

Multiple pairwise comparison of homogeneous data was carried out by the Tukey test: column means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

Laccase was the dominant extracellular enzyme produced in the HN control medium reaching a maximal activity of 0.48 U ml⁻¹ on day 4, while no MnP activity was detected (Figure 2.2.2). The early onset of laccase on this medium was due to the replacement of glucose with fructose. As opposed to the former, in fact, the latter C source does not give rise to the occurrence of catabolite repression phenomena and laccase production has not a tight dependence on the extent of the C source consumption (Gallhaup, 2002).

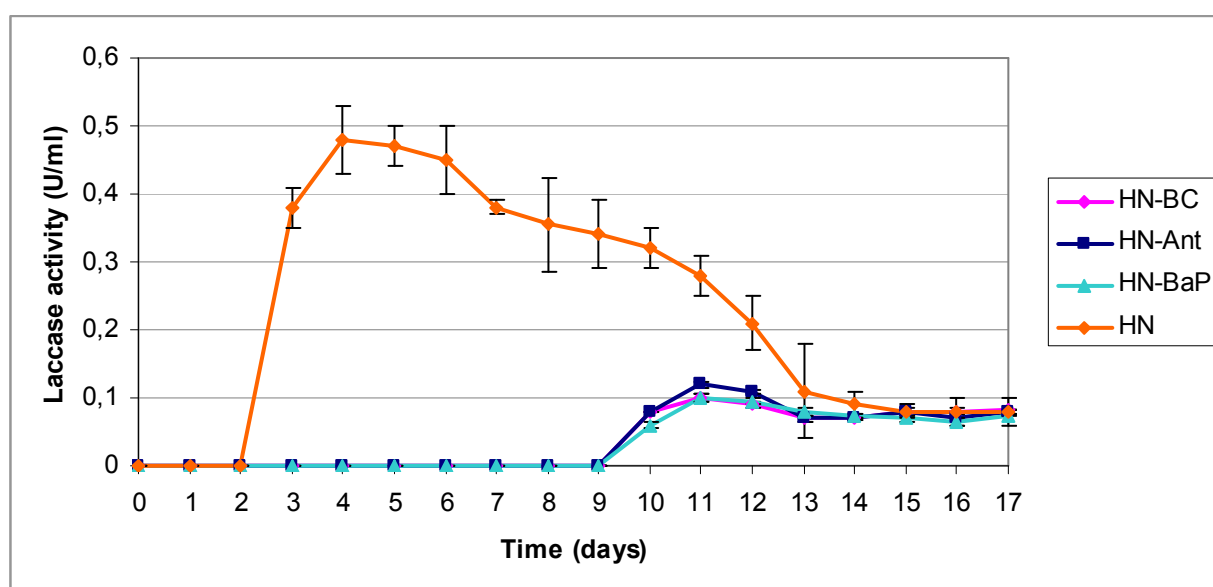


Figure 2.2.2. Time course of MnP activity in HN medium as such and in the HN medium containing: DMS (BC, pink line), DMS + 0.5 mM anthracene (blue line) and DMS + 500 mM benzo[a]pyrene (light blue line). Data are the mean $\pm$ the standard deviation of triplicate experiments.

Nonetheless, the presence of DMS components markedly delayed the onset of laccase activity and led to a significant reduction of its peak. In particular, the onset of laccase activity occurred on day 9 in both BC and PAH-supplemented cultures and an activity peak of approximately 0.1-0.12 U ml⁻¹ was reached after the following 2 days.

In contrast with results observed for MnP production in the LN medium, the presence of the two PAHs did not exert any significant stimulating effect on laccase activity. The time course of laccase activity in the BC paralleled that of cultures spiked with anthracene and benzo[a]pyrene throughout the whole incubation period.

In DMS-containing HN media, no inhibitory effects on biomass production were observed (Table 2.2.2) although the extent of fructose consumption was lower in PAH-containing media than in BC. By contrast, the presence of DMS components led to a marked depression of biomass formation with respect to the control HN medium.

Table 2.2.2. Dry biomass and residual sucrose concentration at the end of the incubation (day 17) in *P. tigrinus* shaken cultures grown on HN medium. Data are mean $\pm$ standard deviation of three replicates.

Culture conditions	Dry Biomass (g l ⁻¹)	Residual C-source (% of original concentration)
HN medium	3,42 ^a $\pm$ 0,09	37,7 ^a $\pm$ 4,2
HN-BC	1,98 ^b $\pm$ 0,03	31,8 ^a $\pm$ 6,1
HN-Ant	1,94 ^b $\pm$ 0,13	46,1 ^b $\pm$ 2,7
HN-BaP	1,81 ^b $\pm$ 0,11	48,5 ^b $\pm$ 4,7

Multiple pairwise comparison of homogeneous data was carried out by the Tukey test: column means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

2.2.3.2. Degradation of anthracene and benzo[a]pyrene

The ability of *P. tigrinus* CBS 577.79 to degrade anthracene and benzo[a]pyrene was examined by reversed-phase HPLC analysis. Figure 2.2.3 reports the extent of degradation of the two PAHs in both LN and HN media at the beginning of the incubation period (day 1), after 10 days (namely, in concomitance with the onset of ligninolytic enzymes activity), and at the end of the experiment (17 days). The recovery of the aromatic contaminants in the non-inoculated IC was higher than 90% in all cases, thus indicating the stability of the micellar phase over the whole incubation period.

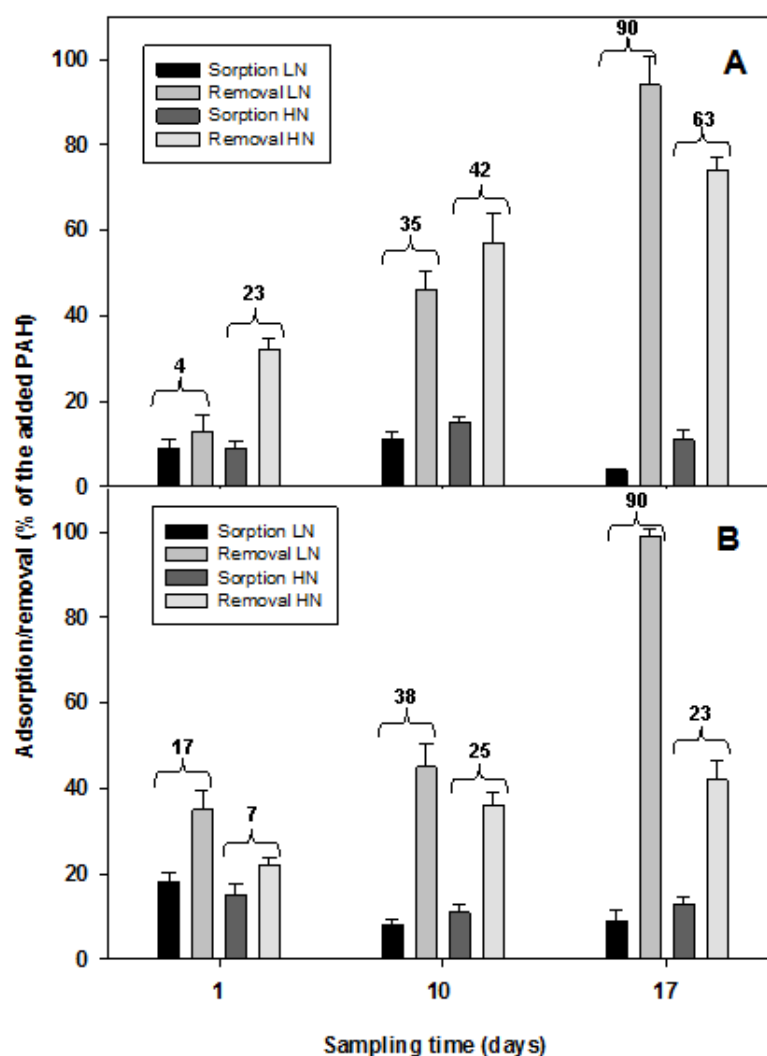


Figure 2.2.3. Percent removal and adsorption onto *P. tigrinus* biomass of anthracene (A) and benzo[a]pyrene (B) with respect to their initial concentration in both LN and HN media. Data are the means $\pm$ standard deviation of three replicates. The numbers on top of the bars represent net removals, which have been calculated by the difference between percent removal and adsorption.

The degradation performances of *P. tigrinus* were largely affected by both the medium and the contaminant examined. These results are illustrated in Figure 2.2.3 in which both percent removal from the media and adsorption of PAHs onto the biomass with respect to their initial concentration are reported. Net removals have been calculated by the difference between percent removal and adsorption. In particular, Figure 2.2.3-A shows that ANT degradation proceeded throughout the

incubation leading to maximal net removals after 17 days of 90 and 63% in LN and HN media, respectively. However, it is interesting to note that ANT was degraded at higher extents in HN media in the first two sampling times (Figure 2.2.3-A).

The LN medium better supported BaP degradation than the HN one and this was evident at all sampling times (Figure 2.2.3-B). Best net removal (90%) was attained at the end of the experiment in the former medium. The delayed onset of lignin-modifying enzymes associated with a non negligible degradation of both PAHs suggests the participation of a non-ligninolytic enzymatic system, such as the cytochrome P-450 monooxygenase/epoxide hydrolase complex (CP-EHC), in the early phases of culture.

Although, the CP-EHC was not assayed for its activity and no experiments were performed with specific inhibitors of this complex, these results seem to indicate the initial predominance of the intracellular degradation. Several authors have reported the involvement of the intracellular cytochrome P-450 and the epoxide hydrolase system (Bezalel *et al.*, 1996; 1996; 1997) in the initial step of PAH degradation by other white rot fungi. According to this hypothesis, it is possible that a part of the aromatic substrates solubilised by the micellar phase was sequestered by the fungal pellets within the early step of incubation and attacked intracellularly via the above cited pathway. In the present study, however, only limited attention has been paid to the role of the intracellular pathway and to the detection of degradation products which could shed some light on the general degradation mechanism. Further insight will be provided by improving the experimental set-up and by focusing the attention on both metabolites of PAH degradation and enzymatic systems involved in their production.

On the other hand, the better degradation results observed with LN than HN cultures at the end of incubation, namely one week later than the onset of lignin-modifying enzyme activities, can be explained by the higher levels of extracellular oxidases observed in the former medium. (Figures 2.2.1 and 2.2.2).

2.2.4. Conclusions

Hydrophobicity of PAHs is one of the crucial factor limiting the microbial degradation of these compounds in natural environments (Haritash and Kaushik, 2009). In the present study, direct micellar systems, generated by the concomitant presence of a surfactant and an oil in solution, have been utilized to improve the solubility of two PAHs and hence to investigate the PAH degrading capabilities of *P. tigrinus* (strain CBS 577.79) at concentrations higher than those usually employed in liquid media containing low amounts of organic solvents. This approach, in fact, allowed PAH

pseudo-solubilization in the millimolar range and it was found to be biocompatible although with some negative effects such as the marked delay in the onset of lignin-modifying enzymes which was more pronounced in HN than LN medium. Degradation performances, however, were interesting in both media although ANT was generally degraded at higher extents than BaP. The PAH removals observed prior to the onset of LME activities might suggest the involvement of CP-EH complex. This, however, would require the assessment of both the presence of this active complex in *P. tigrinus* and of degradation intermediates which might be ascribable to the action of this complex. In this respect, however, the DMS system is not suitable for the purification of the microsomal fraction and the analysis of the degradation intermediates due to the likely interferences of its components during the purification/extraction steps. Thus, these specific objectives have been pursued by a different approach and relative results are shown in sub-section 2.4.3.4 of the present PhD thesis.

2.3. Kinetic and redox properties of MnP II, a major manganese peroxidase isoenzyme from *P. tigrinus* CBS 577.79

2.3.1. Introduction

Among lignin-modifying extracellular enzymes, Mn-dependent peroxidase (E.C. 1.11.1.13 Mn²⁺: hydrogen peroxide oxidoreductase; MnP) is gaining increasing attention due to its possible use in wastewater treatment (Ruiz-Dueñas *et al.*, 2001), bioremediation (Moreira *et al.*, 2003) and biobleaching applications (Heinfling *et al.*, 1998). MnP is a glycosylated heme protein able to catalyse the oxidation of Mn²⁺ into reactive Mn³⁺ which is, in turn, stabilized by fungal organic acids such as oxalate, malonate and tartrate (McEldoon *et al.*, 1995; Lisov *et al.*, 2003). These manganic chelates are highly diffusible mediators with redox potentials ranging from 0.5 to 1.1 V (Armstrong, 2002) able to oxidize phenolic and some non-phenolic lignin substructures (D'Annibale *et al.*, 1996; Quaratino *et al.*, 2007). It has been suggested that the small molecular mass of these chelates (< 0.5 kDa) compared to that of peroxidases (generally higher than 40 kDa) would allow a higher penetration within lignocellulosic substrates which are inaccessible to enzymes (Lisov *et al.*, 2003; Quaratino *et al.*, 2007).

The strain CBS 577.79 of *P. tigrinus* was found to be an efficient producer of both laccase and Mn-dependent peroxidase (Quaratino *et al.*, 2006). In addition, the same strain was capable of degrading and detoxifying olive-mill wastewater, an agro-industrial phenol-containing effluent, even in the presence of high organic loads (D'Annibale *et al.*, 2004). The promising characteristics of *P. tigrinus* CBS 577.79 as well as the need to gain more information on its extra-cellular degradation machinery prompted us to purify a major MnP isoenzyme and to characterize its main physicochemical and biochemical properties. The characteristics of this isoenzyme are comparatively discussed with those described for the reference strain 8/18 of *P. tigrinus* (Maltseva *et al.*, 1991; Golovleva *et al.*, 1993; Leontievsky *et al.*, 1994; Lisov *et al.*, 2003, 2004, 2005).

Knowledge of the redox properties of these enzymes and the understanding of the influence of the microenvironment on the formal redox potential of the active site are crucial to elucidate the catalytic action of peroxidase. Thin film voltammetry has become a valuable approach for studying the redox properties and reactions of proteins and enzymes (Armstrong, 2005; Léger *et al.*, 2008). In this technique, proteins are adsorbed in monolayer film onto an electrode in such a way that electron transfer (ET) to/from the enzyme is direct. Some of the interfacial ET reactions are known to be controlled by a chemical process; in this case the redox reaction is referred to as “gated” (Jeuken, 2003). An important example of ET coupling in proteins is redox-linked proton transfer,

i.e., where a change in oxidation state of a redox center is linked to transfer of a proton (Chen, 2000). A mechanistic problem arises if the proton-binding site is buried and isolated from solvent water molecules, that results in a gated redox reaction.

The steady state current develops when the driving force provided by the electrode potential is high enough that the redox state of the active site is continuously regenerated following the transformation of the substrate. This signal (the “catalytic wave”) is a direct read-out of the activity of the enzyme as a function of driving force (Hirst, 2006). In a previous research, a significant bioelectrocatalytic activity for the reduction of H₂O₂ by a ligninolytic peroxidase immobilized at a graphite electrode was shown (Ferapontova *et al.*, 2006). However, despite the structural similarities and common catalytic cycle, MnP and LiP exhibit differences in their substrate specificity. In the present work, voltammetric experiments with *P. tigrinus* MnP were performed in order to elucidate both its bioelectrochemical properties and its suitability in electron transfer-based biosensor development.

2.3.2. Materials and methods

2.3.2.1 Organism and inoculum preparation

P. tigrinus (strain 577.79) was from the CBS culture collection (Baarn, The Netherlands). During the study, the strain was maintained on potato dextrose agar slants at 4 °C and sub-cultured every month. Inocula were prepared by growing the fungus for 96 h at 28 °C in 1 liter shaken flasks (150 rpm) filled with 200 ml of a medium containing 50 g/l glucose and 2 g/l yeast extract (GYE medium). At the end of incubation, pre-cultures were centrifuged (4,000 x g, 10 min) and washed with sterile deionized water. The mycelium was homogenized (two subsequent steps of 30 s each, at ca. 7,000 rpm) by Ultra-Turrax (IKA Labortechnik, Staufen, Germany) and added with sterile deionized water to yield a biomass concentration of approximately 10 g/l.

2.3.2.2. Enzyme production

Fermentations were carried out in a 3-l jacketed bench-top stirred tank reactor (STR) (Applikon Dependable Instruments, Schiedam, The Netherlands) filled with 2 l of a medium containing: 6.5 g/l sucrose, 0.5 g/l Tween 80, 0.35 g/l (NH₄)₂SO₄, 0.01 g/l yeast extract, 60 mg/l MnSO₄ and 8.3 mM malonic acid at pH 5.0. The STR was equipped with a top stirrer bearing two six-blade Rushton-type turbines. The following probes were installed on the top plate: dissolved oxygen

sensor (Ingold, CH), double reference pH sensor (Phoenix, AZ), PT 100 temperature sensor. Standard bioprocess conditions were as follows: inoculum size 0.25 g/l mycelium dry weight; impeller speed 500 rpm (tip speed = 118 cm/sec), aeration rate 1.0 vvm; temperature 28 °C; initial dissolved oxygen concentration 100% of saturation. Fermentation parameters were monitored in the bioreactor by an adaptative/PID digital controller, ADI 1030 (Applikon Dependable Instruments, Schiedam, The Netherlands).

2.3.2.3. Enzyme purification

Seven-day-old STR cultures were centrifuged (12000 x g, 20 min) and culture supernatants concentrated on a Mini-Sart tangential flow apparatus (Sartorius, Goettingen, Germany) fitted with a membrane cassette (cut-off 10 kDa). The retentate was then dialysed against 10 mM imidazole-HCl buffer containing 0.5 M NaCl, 1 mM CaCl₂ and 1 mM MnCl₂ pH 6.0 (buffer A) prior to its application to a 13 ml Concanavalline-A Sepharose column pre-equilibrated with buffer A at a flow rate of 0.3 ml/min. Unbound proteins and chromophoric substances were eluted by washing the column with 65 ml buffer A. Then, MnP activity was eluted by a linear α -D-methylmannopyranoside gradient from 0 to 0.28 M (4-bed volumes). The pooled active fractions were concentrated again as mentioned above and applied to a Superdex 75 prep-grade column (100 cm x 1.5 cm) equilibrated with 10 mM imidazole-HCl buffer pH 6.0 added with 0.15 M NaCl (flow rate 0.54 ml/min). Active fractions were pooled, desalted, filter-sterilized (0.22 μ m) and stored at – 20 °C.

2.3.2.4. Physico-chemical characterization

The molecular mass of the native enzyme was determined by gel filtration chromatography on Superdex 75 column as described above. The column was calibrated with tyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumine (44 kDa) and myoglobin (17 kDa), vitamin B12 (1.35 kDa). Polyacrylamide (12%) sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was performed at constant voltage (200 V) on a Mini-Protean II apparatus (Biorad, Richmond USA) according to the Laemmli method (1970). Molecular mass determination under denaturing conditions was performed by using low-molecular weight standards (Roche, Mannheim, D). Analytical isoelectric focusing on polyacrylamide gel (IEF-PAGE) in the range 2.5-7.0 was performed on a Mini-IEF apparatus (Biorad, Richmond USA). The pH gradient was measured by using the following standards: human carbonic anhydrase (pI = 6.55), bovine carbonic anhydrase (pI = 5.88), β -glucosidase (pI = 5.20),

soybean trypsin inhibitor (pI = 4.50), glucose oxidase (pI = 4.15), amyloglucosidase (pI = 3.50) and pepsinogen (pI = 2.80). Proteins were visualized on gels by the ultra-sensitive colloidal Coomassie G-250 method (Neuhoff *et al.*, 1988). To quantify the carbohydrate content of *P. tigrinus* MnP, the enzyme (5 μ g) was previously deglycosylated by incubation with 12 IU of *N*-glycosidase F (Roche Mannheim) in 0.2 M phosphate buffer pH 7.2 for 3 h. The quantitation was performed by comparing the relative electrophoretic migrations of native and deglycosylated MnP on an SDS-PAGE gel. UV-vis absorbance spectra of purified MnP (540 μ g/ml 10 mM imidazole-HCl buffer at pH 6.0) were recorded in a Lambda 20 spectrophotometer (Perkin Elmer, Sweden)

2.3.2.5. *Lectin assay*

MnP (1 μ g) and standard glycoproteins (1 μ g each) supplied with the Dig Glycan Differentiation Kit (Roche, Mannheim Germany) were spotted onto a Protran BA 85 nitrocellulose membrane (Schleicher & Schuell, Dassel Germany) and detected immunologically after binding to lectins conjugated with digoxigenin according to the manufacturer's instructions. Lectins used to this purpose were as follows: *Galanthus nivalis* agglutinin, specific for terminal mannose; *Sambucus nigra* agglutinin specific for sialic acid α (2-6)galactose; *Maackia amurensis* agglutinin, specific for sialic acid α (2-3)galactose; peanut agglutinin, specific for galactose β (1-3)*N*-acetylgalactosamine and *Datura stramonium* agglutinin specific for galactose β (1-4)*N*-acetylglucosamine.

2.3.2.6. *Effect of pH and temperature on enzyme activity and stability*

MnP activity as a function of pH was measured at 30 °C using the following buffers: 0.1 M tartrate-NaOH (range 2.5-5.0), 0.05 M malonate-NaOH (3.5-6.5) and 0.1 M phosphate buffer (range 6.5-7.0). The temperature-activity profile was determined in 0.05 M malonate-NaOH buffer pH 5.0 in the range 10-70 °C. To test the pH stability, 10 IU of MnP were incubated at 25°C in 5 ml 0.05 M malonate-NaOH buffer over the pH range 3.5–6.5. Aliquots (10–30 μ l) were withdrawn at specific time-points and immediately assayed for residual MnP activity. Thermal stability experiments were conducted by incubating 40 IU of MnP activity in 4 ml 0.05 M malonate-NaOH buffer pH 5.5 at 50 and 60 and 70°C. Aliquots (50 μ l) were chilled on ice and assayed for residual activity.

2.3.2.7. MnP assay and steady-state kinetic measurements

Manganese-dependent peroxidase activity was routinely determined by the method of Waarishi and collaborators (1992). The assay mixture (1 ml) contained 0.5 mM MnSO₄, 75 μ M H₂O₂ in 50 mM malonate buffer at pH 4.5 and the formation of Mn(III)-malonate complex was kinetically followed at 270 nm (ϵ = 11590 M⁻¹ cm⁻¹). One international unit of enzyme activity (IU) is defined as the amount of enzyme, which produces 1 μ mol of product per min under the assay conditions.

The initial velocity of Mn³⁺ formation with other chelators, including lactate, pyrophosphate, tartrate and oxalate was monitored as described by other investigators (Kuan *et al.*, 1993; Schlosser *et al.*, 2002). Wavelengths and extinction coefficients employed to this purpose are shown in Table 2. Protein determination was performed according to the dye-binding method (Bradford, 1976), using bovine serum albumin as the standard.

Kinetic constants of *P. tigrinus* MnP for Mn²⁺ and H₂O₂ were determined at 35 °C in 0.05 M malonate-NaOH pH 5.5 (buffer B). The same constants were determined for Mn-dependent oxidation of some phenolic substrates using a saturating concentrations of Mn²⁺ and H₂O₂ (1.5 and 0.2 mM, respectively) in malonate-NaOH pH 4.0 and varying the concentration range of phenols from 0.005 to 10 mM. Mn-independent peroxidase activity was determined under the same conditions but omitting MnSO₄. Rates of substrate oxidation were determined by monitoring the absorbance change at 469 nm (ϵ = 55000 M⁻¹ cm⁻¹) for 2,6-dimethoxyphenol (DMOP), 465 nm (ϵ = 12100 M⁻¹ cm⁻¹) for guaiacol, 430 nm (ϵ = 2470 M⁻¹ cm⁻¹) for pyrogallol, 420 nm (ϵ = 36000 M⁻¹ cm⁻¹) for 2,2'-azinobis-3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), 310 nm (ϵ = 9300 M⁻¹ cm⁻¹) for veratryl alcohol (VA) and 340 nm (ϵ = 6300 M⁻¹ cm⁻¹) for NADH. Apparent K_m and V_{max} values were calculated from Lineweaver-Burk plots.

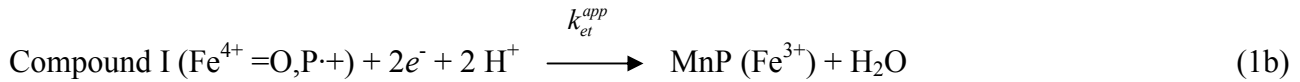
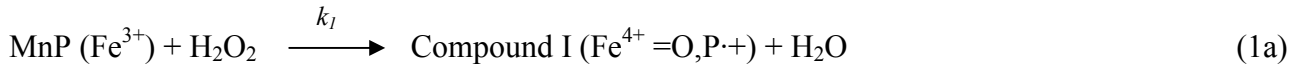
2.3.2.8. Electrochemical experiments

Voltammetric measurements were carried out at different pHs by using the same buffer systems reported in subsection 2.6. Buffer solution was deoxygenated by purging N₂ for at least 20 min and kept under a constant stream of N₂ throughout measurement; electrochemical experiments were carried out in a thermostated glass cell with a μ -Autolab (EcoChemie, The Netherlands) and a typical three-electrode configuration using a current integration method controlled by NOVA software.

A pyrolytic graphite electrode (PGE; Amel, Italy) was used as the working electrode; a standard calomel electrode (SCE; Amel, Italy) was used as reference electrode and a glassy carbon rod as

counter electrode (Metrohm, Switzerland). For the electrokinetics measurements a rotating working electrode (Metrohm, Switzerland) provided with a PGE tip was used. Before the voltammetric measurements, the PGE electrode surface was polished with 1 μm alumina and then sonicated thoroughly to remove the alumina. The electro-active area ($A = 0.12 \pm 0.2 \text{ cm}^2$) was determined as previously reported (Bard and Faulkner, 2002). The protein solution (approximately 5 μg in 0.05 M malonate buffer pH 5.0) was spread out on the electrode surface. After absorption for 20 h at 4 $^\circ\text{C}$, the electrode was washed with Millipore water and transferred into cell solution.

Direct biocatalytic reduction of hydrogen peroxide at a MnP-modified electrode is depicted by the following electro-enzymatic reactions (1a and 1b) where compound I is directly reduced to the native state of MnP and two electrons are transferred from the electrode surface:



Here k_1 is the rate constant for the two-electron oxidation of the ferriheme prosthetic group of MnP by H_2O_2 and k_{et}^{app} represents the apparent rate constant of compound I reduction. The current intensity arising from H_2O_2 reduction at the MnP-modified rotating disc electrode could be influenced by its mass transport to the electrode surface and the kinetics of either reactions.

The measured reduction current I is a combination of the mass transport limiting current I_D and the reaction rate limiting current I_K , as reported in the derivation of the Koutecky-Levich equation where is assumed that the surface kinetics steps are first order (Presnova *et al.*, 2000; Bard and Faulkner, 2002):

$$\frac{1}{I} = \frac{1}{I_K} + \frac{1}{I_D}$$

The overall MnP catalyzed reduction of H_2O_2 at the peroxidase modified electrode, is characteristic of a second order kinetic. The mathematic equation describing this reaction could be expressed as follow:

$$\frac{1}{I} = \frac{1}{nFA\Gamma k_{et}^{app}} + \frac{1}{nFA\Gamma k_1 \left([H_2O_2]^* - I / 0.62nFAD^{2/3} \omega^{1/2} \nu^{-1/6} \right)}$$

Where F is the farady constant (96487 coulomb), A is the electrode surface, n is the number of electron exchanged, D is the coefficient diffusion, Γ is the electroactive protein surface coverage, I is the current, ω is the angular velocity of the electrode and v is the scan rate.

For high ω values:

$$\frac{1}{I_K} = \frac{1}{nFA\Gamma} \left(\frac{1}{k_1[H_2O_2]^*} + \frac{1}{k_{et}^{app}} \right)$$

2.3.3. Results

2.3.3.1. MnP production

P. tigrinus CBS 577.79 cultures from the STR were harvested on day 7 when MnP reached its maximal activity (2 IU/ml) with a specific activity of 40 IU/mg protein and a volumetric productivity of 11.9 IU/(l · h) (Figure 2.3.1).

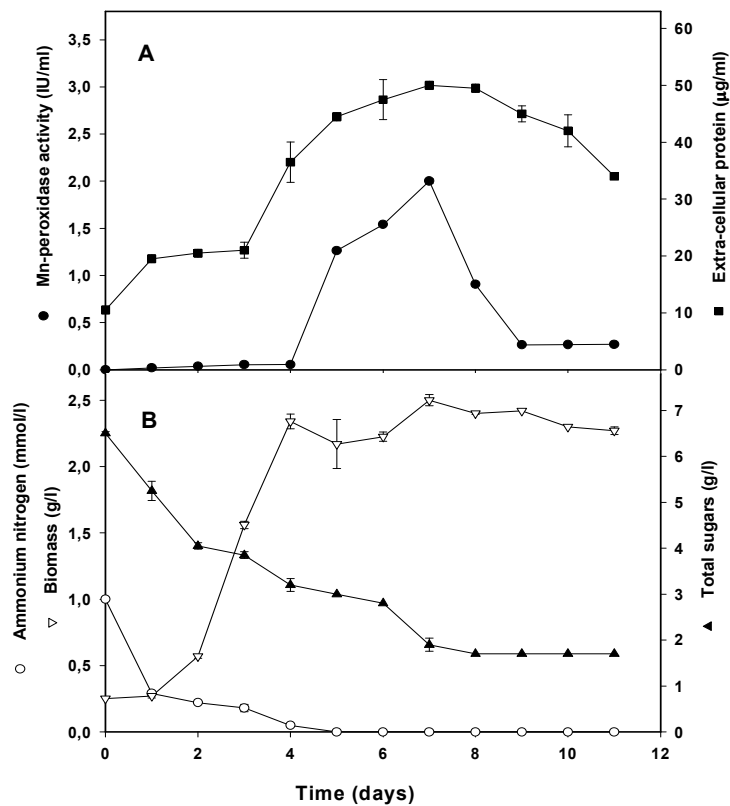


Figure 2.3.1. Time courses of manganese peroxidase activity (●), extra-cellular protein (■), biomass production (▽), nutrient nitrogen (○) and total sugars (▲) when *P. tigrinus* CBS 577.79 was grown in a 3 l stirred-tank reactor. Data are the mean ± standard deviation of data.

The time course of extracellular protein paralleled that of MnP activity. The onset of MnP activity occurred in concomitance with ammonium depletion from the culture broth. No laccase activity was detected under this condition and two MnP isoforms with relative mobilities of 0.45 and 0.50 were detected by polyacrylamide gel electrophoresis followed by activity staining (Quaratino *et al.*, 2007).

2.3.3.2. MnP purification

The purification of *P. tigrinus* CBS 577.79 MnP is summarized in Table 2.3.1. The culture supernatant was concentrated 100-fold by ultra-filtration with a yield of 85%. The retentate was applied to an affinity column packed with Concanavaline-A Sepharose and one of two MnP isoenzymes (MnP I) was recovered in the washthrough with a 25% yield. By contrast, the second one (MnP II) was eluted at 0.28 M α -D-methylmannopyranoside with a purification fold of 4.22 and a specific activity equal to 169 IU/mg protein. MnPII was eluted as a single and symmetric peak ($K_{av} = 0.203$) in the second step involving gel permeation chromatography on Superdex 75 resulting in a final purity-fold and yield of 7.2 and 22%, respectively (Table 2.3.1).

Table 2.3.1. Typical purification scheme of extra-cellular manganese-peroxidase (MnPII) from *P. tigrinus* CBS 577.79.

Step	Total activity (IU)	Total protein (mg)	Specific activity (UI/mg protein)	Yield (%)	Purity (fold)
Culture supernatant	4000	100	40	100	1
Ultra-filtration (cut-off 10 kDa)	3400	82.9	41	85	1.02
Con-A Sepharose chromatography	1200	7.1	169	30	4.22
Superdex 75 chromatography	865	3.0	288	22	7.2

The enzyme, showing a specific activity of 288 IU/mg protein (malonate as a chelator), turned out to be homogeneous by SDS- (Figure 2.3.2-A) and IEF-PAGE (Figure 2.3.2-B).

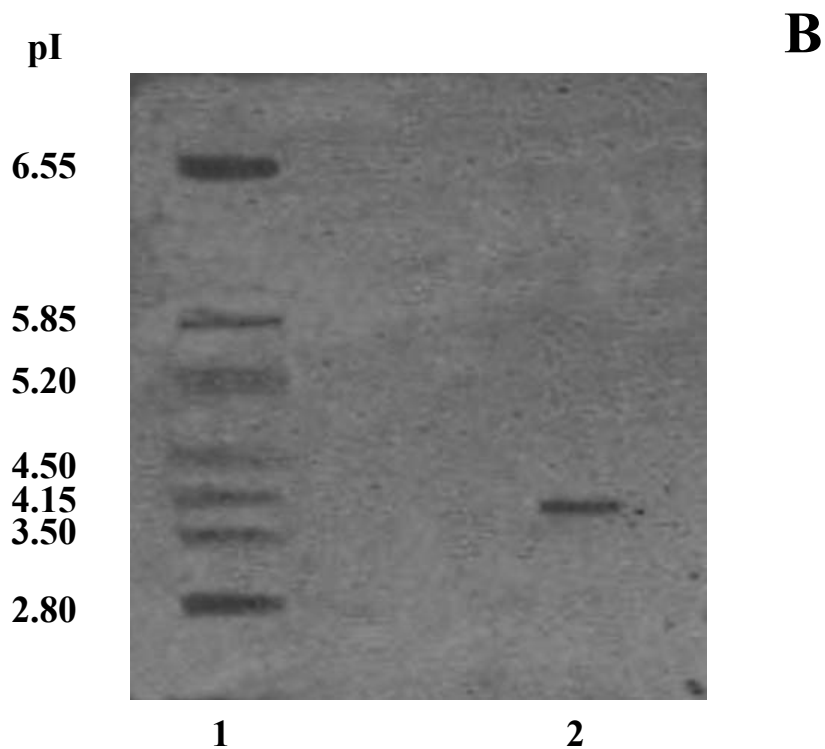
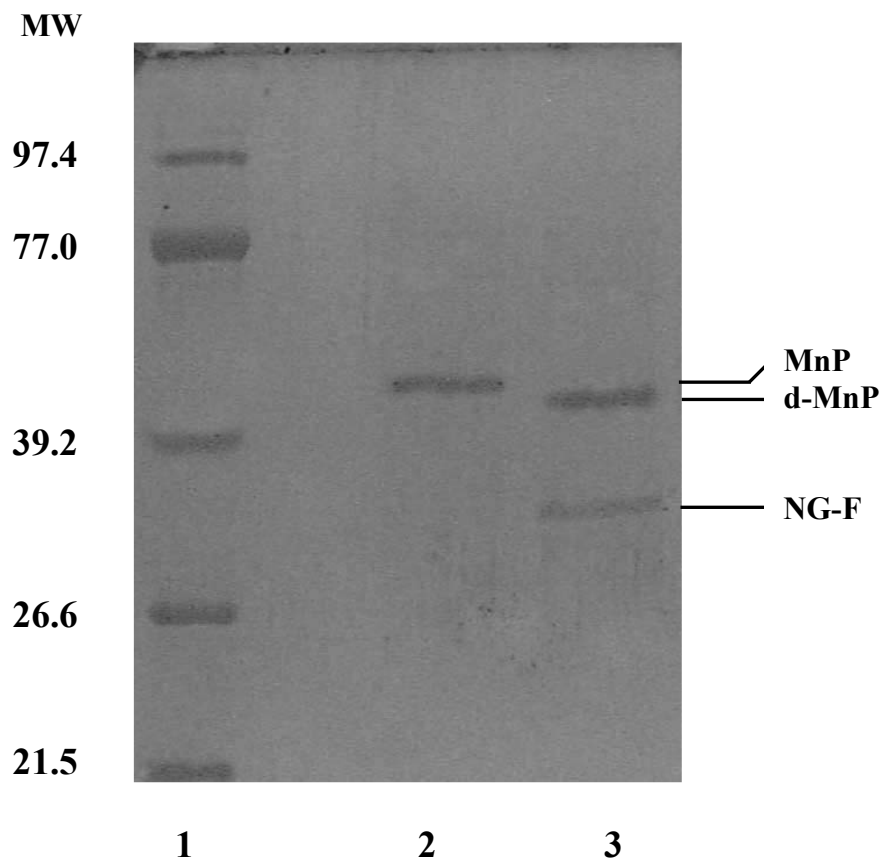


Figure 2.3.2. (A) SDS-PAGE of native (MnP Lane 2, 5 μ g) and deglycosylated (d-MnP Lane 3, 5 μ g) MnP II from *P. tigrinus* CBS 577.79 and molecular weight standards (Lane 1); *N*-glycosidase F, NGF. (B) IEF-PAGE of MnP II (Lane 2, 5 μ g) and pI standards (Lane 1).

2.3.3.3. Physico-chemical properties of *P. tigrinus* MnP II

The determination of the apparent molecular masses of native and denatured enzyme was performed by gel permeation chromatography and SDS-PAGE, respectively, and gave an identical value of 50.5 kDa. MnP II had a carbohydrate content amounting to 5.3% of the molecular mass (Figure 2.3.2-A) and exhibited a pI value of 4.07 (Figure 2.3.2-B).

The analysis of the type of the oligosaccharide bound to the protein showed that the enzyme was specifically recognized by *G. nivalis* agglutinin lectin which is able to bind to terminal mannose residue; lectin specificities suggested the presence of high-mannose-glycans. Negative results obtained with *M. amurensis*, *D. stramonium* and *S. nigra* and peanut agglutinin lectins suggested the absence of both sialylated complex type glycans and *O*-linked oligosaccharide chains containing $\beta(1-3)$ -*N*-acetylgalactosamine at their non-reducing ends.

The UV-vis spectrum of the native enzyme showed the typical absorbance maximum at 405 nm with smaller peaks at 502 and 632 nm (data not shown) and the value of RZ (A_{405}/A_{280}) was equal to 4.8. The presence of the heme was further confirmed by formation of the diagnostic pyridine-hemochromogen complex (PHC). On the basis of the molar extinction coefficient of 33200 M⁻¹ cm⁻¹ for PHC at 556.5 nm, the heme content was calculated to be 0.96 mol per mol of the enzyme.

2.3.3.4. Effect of pH and temperature on activity and stability of MnP

The pH-activity profile for Mn²⁺ oxidation showed an optimum in the range 5.5-5.75 and negligible activity at pHs below 3.5 and above 6.5 (Figure 2.3.3). The optima for both Mn²⁺-dependent and Mn²⁺-independent DMOP oxidation were shifted towards more acidic values (4.0-4.5 and 3.5-4.5, respectively). In the presence and in the absence of Mn²⁺, relative activities for DMOP oxidation at pH 3.0 were about 47 and 53%, respectively, as shown in Figure 2.3.3.

By contrast, the temperature-activity profiles of both Mn²⁺ and DMOP oxidation were very similar with an optimal value of 45 °C and relative activities of 34, 50 and 47% at 10, 40 and 60 °C (data not shown). The determination of MnP stability as a function of pH was conducted at pH values lower than or equal to 6.5, due to the negligible activity exhibited by the enzyme above that threshold. Figure 2.3.4-A shows that the denaturation profiles as a function of time were rather similar in the pH range 4.5-6.5.

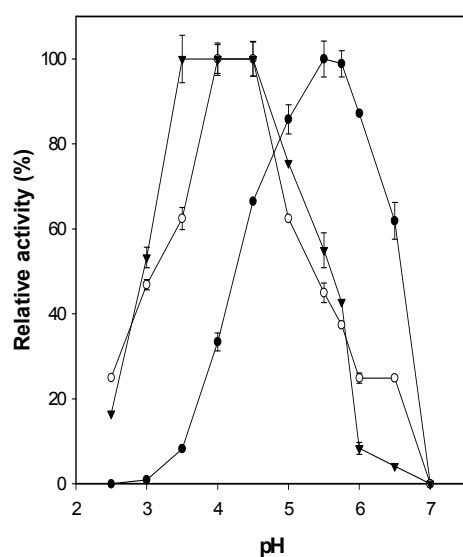


Figure 2.3.3. pH activity profiles in Mn^{3+} -malonate formation (●) and in the Mn-dependent (○) and Mn-independent (▼) oxidation of 2,6-dimethoxyphenol by MnP II of *P. tigrinus* CBS 577.79. Data are the mean of three replicates and standard deviation was less than 10%.

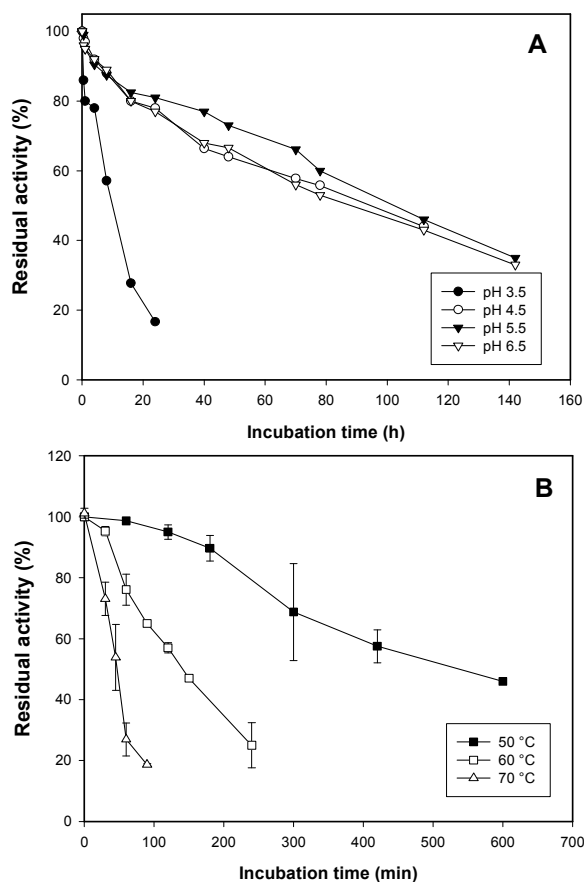


Figure 2.3.4. (A) Residual activity of MnP II from *P. tigrinus* CBS 577.79 at pH 3.5, 4.5, 5.5 and 6.5. Data are the mean $\pm$ standard deviation of three replicates. (B) Residual activity of MnP II at 50, 60 and 70 °C. Data are the mean $\pm$ standard deviation of three replicates.

The half-lives of MnP activity at pH 4.5, 5.0 and 5.5 were 89.5, 99 and 101 h, respectively. By contrast, the enzyme was less stable at 3.5 with a half-life of 8.8 h. Thermal stability experiments showed that the enzyme was rather unstable at 50, 60 and 70 °C, resulting in half-lives of 660, 105 and 41 s, respectively (Figure 2.3.4-B).

2.3.3.5. Kinetic properties of *P. tigrinus* MnP II

Mn²⁺ oxidation by *P. tigrinus* CBS 577.79 MnP II was investigated in the presence of several compounds with reported ability to act as manganese chelators. Table 2.3.2 shows that the highest rate of Mn³⁺ formation was obtained with tartrate (525.9 IU/mg protein). By contrast, pyrophosphate proved to be the worst chelator leading to a specific activity which was about 4.6% of that obtained with tartrate (Table 2.3.2). No inhibition of MnP activity by Mn²⁺ and H₂O₂ was observed in the concentration ranges 0-1.5 mM and 0-0.2 mM, respectively (data not shown).

Table 2.3.2. Effect of chelators on the specific activity of purified *P. tigrinus* CBS 577.79 Mn-peroxidase II.

Chelator	Wavelength (nm)	Extinction coefficient (M ⁻¹ cm ⁻¹)	MnP specific activity ^{a)} (IU/mg protein)	Relative activity (%)
Malonate	270	11590 ^{b)}	288.0 ± 11.5	54.8
Oxalate	270	5500 ^{c)}	340.2 ± 9.7	64.7
Tartrate	290	2860 ^{b)}	525.9 ± 13	100.0
Lactate	290	5890 ^{b)}	254.4 ± 14	48.4
Malate	290	4310 ^{b)}	386.0 ± 15	73.4
Pyrophosphate	258	6200 ^{d)}	24.1 ± 2.2	4.6

Legend: ^{a)} Data are the mean ± standard deviation of four replicates; ^{b)} According to Wariishi *et al.*, 1992; ^{c)} According to Kuan *et al.*, 1993; ^{d)} According to Schlosser *et al.*, 2002.

When testing the oxidation of a terminal phenolic substrate (*i.e.* 2,6-dimethoxyphenol) in the presence of several chelators at a 10 mM concentration, malonate proved to be better than tartrate and oxalate, as shown in Table 2.3.3.

Table 2.3.3. Relative rates of stimulation of the *P. tigrinus* MnP^{II}-catalyzed oxidation of 2,6-dimethoxyphenol in the presence of several organic and inorganic acids.

Compound	Relative rate ^{a) b)} (%)
Citrate	71.1 ± 3.2
Tartrate	43.8 ± 2.4
Lactate	50.8 ± 1.8
Oxalate	48.8 ± 1.6
Pyrophosphate	n.a.
Glyoxylate	21.4 ± 3.2
Malate	44.4 ± 1.6
Malonate	n.a.
Succinate	n.a.
None	n.a.

Legend: n.a., no activity ^{a)} Assays were conducted in 1 ml of 100 mM acetate buffer pH 4.5 containing 0.2 mM 2,6-dimethoxyphenol, 1.0 mM MnSO₄, 10 mM of the tested compound and 1.5 μg MnP II. Reactions were initiated by the addition of H₂O₂ at a 0.15 mM final concentration. ^{b)} Rates of stimulation were calculated with reference to reactions conducted in 100 mM acetate buffer (k_{cat} 13.1 s⁻¹) and expressed as relative rates to those obtained in the presence of malonate, taken as 100;

Figure 2.3.5 shows that double reciprocal plots of initial velocity of Mn(III)-malonate formation vs. $1/[\text{Mn}^{2+}]$ at various fixed H₂O₂ concentrations yielded a set of parallel lines thus suggesting a ping-pong mechanism for the oxidation of Mn²⁺ by *P. tigrinus* MnP II.

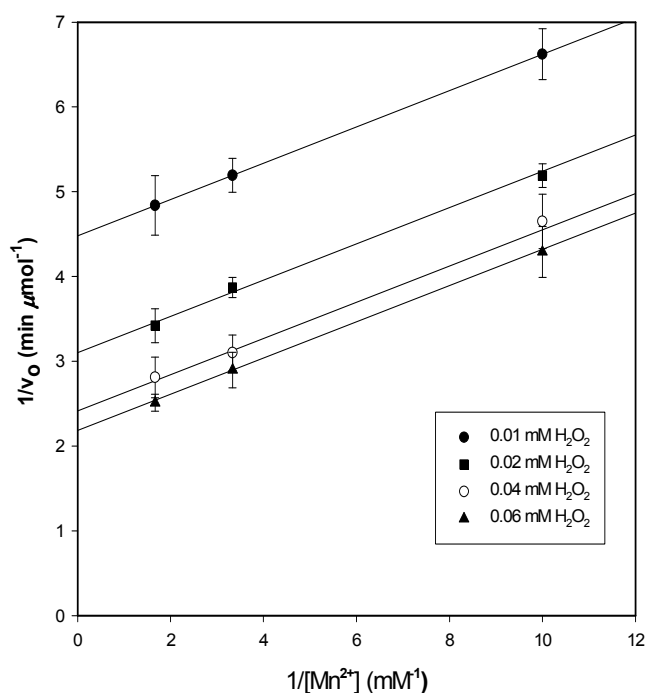


Figure 2.3.5. Double reciprocal plots of initial velocity vs. Mn^{2+} concentration in the presence of fixed H_2O_2 concentrations of 0.01 (●), 0.02 (■), 0.04 (○) and 0.06 (▲) mM. Data are the mean $\pm$ standard deviation of two replicates.

Table 2.3.4 reports kinetic constants for Mn^{2+} and H_2O_2 and those related to both the Mn^{2+} -dependent and Mn^{2+} -independent oxidation of several phenolic and non-phenolic substrates. Turnover numbers (K_{cat}) in the presence of Mn^{2+} were several fold higher than in its absence for each of the substrates tested. Such increases in K_{cat} of Mn^{2+} -dependent over Mn^{2+} -independent reactions ranged from about two-fold for pyrogallol (20.95 vs. 10.18 s⁻¹, respectively) to about twelve-fold in the case of guaiacol (8.92 vs. 0.71 s⁻¹, respectively). In addition, for the majority of the substrates under study, K_m values of MnP II were significantly higher in Mn^{2+} -independent reactions than in the presence of manganese.

The oxidation of the non-phenolic lignin-related compound veratryl alcohol by MnP II was dependent on the presence of both H_2O_2 and Mn^{2+} . Turnover number and K_m for this reaction were 0.45 s⁻¹ and 560 μM , respectively, and the reaction was found to optimally occur at pH 3.5 (Table 4). Opposite to other fungal peroxidases, MnP II was unable to perform the H_2O_2 -independent NADH oxidation.

Table 2.3.4. Kinetic constants (K_m and K_{cat}) and catalytic efficiency (K_{cat} / K_m) of extra-cellular manganese-peroxidase from *P. tigrinus* CBS 577.79. Data are the mean $\pm$ standard deviation of three replicates.

Substrate	K_m (μM)	K_{cat} (s^{-1})	K_{cat} / K_m ($M^{-1} s^{-1}$)
H_2O_2 (1.5 mM Mn^{2+}) ^{a)}	16 ± 1.2	$121.0 \pm 8^b)$	$7.56 \cdot 10^6$
Mn^{2+} (0.2 mM H_2O_2) ^{a)}	124 ± 9.5	242.4 ± 32	$1.95 \cdot 10^6$
ABTS ^{c) d)}	196 ± 8.1	20.20 ± 1.6	$1.03 \cdot 10^5$
ABTS ^{b)} – Mn^{2+} ^{e)}	68.1 ± 2.4	2.05 ± 0.1	$3.01 \cdot 10^4$
2,6-dimethoxyphenol ^{d)}	76 ± 8.2	26.26 ± 0.3	$3.45 \cdot 10^5$
2,6-dimethoxyphenol – Mn^{2+} ^{e)}	7000 ± 234	5.80 ± 0.2	$0.80 \cdot 10^3$
Guaiacol ^{d)}	213 ± 12	8.92 ± 0.6	$4.19 \cdot 10^4$
Guaiacol – Mn^{2+} ^{e)}	2300 ± 125	0.71 ± 0.08	$0.30 \cdot 10^3$
Pyrogallol ^{d)}	293 ± 23	20.95 ± 1.7	$7.15 \cdot 10^4$
Pyrogallol – Mn^{2+} ^{e)}	3700 ± 280	10.18 ± 0.9	$2.70 \cdot 10^3$
Veratryl alcohol ^{f)}	560 ± 45	0.45 ± 0.02	$0.80 \cdot 10^3$
Veratryl alcohol – Mn^{2+}	n.a.	n.a.	n.d.

Legend: n.a. no activity; n.d. not determined; ^{a)} Reactions were conducted in 50 mM malonate-NaOH pH 5.5 (buffer B); ^{b)} K_{cat} for H_2O_2 was calculated according to the reported stoichiometry of two moles of Mn^{3+} produced per mol of H_2O_2 consumed [36]; ^{c)} 2,2'-azinobis-(3-ethylbenzthiazoline)-6-sulfonic acid; ^{d)} Reactions were conducted in 0.05 M malonate-NaOH buffer pH 4.0 (buffer C) containing 0.2 mM H_2O_2 and 1.5 mM Mn^{2+} ; ^{e)} Mn-independent reactions were conducted in buffer C containing 0.2 mM H_2O_2 ; ^{f)} Reactions were conducted in 0.05 M malonate-NaOH pH 3.5 containing 0.2 mM H_2O_2 and 1.5 mM Mn^{2+} .

2.3.3.6. Redox properties

The cyclic voltammograms obtained with the MnP-PGE showed a quasi-reversible redox behaviour (Figure 2.3.6-A). The shape of the peaks is typical of an adsorbed species and does not display any diffusion limitation, which confirms that the peaks are only ascribable to adsorbed MnP.

The redox potential obtained by cyclic voltammograms displayed a pH-dependence as shown in Figure 2.3.6-B indicating that at least two protonating groups are required to account for the observed behaviour.

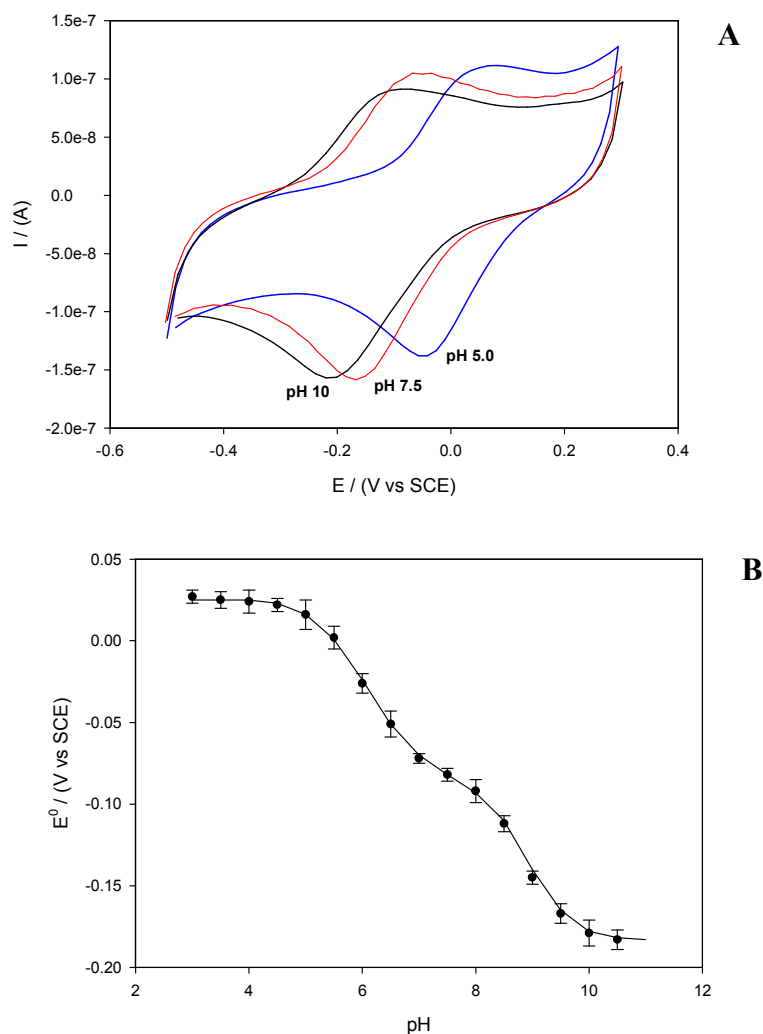


Figure 2.3.6 (A) Cyclic voltammograms at 0.1 V/s of immobilized Mn peroxidase at different pH. (B) pH dependences of the heme pocket reduction potential in *Panus tigrinus* peroxidase. E° values are averages for multiple measurement for scan rate from 5 to 200 mV/s. The solid line is representative of a coupled protonation with the best fitting parameters of Equation (1).

This allowed the determination of the pH values of the controlling ionizations by fitting the data according to equation 1 (Nassar *et al.*, 1997):

$$E = E^0 \left(\frac{1 + K_{b1}^{red} [H^+] + K_{b2}^{red} [H^+]^2}{1 + K_{b1}^{ox} [H^+] + K_{b2}^{ox} [H^+]^2} \right) \quad (1)$$

Where E is the observed redox potential while E^0 (-0.183 ± 0.009 V vs. SCE) is the redox potential of the unprotonated enzyme and K indicates the proton association constant values in the

oxidised (pK_{b1}^{ox} and pK_{b2}^{ox} equal to 9.1 ± 0.2 and 6.2 ± 0.1 , respectively) and in the reduced (pK_{b1}^{red} and pK_{b2}^{red} equal to 8.4 ± 0.3 and 5.5 ± 0.2) forms.

The electroactive protein for the unprotonated enzyme was calculated from the anodic peak integration and was found to be close to 20 pmol cm^{-2} .

Half-height peak width (δ) was 112 mV for MnP at pH 7.5; this value was close to the theoretical δ of 106 mV at 21 °C for Nernstian behaviour and was similar to that reported for proteins immobilized PGE (Armstrong *et al.*, 2000). This almost ideal electron-transfer behaviour was confirmed in the small peak separation between the anodic and cathodic peaks, being close to 81 mV at pH 7.5.

The Butler-Volmer equations (2) and (3), applied to interfacial electron transfer between an electrode and an adsorbed redox couple, were used to define the ET behaviour.

$$k_{red} = k_0 \exp\left\{-\alpha nF(E - E^0)/RT\right\} \quad (2)$$

$$k_{ox} = \exp\left\{(1 - \alpha)nF(E - E^0)/RT\right\} \quad (3)$$

where k_0 is the electron transfer rate constant at zero overpotential and α is the transfer coefficient, representing the degree of symmetry related to E^0 between the rate constants for oxidation and reduction.

Values of k_0 increased with pH; in fact, for the unprotonated form at alkaline (pH=10.5), k_0 was close to $63\pm4 \text{ s}^{-1}$, while for the single protonated and double protonated forms values were $49\pm6 \text{ s}^{-1}$ (pH=7.0) and $37\pm3 \text{ s}^{-1}$ (pH=4.0). In the absence of proton transfer (pH> pK_{b1}), the voltammetric signal was reversible even at 100 V s^{-1} (some pH-independent asymmetry was evident at this high scan rate, although its origin is not known). Conversely, at $pK_{b2}<\text{pH}<pK_{b1}$ and $\text{pH}<pK_{b2}$, where the MnP took up one and two protons, respectively, the voltammetric behaviour was strongly affected by the scan rate.

At low scan rates, the kinetics of the proton transfer reaction is quite close to the scan rate. Consequently, this reaction might be considered close to the equilibrium and under this condition the voltammetric behaviour is reversible and E^0 increases as the pH is lowered. Conversely, at high scan rates, the redox processes, the oxidation in particular, are strongly controlled by the proton transfer reaction. Data show that the electron and proton transfers must be step-wise events. Electron transfer to the active site appeared to drive proton transfer, whereas electron transfer off the active site was “gated” by proton transfer.

2.3.3.7. Bioelectrocatalytic properties of the MnP/PG modified electrode

In Figure 2.3.7 the voltammetric behaviours recorded both in the absence and in the presence of 30 μM H_2O_2 at a MnP/PG modified electrode are compared with those obtained at a bare PGE electrode. Results show the ability of the modified electrode to perform the H_2O_2 electro-reduction thus confirming the catalytic activity retention of the immobilized enzyme.

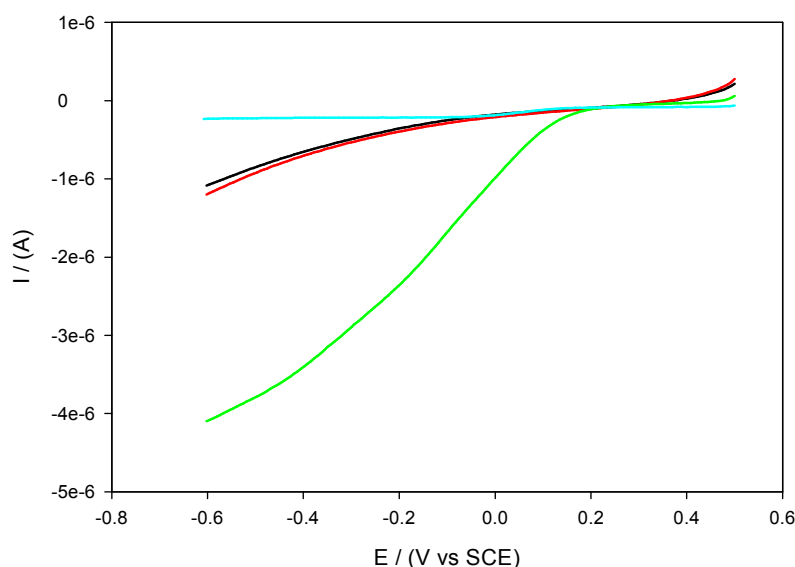


Figure 2.3.7. Catalytic cyclic voltammograms of the reduction of H_2O_2 (30 μM) at bare PGE (red line) and MnP-modified electrode (green line). CV of bare PGE (black line) and MnP-modified electrode (blue line) without H_2O_2 are also reported. Other experimental conditions were: malonate buffer 0.05M pH 4.0, 2000 rpm, 10 mV/s, 25°C.

To evaluate the kinetically limited currents (I_K) obtained in the presence of H_2O_2 , the steady state intensity currents measured by using a RDE at different H_2O_2 concentrations and different electrode rotation speeds were plotted as I^{-1} vs. $\omega^{-0.5}$, as shown in Figure 2.3.8. The slopes and intercepts extrapolated from this plot were plotted against the reciprocal H_2O_2 concentrations (Figure 2.3.9-A and 2.3.9-B, respectively).

From the linear least-squares regression of the dependence of the slopes of the Koutecky-Levich plots on H_2O_2 concentrations, the number of electron transferred was calculated as 2.2 ± 0.3 , which is close to the theoretical value of 2. Kinetically limited reduction currents for H_2O_2 , evaluated from the Koutecky-Levich plots, are reported as a function of the reciprocal $[\text{H}_2\text{O}_2]$. According to the

mathematical expression for I_k , the slope of this plot is proportional to the rate of the reduction of H_2O_2 catalyzed by peroxidase (k_1). The intercept is proportional to the heterogeneous electron transfer rate between oxidised MnP and the PGE surface.

Using the approximate surface concentration of MnP (20 pmol cm^{-2} , evaluated from CV experiments) on the PGE, the rate k_1 of the reaction between H_2O_2 and MnP was calculated as $(0.19 \pm 0.01) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and k_{et}^{app} was $13 \pm 2 \text{ s}^{-1}$ (malonate buffer 0.05M pH 4.0). While the heterogeneous electron transfer between oxidised MnP and the PGE surface increased with the pH (as observed for the rate at zero overpotential in the Butler-Volmer theory), the values of k_1 drastically decrease when the pH was increased; in the single protonated form (pH=7) the value of k_1 was close to $(0.075 \pm 0.012) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and k_{et}^{app} $21 \pm 3 \text{ s}^{-1}$, while for the unprotonated form in alkaline pH (pH=10) we obtained $(0.012 \pm 0.006) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $29 \pm 3 \text{ s}^{-1}$ for k_1 and k_{et}^{app} , respectively.

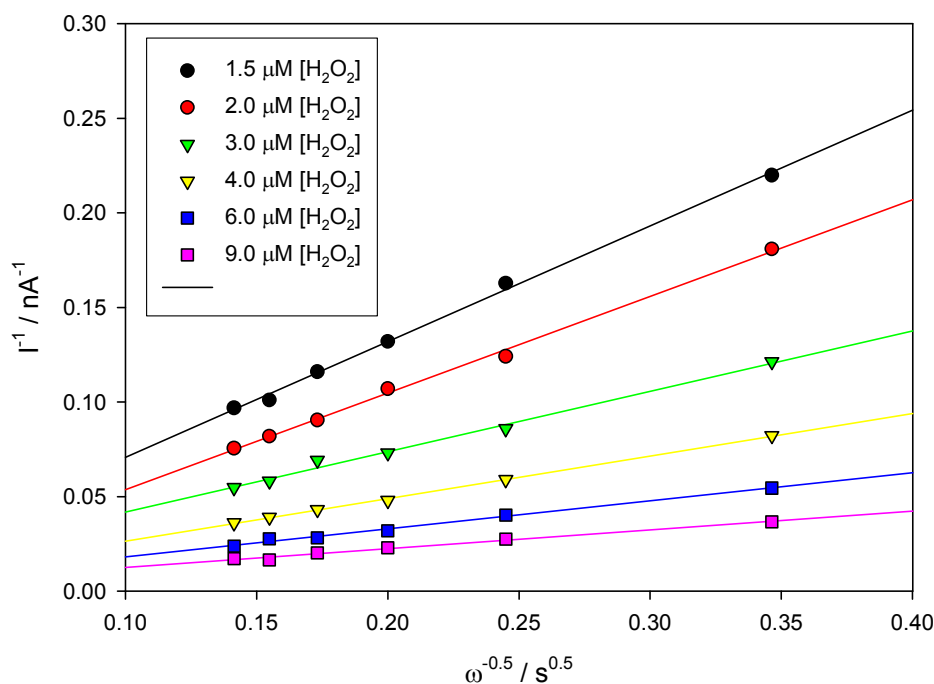


Figure 2.3.8. Koutecky-Levich plots of the RDE modified with MnP in the presence of different concentration of H_2O_2 in 0.05M malonate buffer pH 4. Applied potential: -0.5 V vs SCE.

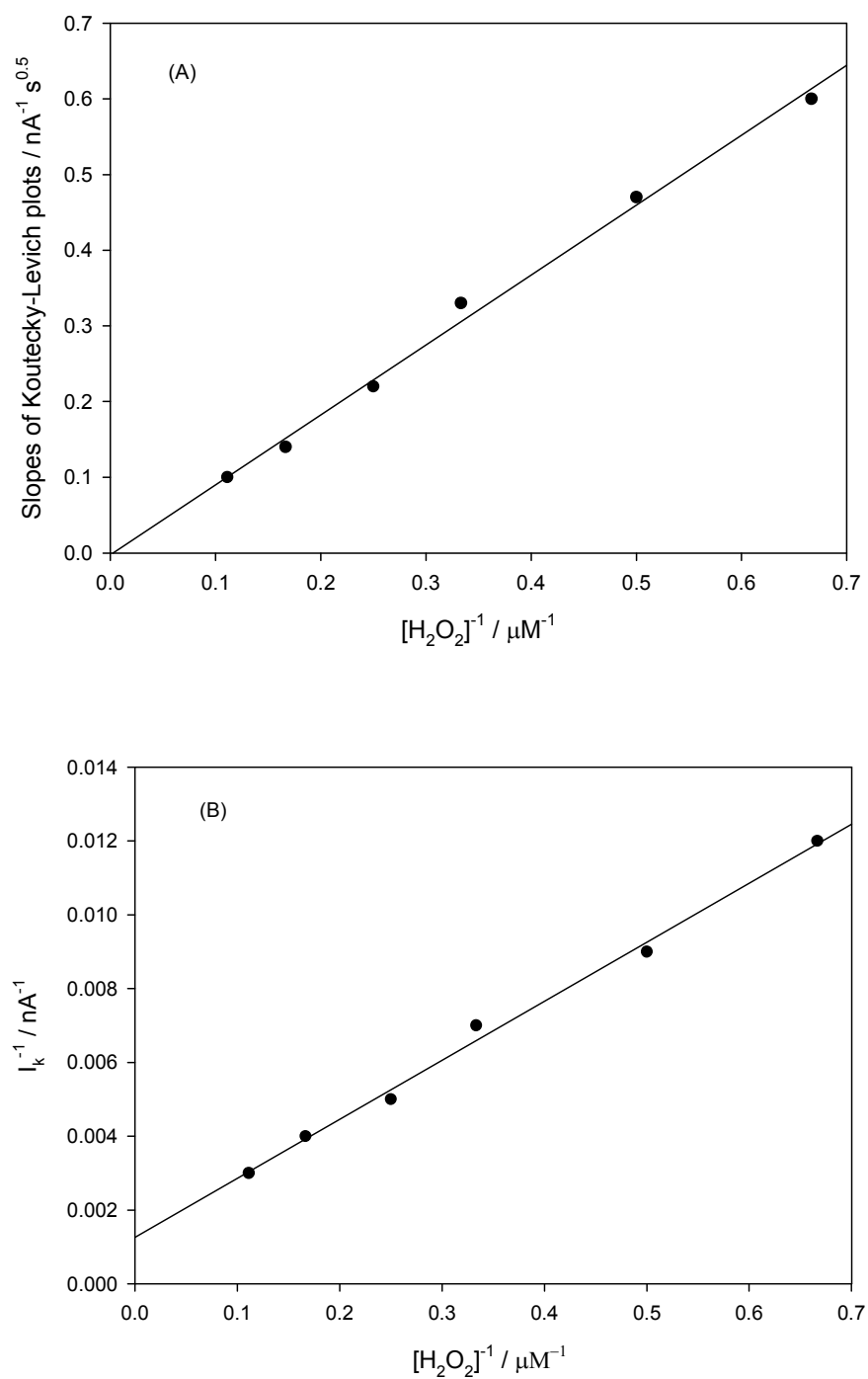


Figure 2.3.9. Dependence of (A) slopes of the Koutecky-Levich plots and (B) intercepts of the Koutecky-Levich plots ($= 1/I_k$) on peroxide concentration.

2.3.4. Discussion

Most of the data available on the lignin-degrading system of the white-rot fungus *P. tigrinus* refer to strain 8/18 (Maltseva *et al.*, 1991; Leontievsky *et al.*, 1994; Lisov *et al.*, 2003; 2005). The total ligninolytic capacity of *P. tigrinus* 8/18 was reported to be highly correlated with MnP production (Leontievsky *et al.*, 1994), which was the main lignin-degrading enzyme in that strain (Leontievsky *et al.*, 1994; Lisov *et al.*, 2003)

By contrast, laccase was the predominant lignin-modifying enzyme in *P. tigrinus* CBS 577.79 (Fenice *et al.*, 2003; D'Annibale *et al.*, 2004) and this strain exhibited a large dephenolization and decolorization capacity of olive mill wastewater even in the presence of initial organic loads higher than 60000 mg/l (D'Annibale *et al.*, 2004). These promising results and the aforementioned differences with the reference strain 8/18 prompted us at purifying and characterizing the physico-chemical, kinetic and stability properties of the main MnP isoenzyme produced by the fungus in liquid cultures. MnP II from *P. tigrinus* CBS 577.79 proved to be a monomeric protein sharing several characteristics with other fungal MnPs, such as a molecular mass around 50 kDa, an acidic *pI* and the presence of *N*-glycosylation of the high-mannose type. However, MnP II was found to greatly differ from manganese-peroxidase purified from solid-state cultures of *P. tigrinus* 8/18 (Golovleva *et al.*, 1993). In fact, MnP from *P. tigrinus* 8/18 had a lower molecular mass (43 kDa), a pH optimum shifted towards more acidic values (2.6) and an optimal value of temperature which was markedly lower than MnP II (25 vs. 45 °C, respectively) (Golovleva *et al.*, 1993). MnP from *P. tigrinus* 8/18 and MnP II also greatly differed for their K_m values for both H_2O_2 (43.5 vs. 16 μM , respectively) and Mn^{2+} (64 vs. 124 μM , respectively).

In this study, the highest rate of Mn^{3+} formation in MnP II-catalyzed reactions was observed using tartrate as the chelator. However, it is widely known that Mn^{3+} -tartrate, like Mn^{3+} -lactate, significantly reacts with H_2O_2 with the subsequent formation of Mn^{2+} and molecular oxygen (Aitken and Irvine, 1990; Schlosser and Hofer, 2002), thus leading to a change in the reported stoichiometry of 2 moles Mn^{3+} per mol of H_2O_2 consumed.

By contrast, this reaction is minimal with malonate and oxalate (Wariishi *et al.*, 1992), the only organic acids to be secreted in significant amounts by white-rot fungi (Kuan *et al.*, 1993; Hofrichter 2002), which both were effective chelators for *P. tigrinus* CBS 577.79 MnP II. In this respect, k_{cat} value for Mn^{3+} -malonate formation was very close to that of the *Phanerochaete chrysosporium* MnP H4 isoenzyme (Kuan *et al.*, 1993). Interestingly, the stimulating action on DMOP oxidation was significant even in the presence of physiological concentrations of the chelators (*i.e.* 0.5 and 2.0 mM). Further investigations on the mechanism of Mn^{2+} oxidation by MnP II were conducted using malonate as the chelator. The parallel trend of double reciprocal plots of initial velocity vs. $[Mn^{2+}]$

at different H_2O_2 concentrations indicated a ping-pong mechanism, as reported for other peroxidases (Aitken and Irvine, 1990; Dunford *et al.*, 1991; Wariishi *et al.*, 1992; Kang *et al.*, 1993).

The present study shows that although all of the tested substrates were oxidized by the enzyme in the absence of Mn^{2+} , the relative kinetic constants markedly differed from those of Mn-dependent reactions. The reason underlying the reduced K_{cat} in Mn-independent reactions was clearly explained by Wariishi and collaborators (1988; 1992) showing that both Mn^{2+} and phenols were able to reduce the catalytic intermediate Compound I to Compound II, although the rate of reduction was markedly faster with Mn^{2+} . In the same study, it was also observed that the half-life for further conversion of Compound II to the native enzyme was less than 10 s in the presence of one equivalent of Mn^{2+} , while the same conversion in the presence of 20 equivalents of either syringic acid or guaiacol required 72 and 160 s, respectively (Schlosser and Hofer, 2002).

Study of absorption spectra of the intermediates of the catalytic cycle of the hybrid peroxidase from *P. tigrinus* 8/18 revealed that the enzyme was able to complete the redox cycle by the use of hydroquinone in alternative to Mn^{2+} to perform the one-electron reduction of Compound II (Lisov *et al.*, 2003). In this respect, the enzyme turned out to be a “hybrid” Mn-peroxidase (h-MnP), differing from conventional MnPs (Lisov *et al.*, 2003). The *P. tigrinus* 8/18 h-MnP brought about the Mn-independent oxidation of hydroquinone, ABTS and *p*-phenylenediamine while it was unable to oxidize guaiacol and showed negligible activity on DMOP in the absence of Mn^{2+} (Lisov *et al.*, 2003). Conversely, MnP II from *P. tigrinus* CBS 577.79 oxidized both guaiacol and DMOP in the absence of Mn^{2+} albeit their turnover numbers were about twelve- and four-fold lower, respectively, than in the presence of Mn^{2+} . In addition, apparent K_m values for these compounds in Mn-independent reactions were markedly higher than those in the presence of the cation.

The capability of MnP of catalyzing the oxidation of phenolics both in the presence and in the absence of Mn^{2+} has already been reported for MnP isoenzymes from *Pleurotus eryngii* (Martinez *et al.*, 1996), *Bjerkandera adusta* (Heinfling *et al.*, 1998), *Pleurotus ostreatus* (Giardina *et al.*, 2000) and *Bjerkandera* sp. strain B33/3 (Moreira *et al.*, 2006). With regard to *P. eryngii* and *B. adusta* MnP isoenzymes, the non-competitive inhibition exerted by Mn^{2+} in the oxidation of the dye Reactive black 5 suggested that the dye and the metal cation bound to different sites (Heinfling *et al.*, 1998).

In the present study, optimal pH value for Mn-independent DMOP oxidation by MnP II was lower than that required for Mn^{2+} oxidation in agreement with another study (Martinez *et al.*, 1996). In addition, MnP II was able to oxidize the non-phenolic compound VA with a low turnover number and the reaction showed a dependence on both Mn^{2+} and H_2O_2 . In this respect, the enzyme

behaved similarly to *Lentinula edodes* MnP isozymes (Dunford *et al.*, 1991; Buswell *et al.*, 1995; D'Annibale *et al.*, 1996), which, unlike *P. chrysosporium* MnP, did not necessarily require the presence of thiol-containing compounds (Wariishi *et al.*, 1989). VA oxidation rate by *P. tigrinus* MnP II was highest at pH 3.0-3.5 as already observed for both plant (Mc Eldoon *et al.*, 1995) and fungal (Martinez *et al.*, 1996) peroxidases and might be likely due to increased redox potential of the oxidized heme at low pH (Mc Eldoon *et al.*, 1995; Heinfling *et al.*, 1998). Also in this respect MnP II differed from *P. tigrinus* 8/18 h-MnP due to the inability of the latter to perform VA oxidation (Lisov *et al.*, 2004). However, another h-MnP purified from *P. tigrinus* 8/18 solid-state cultures displayed the capability to oxidize VA in the presence of Mn^{2+} (Maltseva *et al.*, 1991). The reasons for this difference in activity was ascribed to the presence of two isoforms, the expression of which was possibly affected by culture conditions (Maltseva *et al.*, 1991; Lisov *et al.*, 2004).

A further difference of MnP II from *P. tigrinus* 8/18 h-MnP was its inability to catalyze the oxygen-dependent NADH oxidation, regardless of chelator, pH conditions and reaction times employed (Lisov *et al.*, 2003; 2005). This oxidase-like reaction has been reported for several peroxidase systems (Martinez *et al.*, 1996; Yokota and Yamazaki, 1977; Lisov *et al.*, 2005).

Although *P. tigrinus* MnP II was not strictly dependent on Mn^{2+} in the oxidation of phenols, it can be considered as a manganese peroxidase since Mn^{2+} was its best substrate. However, as suggested for other hybrid peroxidases, the versatility of MnPII isoenzyme might increase the adaptation potential of the strain to the widely fluctuating conditions generally characterizing the natural habitats of the white-rot fungi.

From the electrochemical experiments the direct electron transfer phenomena of the MnP/PG modified electrode has been observed. The results obtained evidence the strictly connection between the MnP electron transfer and the pH values. In particular we can hypothesize that electron transfer to the active site could drive the proton transfer, whereas electron transfer off the active site could be “gated” by the proton transfer. This interdependence between the electron and proton transfer could be ascribed to the influence of the aminoacidic residues present in the redox centre surrounding. Furthermore voltammetric experiments performed at different pH and scan rate values suggest that at low pH values (3÷5) the reversible behaviour is not depending on the pH variation. This could be explained by considering the possibility that the rate of the electron transfer from the electrode surface to the redox centre of the protein could be quite similar to the rate of the proton transfer from the solution to the aminoacidic residue involved in the MnP bioelectrocatalytic activity. Moreover in the case of voltammetric experiments performed at high scan rate values (20-30 V/s) in the pH range of 8÷11, we observed an irreversible behavior of the immobilized MnP, suggesting the hypothesis that the electron transfer from the electrode surface to the redox centre of

the protein and the proton transfer from the solution to the aminoacidic residue take place at different rates. In particular the lower value of the proton transfer rate could be explained with the involvement of an aminoacidic residue embedded into the protein structure.

Finally, another aspect to be taken into account is the catalytic activity retention of the immobilized MnP that opens interesting possibility of applications in the electron transfer based biosensors development.

2.4. *In Vivo* and *In Vitro* Polycyclic Aromatic Hydrocarbons degradation by *Panus tigrinus* CBS 577.79

2.4.1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are ubiquitous environmental pollutants consisting of two or more fused benzene rings in linear, angular, or cluster arrangements. The persistence of these compounds in the environment is mainly due to their low solubility in water and stable polycondensed aromatic structure. Hydrophobicity and recalcitrance to microbial degradation generally increase as the molecular weight increases (Cerniglia, 1992; Bezalel *et al.*, 1996). Moreover, many PAHs are toxic to animals and some of the compounds with four or more benzene rings, like benzo[*a*]anthracene, crysene and benzo[*a*]pyrene, have been shown to be carcinogenic (Cerniglia *et al.*, 1992). In the last few decades, different approaches to PAHs biodegradation have been investigated. Among the microorganisms tested, white rot fungi (WRF) have captured attention of research groups since the early studies with *P. chrysosporium* (Haemmerli *et al.*, 1986; Hammel *et al.*, 1986). WRF are the most efficient lignin degraders in nature and are capable of degrading and, to some extent, mineralizing a wide range of xenobiotics by means of their extracellular enzyme system (Hammel *et al.*, 1986; Bogan & Lamar 1996; Bezalel *et al.*, 1996; Scheibner *et al.*, 1997; Hofrichter *et al.*, 1998; Cajthaml *et al.*, 2006). In this respect, laccases and ligninolytic peroxidases produced by WRF can degrade PAHs under *in vitro* conditions (Haemmerli *et al.*, 1986; Majcherczyk *et al.*, 1998; Eibes *et al.*, 2006; Baborova *et al.*, 2006). Nevertheless, the use of natural or synthetic mediators can extend both the range of PAHs oxidized by ligninolytic enzymes and enhance their degradation rates (Sack *et al.*, 1997; Johannes & Majcherczyk, 2000; Cañas *et al.*, 2007; Camarero *et al.*, 2009). Besides the extracellular degradation pathway, there is an intracellular one mainly involving cytochrome P-450 monooxygenase and epoxide hydrolase in the initial degradation steps (Bezalel *et al.*, 1996, 1997).

The WRF *P. tigrinus* (strain CBS 577.79) was found to be an efficient producer of both laccase and Mn-peroxidase (MnP) in both solid-state (Fenice *et al.*, 2003) and liquid (Quaratino *et al.*, 2006; Quaratino *et al.*, 2007) cultures. Major isoenzymes of both laccase and MnP from *P. tigrinus* CBS 577.79 were purified from liquid cultures and their main biochemical and redox properties characterized (Quaratino *et al.*, 2007; Petruccioli *et al.*, 2009). In addition, the same strain was capable of degrading and detoxifying olive-mill wastewater, an agro-industrial phenol-containing effluent, and to withstand high organic loads (D'Annibale *et al.*, 2006).

Objectives of the present study were: (i) to assess PAH-degradation capability of *P. tigrinus* strain CBS 577.79 in liquid cultures, (ii) to clarify the possible involvement of laccase and MnP in the degradation process and (iii) to identify the fungal PAH degradation products. The first aim was pursued by growing the fungus on both N-rich and N-limited standard media spiked with selected PAHs under stationary and shaken conditions. *In vitro* degradation experiments with purified laccase and MnP preparations from the strain were performed to pursue the second aim. With regard to the third objective, the possible presence of active forms of cytochrome P-450 monooxygenases was investigated due to the presence of certain fungal degradation intermediates which have been ascribed to the action of this enzyme (Bezalel *et al.*, 1996; Bezalel *et al.*, 1997).

2.4.2. Materials and methods

2.4.2.1 Materials

Anthracene (ANT), phenanthrene (PHE), fluoranthene (FLT), pyrene (PYR), benzo[*a*]anthracene (BaA), chrysene (CHR), benzo[*a*]pyrene (BaP) were purchased from Fluka (Germany). Purity of the chemicals was higher than 96-98%. A stock solution of PAH mixture (PAHM) was prepared by dissolving each PAH in DMSO at a final concentration of 5 g l⁻¹. *N*-hydroxybenzotriazole (HBT) and reduced glutathione (GSH) were purchased from Sigma (Germany). All the compounds were in dimethylsulfoxide (*Sigma*, Germany). All solvents of p.a. quality, trace analysis quality or gradient grade were purchased from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was from Sigma (Germany). *P. tigrinus* laccase (*PtL*) and MnP II (*PtM* II) isoenzymes were purified as reported elsewhere (Quaratino *et al.*, 2007; Petruccioli *et al.*, 2009).

2.4.2.2. Organism, culture media and inocula preparation

As reported above, *P. tigrinus* (strain 577.79) was obtained from CBS culture collection (Baarn, The Netherlands). The fungus was maintained on Malt Extract Agar plates at 4°C and sub-cultured every 21 days. Two different liquid media were used in this study: Low-N Kirk's medium (LNKM) containing 2.4 mM diammonium tartrate (Tien and Kirk, 1988), and complex malt extract glucose medium (MEG), containing 5 g and 10 g l⁻¹ malt extract broth (Oxoid, Basingstoke, UK) and glucose, respectively. Inocula were prepared in 250 ml Erlenmeyer flasks containing 20 ml of MEG or LNKM starting from 2 mycelial plugs (0.7 mm Ø). Cultures grown for 7 days at 28 °C

were homogenized by Ultraturrax-T25 (IKA-Labortechnik, Staufen, Germany), and 1 and 2 ml aliquots of the mycelial suspension were used to inoculate 20 ml static and 40 ml submerged cultures, respectively.

2.4.2.3 Culture conditions

Experiments were conducted at 28 °C under both stationary and reciprocal shaking conditions (100 rpm) in 250 ml Erlenmeyer flasks either containing 20 ml of MEG or LNKM. Immediately after the inoculation, stationary and shaken cultures were spiked with 100 and 200 µl of PAHM in dimethylformamide, respectively, to yield a final concentration of 25 mg l⁻¹ for each compound. Two harvests were performed during the fermentation (after 2 and 4 weeks for shaken flasks and 3 and 6 weeks for stationary flasks). Parallel degradation experiments with single compounds (25 mg l⁻¹) were performed under the same conditions. Heat-killed controls (HKC) were run with mycelia previously sub-cultured for 7 days, then killed by autoclaving (121 °C, 20 min) and consequently contaminated with the appropriate amount of PAHs. Biotic controls (BC) were prepared by spiking stationary and shaken cultures with 100 and 200 µl of DMSO alone. All the degradation experiments were carried out in triplicate in the dark.

2.4.2.4 Detection and quantitation of cytochrome P-450

Microsomal fractions were extracted from 7- and 20-day-old shaken cultures in MEG medium (Cajthaml *et al.*, 2008). Briefly, the mycelial pellets were filtered on nylon cloth, washed with cold potassium phosphate buffer (100 mM, pH 7.2) and disrupted in a Virtis 45 blender at 375 Hz in the same buffer supplemented with glycerol (200 g l⁻¹) and BSA (1.5 g l⁻¹). The supernatant was centrifuged (10000 x g, 15 min, and 100000 x g, 90 min) and the second supernatant was referred to as the cytosolic fraction. The pellets were then suspended in the same buffer, centrifuged again (100000 x g, 90 min) and then resuspended and stored in phosphate buffer (50 mM, pH 7.2) containing glycerol (300 g l⁻¹), EDTA (0.1 mM) and GSH (0.1 mM). The pellets thus obtained were referred to as the microsomal fraction. Total protein content was determined by the dye-binding method using BSA as the standard (Bradford, 1976). Cytochrome P-450 was determined in both cytosolic and microsomal fractions using CO-binding spectra (Omura and Sato, 1964).

2.4.2.5 Enzyme Assays

During fungal cultivation with the PAHs, MnP and Mn-independent peroxidase (MIP) activities were spectrophotometrically determined at 469 nm by using 2,6-dimethoxyphenol ($\epsilon = 49600 \text{ M}^{-1} \text{ cm}^{-1}$) as the substrate and corrected for the laccase activity by omitting H_2O_2 (Kaal *et al.*, 1993). One unit of enzyme activity (U) is defined as the amount of enzyme which produces 1 μM of product per minute under the assay conditions.

2.4.2.6. Sample preparation and analytical methods

The whole content of each flask was homogenized with Ultraturrax-T25 (IKA-Labortechnik, Staufen Germany), then acidified to $\text{pH} \approx 2$ and extracted five times with ethyl acetate (20 ml portions). Microbial biomass concentration was gravimetrically determined: cultures were filtered on pre-weighed Whatman GF/C discs (diameter, 47mm), the harvested biomass was washed twice with distilled water and the filter was dried at 105°C for 24 h, cooled in a desiccator and weighed.

The ethyl acetate extracts were dried with anhydrous sodium sulphate columns (3 g), concentrated with a rotary evaporator diluted again to exactly 10 ml of ethyl acetate. To enable HPLC analysis, an aliquot of extract was mixed with acetonitrile at a ratio of 1:10, this mixture being used for injection. PAH degradation was monitored by reversed-phase high performance liquid chromatography (RP-HPLC) using a HPLC system consisting of a 2695 Separations Module (Waters) equipped with a LichroCart-PAH column filled with LichroSphere ($250 \times 5 \text{ mm}$, particle $\text{Ø } 5 \mu\text{m}$; Merck, Germany) and a 2996 diode-array detector (Waters). An isocratic program was applied with acetonitrile–water 9 : 1 (v/v) and PAHs were quantified at their respective UV maxima.

PAH degradation intermediates were separated and identified by gas chromatography-mass spectrometry (450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA). The ethyl acetate extracts were both directly injected without any derivatization and also after trimethylsilylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Merck, Germany) and methylation with diazomethane (Cajthaml *et al.*, 2002). The GC instrument was equipped with split/splitless injector and a DB-5MS column was used for separation (30 m, 0,25 mm I.D., 0,25 μm film thickness). The temperature program started at 60°C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 120°C at a rate of $25^\circ\text{C min}^{-1}$. The second temperature ramp was up to 240°C at a rate of $10^\circ\text{C min}^{-1}$, this temperature being maintained for 20 min. The solvent delay time and transfer line temperature were set at 6 min and 240°C , respectively. Mass spectra

were recorded at 3 scan s⁻¹ under electron impact at 70 eV and mass range 50-450 amu. The excitation potential for the MS/MS product ion mode applied was 0.2 V and it was increased to 0.8 V in the case of more stable ions. Acetonitrile was used as the medium for chemical ionization, where the maximum time for ionization was 2000 µs and 40 µs for reaction.

2.4.2.7. *Stability of laccase and MnP activity in different water:solvent mixtures*

All experiments (1 ml reaction mixtures) were performed in triplicate in 2 ml reaction tubes endowed with teflon-lined screw caps. The stability of laccase activity (2 U) was assessed in 0.1 M acetate buffer (pH 4.5) containing Tween 80 (1%, w/v) and acetone (5%, v/v), either in the presence or in the absence of the synthetic mediator HBT (1 mM), over a period of 168 hours. Similarly, the activity of MnP (2 U) was monitored in 0.05 M malonate-NaOH buffer (pH 4.5) and in different solvent systems (*i.e.*, 10 and 20% acetone in 0.05 M malonate-NaOH buffer, pH 4.5). In both cases GSH (5 mM) was either omitted or added.

2.4.2.8. *In vitro oxidation of PAHs with purified enzymes*

All experiments were performed in triplicate in 2 ml reaction tubes endowed with teflon-lined screw caps. Degradation experiments with laccase were conducted in 1 ml reaction mixtures containing 0.1 M acetate buffer pH 4.5 added with 1% (w/v) Tween 80, 20 µl of a stock solution of each PAH (0.15 g l⁻¹ in acetone) and 2 U laccase. Incubations were performed at 28 °C for 168 h on a rotary shaker (80 rpm). In mediated reactions, HBT was added at a 1 mM final concentration.

In vitro degradation tests with MnP were conducted in 1 ml reaction mixtures containing 0.05 M malonate-NaOH buffer pH 4.5, 0.01 M MnSO₄ and 2 U MnP. Glucose (0.03 M) and glucose-oxidase (0.06 U) were included in the reaction mixture to ensure a gradual and continuous H₂O₂ production. The reaction mixture was then spiked with 200 µl of each PAH (15 mg l⁻¹ in acetone) and then incubated as above. GSH was either omitted or added at a final concentration of 5 mM.

Relative abiotic controls were performed by adding either heat-denatured laccase or MnP to reaction mixtures. Quantitative analyses were initially performed after 0, 1.5, 3, 5, 6 and 24 h incubation. Thereafter, samplings were done on a daily basis. On the one hand, and as for quantitative analyses, the direct injection of reaction mixtures into HPLC was allowed by the quantitative solubilisation of PAHs. Recoveries of each of the PAHs in the abiotic controls were higher than 90% with the only exception of anthracene (70-75%). On the other hand, prior to qualitative analyses, samples were acidified with 1 N HCl to pH ≈3 and then extracted with four 1-

ml portions of ethyl acetate. The solvent was then concentrated under N₂ stream and injected into GC-MS for the detection of possible metabolites.

2.4.3. Results and Discussion

2.4.3.1 Enzymatic activities in *P. tigrinus* liquid cultures

Laccase and MnP from *P. tigrinus* have been shown to undergo distinct physiological regulation mechanisms. In this respect, laccase activity is stimulated in N-rich media and expression levels of one of the two constitutive genes are enhanced in the presence of an aromatic inducer (Quaratino *et al.*, 2007). By contrast, the onset of MnP occurs in concomitance with ammonium depletion and its production is boosted by the presence of Mn²⁺ ions and a phenols mixture (Quaratino *et al.*, 2006).

These previous findings were partially confirmed by the present study. Table 2.4.1, in fact, shows that in the N-rich medium (*i.e.*, MEG), laccase was the predominant ligninolytic enzyme produced by *P. tigrinus* both under stationary and shaken conditions. MnP was mainly produced in low-N media and the highest activity (60 U l⁻¹) was reached under stationary conditions during the third week of cultivation.

Table 2.4.1. Biomass production at the first and second harvest and maximal activities of laccase and MnP in *P. tigrinus* CBS 577.79 cultures.

Medium	Culture conditions	Spiking	Biomass ^{a)} (g l ⁻¹)		Laccase ^{b)} (U l ⁻¹)	MnP ^{b)} (U l ⁻¹)
			1st	2nd		
MEG	Stationary	BC	2.4±0.1	2.2±0.2	8.2±4.6 (17)	6.0±2.3 (13)
		PAHM	5.2±0.3	4.8±0.3	63.9±17.8 (9)	39.4±22.1 (9)
	Shaken	BC	2.2±0.2	2.0±0.1	24.3±6.3 (24)	13.4±6.8 (13)
		PAHM	1.6±0.1	1.6±0.1	56.6±4.3 (9)	19.5±7.4 (9)
LNKM	Stationary	BC	1.5±0.1	1.4±0.2	5.2±3.8 (13)	23.5±9.2 (20)
		PAHM	0.8±0.1	0.7±0.1	19.2±2.1 (9)	60.0±12.5 (28)
	Shaken	BC	0.6±0.1	0.6±0.1	2.3±5.1 (20)	13.2±5.4 (17)
		PAHM	1.5±0.1	1.6±0.1	16.6±4.6 (28)	40.8±9.6 (20)

a) Biomass was determined in concomitance with harvests set for PAH analyses (*i.e.*, 2 and 4 weeks for shaken cultures and 3 and 6 weeks for stationary cultures; b) days required to attain the activity peaks are reported between round brackets.

In shaken cultures, MnP activity peak (41 U l^{-1}), albeit lower than stationary cultures, was anticipated by one week. Both enzyme activities were significantly stimulated in cultures that had been spiked with the PAHM with respect to the relative biotic controls. In particular, spiking of MEG with PAHM gave rise to two activity peaks after 9 and 28 d which were from 5 to 8 fold higher than those of the relative BC (Tab. 2.4.1). Same results were also observed for MnP although the stimulatory effect of PAHs were generally lower than for laccase. These results are in agreement with other studies (Cajthaml *et al.*, 2006, Cajthaml *et al.*, 2008) suggesting that either PAHs or their degradation intermediates might stimulate the activity of ligninolytic enzymes. In addition, they confirm the stimulatory effect of aromatic compounds on the production of *P. tigrinus* ligninolytic enzymes (Quaratino *et al.*, 2006; Quaratino *et al.*, 2007). No MIP activity was detected, regardless of both media and growth conditions.

2.4.3.2 In vivo degradation of PAHs

PAH degradations in shaken and stationary *P. tigrinus* cultures are reported in Figure 2.4.1 and 2.4.2., respectively. Although after two-week incubation the extent of PAH degradation in shaken cultures was limited, some compounds, including Phe, Ant and Pyr, were more degraded in MEG than in LNKM.

Regardless of the medium, PHE was quantitatively removed from 4-week-old shaken cultures despite its high ionization potential ($IP = 7.91 \text{ eV}$), while CHR ($IP \approx 7.59 \text{ eV}$). PAH residual concentrations under stationary conditions are reported in Figure 2.4.2. Although the overall PAH removal observed in the first harvest of stationary cultures was higher than shaken ones, a mass balance in 6 week-old cultures showed a PAH recovery of 47 and 49% with respect to the original amount added to MEG and LNKM, respectively; with this regard, and on the same media, this recovery was equal to 8.7 and 3.2% in 4-week-old shaken cultures, respectively. CHR confirmed to be the most recalcitrant PAH, with only 24 and 16% being degraded in high-N and N-limited cultures, respectively. Recovery of PAHs in HKC was higher than 90% in all cases.

Regardless of incubation time and growth medium, ANT and CHR were the most and the least susceptible PAHs to degradation. In general, PAH degradation was neither related with biomass production nor with ligninolytic enzyme activities. In fact, biomass concentration remained constant between the two harvests and in the majority of the conditions activity peaks of both laccase and MnP were attained prior to the first harvest (Table 2.4.1).

In spite of a relatively low activity of the ligninolytic enzymes in our set of cultures, the resulting degradation of the PAHM was substantial. Although both biomass production and ligninolytic

activities were generally higher in stationary than in shaken cultures (Table 2.4.1), best PAH degradation was observed in the latter. Less than 10% recovery of the 7 PAHs added was observed in MEG and almost complete disappearance was achieved in LNKM after 4 weeks of cultivation. Such removal efficiencies were akin to, and in some case higher than those reported for *P. chrysosporium*, *Pleurotus ostreatus*, *Bjerkandera adusta*, *Trametes versicolor* and *Irpex lacteus* tested in similar degradation studies (Field *et al.*, 1992; Vyas *et al.*, 1994; Cajthaml *et al.*, 2008).

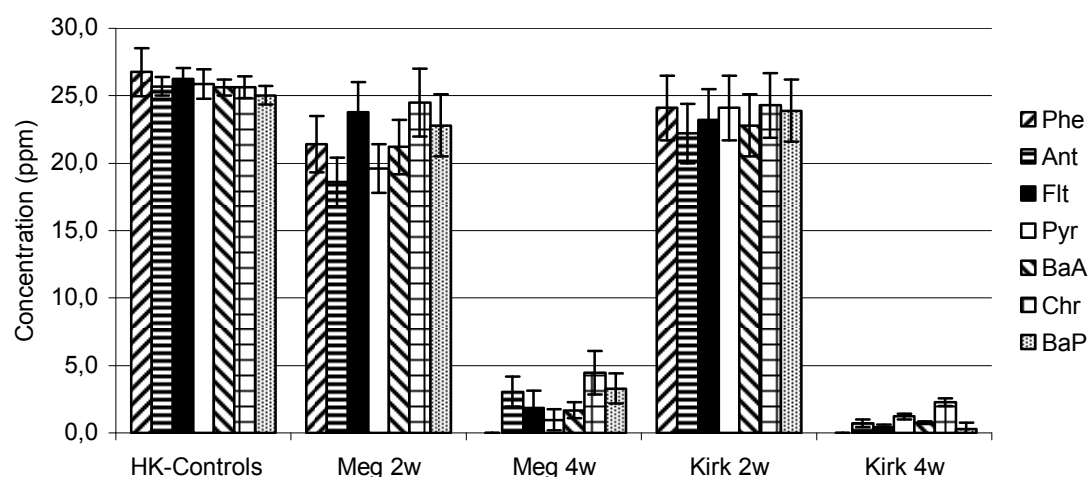


Figure 2.4.1. Degradation of PAHs (recovery, %) by *Panus tigrinus* submerged cultures in MEG and LNM (Kirk). Recovery in the heat killed controls are also reported.

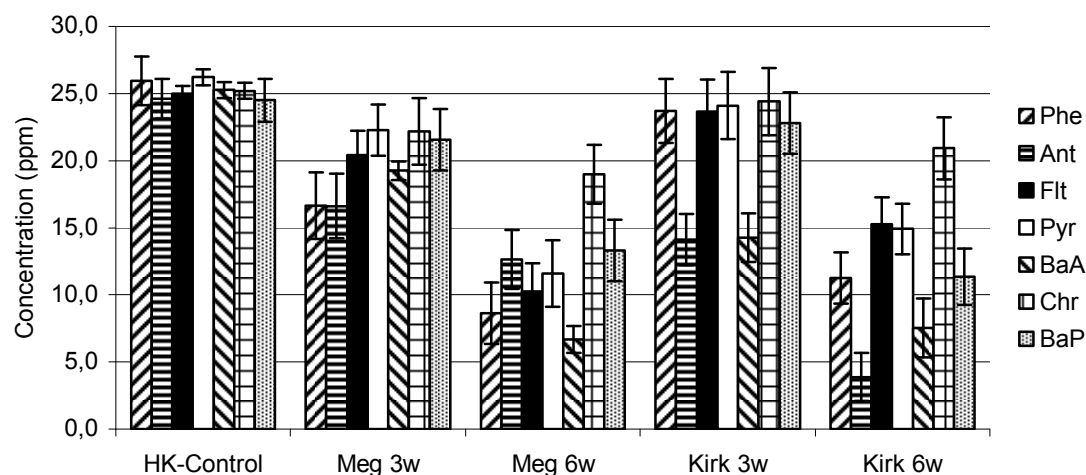


Figure 2.4.2 Degradation of PAHs (recovery, %) by *Panus tigrinus* static cultures in MEG and LNM (Kirk). Recovery in the heat killed controls are also reported.

2.4.3.3 Detection of PAH degradation products

The detection of several intermediates resulting from the degradation of the PAH mixture prompted us to perform an experiment with individual PAHs, in order to correlate metabolites to their parental molecules. Table 2.4.2 lists retention times and mass spectral characteristics of the detected degradation products. The structures signed with asterisks were later confirmed with respective chemical standards. Some of the compounds were detected after derivatization with BSTFA or diazomethane or without derivatization. In the latter case, non-derivatized compounds were detected as the corresponding dehydrated forms (*i.e.*, anhydrides and lactones) due to dehydration occurring during injection into GC-MS. The degradation intermediates were identified by comparing the mass spectra with data in the NIST 05 library, and independently by interpreting the fragmentation pattern. The structures of metabolites were explored using MS/MS (product ion scan) to clarify the fragmentation sequence and chemical ionization was employed to find molecular weight. Most of the compounds were later confirmed with authentic chemical standards. The detailed MS/MS characteristic of the suggested intermediates were published in previous works (Cajthaml *et al.*, 2002; Cajthaml *et al.*, 2006).

Decomposition of ANT by *P. tigrinus* coincides with previously reported results of ligninolytic fungi (Hammel *et al.*, 1991, Cajthaml *et al.*, 2002). Anthrone and 9,10-anthraquinone were the major metabolites of anthracene in both media, although only low amount was detected with GC-MS, suggesting that none of them was a dead-end product. A further cleavage of aromatic structure was observed when 2-(2'-hydroxybenzoyl)-benzoic acid was detected. This ring fission could be attributed to the action of *P. tigrinus* MnP as it was reported for another fungus (Baborová *et al.*, 2006). Other consequent intermediates were phthalic acid and 2-hydroxymethyl-benzoic acid, that were detected as dehydrated forms of the original compounds or as (suggesting) further cleavage of 2-(2'-hydroxybenzoyl)-benzoic acid. Other ANT oxidation products *i.e.* hydroxylated anthron and hydroxylated anthracene show a possible involvement of hydroxylation by cytochrome P-450 monooxygenase complex. All the anthracene degradation products were detected in both of the media.

Phenanthrene-9,10-dihydrodiol, which was already found in other studies with *P. ostreatus* (Bezalel *et al.*, 1996), *P. chrysosporium* (Sutherland *et al.*, 1991) and *I. lacteus* (Cajthaml *et al.*, 2002), indicates also the possible involvement of the intracellular cytochrome P-450-epoxide hydrolase system in the initial oxidation of the parental compound. The formation of the ring cleavage metabolite 2,2'- diphenic acid, that was detected as dimethyl derivative after application of diazomethane, was observed in our study as well.

Table 2.4.2. Retention data and electron impact mass spectral characteristics of PAH metabolites

t_R (min)	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion	Possible origin	Notes
10.155	134	134 (14), 105 (100), 77 (42), 51 (9)	phthalide*	ANT	dehydrated form
10.218	166	148 (2), 104 (100), 76 (42), 50 (23)	phthalic anhydride*	ANT	dehydrated form; detected also as di-TMS derivative
27.141	198	198 (80.1), 154 (87.9), 126 (100)	1,2-naphthalic anhydride	BaA	dehydrated form; detected also as dimethylated derivative with diazomethan
29.708	270	211 (100), 196 (14), 180 (11), 168 (9), 152 (15), 139 (11), 104 (7), 76 (8)	2,2'-dimethylbifhenic acid*	PHE	methylated derivative with diazomethan
31.596	194	194 (100), 165 (98), 139 (47), 81 (36)	anthrone*	ANT	
32.370	208	208 (100), 180 (63), 152 (56), 126 (6), 76 (6)	9,10-anthracenedione*	ANT	
34.414	198	198 (67), 154 (100), 126 (65), 74 (11)	1,8-naphthalic anhydride*	FLT	dehydrated form; detected also as dimethylated derivative with diazomethan
34.464	224	224 (100), 208 (28), 196 (69), 168 (70), 139 (36)	?-hydroxy-9,10-anthracenedione	ANT	
36.088	242	256 (36), 225 (34), 224 (100), 223 (76), 196 (76), 168 (34), 139 (57), 121 (55), 77 (36)	2-(2'-hydroxybenzoyl)-benzoic acid methyl ester	ANT	methylated derivative with diazomethan
36.274	210	210 (100), 193 (52), 180 (78), 165 (73), 152 (61)	?-hydroxyanthrone	ANT	
37.027	212	212 (100), 194 (35), 181 (54), 165 (68), 153 (14), 152 (13), 77 (23)	phenanthrene-9,10-dihydrodiol*	PHE	
43.134	220	220 (100), 192 (32), 163 (47)	lacton of 4-hydroxy-5-phenanthrenecarboxylic acid	PYR	
52.496	258	258 (100), 230 (43), 202 (45), 174 (5), 150 (6)	benz(a)anthracene-7,12-dione*	BaA	

* structures were later identified with authentic standard.

A possible explanation could be an activity of either some intracellular ring opening enzyme *e.g.*, protocatechuate 3,4-dioxygenase (Bezalel *et al.*, 1997) or an involvement of ligninolytic system (Baborová *et al.*, 2006).

BaA was metabolized by *P. tigrinus* at the first step probably via a typical pathway of ligninolytic fungi with formation of appropriate quinone (Vyas *et al.*, 1994). Benzo[a]anthracene-7,12-dione and another degradation product naphthalene-1,2-dicarboxylic acid were detected in the ethyl acetate extract of *P. tigrinus* static cultures grown in LNMM. Single PAH cultures confirmed the latter one was a degradation product of BaA via cleavage of the aromatic ring, as it was already demonstrated by Cajthaml and coworkers (2006). The only degradation product of FLT that was found in this study was naphthalene-1,8-dicarboxylic acid, detected as its dehydrated anhydride form. The same compound was already identified in a previous work after degradation with *I. lacteus*. A similar situation was with PYR decomposition product, where the degradation resulted in lactone of 4-hydroxy-5-phenanthrenecarboxylic acid representing a dehydrated form of the respective hydroxylated acid (Cajthaml *et al.*, 2002).

2.4.3.4 Detection of cytochrome P-450

Cytochrome P-450-epoxide hydrolase is a constitutive enzymatic system present in all eukaryotic organisms. It is known that it plays a key role in the oxidation of xenobiotics in fungi (Sutherland, 1992; Sutherland *et al.*, 1993; Masaphy *et al.*, 1995; Bezalel *et al.*, 1996, 1997; Cajthaml *et al.*, 2008). Cytochrome P-450 was mainly detected in the microsomal fraction of *P. tigrinus* biomass grown on MEG medium, probably because of the scant growth in LNKM. In particular, about 560 and 2.2 pmol mg⁻¹ protein were found in the microsomal and cytosolic fraction, respectively, from 7-day-old cultures grown on MEG (Figure 2.4.3). By contrast, no active cytochrome P-450 was detected in the above fractions of 20-day-old cultures (data not shown). In fact, spectral scans performed on the microsomal fraction isolated from the cultures incubated for longer times than 7 days showed the presence of a major peak at 420 nm and no significant peak at 450 nm, suggesting that almost all the cytochrome P-450 was converted into inactive form. Similar results were reported by Cajthaml *et al.* (2008) for the WRB *I. lacteus* grown on the same media. This result seems to be in agreement with the hypothesis that the intracellular pathway may play a role in the initial hydroxylation of PAHs in fungi (Bezalel *et al.*, 1997).

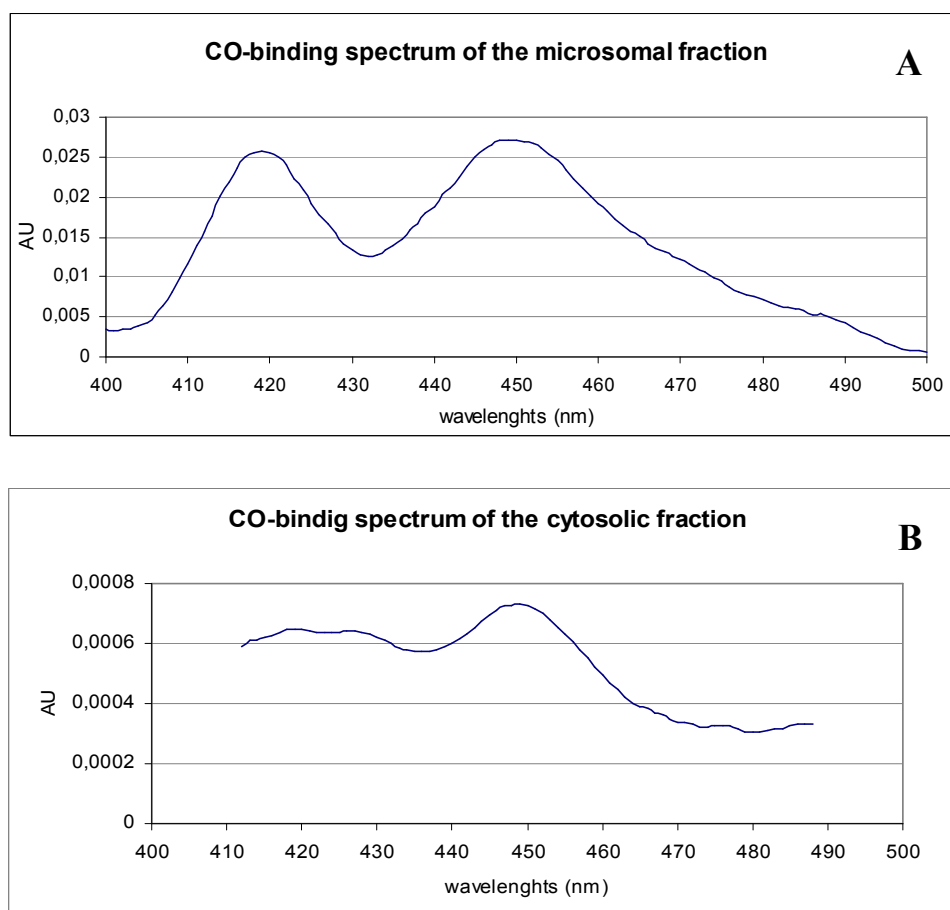


Figure 2.4.3. CO- binding spectra of the microsomal (A) and cytosolic (B) fraction from 7-day-old *P. tigrinus* cultures on MEG. Both samples contained 1 mg ml⁻¹ of dithionite-reduced total proteins.

2.4.3.5 In vitro degradation of PAHs with laccase from *P. tigrinus*

The ability *P. tigrinus* laccase (*PtL*) to degrade PAHs *in vitro* was assessed both in the presence and in the absence of HBT, as the mediator (Figures 2.4.4 and 2.4.5, respectively). Tween 80 was added to reaction mixtures to enhance PAHs solubility, to provide a source of unsaturated fatty acids and to preserve enzyme activity throughout incubation. With regard to the last criterion, about 50% of initial laccase activity was retained after 120 h incubation and inactivation profiles in mediated and non-mediated systems did not significantly differ (Figure 2.4.4). Thus, the selected solvent system, containing 5% acetone and 1% Tween 80, proved to be much milder than any other organic solvent/water mixtures employed for *in vitro* PAH degradation studies (Pickard *et al.*, 1999; Eibes *et al.*, 2005).

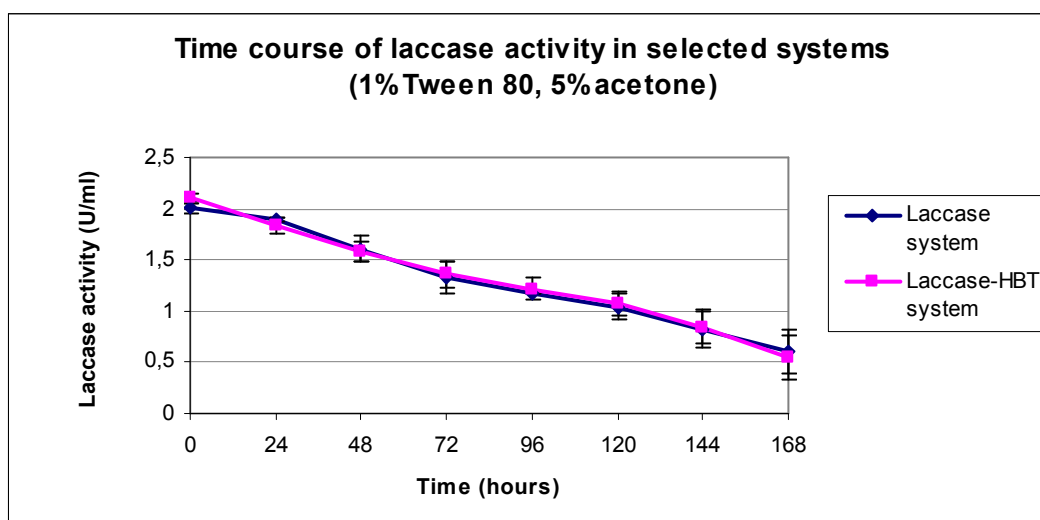


Figure 2.4.4. Time course of laccase activity (2 U ml^{-1}) in acetate buffer 0.1 M ($\text{pH } 4.5$) containing 1% Tween 80 and 5% acetone, either in the presence or in the absence of the redox mediator HBT.

Whereas *Nematoloma frowardii* and *Pleurotus ostreatus* laccases were unable to degrade ANT without mediators (Gunther *et al.*, 1998; Pozdnyakova *et al.*, 2006), this compound was quantitatively oxidized by *PtL* within 96 h incubation (Figure 2.4.5).

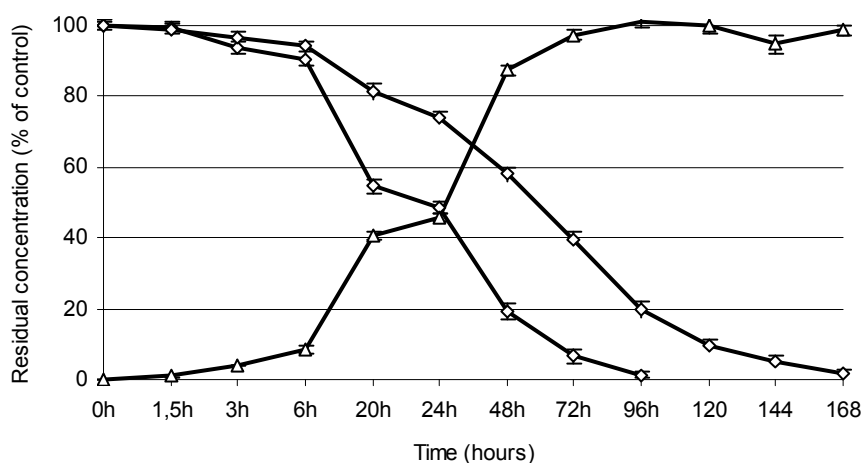


Figure 2.4.5. Single PAH degradation with laccase from *P. tigrinus* CBS 577.79.

In addition, and in agreement with other studies (Collins *et al.*, 1996; Majcherczyk *et al.*, 1998), ANT oxidation proceeded with the stoichiometric accumulation of 9,10-anthraquinone (AQ). In addition, BaP, the concentration of which was halved after about 60 h, was quantitatively removed after 168 h by *PtL*; the ability of *Trametes versicolor* laccase (*TvL*) to oxidize this compound in non-mediated reactions was previously reported by Majcherczyk and colleagues (1998); the extents of BaP oxidation by *PtL*, however, were significantly higher than *TvL* and this might be due to the

lower initial concentration of the PAH (11.9 vs. 25 μ M, respectively) and to the aforementioned presence of Tween 80. In this respect, in fact, it was suggested that the concomitant presence of Tween 80 and laccase leads to the generation of peroxy radicals which behave as strong oxidizers, thus enhancing transformation of aromatic structures (Camarero *et al.*, 2008). The other PAHs under study (*i.e.*, PHE, FLT, PYR) were not oxidized at all, probably due to their high ionization potential (IP) and half-wave oxidation potential ($E_{1/2-ox}$) values and this study further confirms that these compounds are not natural substrates of fungal laccases (Johannes *et al.*, 1996; Gunther *et al.*, 1998; Majcherczyk *et al.*, 1998; Pickard *et al.*, 1999).

For further improvement of the *in vitro* degradation studies, HBT was selected as the laccase mediator because of its high redox potential and reported ability to stimulate PAH oxidation by laccase (Majcherczyk *et al.*, 1998). Figure 2.4.6 shows that the degradation kinetics of ANT and BaP by the *PtL*-HBT system were markedly accelerated with a quantitative oxidation of both compounds within the first 24 h of incubation.

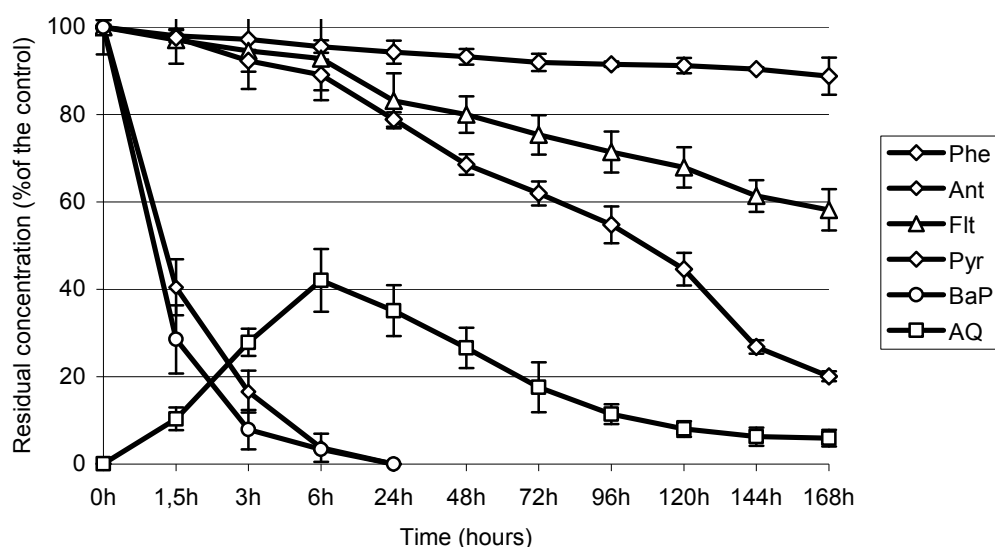


Figure 2.4.6. Single PAH degradation with laccase and 1 mM HBT as a mediating agent.

On the one hand, and with respect to the non-mediated reactions (Figure 2.4.5), ANT oxidation resulted in a lower conversion into AQ and the concentration of this degradation intermediate decreased as the incubations were further extended after the complete ANT removal (Figure 2.4.6). On the other hand, and unexpectedly, no BaP degradation intermediates, such as dione derivatives, were detected by GC-MS method. This might be due to the fact that BaP might have been converted into polymeric products as demonstrated by Majcherczyk *et al.* (1998) through the concomitant use of the radio-labelled compound and size-exclusion chromatography analysis of reaction products. In

contrast to the same study, however, oxidation of FLT and PHE was markedly stimulated in the *PtL*-HBT system with removal extents amounting to 41.8 and 11.2%, respectively, after 168 h incubation. These findings are particularly relevant for FLT and, to a lesser extent, for PHE since these PAHs are characterized by high values of both IP (7.95 and 7.91 eV, respectively) and $E_{1/2-ox}$ (1.45 and 1.50 V *vs.* SCE, respectively) and thus less prone to oxidation than other PAHs. In this respect, a good correlation between IP and oxidation efficiency by the laccase/mediator system was only found for those PAHs with IP < 7.45 and it was suggested that a valuable prediction based on this parameter might be only valuable for alternating PAHs (Majcherczyk *et al.*, 1998).

2.4.3.6 In vitro degradation of PAHs with Mn-peroxidase from *P. tigrinus*

The utilization of different water-solvent mixtures for similar *in vitro* experiments with MnP was already found to be effective and relatively mild towards the enzyme (Eibes *et al.*, 2005, 2006; Baborova *et al.*, 2006). Nevertheless, preliminary tests were run to check the stability of the enzymatic activity in the buffer-solvent mixture

In the presence of 20% acetone (reaction mixture which allowed the complete solubilisation of individual PAHs tested at a final concentration of 3 mg l⁻¹), MnP showed a biphasic inactivation profile with around 30% activity losses within the early 48 h, followed by a slower decline during the subsequent phases of incubation (Figure 2.4.7).

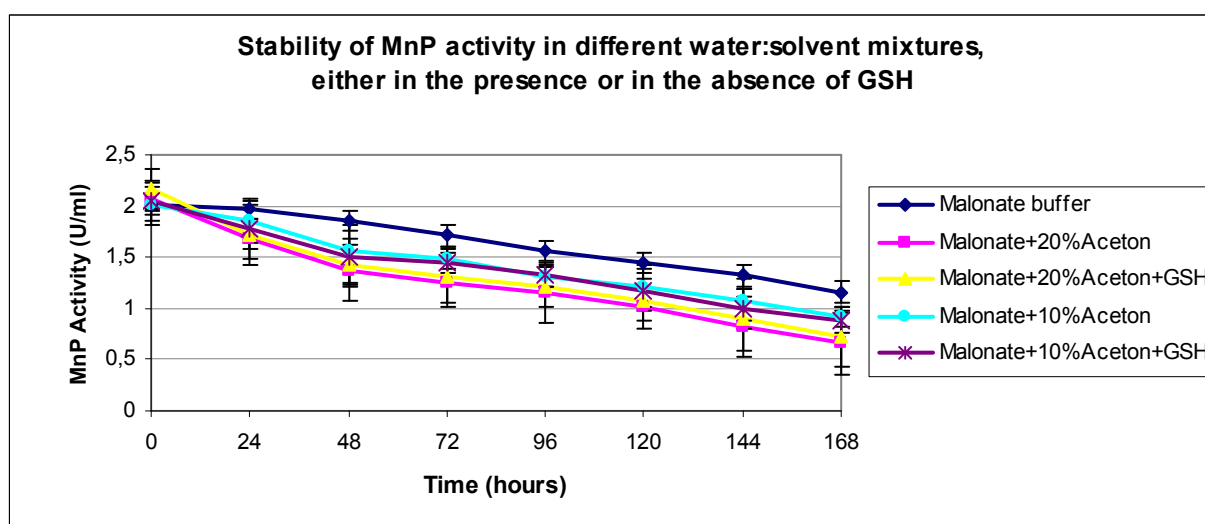


Figure 2.4.7. Time course of MnP activity (2 U ml⁻¹) in malonate buffer 0.05 M (pH 4.5) and in the same buffer containing 10 and 20% acetone, either in the presence or in the absence of the redox mediator GSH.

Experiments conducted with *P. tigrinus* MnP II showed the ability of the enzyme to oxidize all the PAHs under study albeit with different efficiencies and time courses (Figure 2.4.8).

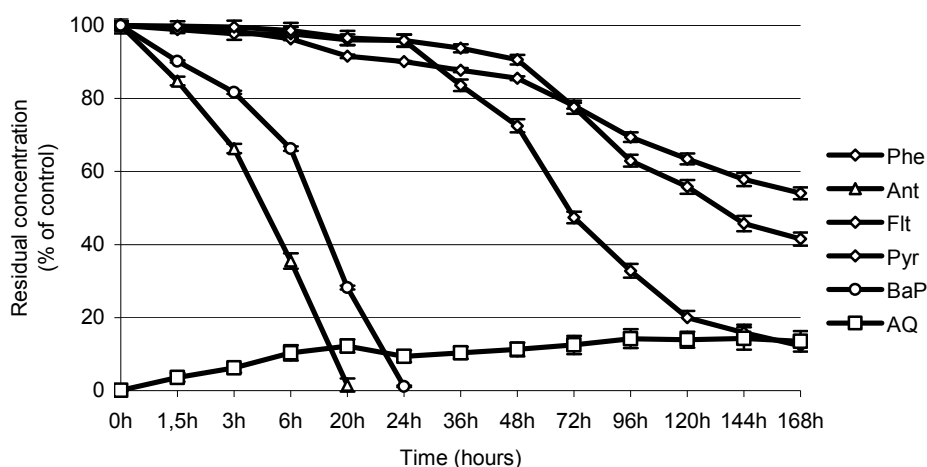


Figure 2.4.8. Single PAH degradation with MnP from *P. tigrinus* CBS 577.79.

ANT and BaP degradation started from the early phases of incubation and these compounds were quantitatively removed within 20 and 24 h. By contrast, a lag phase ranging from 24 to 36 h was observed for PYR, FLT and PHE, the removal extents of which were 88, 59 and 46%, respectively, after 168 h. In this respect, with the sole exception of PHE, MnP proved to be more effective in degrading PAHs than *N. frowardii* MnP which was used under similar experimental conditions (Sack *et al.*, 1997).

In the presence of 5 mM GSH, *P. tigrinus* MnP was able to oxidize all the PAHs tested more extensively than the laccase-HBT system as shown in Figure 2.4.9.

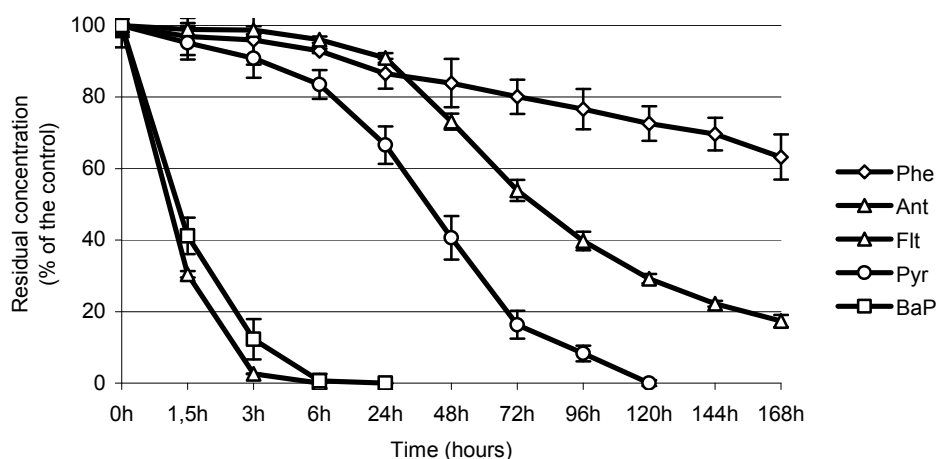


Figure 2.4.9. Single PAH degradation with MnP and 5 mM reduced glutathione as mediator.

The degradation kinetics of ANT, BaP and PYR were considerably enhanced in the presence of GSH and quantitative removal was attained within 4, 9 and 120 h, respectively. FLT removal was also increased under these conditions, this compound being removed by 82.4% after 168 h. By contrast, PHE degradation was not stimulated in the presence of GSH. Thus, in general, the inclusion of GSH as a thiyl radical generator significantly improved the degradation performances of MnP II in agreement with other *in vitro* studies conducted with other MnP systems (Sack *et al.*, 1997; Hofrichter *et al.*, 1998). No significant accumulation of PAH quinone derivatives was detected during oxidation. Unlike laccase oxidation of PAHs, which commonly generates quinones (Collins *et al.*, 1996; Majcherczyk *et al.*, 1998), it has been demonstrated that MnP from *I. lacteus* can cleave the aromatic ring (Baborova *et al.*, 2006).

2.4.4. Conclusions

The structural identification of PAH degradation products clearly showed the combined action of both the extracellular and the intracellular enzyme systems of *P. tigrinus* CBS 577.79. N-rich and N-limited standard media were conducive to the preferential production of laccase and MnP, respectively, but *in vitro* PAH degradation studies with the purified enzymes clearly showed that the latter had wider substrate PAH range and higher oxidation ability than the former. This might suggest a predominant role of MnP under *in vivo* conditions with important implications in practical remediation cases where both N-limiting conditions and absence of mediators are common scenarios.

3. DEGRADATION OF POLYCHORINATED BIPHENYLS (PCBs) AND CHLOROBENZOIC ACIDS (CBAs) BY *Panus tigrinus* CBS 577.79

3.1. Chlorinated Organopollutants

Chlorinated aromatics (*i.e.*, chlorobenzenes, chlorophenols, chlorobenzoates, polychlorinated biphenyls, dioxins and furans) may occur in great number. The number of persistent, ecotoxicologically or human toxicologically alarming chloroaromatics relevant to contaminated sites is comparatively high.

In general, highly chlorinated compounds are classified as hardly degradable under the usually prevailing biogeochemical conditions. Preferably, the highly chlorinated aromatic compounds are degraded anaerobically while the lower chlorinated ones aerobically (ICSS 2006).

Within the individual substance groups volatility, water solubility and mobility decline with the number of chlorine substituents. Owing to their lipophilicity, chloroaromatics are stored by organisms and tend to be accumulated along the trophic chain (bio-magnification). Persistent chloroaromatics are ubiquitous contaminants even though their production has long been stopped or their use (*e.g.* use of phenol containing wood preservatives and production of lindane) in the OECD countries has long been prohibited (ICSS 2006).

3.1.1. Polychlorinated biphenyls (PCBs)

PCBs consist of a “dumbbell-shaped” skeleton with two benzene rings being linked with each other through a carbon atom (biphenyl). The aromatic structure may be substituted with one to ten chlorine atoms. Theoretically, altogether 209 individual compounds designated as congeners are obtained even though only about 130 could be found in commercial PCB mixtures. PCB congeners differ among each other as a function of the number and the position of the chlorine atom substitution on the biphenyl structure. Such differences have a deep influence on the physico-chemical properties of the congener and thus on their recalcitrance to microbial degradation.

PCBs were synthesized for the first time in 1881, even though their use at the industrial scale was started in 1929 by Monsanto Company in the United States. Since then, many other countries started producing their own PCB mixtures, composed by various combinations of congeners (Clophen in Germany, Aroclor and Pyroclor in the UK, Fenclor and Apirolino in Italy, Phenoclor and Pyralene in France, Delor in the former Czechoslovakia, etc.). The production of PCBs, which was approximately 1000 tons/year in the early 30s, increased up to 200,000 tons/year in 1975 (Abraham

et al., 2002). At the end of the 70s - beginning of the 80s the production and use of PCB mixtures was stopped in all countries due to their toxicity and classification as “persistent organic pollutants” (POP).

3.1.1.1. Physico-chemical properties of PCBs

PCB congeners are odorless, tasteless, clear to pale-yellow, viscous liquids (the more highly chlorinated mixtures are more viscous and deeper yellow). PCBs have low water solubility and low vapour pressures at room temperature, but they have high solubility in most organic solvents, oils, and fats. They have high dielectric constants, very high thermal conductivity, high flash points (from 170 to 380 °C) and are chemically fairly inert, being extremely resistant to oxidation, reduction, addition, elimination, and electrophilic substitution. Their density varies from 1.182 to 1.566 kg l⁻¹. Other physical and chemical properties vary widely across the class. As the degree of chlorination increases, melting point and lipophilicity increase, but vapour pressure and water solubility decrease.

3.1.1.2. Sources and distribution of PCBs

To date, there is no evidence about point pollution sources of PCBs, the production of which has been stopped several years ago. However, due to their stability to weathering forces, PCBs still dominate in soil or sediments surrounding the places where they were produced in the past. The range of PCB concentration in the above-mentioned sites has been reported to fluctuate between 10 and 104 mg·Kg⁻¹ of soil. Such values are markedly higher than the limits set by the various national regulatory agencies, which range between 0,01 and 50 mg·Kg⁻¹.

The most volatile congeners have been shown to be transported for long distances by natural agents (wind, river and marine stream). In this respect, Abraham and co-workers (2002) claimed that PCBs have a worldwide distribution, since traces of these contaminants have even been found in remote Antarctic regions.

3.1.1.3. Toxicity of PCBs

It has been demonstrated that PCBs are highly toxic to humans and animals, although the effects observed become apparent only after long-term exposure. The toxicity of such molecules is directly correlated with both number and position of the chlorine substituents. The coplanar PCBs, known as non-ortho PCBs because they are not substituted at the ring positions *ortho*-, tend to have dioxin-like properties, and generally are among the most toxic congeners.

PCBs enter the human body *via* inhalation, ingestion or sorption through the skin (Clark, 1997). They are then transported by the blood stream to the organs (mainly liver and kidneys) and to the muscle and adipose tissues, where they are accumulated.

Studies have shown that PCBs alter estrogenic levels in the body and contribute to reproduction problems. Therefore, they have been described as “endocrine disruptors”. A few studies indicate that PCBs are associated with specific types of cancer in humans, such as the liver and the biliary tract cancers. Moreover, they have been shown to mimic the action of estrogens in breast cancer cells and can enhance breast carcinogenesis.

3.1.2. Chlorobenzoic acids (CBAs)

The chemical structure of the CBAs consists of a benzoic acid with different degree of chlorination in the aromatic ring. Therefore, depending on the number and position of chlorine substituents, their family comprises mono-, di-, tri-, tetra and penta-chlorobenzoates.

The scientific interest towards this class of molecules is mainly due to the fact that CBAs are often found in association with other persistent chlorinated organo-pollutants, PCBs above all. It is noteworthy, in fact, that chlorobenzoates enter the environment as metabolites of other halogenated compounds such as the fungicide pentachlorobenzyl alcohol (Ishida, 1972), *via* aerobic degradation of polychlorinated biphenyls (Seeger *et al.* 1997; Flanagan and May, 1993; Fava *et al.*, 1994; Bedard, 2003), or as intermediates in the anaerobic degradation of 2-chlorophenol (Becker *et al.* 1999). Moreover, some of the compounds belonging to this class, such as 2,3,6-TCB and *dicamba* (2-methoxy-3,6-dichlorobenzoate) have been used as herbicides (Horvath, 1971). Although CBAs are considered less recalcitrant and hazardous than other chlorinated organo-pollutants, their solubility in water (and therefore their diffusion) is several order of magnitude higher than that of PCBs.

However, although the anthropic contribution to CBA pollution is thought to be the most relevant, a natural formation of chlorobenzoates has been reported. Pereira and co-workers (1980)

revealed the presence of CBAs in volcanic ashes, possibly due to pyrolytic processes. Hydroxylated CBAs are also known as oxidative degradation products of naturally occurring fulvic acids and as metabolites produced by some fungal strains. Also, the natural formation of 2,4-DCB and 2,5-DCB was demonstrated in bog samples, possibly *via* a reaction involving chloroperoxidase (Niedan and Scholer, 1997).

3.2. Biological degradation of PCBs and CBAs

The microbial metabolism of PCBs has been extensively studied over the past decades due to their physico-chemical and toxicological properties. It is known that several factors can influence the microbial conversion of such recalcitrant compounds. Chemical structure, namely number and position of chlorine substituents, concentration and toxicity of the contaminant, as well as other environmental parameters (pH, nutrients, inhibitors, electron acceptors, and other microorganisms) regulate the overall degradation process.

The degradation of highly chlorinated PCBs in soil or sediments is effective only under anaerobic conditions, where reductive de-halogenation of the biphenyl structures occur (Brown *et al.*, 1988). Although bacterial strains able to de-chlorinate PCB anaerobically have not been isolated, their activity in anoxic sediments has been detected by molecular approaches (*i.e.*, denaturing gradient gel electrophoresis, DGGE) (Cutter *et al.*, 2001).

On the contrary, lower chlorinated PCBs degradation is a co-metabolic process operated by biphenyl catabolic enzymes (Furukawa and Fujihara, 2008). Bacterial strains that degrade PCBs oxidatively include various gram-negative genera such as *Pseudomonas*, *Alcaligenes*, *Achromobacter*, *Burkholderia*, *Comamonas*, *Sphingomonas*, *Ralstonia* and *Acinetobacter* and gram-positive genera such as *Rhodococcus*, *Corynebacterium*, and *Bacillus* (Furukawa and Fujihara, 2008).

However, PCB degradation by both pure bacterial cultures and co-cultures is generally incomplete with few exceptions (Potrawfke *et al.*, 1998; Kim and Picardal, 2000; 2001), and results in the formation of isomeric mixtures of CBAs (Adebusoye *et al.*, 2008). In fact, soil bacteria that co-metabolize PCBs tend to accumulate CBA as dead-end products since they are unable to grow on these substrates (Sondossi *et al.*, 1992; Kobayashi *et al.*, 1996). The build up of these metabolites in the growth medium may result in a feed-back inhibition and impede or slow down PCB biotransformation (Adebusoye *et al.*, 2008). Several studies have reported that PCB-degrading and CBA-degrading bacterial co-cultures are able to perform complete mineralization of lightly substituted biphenyls, but the rate of CBA removal dictated the rate of the PCB degradation

(Adriaens *et al* 1989; Fava *et al.*, 1994). Whereas PCB-degrading bacteria and their applicability to bioremediation strategies have been extensively studied, the knowledge about fungal degradation of PCBs is still unsatisfactory (Tigini *et al.*, 2009). In spite of their remarkable potential in organo-pollutants biodegradation, a relatively small number of white-rot species (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Lentinus edodes*, *Pleurotus ostreatus*, *Grifola frondosa*, *Coriolopsis polyzona* and *Bjerkandera adusta*) have been tested for their ability to attack chlorinated biphenyls. The PCB degradation mechanism in fungi is not yet clear (Kamei *et al.*, 2006), although successful removal of single congeners and mixtures has been reported (Vyas *et al.*, 1994; Yadav *et al.*, 1995; Beaudette *et al.*, 1998). Also, it has been indicated that substantial amounts of PCBs can be abiotically removed through adsorption onto fungal mycelia (Eaton 1985; Thomas *et al.*, 1992; Dietrich *et al.*, 1995). Some studies also indicated the involvement of the non-specific ligninolytic systems (e.g., laccases and peroxidases) in PCB degradation by fungi, basidiomycetes in particular (Singh 2006; Novotny *et al* 1997). Shultz and co-workers (2001) demonstrated that *Pycnoporus cinnabarinus* laccase was able to dechlorinate mono-hydroxylated chlorobiphenyls. However, the involvement of other enzymes, such as mono- and dioxygenases, (Kremár *et al.*, 1999; Gesell *et al.*, 2001) or cytochrome P450 monooxygenases (Kamei *et al.*, 2006) has also been hypothesized.

The microbial degradation of chlorobenzoates has been recently reviewed by Field and Sierra-Alvarez (2008). Ample evidence is available indicating biodegradation of CBAs in the environment under aerobic as well as anaerobic conditions. Under the latter condition, chlorinated benzoates are subjected to reductive de-chlorination when suitable electron-donating substrates are available. Several halo-respiring bacteria are known which can use chlorobenzoates as electron acceptors to support growth. Lower chlorinated benzoates are also used as a carbon and energy source by anaerobic bacteria.

Aerobically, lower chlorinated benzoates (3-CBA, in particular) can serve as sole electron and carbon sources thus supporting the growth. Additionally, there are several examples of chlorobenzoates that are aerobically co-metabolized. In this respect, benzoate, *ortho*-anisate, toluate and benzene have been extensively used as primary substrates supporting the co-metabolism of several dichlorobenzoates and 2,3,6-trichlorobenzoic acid. The microbial degradation of CBAs has an important linkage to the aerobic biodegradation of polychlorinated biphenyls (PCB). CBAs are common metabolites accumulating from the aerobic bacterial metabolism of PCBs and are further metabolized by the PCB-degrading bacteria. Therefore, improvements in PCB degradation have been achieved by co-culture with CBA-degrading bacteria (Adriaens *et al.* 1989; Fava *et al.* 1994)

or by incorporating CBA-degrading genes into PCB-degrading strains via genetic engineering (Pieper, 2005; Rodrigues *et al.* 2006, Wittich and Wolff, 2007).

To date, there is no evidence in the literature about the use of fungi for the biotreatment of CBAs. Nevertheless, our belief is that the CBA-degrading capabilities of fungi, white rots in particular, should be investigated and taken into account for an integrated bioremediation approach. So far this group of contaminants is strictly linked with PCBs, thus the aim of the present chapter is to preliminarily investigate the ability of the WRF *Panus tigrinus* CBS 577.79 to degrade mixture of both PCBs and CBAs. *In vitro* degradation experiments with purified laccase and MnP towards individual compounds of the above-mentioned mixtures have been performed to elucidate the role/involvement of LME in the general degradation mechanism. Moreover, an attempt to identify degradation products has also been performed.

3.3. Materials and methods

3.3.1. Materials

The PCB commercial mixture Delor 103, having from 2 to 5 chlorosubstituents, was obtained from a former Czechoslovak producer (Chemko Stráské, Slovakia). In previous investigations the congener composition of Delor 103 was described in detail (Káš *et al.*, 1997). Chlorobenzoic acids (CBAs) 2-CBA, 3-CBA, 4-CBA, 2,3-CBA, 2,4-CBA, 2,5-CBA, 2,6-CBA, 3,4-CBA, 3,5-CBA, 2,3,5-CBA, 2,3,6-CBA, 2,4,6-CBA, were obtained from Sigma (Darmstadt, Germany).

A stock solution of PCBs (PCBM) was prepared by dissolving Delor 103 in DMSO (Sigma, Germany) at a final concentration of 20 g·l⁻¹. Analogously, a stock solution of the CBA mixture (CBAM) was prepared by dissolving each compound in DMSO at a final concentration of 2 g·l⁻¹.

Hexachlorobenzene (HCB), *N*-hydroxybenzotriazole (HBT), violuric acid (VLA) were from Fluka (Germany); reduced glutathione (GSH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 2,2',6,6'-tetramethylpiperidine-1-oxyl radical (TEMPO) were from Sigma (Darmstadt, Germany).

All solvents of p.a. quality, trace analysis quality or gradient grade were from Merck (Darmstadt, Germany). Purified *P. tigrinus* CBS 577.79 laccase (*PtL*) and MnP II (*PtM* II) isoenzymes were obtained as reported elsewhere (Quaratino *et al.*, 2007; Petruccioli *et al.*, 2009).

3.3.2. Organism, culture media and inocula preparation

Fungal strain, *P. tigrinus* CBS 577.79, culture media, MEG and LNKM, and inocula preparation were as described in subsection 2.4.2.2 of the present PhD thesis.

3.3.3. Culture conditions

All the experiments were conducted at 28 °C under both stationary and reciprocal shaking conditions (100 rpm) in 250 ml Erlenmeyer flasks containing 20 ml and 40 ml, respectively, of either MEG or LNKM. The PCB mixture was added to *P. tigrinus* stationary and shaken cultures by spiking 100 and 200 µl of PCBM, respectively, immediately after inoculation. The final concentration of the PCBM was 100 mg l⁻¹. Two harvests were performed during the fermentation (after 3 and 6 weeks for stationary cultures and 2 and 4 weeks for shaken cultures).

On the contrary, 1 week after inoculation stationary and shaken cultures were spiked with 100 and 200 µl of the CBAM, respectively, to yield a final concentration of 10 mg l⁻¹ of each compound. Five harvests were performed during the incubation with the CBAM: after 5, 10, 15, 20 and 42 days from spiking for static cultures and 5, 10, 15, 20 and 28 days for shaken cultures.

Heat-killed controls (HKC) were run with mycelia previously sub-cultured for 7 days, then killed by autoclaving (121 °C, 20 min) and subsequently contaminated with the appropriate amount of PCBM and CBAM. Biotic controls (BC) were prepared by spiking stationary and shaken cultures with 100 and 200 µl of DMSO alone. All the degradation experiments were carried out in triplicate in the dark.

3.3.4. Enzymes assays

The activities of the lignin modifying enzymes (laccase, MnP and MIP) were determined using DMP as described in section 2.4.2.5.

3.3.5. Sample preparation and analytical methods

Sample preparation and biomass determination were as described in sub-section 2.4.2.6. PCB congeners were separated and identified by gas chromatography-mass spectrometry (450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA). Hexachlorobenzene (HCB) was added as the internal standard at a final concentration of 10 mg l⁻¹. The ethyl acetate extracts were directly injected in the GC instrument, which was equipped with split/splitless injector and a DB-5MS

column (Agilent, Prague, Czech Republic, 30 m, 0.25 mm I.D., 0.25 μm film thickness). The temperature program started at 60 $^{\circ}\text{C}$ and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 120 $^{\circ}\text{C}$ at a rate of 25 $^{\circ}\text{C min}^{-1}$. The second temperature ramp was up to 240 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C min}^{-1}$, this temperature being maintained for 20 min. Solvent delay time and transfer line temperature were set at 6 min and 280 $^{\circ}\text{C}$, respectively. Mass spectra were recorded at 3 scan s^{-1} under electron impact at 70 eV and mass range 50-450 amu.

Quantitative analysis of the CBA mixture were performed using the RP-HPLC system described in sub-section 2.4.2.6, equipped with an X-Bridge C-18 column (4.6 x 250 mm, particle $\text{\O}$ 3.5 μm , Waters, MA). A two-step gradient program was applied, with A (acetonitrile) and B (0.1% TFA in 10% acetonitrile): 0-25 min from 85 to 78% A, 25-40 min from 78 to 62% A.

CBA degradation products were separated and identified by gas chromatography-mass spectrometry using the equipment described above. The ethyl acetate extracts were both directly injected without any derivatization and after either trimethylsilylation with BSTFA or methylation with diazomethane (Cajthaml *et al.*, 2002). Non-derivatized and trimethylsilylated samples were injected in the same GC-MS described above and analyzed with the following program: after the splitter was opened the oven was heated from 60 to 120 $^{\circ}\text{C}$ at a rate of 25 $^{\circ}\text{C min}^{-1}$, this temperature being held for 3.4 min; then a second temperature ramp from 120 $^{\circ}\text{C}$ to 240 $^{\circ}\text{C}$ at a rate of 25 $^{\circ}\text{C min}^{-1}$, held for 28.6 min. The program for diazomethane-derivatized samples consisted of the following gradient: 60-100 $^{\circ}\text{C}$ in 1.6 min, 100-135 $^{\circ}\text{C}$ in 35 min and 135-240 $^{\circ}\text{C}$ in 10.5 min, this temperature being held for 20 min. In all cases, solvent delay time and transfer line temperature were set at 6 min and 280 $^{\circ}\text{C}$, respectively, and mass spectra were recorded at 3 scan s^{-1} under electron impact at 70 eV and mass range 50-450 amu.

3.3.6. *In vitro* oxidation of CBAs with purified enzymes

All experiments were performed in triplicate in 2 ml reaction tubes with teflon-lined screw caps. Degradation experiments with purified laccase were conducted in 1 ml reaction mixtures containing 0.1 M acetate buffer pH 4.5 added with 1% (w/v) Tween 80, 20 μl of a stock solution of each CBA (0.25 g l^{-1} in acetone) and 2 U of laccase. Incubations were at 28 $^{\circ}\text{C}$ for 168 h on a rotary shaker (80 rpm). In mediated reactions HBT, ABTS, VLA and TEMPO were added at a 1 mM final concentration.

In vitro degradation tests with purified MnP were conducted in 1 ml reaction mixtures containing 0.05 M malonate-NaOH buffer pH 4.5, 0.01 M MnSO_4 and 2 U MnP. Glucose (0.03 M) and glucose-oxidase (0.06 U) were included in the reaction mixture to ensure a gradual and continuous

H₂O₂ production. The reaction mixture was then spiked with 100 µl of each CBA (50 mg l⁻¹ in acetone) and then incubated as above. GSH, VLA and TEMPO were either omitted or added at a final concentration of 5, 1 and 1 mM respectively. Relative abiotic controls were performed by adding either heat-denatured laccase or MnP to reaction mixtures. Quantitative analyses were initially performed after 0, 3, 6, 9 and 24 h. Thereafter, samplings were done on a daily basis. As for quantitative analyses, the direct injection of reaction mixtures into HPLC was allowed by the quantitative solubilization of CBAs. Recoveries of each of the CBAs in the abiotic controls were higher than 95%.

3.3.7. Ecotoxicology test with *Vibrio fischeri* (luminescent bacteria test)

The inhibitory effect on the light emission of *Vibrio fischeri* was measured using the luminometer Lumino-M90a (ZD Dolni Ujezd, Czech Republic) according to the normalized protocol ISO 11348-3 (1998), modified as follows: the buffer (buffer A) used for measurements was 2%NaCl-1% DMSO. Lyophilized bacteria stored at -20°C were previously revitalized in 2%NaCl (cooled to 0-2°C); the “working bacteria suspension” thus obtained was maintained in a water bath thermostated at 15° C throughout the whole period of measurements.

The ethyl acetate extracts of HKC samples, evaporated to dryness under N₂ stream and resuspended in buffer A, were prepared in serial dilutions. The concentration of CBAM in HKC which was giving a 50-70% decrease in the luminescence at the end of the incubation period (30 min) was selected for further analyses. Fungal-treated samples were prepared at the same concentration of the relative HKC.

A preliminary test, aiming at the viability assessment of the working bacteria suspension, was performed in 0.5 ml of buffer A, measuring the luminescence emission at the times 0, 15 and 30 minutes. Successively, the inhibition of light emission caused by the extracts of fungal-treated samples (dried and resuspended in 0.5 ml of buffer A) was monitored at the same time intervals.

The inhibition of luminescence, I(%), was calculated as follows:

$$I (\%) = 100 - (L_{v15 / 30} / f * L_{v0}) * 100$$

where:

L_{v0} = lum. at time 0 in the presence of the sample; L_{v15} = lum. at time 15 min in the presence of the sample; L_{v30} = lum. at time 30 min in the presence of the sample; f = (L_{k15}/L_{k0}) is a correction factor; L_{k0} = luminescence at time 0 in the absence of the sample; L_{k15} = luminescence at time 15 min in the absence of the sample

3.4. Results and discussion

3.4.1. Enzymatic activities and biomass production during *in vivo* degradation of PCBM

The maximal values of ligninolytic enzyme activities during incubation with Delor 103, as well as the biomass production in concomitance with the harvests programmed for contaminant analyses, are reported in Table 3.1. The PCBM, which was spiked immediately after inoculation, markedly inhibited the growth of the fungus under shaking conditions. The toxicity effect of the PCB mixtures was particularly evident in LNKM shaken cultures, where also the activity of extracellular oxidases was negligible during the whole incubation period. On the contrary, the fungal growth in MEG stationary cultures was evidently more abundant in the presence of the PCBM than in the relative BC.

Regardless of the culture conditions and the presence or the absence of contaminants, laccase was the predominant ligninolytic enzyme produced by *P. tigrinus* in MEG medium, while the production of MnP was mainly stimulated in low-N medium (Table 3.1). This result is in agreement with previous studies (Quaratino *et al.*, 2006; 2007) thus confirming the distinct physiological regulation of the two extracellular enzymes in *P. tigrinus* CBS 577.79.

Table 3.1. Biomass production at the first and second harvest and maximal activities of laccase and MnP in *P. tigrinus* CBS 577.79 cultures.

Medium	Culture conditions	Spiking	Biomass ^{a)} (g l ⁻¹)		Laccase ^{b)} (U l ⁻¹)	MnP ^{b)} (U l ⁻¹)
			1st	2nd		
MEG	Stationary	BC	2.4±0.1	2.2±0.2	8.2±4.6 (17)	6.0±2.3 (13)
		PCBM	3.4±0.6	3.4±0.1	93.7±7.6 (10)	39.4±22.1 (9)
	Shaken	BC	2.2±0.2	2.0±0.1	27.3±6.3 (24)	13.4±2.8 (13)
		PCBM	1.6±0.2	1.3±0.1	39.2±4.3 (17)	19.5±7.4 (9)
LNKM	Stationary	BC	1.5±0.1	1.4±0.2	5.2±3.8 (13)	23.5±9.2 (20)
		PCBM	1.3±0.3	1.1±0.1	44.5±2.1 (24)	60.0±12.5 (28)
	Shaken	BC	0.7±0.1	0.6±0.1	2.3±5.1 (20)	13.2±5.4 (17)
		PCBM	0.5±0.1	0.4±0.1	1.8±1.2 (24)	2.6±1.9 (20)

Biomass was determined in concomitance with harvests set for PCB analyses (*i.e.*, 2 and 4 weeks for shaken cultures and 3 and 6 weeks for stationary cultures; b) days required to attain the activity peaks are reported within round brackets.

The presence of the PCB mixture gave rise to different effects on the activity of LME (Table 3.1). In PCB-spiked MEG medium, laccase activity peaks were anticipated by one week and were 9- and 1.5-fold higher than those of the relative BC under stationary and shaken condition, respectively. In the same high-N medium spiked with the PCBM, also the MnP activity peaks resulted to be enhanced and reached 1 week in anticipation with respect to BC; this result, however, was mainly observed under stationary conditions.

The PCBM stimulated both MnP and laccase production in LNKM stationary cultures, the activity peaks of which were 9- and 2.5-fold higher than those of the relative BC, respectively. On the contrary, as already reported above, the production of ligninolytic enzymes was completely suppressed by the PCBM in LNKM shaken cultures.

3.4.2. PCB degradation analysis

The ethyl acetate extracts (HKC and samples) were analysed by gas chromatography-mass spectrometry using hexachlorobenzene (HCB) as internal standard. Thus, the amount of each congener belonging to the Delor 103 commercial mixture was reported as relative abundance (peak heights) with respect to the amount of HCB.

In general, *P. tigrinus* CBS 577.79 was not effective in the degradation of the PCB mixture. This was particularly evident for the fungal cultures incubated under shaking conditions, where no differences were detected among the concentration of individual congeners in HKC and in samples incubated for 4 weeks in the presence of active mycelia.

Under stationary conditions, partial removal of less chlorinated congeners was observed in both media (Figure 3.1, A and B).

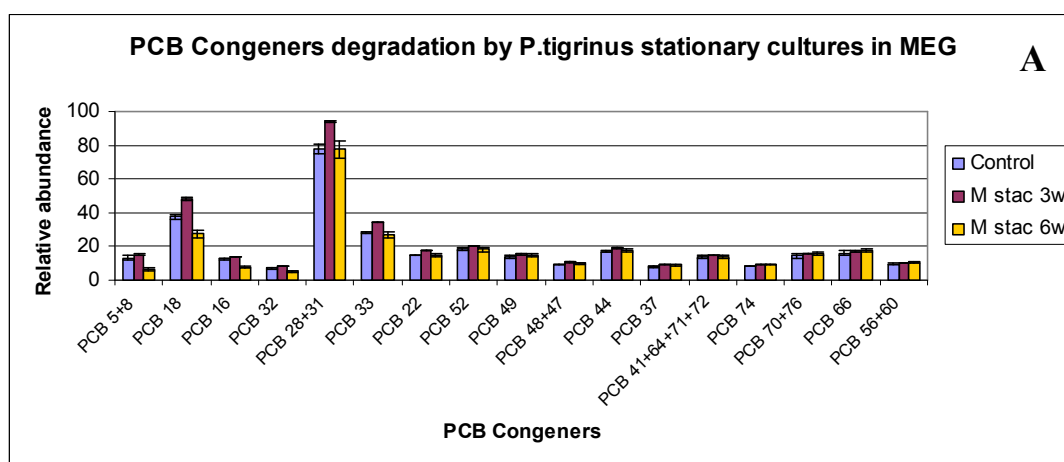


Figure 3.1-A. Relative abundance (relative heights with respect to the internal standard HCB) of individual congeners of the PCBM after 3 and 6 weeks of static incubation in MEG and in the relative HKC.

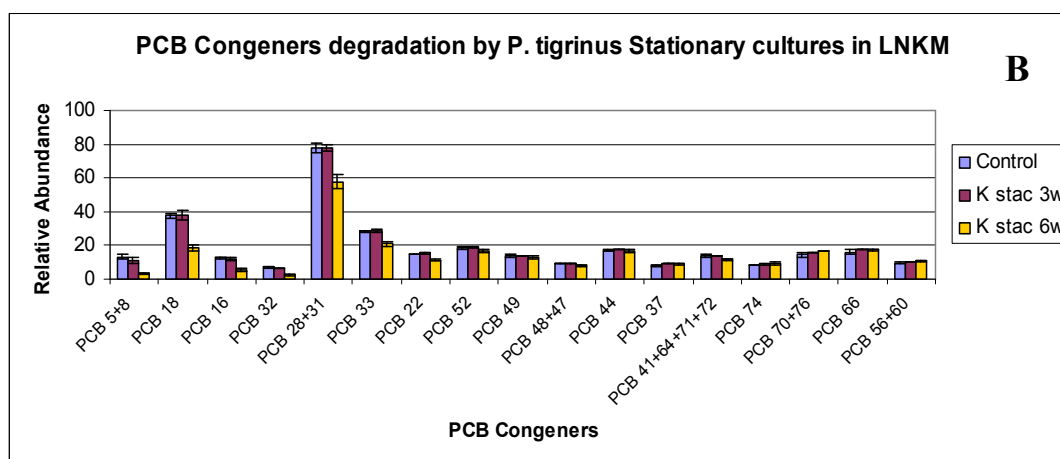


Figure 3.1-B. Relative abundance (relative heights with respect to the internal standard HCB) of individual congeners of the PCBM after 3 and 6 weeks of static incubation in LNKM and in the relative HKC.

The chemical structure and the residual concentration of lower chlorinated biphenyls, which resulted to be significantly depleted after 6 weeks of incubation, are reported in Table 3.2.

Table 3.2. Residual concentration (% of HKC) of individual CB congeners detected in MEG and LNKM static cultures at the end of incubation (*i.e.*, 6 weeks)

Individual congeners		Residual Concentration * (% of HKC)	
IUPAC No.	Substitution	MEG 6 weeks	LNKM 6 weeks
5+8	2,3+2,4'	50±4	26±6
18	2,2',5	72±3	48±4
16	2,2',3	60±5	44±3
32	2,4',6	72±6	35±7
28+31	2,4,4'+2,4',5	100±5	74±3
33	2',3,4	100±3	73±4

(* Data are the mean of triplicate experiments ± standard deviation)

The PCB-degrading capabilities of the strain CBS 577.79 differed from those reported for other white rot species such as *Pleurotus ostreatus*, *Trametes versicolor*, *Bjerkandera adusta* and the model organism *Phanerochaete chrysosporium* (Dietrich *et al.*, 1995; Beaudette *et al.*, 1998; Novotny *et al.*, 1999; Kubatova *et al.*, 2001; Kamei *et al.*, 2006).

Due to the limited PCB degradation observed under *in vivo* conditions, our investigation was not further extended to *in vitro* degradation tests with the purified extracellular enzymes from *P. tigrinus*.

3.4.3. Biomass production and enzymatic activities during *in vivo* degradation of CBAM

P. tigrinus biomass was determined in the cultures spiked with the CBAM as well as in the relative BC, in concomitance with the harvests set for contaminants analyses (Table 3.3).

Table 3.3. Time course of *P. tigrinus* biomass production (g l^{-1}) in LN and HN media, either in the presence or in the absence of the CBA mixture.

Sampling (days)	MEG				LNKM			
	Stationary		Shaken		Stationary		Shaken	
	BC	CBAM	BC	CBAM	BC	CBAM	BC	CBAM
7	1.9±0.2	1.8±0.2	2.1±0.3	2.0±0.3	0.8±0.1	0.9±0.1	0.9±0.1	1.0±0.1
12	3.2±0.4	3.0±0.1	3.0±0.3	3.1±0.2	1.3±0.2	1.2±0.2	1.2±0.2	1.2±0.1
17	3.5±0.4	3.6±0.2	3.3±0.4	3.7±0.3	2.1±0.2	2.0±0.1	1.9±0.1	1.7±0.1
22	2.8±0.3	2.7±0.3	3.0±0.4	2.9±0.5	1.8±0.2	1.6±0.2	1.7±0.3	1.5±0.2
27	2.5±0.3	2.5±0.6	2.5±0.2	2.3±0.5	1.7±0.2	1.5±0.3	1.5±0.2	1.4±0.3
35	2.4±0.2	2.3±0.4	2.0±0.1	2.6±0.1	1.5±0.1	1.3±0.2	1.0±0.2	1.1±0.1
49	2.2±0.2	2.1±0.1			1.4±0.2	1.2±0.1		

Data are the means $\pm$ standard deviation of three replicates.

The presence of CBAM did not significantly inhibit the fungal growth in any of the conditions tested. On the contrary, in the case of the shaken cultures in CBAM-spiked MEG medium the biomass production was higher than in the relative BC (2.6 vs. 2.0 g l^{-1}) at the end of the incubation period.

The ligninolytic enzymes of white-rot fungi have a broad substrate specificity and have thus been implicated in the transformation and mineralization of organopollutants with structural similarities to lignin (Pointing, 2001). *P. tigrinus* is a white rot basidiomycetes which produces high yields of laccase and MnP in liquid cultures (Quaratino *et al.*, 2006; 2007). Therefore, in the present study, which represents a pioneering investigation on fungal degradation of CBAs, particular attention has been paid to the activity of its extracellular ligninolytic enzymes (*i.e.*, laccase and MnP).

Table 3.4 reports the activity peaks of laccase and MnP of fungal cultures incubated in the presence and in the absence of the CBAM.

Table 3.4. Maximal values of laccase and MnP activity attained by *P. tigrinus* cultures in MEG and LNKM media in the presence or the absence of the CBAM. Data between round brackets indicate the time (days) required to attain maximal activity.

Medium	Culture conditions	Spiking	Laccase (U l ⁻¹)	MnP (U l ⁻¹)
MEG	Stationary	BC	8.2±4.6 (30)	6.0±2.3 (13)
		CBAM	61.1±5.5 (33)	9.3±1.7 (30)
	Shaken	BC	24.3±6.3 (24)	13.4±6.8 (13)
		CBAM	78.4±2.6 (28)	8.2±1.7 (8)
LNKM	Stationary	BC	5.2±3.8 (13)	23.5±9.2 (20)
		CBAM	35.9±2.7 (14)	28.8±0.2 (11)
	Shaken	BC	2.3±5.1 (20)	13.2±5.4 (17)
		CBAM	1.3±0.3 (12)	2.3±0.4 (14)

As already reported in a previous study (Quaratino *et al.*, 2007), laccase was the main LME produced by the fungus in N-rich medium (MEG). Moreover, the spiking of the CBAM in 7 day-old cultures gave rise to a sudden and continuous enhancement of laccase activity with respect to the relative BC, under both stationary and shaken cultures (data not shown).

Laccase activity peaks in MEG medium spiked with the CBAM was approximately 8- and 3-fold higher than those of the relative BC under stationary and shaken conditions, respectively. On the other hand, MnP production by *P. tigrinus* is known to be mainly stimulated in low-N media (Quaratino *et al.*, 2006). In LNKM BC, in fact, under both stationary and shaken conditions, MnP was the main LME produced by the fungus. The addition of the CBAM to LNKM stationary cultures, however, markedly boosted the laccase activity, the peak of which was 7-fold higher than the relative BC. On the contrary, a certain inhibitory effect on LME production upon the spiking of the CBAM was observed in LNKM shaken cultures.

3.4.4. In vivo degradation of CBAs

The concentration of individual CBAs in concomitance with the harvests set for their analyses is reported in Figure 3.2 (A,B,C,D). Reversed phase-HPLC analyses of culture extracts revealed that *P. tigrinus* was capable of transforming the majority of CBAs within the early phases of incubation. In general, the bioconversion process was faster under stationary than shaken conditions in both media (Fig 3.2-A and 3.2-B). The three mono-CBAs (2-, 3-, and 4-), in fact, were degraded within 5 days from the spiking of the CBAM in stationary cultures, while the same result was attained under shaken conditions on day 10. Similarly, the complete removal of 2,4-, 2,5-, 3,4-, 3,5-DCBAs and 2,3,5-TCBA was achieved in both media after 10 and 15 days under stationary and shaking conditions, respectively.

Although the CBA-degradation was faster under stationary conditions (Fig 3.2-A, and 3.2-C), best results in terms of overall depletion were achieved under shaken conditions in MEG medium (Fig 3.2-B).

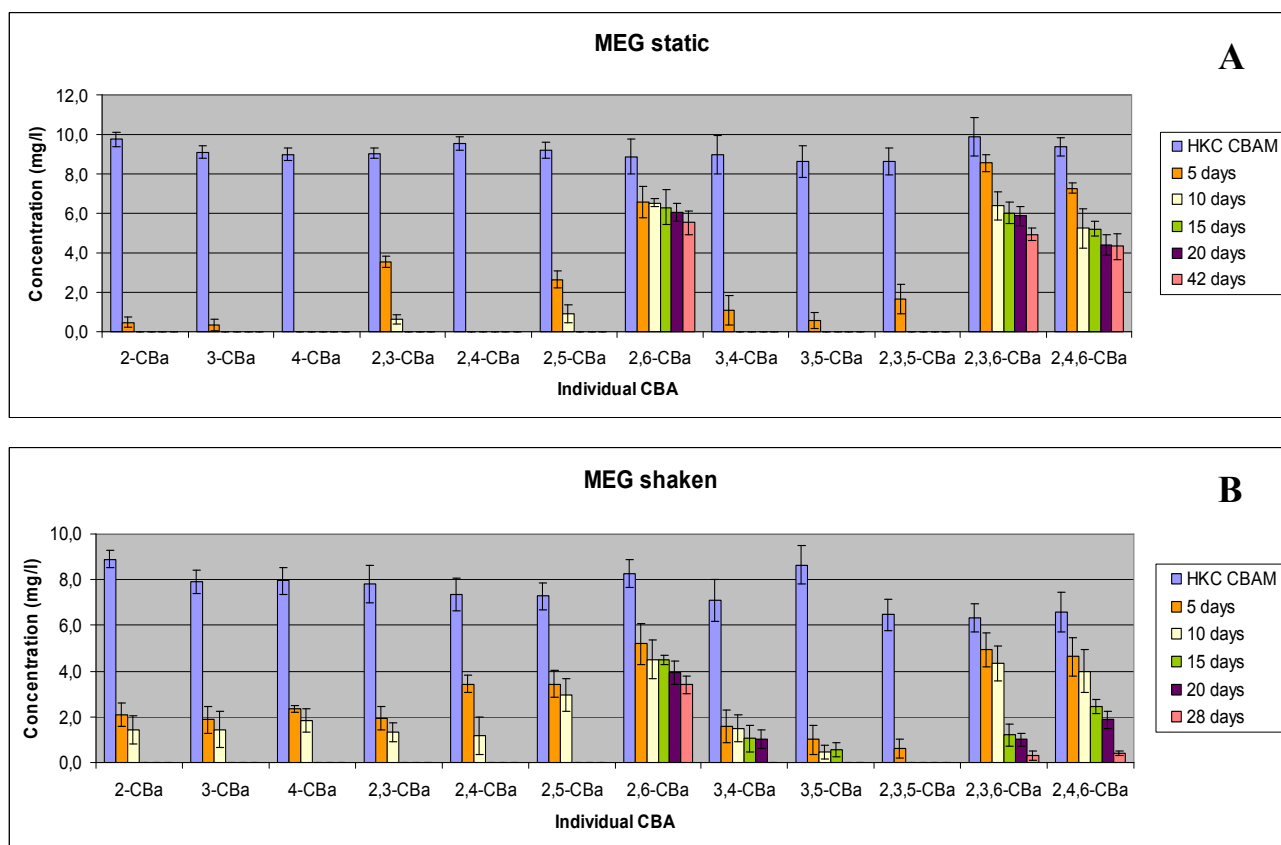


Figure 3.2. (A and B) Residual concentrations (mg l^{-1}) of individual CBAs of the CBAM in MEG stationary (A), MEG shaken (B) cultures, and in the relative HKC. Data are the means $\pm$ standard deviation of three replicates.

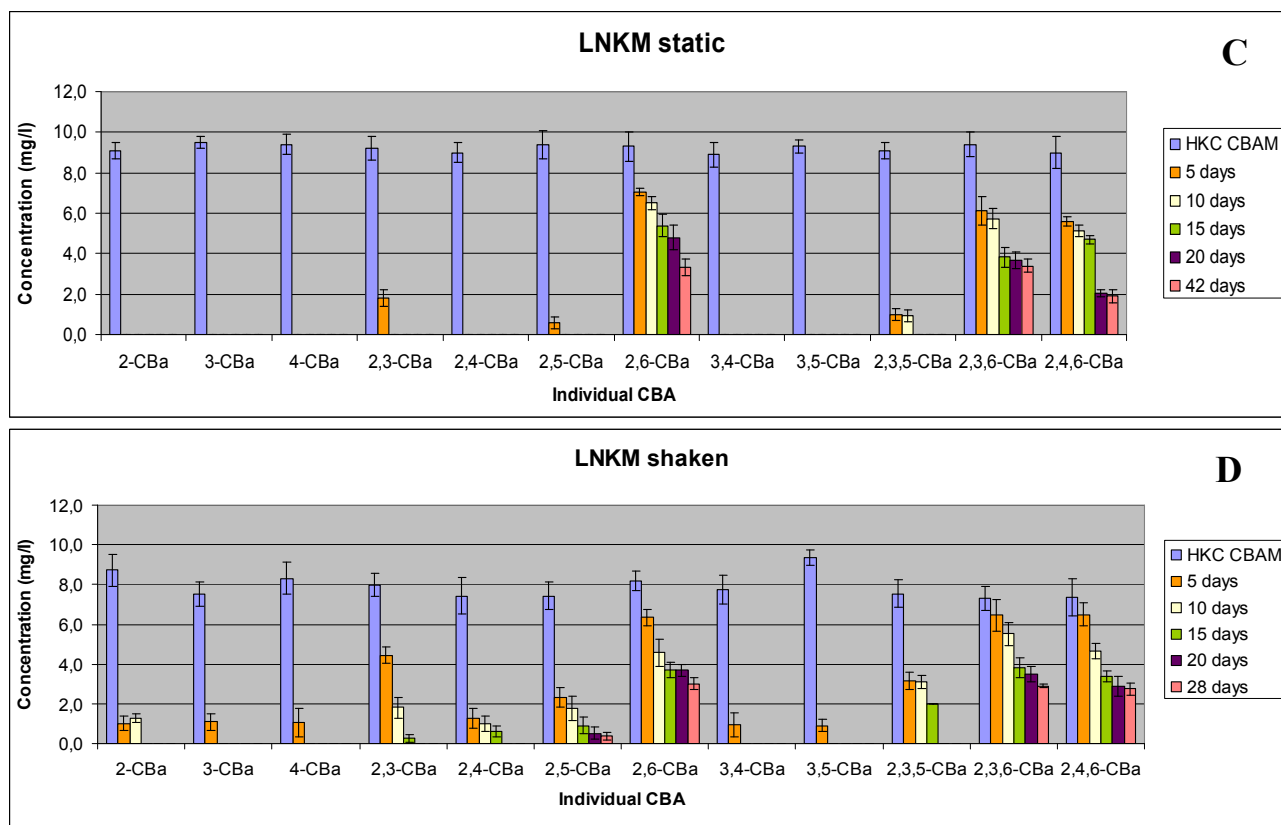


Figure 3.2. (C and D) Residual concentrations (mg l^{-1}) of individual CBAs of the CBAM in LNKM stationary (C) and LNKM (D) cultures, and in the relative HKC. Data are the means $\pm$ standard deviation of three replicates.

Regardless of the medium and the cultural conditions, the most recalcitrant compound was 2,6-DCBA, followed by 2,3,6- and 2,4,6-TCBA. All these three compounds present two chloro-substituents adjacent to the carboxyl moiety; therefore, the electron-withdrawing effect of the two chlorine substituents might deactivate the aromatic ring and thus its reactivity.

At the end of the incubation period (28 days after the spiking of CBAM), in fact, the residual concentrations of 2,6-DCBA, 2,4,6-TCBA and 2,3,6-TCBA were 41.6 and 5% of the relative HKC, respectively, while all the other CBAs were completely removed.

3.4.5. Detection of CBA degradation products

Several intermediates of the CBA degradation were detected in the ethyl acetate extracts of *P. tigrinus* cultures. Some compounds were detected by injecting the solvent extracts directly into the GC-MS instrument (Table 3.5); some other structures were detectable only after derivatization with BSTFA (trimethylsilylation) or diazomethane (methylation) (Tables 3.6 and 3.7).

Table 5. Degradation intermediates of CBAs in non-derivatized ethyl acetate extracts with relative retention times (tR), molecular weight (MW) and fragmentation patterns.

t _R (min)	MW according to CI	m/z of fragment ions (relative intensity, %)	Structural suggestion
6,609	160	162(10,4), 160(17,6), 127 (29,6), 126(9,4), 125(99,9), 114(8,4), 99(9,5), 89(31,7)	? ? -dichloro-?-methylbenzene (?, ? -dichlorotoluene)
6,885	160	162(8,3), 160(18,3), 127(33), 125(99,9), 99(7,5), 89(28,4), 63(16,6), 62(6,2)	? ? -dichloro-?-methylbenzene (?, ? -dichlorotoluene)
6,972	160	162(8,5), 160(14,1), 127(30,8), 126(7,3), 125(99,9), 99(6,7), 89(25,7), 63(13,5)	? ? -dichloro-?-methylbenzene (?, ? -dichlorotoluene)
7,666	160	162(12,4), 160(18,7), 127(31,2), 125(99,9), 89(32,9), 87(13,6), 63(13,9), 55(10,2)	? ? -dichloro-?-methylbenzene (?, ? -dichlorotoluene)

Table 6. Degradation intermediates of CBAs in ethyl acetate extracts derivatized with BSTFA (trimethylsilylated) with relative retention times (t_R), molecular weight (MW) and fragmentation patterns.

t _R (min)	MW according to CI	m/z of fragment ions (relative intensity, %)	Structural suggestion
8,882	214	201(34), 199 (99,9), 179(18,5), 163(30,7), 127(25,4), 125(82,5), 89(25,1), 73(18,6)	?,-monochlorobenzyl alcohol
9,388	214	201(31,8), 199(90,9), 179(33,4), 171(19,9), 169(60,1), 127(30,2), 125(99,9), 89(32,7)	?,-monochlorobenzyl alcohol
9,664	214	201(20,8), 199(58,7), 179(24,2), 169(20,5), 127(31,9), 125(99,9), 89(24,2), 73(12,5)	?,-monochlorobenzyl alcohol
13,17	248	235(67,6), 233(99,9), 205(18,5), 203(25,6), 161(41,4), 159(64,2), 123(18,4), 103(18,9)	?,?-dichlorobenzyl alcohol
13,344	248	247(14,5), 235(58), 233(84,2), 161(61,1), 159(99,9), 123(13,8), 103(29,1), 73(12,7)	?,?-dichlorobenzyl alcohol
13,596	248	235(70,9), 233(99,9), 205(32,3), 203(45,8), 161(58,5), 159(89,7), 147(27,7), 123(21,1)	?,?-dichlorobenzyl alcohol
14,339	248	235(69,6), 233(99,9), 205(17,8), 203(25,3), 161(46,3), 159(71,1), 123(17,5), 103(22,8)	?,?-dichlorobenzyl alcohol
15,045	248	235(68,5), 233(94,6), 203(16,7), 161(67,3), 159(99,9), 75(27,5), 73(28,5), 59(34,2)	?,?-dichlorobenzyl alcohol
18,208	210	212(50,4), 210(47,6), 177(50,9), 175(99,9), 173(31,5), 111(97,3), 75(52,6), 73(43)	?,?,?-trichloro-?-hydroxy-?-methylbenzene (?,?,?-trichloro cresol)

Table 7. Degradation intermediates of CBAs in ethyl acetate extracts derivatized with diazomethane with relative retention times (tR), molecular weight (MW) and fragmentation patterns.

t _R (min)	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion
7,548	140	142(23,8), 141(36,6), 140(73,7), 139(99,9) 111(55), 75(32), 51(19,6), 50(29,8)	?-monochlorobenzaldehyde
7,623	140	142(20,6), 141(36,1), 140(66,6), 139(99,9), 113(18,4), 77(22,7), 75(33,5), 74(19,1)	?-monochlorobenzaldehyde
7,834	140	142(16), 141(37,1), 140(49,4), 139(99,9), 113(16,8), 111(49,6), 77(15,1), 74(16,8)	?-monochlorobenzaldehyde
9,937	176	178(51), 176(92,9), 163(53), 161(99,9), 135(64,4), 133(90,5), 75(25,9), 63(31,5)	?,?-dichloro-?-methoxy benzene
12,013	176	178(65,7), 175(99,9), 148(49,5), 146(75,4), 135(26,5), 133(41,2), 111(31,6), 63(47,3)	?,?-dichloro-?-methoxy benzene
12,488	174	175(62,4), 174(70,3), 173(99,9), 145(47), 139,1(54,2), 111,1(50,1), 75(61,1), 74(52,6)	? ,?-dichlorobenzaldehyde
12,646	174	176(39,5), 175(70,2), 174(61,4), 173(99,9), 147(16,9), 145(25,9), 75(18), 74(15)	? ,?-dichlorobenzaldehyde
13,149	174	176(38,2), 175(68,4), 174(61,3), 173(99,9), 111(25), 75(61), 74(45,9), 50(25,3)	? ,?-dichlorobenzaldehyde
13,613	176	178(55,8), 176(99,9), 163(12,6), 161816,2), 146(31,5), 137(26,1), 135(81,9), 133(97,2)	?,?-dichloro-?-methoxy benzene
14,1	174	176(38,9), 175(69,5), 174(64,9), 173(99,9), 147(28,1), 145(43), 75(29,1), 74(24,9)	? ,?-dichlorobenzaldehyde
14,729	174	176(38,9), 175(69,5), 174(64,9), 173(99,9), 147(28,1), 145(43), 75(29,1), 74(24,9)	? ,?-dichlorobenzaldehyde
14,914	174	176(53,8), 175(74,9), 174(65,3), 173(99,9), 147(49), 145(49), 75(45,1), 74(40,3)	? ,?-dichlorobenzaldehyde
15,804	176	178(50,8), 176(65,9), 163(28,8), 160(28,8), 135(67,8), 133(99,9), 113(18,7), 11(15,1)	?,?-dichloro-?-methoxy benzene

Metabolites from CBA degradation were identified by comparing the mass spectra with data in the NIST 08 library and, independently, by interpreting the fragmentation pattern. Product ion scan (MS/MS) was not needed to clarify the fragmentation sequence and all the molecular weights were estimated according to chemical ionization. Nevertheless, the position of chlorine-, hydroxyl- and methyl- substituents on the aromatic ring was not determined by injecting authentic chemical standards. Therefore, several isomers of the same molecules, showing different fragmentation pattern and relative abundances, are listed in Tables 3.5, 3.6, and 3.7.

Derivatization with diazomethane (Table 3.7) allowed the identification of 3 isomers of monochlorobenzaldehyde and 6 isomers of dichlorobenzaldehyde. Moreover, 3 isomers of monochlorobenzyl alcohol and 5 dichlorobenzyl alcohol were detected after trimethylsilylation with BSTFA (Table 3.6). The concomitant presence of benzaldehydes and benzyl alcohols suggests the occurrence of a reductive mechanism operating on the carboxylic group of CBAs. This two-step process could be mediated (intracellularly) by enzymes such as aryl-aldehyde dehydrogenase (AAldD, converting chlorobenzoic acids into benzoaldehydes) and aryl-alcohol dehydrogenase (AAD, transforming benzoaldehydes into benzyl alcohols). The latter enzyme was purified from the white rot fungus *P. chrysosporium* (Muheim *et al.*, 1991) and detected in *Pleurotus eryngii* (Varela *et al.*, 1992), while the presence of the former enzyme was discussed in other ligninolytic fungi (Shoemaker *et al.*, 1989; Lundell *et al.*, 1990). AAD from *P. chrysosporium* exhibits a wide substrate specificity and was shown to reduce different aromatic aldehydes (Muheim *et al.*, 1991).

Moreover, it is noteworthy that AAldD and AAD are enzymes involved in a multi-enzymatic cyclic system in which H_2O_2 is produced extracellularly by the action of aryl-alcohol oxidase (AAO) (Guillen *et al.*, 1994). In particular, AAO catalyzes the oxidation of benzyl alcohols to benzaldehydes with the concomitant production of hydrogen peroxide which, in turn, might support the catalytic cycle of peroxidases.

Due to the intracellular location of both AAldD and AAD, the disappearance of CBAs from both media in the early steps of incubation (Figure 3.2-A, B, C, D) might imply the intracellular uptake of these compounds.

In the case of direct injection of ethyl acetate extracts (Table 3.5, without derivatization) four isomers of dichloromethylbenzene (dichlorotoluene) were detected. The formation of chlorotoluenes could be due to an additional reductive step involving the hydroxyl group of benzyl alcohols. With this regard, it was shown that the first step of toluene oxidation by *Pseudomonas putida* cultures was the formation of benzyl alcohol (Shaw *et al.*, 1992); in our case, obviously, the occurrence of toluene derivatives might imply reductive reactions occurring on chloro-substituted benzyl alcohols.

Derivatization with diazomethane allowed the detection of 4 isomers of dichlorophenols. These compounds are shown in Table 3.7 in their respective methoxylated form owing to esterification (methylation) of the hydroxyl group. The presence of these degradation intermediates might be explained by the action of cytochrome P-450 monooxygenase (Sono *et al.*, 1996) rather than by the occurrence of oxidative dehalogenation reactions known to be brought about by both laccases and peroxidases (Hammel and Tardone, 1988). Oxidative dehalogenation, in fact, requires the presence of either a hydroxyl or an amino substituent on the benzene ring in order to take place. In this respect, the detection of a single isomer of the TMS-derivative of trichloro-hydroxy-methylbenzene (trichloro-cresol) confirms the above considerations. It is noteworthy that this chloro-cresol derivative was detected among degradation intermediates of the early phases of culture.

Consequently, it might be postulated that this hydroxylated derivative was oxidized by laccase and MnP that are known to be able to perform the oxidation of chlorophenols (Stazi *et al.*, 1994; 2002; Leontievsky *et al.*, 2002; Lisov *et al.*, 2007).

3.4.6. *In vitro degradation of individual CBAs by purified laccase and MnP from P. tigrinus*

Results obtained in *in vivo* experiments might rule out the involvement of LMEs in the oxidation of parent compounds. It is known, however, that the substrate range of these enzymes can be expanded by the inclusion in reaction mixtures of appropriate organic compounds able to act as electron shuttles between the enzyme and the target molecule, the so called mediators. Among them, ABTS, HBT, VA and TEMPO were selected for laccase while GSH, VA and TEMPO for MnP. *In vitro* reactions were also performed in the absence of mediators. Both enzymes were unable to oxidize CBAs even under mediated conditions thus confirming the absence of their involvement in the initial phases of the degradation process.

3.4.7. *Ecotoxicology test with Vibrio fisheri (bioluminescent bacteria test)*

The toxicity of culture extracts, during and after *in vivo* fungal treatment of the CBAM, was evaluated with the “luminescent bacteria (*Vibrio fisheri*) test”, according to the protocol ISO 11348-3 (1998). The results of the ecotoxicology test are reported in Figure 3.3.

In general, incubation of the CBAM with the fungus did not decrease the toxicity of the mixture towards the *V. fisheri* suspension: the majority of the inhibition values measured, in fact, were not significantly different with respect to the relative HKC.

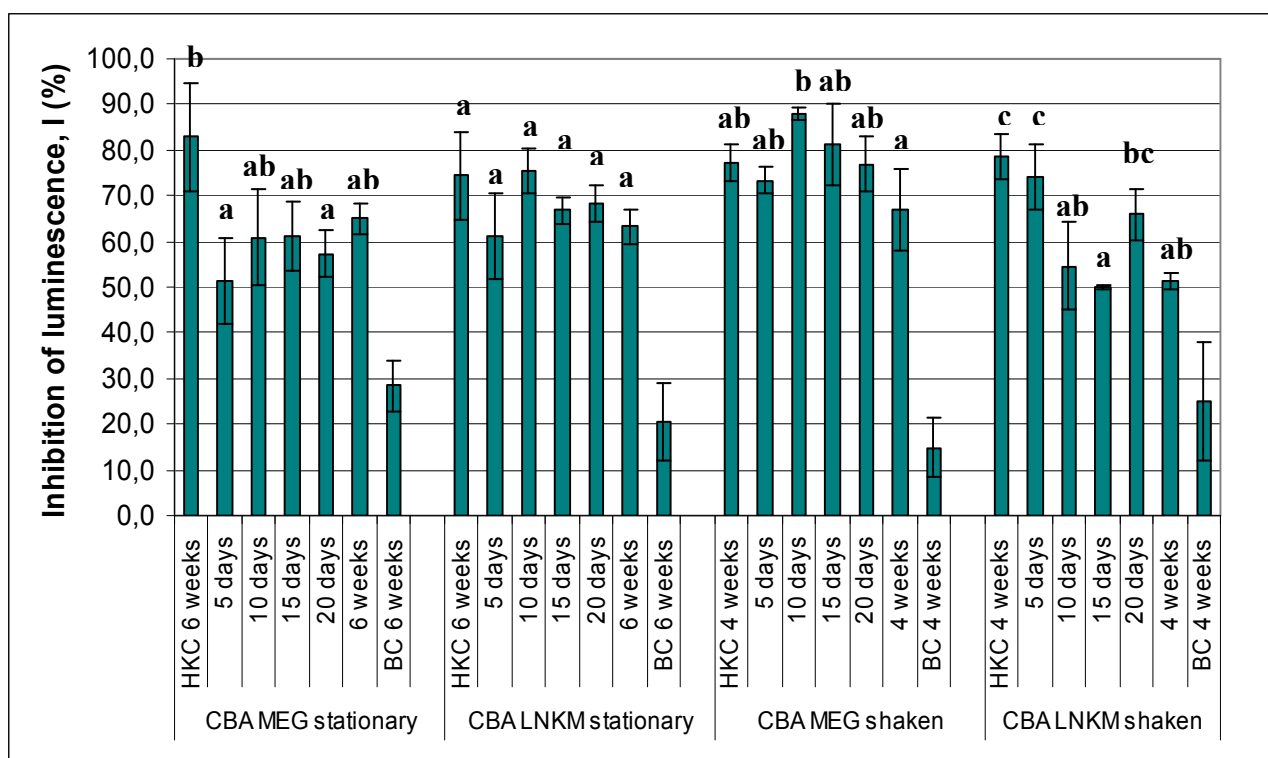


Figure 3.3. Inhibition of light emission of the luminescent bacterium *V. fischeri* caused by *P. tigrinus* culture extracts. Values of inhibition I (%) were calculated according to the equation reported in section 3.3.7. Multiple pair-wise comparisons were performed by the Tukey test ($P < 0.05$); same letters above bars indicate that differences between samples within the same culture condition were not significant.

This result could be explained by the fact that some metabolites (*e.g.*, chloro-toluenes), produced by the fungus during the degradation of the CBAM, might be more toxic than the parent compounds. However, a transient detoxification towards *V. fischeri* suspension was observed in concomitance with the early phases of incubation in MEG stationary cultures (5 days). The best results, in terms of detoxification of the CBAM, were achieved by LNKM shaken cultures. Under this condition, in fact, values of inhibition of the luminescence (I%) after 10-, 15- and 28-days (4 weeks) treatment were significantly lower than the relative HKC, which means that the fungus partially detoxified the original mixture of contaminants.

3.5. Conclusions

The aim of the present work was to investigate the ability of the WRF *Panus tigrinus* CBS 577.79 to degrade mixture of both PCBs and CBAs, the latter representing stable metabolites of (bacteria-mediated) PCB degradation. Although few lower-chlorinated PCB congeners were

partially removed by static cultures of *P. tigrinus* after 6 weeks of cultivation, the PCB-degrading capabilities of this fungus were markedly lower than those reported for other representative white rot species (*i.e.*, *P. ostreatus*, *T. versicolor*, *B. adusta* and *P. chrysosporium*). On the contrary, the majority of CBAs included in the mixture were promptly removed by *P. tigrinus* cultures; only exceptions were 2,6-DCBA, 2,3,6- and 2,4,6-TCBA, the reactivity of which is probably reduced by the steric hindrance of two chlorine substituents adjacent to the carboxyl moiety. Moreover, with the sole exception of LNKM shaken cultures, the spiking of the CBAM stimulated laccase production in all *P. tigrinus* cultures, with respect to the relative BC. However, *in vitro* experiments with purified laccase and MnP, under both mediated and non-mediated conditions, showed that these enzymes are unable to degrade individual CBAs, thus indicating that other enzymatic systems are involved in the initial attack to chlorobenzoates under *in vivo* conditions.

The structural identification of CBA degradation products allowed us to hypothesize that the degradation of these compounds by *P. tigrinus* was the result of a combined action of both extracellular and intracellular enzyme systems. The concomitant presence of chlorinated benzaldehydes and benzyl alcohols in culture extracts might be due to the action of a multi-enzymatic cyclic system (intra- and extracellular) which is used by the fungus to produce the H₂O₂ necessary for the catalytic cycle of peroxidases. The presence of chlorotoluenes could be due to an additional reductive step involving the hydroxyl group of benzyl alcohol, although, to date, such an enzymatic reaction have not yet been reported in the literature. In addition, 4 dichlorophenols were detected; the formation of these compounds might be ascribed to the action of intracellular enzymatic systems (*e.g.*, cytochrome P-450) and is likely to be susceptible to oxidation by LMEs (*i.e.*, laccase and MnP).

Moreover, the toxicity of culture extracts was tested using the luminescent bacterium *Vibrio fischeri*; the test revealed that the fungus, in the majority of the conditions under study, was not effective in removing the toxicity associated with the CBAM; partial detoxification was achieved in LNKM shaken conditions and, transiently, in the early phases of static incubation in MEG medium. In this respect, it is not possible to exclude that metabolites produced during the *in vivo* treatment of the CBA mixture might have similar or higher toxicity than the parents compounds.

The present study is the first report on fungal degradation of CBAs; white rot fungi, besides their ability to degrade PCBs, are supposed to have a great potential to degrade CBAs and therefore should be taken into account for an integrated bioremediation approach. However, the results achieved emphasize the need for further research: future works will be addressed to the identification of the enzymatic systems involved in the production of the metabolites detected, assessment of the toxicological risks related to CBA degradation products.

4. *IN VIVO* AND *IN VITRO* DEGRADATION OF ENDOCRINE DISRUPTING COMPOUNDS (EDCs) BY *Panus tigrinus* CBS 577.79

4.1. Endocrine disrupting compounds (EDCs)

A growing body of evidence indicates that several anthropic chemicals and pesticides may mimic or antagonize natural hormones in human and wildlife endocrine systems. A hormone is defined as any substance in the body that is produced by one organ and then carried by the bloodstream to have an effect in another organ. The primary function of hormones, or the endocrine system, is to maintain a stable environment within the body (homeostasis). The endocrine system also controls reproduction and growth. Recently, public concern has focused on the possible hormonal effects of some environmental pollutants on wildlife and humans. These chemicals, collectively referred to as endocrine disruptors, comprise a wide range of substances including: pesticides (methoxychlor), surfactants (nonylphenols), plasticizers (diethylphthalate, Bisphenol A), organohalogens (PCBs, dioxin and furans), natural and synthetic estrogens (estrone (E1), 17 β -estradiol (E2), estriol (E3) and 17 α -ethynylestradiol (EE2)) and personal care products ingredients (Triclosan). Many industrial chemicals and pesticides have undergone extensive toxicological testing. However, since the purpose of this testing was not to find some subtle endocrine effects these potential effects may not have been revealed (US-EPA, 2001). Much attention has to be paid to xenobiotics which induce endocrine disruption at typical environmental concentrations (*e.g.*, disruption of sexual behaviour in aquatic organisms), although no toxicity is exhibited at such low levels.

4.1.1. 17 α -Ethynylestradiol (EE2)

The presence of natural (E1, E2, E3) and synthetic (EE2) steroid estrogens in sewage effluents and surface water is a common scenario (Fernandez *et al.*, 2007; Cabana *et al.*, 2007). These compounds are considered to be major contributors to the estrogenic activity associated with wastewater treatment plant effluents (Clouzot *et al.*, 2008) and are known to have a negative effect on aquatic organisms (Cajthaml *et al.*, 2009).

The synthetic compound 17 α -ethynylestradiol is widely used as ovulation inhibitor in contraceptive pills. Non-metabolized EE2 and its conjugates are excreted into wastewaters and, owing to its hydrophobicity and recalcitrance to biodegradation, it is not efficiently degraded by municipal wastewater treatment plants (Jonson and Sumpter, 2001). The K_{ow} of EE2 is particularly

high (4.2) and one of the major removal factor is sorption onto particulate matter (Cajthaml *et al.*, 2009). Concentration of EE2 in effluents ranges approximately between 10 and 78 ng l⁻¹ (Fernandez *et al.*, 2007).

In this respect, Versonnen and co-workers (2004) demonstrated that the exposure of zebrafish (*Danio rerio*) to environmentally relevant concentration of EE2 (100 ng l⁻¹) was toxic to larvae, embryos and juveniles, while vitellogenin production was induced in adult fishes. Previous studies indicated that much lower concentrations (0.1 ng l⁻¹) were needed to induce vitellogenesis in male rainbow trout (*Oncorhynchus mykiss*) (Purdom *et al.*, 1994; Aerni *et al.*, 2004). Furthermore, the bioaccumulation of EE2 by the endobenthic oligochaete *Lumbriculus variegatus* was shown by Liebig and co-worker (2005), thus suggesting that a secondary poisoning of predators (biomagnification) might occur in polluted environments.

The microbial transformation of EE2 has been recently reviewed by Cajthaml and colleagues (2009). EE2 was found to be slowly decomposed by bacteria under anaerobic conditions (Czajka and Londry, 2006): the “dissipation time” can exceed 1000 days and the degradation is attributed to sulfate-, nitrate-, and iron-reducing conditions. Faster degradation can be recorded for dissolved EE2 by bacteria under aerobic conditions (Sarmah and Northcott, 2008) and also seawater microbes were found to degrade EE2 after acclimation (Ying and Kookana, 2003). More rapid degradation of EE2 was also linked to the presence of ammonium-oxidizing bacteria from activated sludge (Shi *et al.*, 2004). Ligninolytic fungi are also promising candidates for EE2 decomposition and degradation; several fungal strains, in fact, were shown to efficiently degrade EE2 and other estrogens (Cajthaml *et al.*, 2009). Interestingly, even isolated ligninolytic enzymes were able to extensively oxidize EE2 within a relatively short time (Tanaka *et al.*, 2000, 2001; Suzuki *et al.*, 2003).

4.1.2. Bisphenol A (BPA)

BPA is a key component (strengtheners) of polycarbonate plastics and epoxy resins, although several other uses are reported (*e.g.*, in dental sealants, orthodontic products, water pipes, sport equipments and CD-DVD). Its ubiquitous presence in the environment is mainly due to installations that incorporate BPA into plastics (Staples *et al.*, 1998), leaching of plastic wastes (Sajiki, 2003) and landfill sites (Asakura *et al.*, 2004), and from leaching of the flame retardant tetrabromobisphenol A (Ronen and Abeliovic, 2000).

Solubility of BPA in unbuffered water at pH 6.7 is relatively high (89 mg l⁻¹) and the value of K_{ow} for BPA is 3.4. Consequently, these properties confer a higher propensity to this compound

than other EDCs to partition in water. On the basis of this information, it is generally assumed that BPA has a moderate potential of bioaccumulation (Cabana *et al.*, 2007). The maximal BPA concentrations detected in marine water and sediments are reported to be 21 mg l⁻¹ and 191 ng l⁻¹, respectively (Cabana *et al.*, 2007). In sewage effluents and sludge, instead, maximal BPA concentrations range from 2.2 µg l⁻¹ to 2.89 µg g⁻¹ (Cabana *et al.*, 2007).

There is a great concern about the impact of the low-dose BPA effect on living organisms in different environmental matrices. Most of the published *in vivo* studies report significant effects of this EDC on organisms (Vom Saal *et al.*, 2007). BPA has been reported to interact with estrogens, androgens and thyroid receptors (Cabana *et al.*, 2007). The hormonal dysfunctions such as morphological and functional alterations of genitals and mammalian glands (Jobling *et al.*, 2004; Kanno *et al.*, 2004; Li *et al.*, 2004; Mohri *et al.*, 2005; Maffini *et al.* 2006).

Bacteria able to degrade BPA have been isolated from a sewage treatment plant (Lobos *et al.*, 1992; Kang *et al.*, 2002) and river water (Kang *et al.*, 2004). Furthermore, BPA has been shown to be efficiently degraded in both soil and aqueous matrices by white rot fungi (Hirano *et al.*, 2000; Lee *et al.*, 2005; Cajthaml *et al.*, 2009) and free ligninolytic enzymes (Cabana *et al.*, 2007; Okazaki *et al.*, 2002; Tsutsumi *et al.*, 2001; Kimura *et al.*, 2004; Hirano *et al.*, 2000; Tanaka *et al.*, 2001; Lee *et al.*, 2005; Kim and Nicell, 2006) and by the action of more industrially relevant treatments such as immobilized enzymes (Nicotra *et al.*, 2004; Iida *et al.*, 2002; Iida *et al.*, 2003; Diano *et al.*, 2007), laccase secreting transgenic plants (Sonoki *et al.*, 2005), or combination of laccase and activated sludge (Nakamura and Mtui, 2003).

4.1.3. Nonylphenols (NP)

NP isomers are present in the aquatic environment owing to the partial biodegradation of the surfactants nonylphenol etoxylates (NPEs) in municipal sewage treatment plants. NPEs and other alkylphenol etoxylates (APEs) are the primary active ingredients in industrial chemicals that are used as cleaning and sanitizing agents. Moreover, NPs are also used as plasticizers, in the preparation of phenolic resins, polymers, heat stabilizers, antioxidants, and curing agents (US-EPA, 2001).

The degradation of NP is known to proceed slowly under aerobic conditions, due to their hydrophobic nature ($K_{ow} = 4.48$) (Ying *et al.*, 2002). Therefore, NP concentrations up to 1 mg l⁻¹ and 500 mg kg⁻¹ were detected in STP effluents and sludges, respectively (Loos *et al.*, 2007; Cabana *et al.*, 2007).

Among the microorganism capable of utilizing NP as sole carbon and energy source there are few species of aerobic bacteria (*i.e.*, *Sphingomonas*) (Tanghe *et al.*, 1999; Fujii *et al.*, 2001; De Vries *et al.*, 2001; Gabriel *et al.*, 2005), anaerobic bacterial isolates from sludges and river sediments (Chang *et al.*, 2004; 2005) and the yeast *Candida aquatextoris*, (Vallini *et al.*, 1997). Recent studies have shown the ability of selected fungi, including white rot fungi, to degrade this chemical, albeit to various extents (Soares *et al.*, 2005, 2006; Dubroca *et al.*, 2005; Cajthaml *et al.*, 2009). Extracellular oxidases (laccases) have been implicated in the NP fungal oxidation (Tanaka *et al.*, 2000, 2001; Saito *et al.*, 2004; Junghanns *et al.*, 2005; Cabana *et al.*, 2007).

NPs are known to bind to estrogens receptor, thereby mimicking the effects of endogenous hormones, and has been shown to induce synthesis of vitellogenin and inhibit testicular growth in rainbow trout (White *et al.*, 1994; Sumpter *et al.*, 1995; Jobling *et al.*, 1996). Similar effects with respect to vitellogenin production were observed also in frogs (*Xenopus laevis*) (Lutz and Kloas, 1999; Kloas *et al.*, 1999) while multi-generational reproductive process disruptions were detected in the planctonic crustacean *Daphnia magna* (Brennan *et al.*, 2006). These observations has led to increased interest in the biodegradation and possible elimination of this class of xenobiotic surfactants from the environment.

4.1.4. Triclosan (TCS)

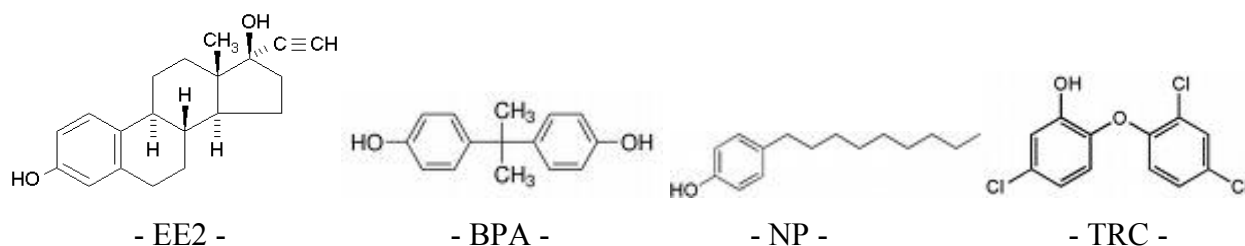
TCS (or Irgasan) is a broad-spectrum antimicrobial agent widely used in dental preparations, toothpastes, deodorant sticks, soap and other personal care products (Cabana *et al.*, 2007; Cajthaml *et al.*, 2009). Its hydrophobicity ($K_{ow} = 4.8$) determines the adsorption of TCS to particles and sediments in the aquatic environment and thus its bioaccumulation in aquatic organisms (Adolfsson-Erici *et al.*, 2002). TCS concentrations can reach up to $4.1 \mu\text{g l}^{-1}$ in STP effluents and 55 mg l^{-1} in the sludge (Cabana *et al.*, 2007).

Although TCS presents some structural similarity with BPA and dioxins, its endocrine disrupting activity has not yet been investigated extensively. TCS is suspected to induce weak estrogenic or androgenic activity and to disrupt gene expression linked with the thyroid hormone (Veldohen *et al.*, 2006; Foran *et al.*, 2000). Moreover, it has been shown that this EDC induces vitellogenin production in *Oryzias latipes* (Ishibashi *et al.*, 2004).

To date, relatively few studies have been performed targeting TCS degradation by white rot fungi. Hundt and co-workers (2000) reported the transformation of TCS by *T. versicolor* and *P. cinnabarinus* and the detection of several intermediates (*e.g.*, 2,4-dichlorophenol and other glucoside conjugates) with much lower cytotoxicity. In a recent study (Cajthaml *et al.*, 2009), 8

fungal strains were tested for their ability to degrade TCS; all the fungi, with the exception of *B. adusta*, were capable of extensively degrading the EDC and to reduce its original ED activity.

Figure 4.1. Chemical structure (from left to right) of ethynylestradiol (EE2), bisphenol A (BPA), nonylphenols (NP) and triclosan (TCS).



4.2. Aim of the study

The removal of EDCs from several environmental matrices has been achieved by using different strains of white rot fungi and the elimination efficiency was found to be strain- and cultivation condition-dependent (Cabana *et al.*, 2007). Objectives of the present study were to investigate the degradation capacity of *P. tigrinus* towards four representative EDCs (*i.e.*, (EE2, BPA, NP and TRC) and to assess the possible involvement of its lignin-modifying enzymes in the degradation process. The first aim was followed by performing *in vivo* studies in both stationary and shaken liquid cultures on two standard media mainly differing for their N content. The second aim was pursued by performing *in vitro* studies with purified laccase and MnP isoenzymes from the same strain both in the presence and in the absence of redox mediators. The outcome of *in vitro* and *in vivo* incubations was assessed by determining the residual estrogenic activity of ethyl acetate-extracted reaction mixtures.

4.3. Materials and methods

4.3.1. Materials

Technical nonylphenol mixture, 17 α -ethynylestradiol ($\geq 98\%$), and triclosan ($\geq 97\%$) were purchased from Fluka (Germany). Bisphenol A ($\geq 99\%$) and chlorophenol red- β -D-galactopyranoside, (CPRG) were from Sigma, (Germany).

Stock solutions of EE2 (EE2S), BPA (BPAS), NP (NPS) and TRC (TRCS) were prepared by dissolving individual compounds in DMSO (Sigma, Germany) at a final concentration of 2, 2, 0.6 and 0.4 g·l⁻¹, respectively.

N-hydroxybenzotriazole (HBT), was from Fluka (Germany), while reduced glutathione (GSH) was from Sigma (Darmstadt, Germany). All solvents of p.a. quality, trace analysis quality or gradient grade were from Merck (Darmstadt, Germany). Purified *P. tigrinus* CBS 577.79 laccase (PtL) and MnP II (PtM II) isoenzymes were obtained as reported elsewhere (Quaratino *et al.*, 2007; Petruccioli *et al.*, 2009).

4.3.2. Organism, culture media and inocula preparation

Fungal strain, *P. tigrinus* CBS 577.79, culture media (MEG and LNKM) and inocula preparation were already described in sub-section 2.4.2.2 of the present PhD thesis.

4.3.3. Culture conditions

All the experiments were conducted at 28 °C under both stationary and reciprocal shaking conditions (100 rpm) in 250 ml Erlenmeyer flasks containing 20 ml and 40 ml, respectively, of either MEG or LNKM. Individual EDCs were added to *P. tigrinus* stationary and shaken cultures by spiking 100 and 200 µl of either EE2S, BPAS, NPS or TRCS, respectively, immediately after inoculation. The final concentration of the EE2 and BPA was 10 mg l⁻¹, while those of NP and TRC were 6 and 3 mg l⁻¹, respectively. Three harvests were performed after 5, 10 and 15 days of incubation for both the stationary and the shaken cultures.

Heat-killed controls (HKC) were run with mycelia previously sub-cultured for 7 days, then killed by autoclaving (121 °C, 20 min) and subsequently contaminated with the appropriate amount of individual EDC stock solutions (EE2S, BPAS, NPS, and TRCS). Biotic controls (BC) were prepared by spiking stationary and shaken cultures with 100 and 200 µl of DMSO alone. All the degradation experiments were carried out in triplicate in the dark.

4.3.4. Enzymes assays

The activities of the lignin modifying enzymes (laccase, MnP and MIP) were determined as described in sub-section 2.4.2.5 of the present PhD thesis.

4.3.5. Sample preparation and analytical methods

Sample preparation and biomass determination were as described in sub-section 2.5.2.6. Quantitative analysis of the EDCs were performed using the RP-HPLC system (Waters 2695 Separations Module) equipped with a diode-array detector (Waters 2996) already described in the above cited sub-section. Isocratic programs were applied for all compounds under analysis. NP was analyzed at 35 °C, using 85% of methanol and 15% of water (v/v) with detection at 224 nm. BPA and EE2 were separated with 48% of acetonitrile at 38 °C. An isocratic program was used with 65% of acetonitrile and 35% of formic acid (0.1% in water). The compounds were separated on a LichroCart column filled with LichroSphere RP-18e (250 mm × 5 mm, particle Ø 5 µm), provided by Merck (Germany), with a flow rate of 1 ml min⁻¹. Due to UV interference with an unknown compound in the extract, TRC was analyzed using HPLC-MS (LCQ Advantage, Thermo) with the same HPLC conditions. The mass spectrometer was operated in negative mode electrospray ionization. The discharge voltage was set 3 kV, capillary voltage maintained at -12.5 V and the capillary temperature was 300 °C. The sheath and sweep gas were supplied with nitrogen. The sheath and sweep gases were adjusted to 52 and 3 arbitrary units, respectively. The data were collected in SIM mode when ion 287 amu was monitored. The detection limits for BPA, EE2, NP, 4-n-NP and TRC were 0.23, 0.44, 0.21, 0.20 and 0.1 mg l⁻¹, respectively.

4.3.6. In vitro oxidation of EDCs with purified enzymes

All experiments were performed in triplicate in 2 ml reaction tubes with teflon-lined screw caps. Degradation experiments with purified laccase were conducted in 1 ml reaction mixtures containing 0.1 M acetate buffer pH 4.5 added with 1% (w/v) Tween 80, 20 µl of a stock solution of each EDC (0.25 g l⁻¹ in acetone) and 1 U of laccase. Incubations were at 28 °C for 168 h on a rotary shaker (80 rpm). In mediated reactions, HBT was added at a final concentration of 1 mM.

In vitro degradation tests with purified MnP were conducted in 1 ml reaction mixtures containing 0.05 M malonate-NaOH buffer pH 4.5, 0.01 M MnSO₄ and 1 U MnP. Glucose (0.03 M) and glucose-oxidase (0.06 U) were included in the reaction mixture to ensure a gradual and continuous H₂O₂ production. The reaction mixture was then spiked with 100 µl of each EDC (50 mg l⁻¹ in acetone) and then incubated as above. GSH was either omitted or added at a final concentration of 5 mM. The final concentration of individual EDCs was added at a 5 mg l⁻¹ in all cases. Relative abiotic controls were performed by adding either heat-denatured laccase or MnP to reaction mixtures. Quantitative analyses were initially performed after 0, 3, 6, 9 and 24 h. Thereafter,

samplings were done on a daily basis. As for quantitative analyses, the direct injection of reaction mixtures into HPLC was allowed by the quantitative solubilization of EDCs.

At the end of incubation, each sample was extracted with 1 ml of ethyl acetate (3 times) in order to allow the measurements of the residual estrogenic activity.

4.3.7. Determination of estrogenic activity

Estrogenic activity of ethyl acetate extracts (from both *in vivo* and *in vitro* samples) was assessed using a recombinant yeast screen according to Routledge and Sumpter (1996). The assay uses *Saccharomyces cerevisiae* with the integrated human estrogen receptor (hER) into the DNA sequence of the yeast genome, also containing the expression plasmids that carry estrogen-responsive sequences (ERS) controlling the expression of the reporter gene *lacZ* (encoding the enzyme β -galactosidase). Thus, in the presence of estrogens, β -galactosidase is synthesized and secreted into the medium, where it metabolizes the chromogenic substrate chlorophenol-red- β -D-galactopyranoside (CPRG) that turns into a red product, the absorbance of which can be measured at 540 nm. In order to assay the EE2 samples, the ethyl acetate extracts were diluted 2,000 times in methanol and 5 μ l were used for the test. In cases of other EDCs, 20 μ l of the extracts were applied directly for the assay. The concentrations were then fitting to the linear part of respective calibration curves when regression coefficient were better than 0.93 for all the four compounds. Simultaneously, representative EDCs (same amounts as added to the cultures) were tested using the same dilution and their endocrine activity was compared to that of 17 β -estradiol (E2). The estrogenic activity of the samples was then expressed as a percentage of the estrogenic activity of the original EDCs. The estrogenic activity of HKC samples was equal to that of EDC standard chemicals and therefore is not reported. BC, cultivated in the absence of EDCs, did not show estrogenic activity (data not shown).

4.4. Results and discussion

The toxicity of EDCs towards aquatic and terrestrial organisms is well known and documented (Hundt *et al.*, 2000; Kollmann *et al.*, 2003; Corvini *et al.*, 2006; Cabana *et al.*, 2007; Cajthaml *et al.*, 2009; Subramanian and Yadav, 2009). Although white rot fungi showed to be resistant to high concentrations of xenobiotics (Leontievsky *et al.*, 2002), previous studies showed that high concentrations of NP and TRC, in particular, cause strong inhibition of fungal growth and alteration of metabolism (Subramanian and Yadav, 2009; Cajthaml *et al.*, 2009). Therefore, the applied

concentration of individual EDCs (see section 4.2.3) was adjusted according to previous observations (Cajthaml *et al.*, 2009). In this respect, no suppression of fungal growth was detectable in *P. tigrinus* cultures in the presence of single EDCs with respect to BC. Notable exception was TRC in LNKM shaken cultures, which negatively affected biomass production (data not shown).

4.4.1. Enzymatic activities

Laccase and MnP activity peaks detected in *P. tigrinus* cultures, either in the presence or the absence of EDCs, are reported in Table 4.1. As for the former enzyme, it was confirmed to be the predominant LME produced by the fungus in the high-N medium (MEG) in accordance with previous studies (Quaratino *et al.*, 2007). Moreover, the maximal values of laccase activity in the presence of NP were approximately 2- and 3- fold higher than those of the relative BC, in MEG stationary and MEG shaken cultures, respectively. Similarly, laccase activity in *T. versicolor* cultures was clearly triggered by the presence of NP (Soares *et al.*, 2005; Cajthaml *et al.*, 2009) although it was only observed with stationary cultures (Soares *et al.*, 2006). In addition, the technical NP mixture had stimulated laccase activity also in *P. cinnabarinus* and Mn-independent peroxidase in *B. adusta* (Cajthaml *et al.*, 2009). The other EDCs spiked in *P. tigrinus* cultures did neither increase nor suppress laccase activity in static cultures grown in MEG. On the contrary, lower laccase activity peaks with respect to the relative BC were achieved in MEG shaken cultures when either BPA or TRC were spiked.

The production of MnP by *P. tigrinus* is known to be mainly stimulated in N-limiting conditions (Quaratino *et al.*, 2006). This result was confirmed in the LNKM BC cultures, under both stationary and shaken conditions. On the contrary, a certain inhibitory effect on MnP production in EDCs-containing cultures was observed in all *P. tigrinus* cultures. With this regard, the levels of expression of peroxidase genes and other genes specific to the ligninolytic condition (*i.e.*, glioxal oxidase) were found to be lowered by the presence of NP (100 mg l⁻¹) in *P. chrysosporium* cultures grown in low-N medium (Subramanian and Yadav, 2009). Moreover, in LNKM static cultures supplied with TRC and EE2 laccase activity peaks were slightly higher than those of the BC.

In conclusion, although the degradation of various EDCs and the concomitant decrease in the estrogenic activity have been successfully achieved *in vitro* using LMEs (Tanaka *et al.*, 2000; Hirano *et al.*, 2000; Saito *et al.*, 2004; Cabana *et al.*, 2007; Auriol *et al.*, 2008), it is noteworthy that the elimination of such compounds can be brought about by other enzymatic systems (Lee *et al.*, 2005; Hundt *et al.*, 2000; Subramanian and Yadav, 2009).

Table 4.1. Maximal values of laccase and MnP activities in *P. tigrinus* cultures either in the presence or in the absence of individual EDCs. Data are means of triplicate experiments $\pm$ standard deviations; days required to attain the activity peaks are reported within round brackets.

Medium	Culture conditions	EDCs	Lacc (U l ⁻¹)	MnP (U l ⁻¹)
MEG	stationary	BC	21.5 $\pm$ 0.3 (6)	1.6 $\pm$ 1.2 (10)
		EE2	16.6 $\pm$ 4.4 (8)	1.6 $\pm$ 0.8 (6)
		BPA	21.7 $\pm$ 5.2 (6)	1.7 $\pm$ 1.1 (10)
		NP	44.9 $\pm$ 5.2 (8)	0.9 $\pm$ 0.3 (4)
		IRG	15.8 $\pm$ 1.1 (10)	0.8 $\pm$ 0.5 (4)
MEG	shaken	BC	20.3 $\pm$ 1.3 (14)	10.4 $\pm$ 2.8 (13)
		EE2	17.9 $\pm$ 3.5 (6)	2.5 $\pm$ 1.7 (2)
		BPA	15.8 $\pm$ 2.1 (6)	3.2 $\pm$ 2.6 (8)
		NP	56.5 $\pm$ 6.1 (10)	0.5 $\pm$ 0.3 (6)
		IRG	8.3 $\pm$ 1.4 (8)	2.6 $\pm$ 1.8 (4)
LNKM	stationary	BC	7.4 $\pm$ 2.8 (13)	21.1 $\pm$ 2.3 (15)
		EE2	14.4 $\pm$ 1.7 (10)	0.8 $\pm$ 0.7 (6)
		BPA	4.6 $\pm$ 0.3 (14)	11.8 $\pm$ 1.2 (12)
		NP	4.8 $\pm$ 1.8 (8)	2.8 $\pm$ 0.7 (6)
		IRG	17.9 $\pm$ 3.7 (8)	8.7 $\pm$ 4.4 (6)
LNKM	shaken	BC	4.9 $\pm$ 2.3 (10)	17.2 $\pm$ 4.4 (14)
		EE2	1.5 $\pm$ 0.5 (6)	2.7 $\pm$ 0.8 (6)
		BPA	1.7 $\pm$ 0.4 (8)	2.1 $\pm$ 1.4 (8)
		NP	2.9 $\pm$ 1.8 (8)	0.7 $\pm$ 0.4 (6)
		IRG	1.4 $\pm$ 1.1 (6)	0.3 $\pm$ 0.2 (8)

Data are the means $\pm$ standard deviation of three replicates.

4.4.2. In vivo degradation of individual EDCs and removal of their estrogenic activity

The time course of degradation of individual EDCs (EE2, BPA, NP and TRC) by *P. tigrinus* cultures and the values of estrogenic activity of the ethyl acetate extracts are reported in Figure 4.2 and Table 4.2, respectively. The synthetic estrogen EE2 was extensively degraded by the fungus

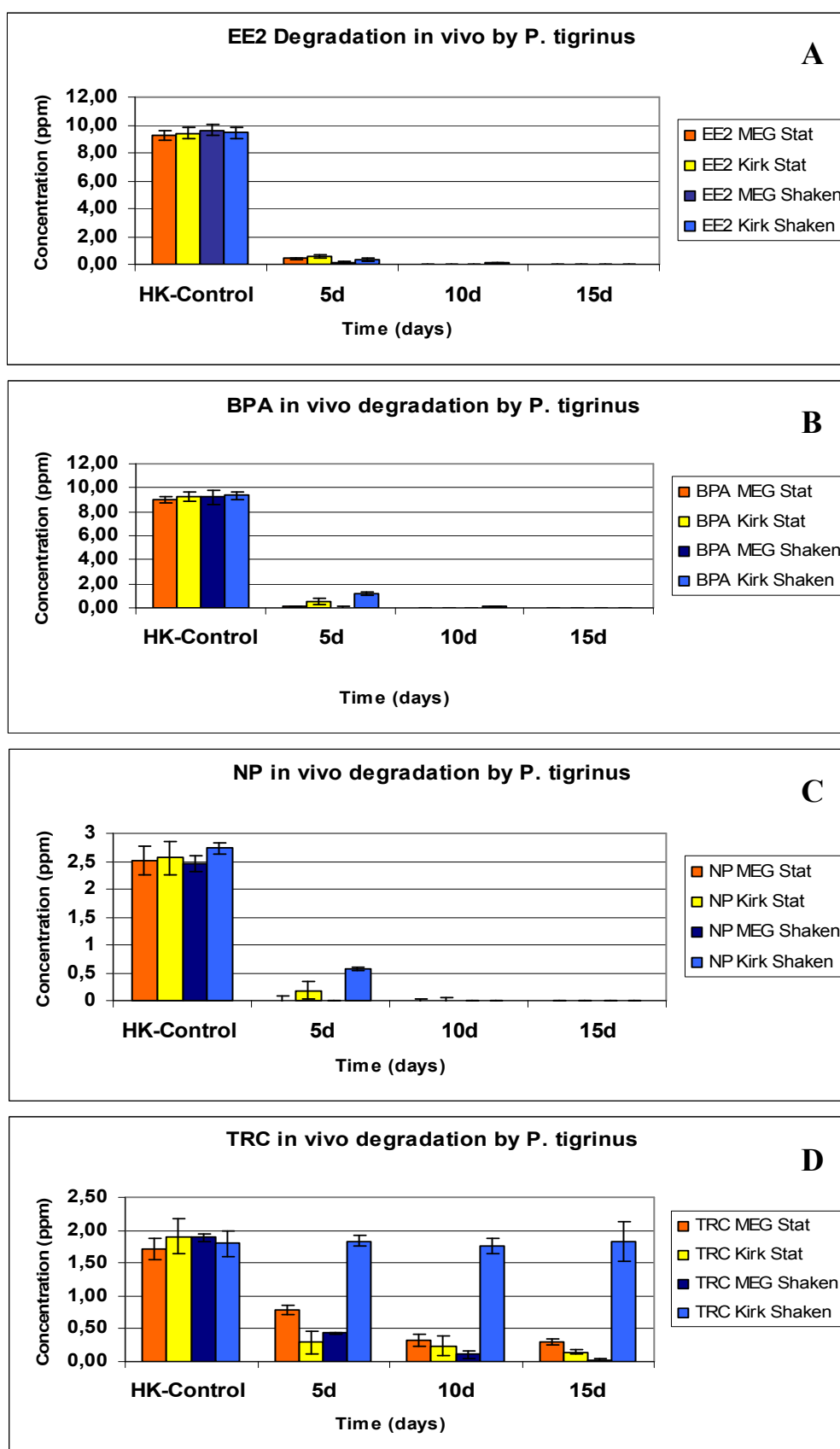


Figure 4.2. Time course of degradation of EE2 (A), BPA (B), NP (C) and TRC by *P. tigrinus* in MEG and LNKM media. Data are means $\pm$ standard deviations of triplicate experiments.

within the first 5 days of incubation; its residual concentration, in fact, ranged from 1 to 6% of that of the relative HKC under both stationary and shaken conditions, while it was reduced to below the detection limit after 10 days. The degradation of EE2 in the early phases of incubation with the fungus was accompanied by the concomitant decrease in the estrogenic activity of the culture extracts under all the conditions tested (Table 4.2). Concerning MEG stationary cultures, the rate of EE2 degradation by *P. tigrinus* ($1.9 \text{ mg l}^{-1} \text{ d}^{-1}$) was lower than those reported for *P. ostreatus*, *I. lacteus* and *P. cinnabarinus*, which completely removed 10 mg l^{-1} of the synthetic estrogen in 3 days of incubation, (Cajthaml *et al.*, 2009). Other white rot strains (*P. chrysosporium*, *P. magnoliae* and *B. adusta*) investigated in the same work, however, were less efficient than *P. tigrinus* in removing EE2 and its estrogenic activity. Moreover, Blanquez and Guieysse (2008) have recently reported that batch cultures of *T. versicolor* removed 97% of 10 mg l^{-1} initially supplied EE2 in 24 hours (yielding a volumetric rate of $0.44 \text{ mg l}^{-1} \text{ h}^{-1}$).

The EDC BPA was also efficiently degraded by *P. tigrinus*. After 5 days of incubation in MEG medium, under both stationary and shaken conditions, only trace amounts of the initially supplied BPA (10 mg l^{-1}) were detected. The resulting volumetric removal rate in such cultural conditions was $1.99 \text{ mg l}^{-1} \text{ d}^{-1}$. In similar experiments (Cajthaml *et al.*, 2009), faster BPA conversion rate ($\geq 3.3 \text{ mg l}^{-1} \text{ d}^{-1}$) were reported for MEG stationary cultures of *I. lacteus* and *P. ostreatus*, while other fungi investigated were less efficient (*P. chrysosporium* and *B. adusta*). Moreover Lee and co-workers (2005) showed that two fungi (*Stereum hirsutum* and *Heterobasidion insulare*) were able to degrade 200 mg l^{-1} of BPA and to remove their estrogenic activity within 14 days; it must be mentioned, however, that in their case the EDC was supplied to 7-day-old cultures. Best BPA degradation was reported for a Korean strain of *I. lacteus* (Shin *et al.*, 2007), which removed 50 mg l^{-1} of the EDC in 12 h (volumetric removal rate $100 \text{ mg l}^{-1} \text{ d}^{-1}$). The degradation of BPA by *P. tigrinus* was slightly slower on LNKM than MEG: the residual concentrations of the EDC on day 5 were about 5 and 11% in stationary and shaken cultures, respectively. An evidence of better performances in nutrient-rich medium with respect to Kirk's mineral medium (LNKM) was already reported for *I. lacteus* (Shin *et al.*, 2007). Despite this small differences in the time course of BPA-degradation by *P. tigrinus*, the estrogenic activity was markedly decreased under all the conditions tested since the early phases of incubation.

In this study, we chose to use technical-grade NP, a formulation containing multiple isomers, as opposed to the model compound 4-*n*-NP, which consists of a single congener. As already reported by other authors (Subramanian and Yadav, 2009), this mixture of congeners is more difficult to degrade than the single one. Consequently, technical-grade NP is a more environmentally representative form of this chemical.

Table 4.2. Detection of estrogenic activities during biodegradation of EDCs using a recombinant yeast assay according to Routledge and Sumpter (1996).

EDC	Culture conditions	Residual estrogenic activity ^{a)} (%) $\pm$ std. dev.		
		5 days	10 days	15 days
EE2	MEG stationary	5.5 $\pm$ 1.1	5.8 $\pm$ 0.8	6.3 $\pm$ 1.1
	MEG shaken	0.9 $\pm$ 5.0	0.0 $\pm$ 0.5	0.0 $\pm$ 0.4
	LNKM stationary	8.7 $\pm$ 3.5	1.4 $\pm$ 0.4	5.1 $\pm$ 0.8
	LNKM shaken	0.0 $\pm$ 0.4	0.8 $\pm$ 1.2	0.2 $\pm$ 1.6
BPA	MEG stationary	2.5 $\pm$ 1.1	8.1 $\pm$ 7.5	3.1 $\pm$ 2.8
	MEG shaken	2.1 $\pm$ 0.8	2.6 $\pm$ 1.8	2.5 $\pm$ 1.1
	LNKM stationary	2.0 $\pm$ 0.1	0.0 $\pm$ 0.6	3.5 $\pm$ 1.6
	LNKM shaken	9.1 $\pm$ 5.2	0.3 $\pm$ 3.2	0.0 $\pm$ 1.7
NP	MEG stationary	0.9 $\pm$ 0.2	1.2 $\pm$ 0.5	9.3 $\pm$ 3.0
	MEG shaken	74.5 $\pm$ 12.9	46.8 $\pm$ 4.9	3.1 $\pm$ 2.0
	LNKM stationary	10.2 $\pm$ 0.1	6.2 $\pm$ 1.8	0.5 $\pm$ 0.5
	LNKM shaken	45.6 $\pm$ 18.4	28.4 $\pm$ 13.8	2.3 $\pm$ 1.5
TRC	MEG stationary	105.6 $\pm$ 34.7	81.2 $\pm$ 20.2	13.3 $\pm$ 4.4
	MEG shaken	60.2 $\pm$ 13.7	8.1 $\pm$ 2.2	9.6 $\pm$ 2.5
	LNKM stationary	120.6 $\pm$ 8.9	129.7 $\pm$ 22.8	49.5 $\pm$ 2.8
	LNKM shaken	188.5 $\pm$ 29.6	192.1 $\pm$ 32.4	163.8 $\pm$ 44.5

a) estrogenic activities are expressed as percentage of activity detected in HKC conducted in the presence of either 10, 10, 3 and 2 mg l⁻¹ of EE2, BPA, NP and TRC, respectively.

P. tigrinus cultures in MEG degraded NP within 5 days from the spiking, under both shaken and stationary conditions (removal rate of 0.6 mg·l⁻¹·d⁻¹). At the same time of incubation residual concentrations of NP in LNKM were 6 and 19% of the amount originally added in stationary and shaken cultures, respectively. Successful degradation of NP in soil and aqueous media have already been reported for other white rot fungi (Soares *et al* 2005, 2006; Cajthaml *et al.*, 2009; Subramanian and Yadav, 2009) although the performances depend on the strain, the culture conditions and the degradation pathway used (Cabana *et al.*, 2007). In this respect, Soares and co-workers found that *B. adusta* was able to eliminate NP at a rate of 9.7 mg l⁻¹ d⁻¹ under shaking conditions, while the conversion in stationary cultures was slower (3.8 mg l⁻¹ d⁻¹). Conversely, *T. versicolor* degraded technical-grade NP at a rate of 2.8 mg l⁻¹ d⁻¹ in stationary cultures whereas it was unable to grow

under agitation. In a recent work (Subramanian and Yadav, 2009), it was clarified that *P. chrysosporium* is able to degrade technical-grade NP under both ligninolytic and non-ligninolytic conditions. In particular, under N-limiting conditions (ligninolytic) the degradation of NP was ascribed to the action of peroxidases (although their expression was found to be down-regulated by the EDC), while in N-rich medium (non-ligninolytic) cytochrome P450 monooxygenases were responsible for the bioconversion.

At the end of the incubation (15 days), no estrogenic activity was detectable in NP-spiked *P. tigrinus* cultures. Interestingly, the estrogenic activity of samples from stationary cultures decreased consistently with the elimination of NP by the fungus; in shaken cultures, instead, a non proportional decrease in estrogenic activity was observed (Table 4.2). In this respect, it might be postulated that intermediates of degradation showing residual estrogenic activity were produced under shaken conditions (Tsustumi *et al.*, 2001; Cajthaml *et al.*, 2009). In the particular case of MEG shaken cultures, the delayed removal of estrogenic activity could be ascribed to the action of laccase, the maximal values of which occurred during the second week of incubation in the presence of NP (see table 4.1). Laccases from several WRF, in fact, were found to be competent in the *in vitro* degradation of NP (Tsutsumi *et al.*, 2001; Junghanns *et al.*, 2005; Cabana *et al.*, 2007).

Among all the EDCs tested in this study, TRC was the most recalcitrant (Fig. 4.2-D). Although its concentration was relatively low (2 mg l⁻¹) TRC was not degraded by *P. tigrinus* in LNKM shaken cultures: under this condition, in fact, both biomass growth (not shown) and the production of ligninolytic enzymes were strongly inhibited by the presence of this compound with respect to the relative BC.

The highest TRC degradation by *P. tigrinus* (>98% of the concentration in the relative HKC) was achieved after 15 days of incubation in shaken cultures grown on MEG. Residual TRC concentrations of 15 and 7% were detected in MEG static and LNKM shaken cultures, respectively. Other authors (Cajthaml *et al.*, 2009) reported similar results concerning the time course of TRC-removal by representative white rot species. On the contrary, the first study investigating the fungal degradation of TRC (Hundt *et al.*, 2000) revealed that *T. versicolor* degraded 90 % of a 0.25 mM solution of TRC (approx. 70 mg l⁻¹) in 4 weeks of cultivation. The initial toxicity of the antimicrobial agent disappeared after 3-5 days due to the conversion of the EDC into conjugation products (Hundt *et al.*, 2000). 2,4-dichlorophenol was also detected as xylosilated derivative, whereas no ring-cleavage products were identified. In this respect, the authors hypothesized that the two chlorine atom in *para*-position with respect to the oxygen involved in the ether bond might prevent the formation of an intermediate with three adjacent hydroxyl groups, the occurrence of

which seems to be essential for the formation of a hydroxyphenoxymuconic acid as a ring cleavage product from diphenyl ether (Hundt *et al.*, 2000).

The value of the estrogenic activity of this compound was found to be very close to that of NP. The loss of estrogenic activity in 15-day-old *P. tigrinus* cultures was higher in MEG than LKKM (9 and 13% in shaken and stationary cultures, respectively). In LNKM, the estrogenic activity of TRC was approximately halved after 15 days in static cultures while it was increased by 15 days of incubation under shaken conditions. This result might probably be due to the higher laccase production achieved by stationary cultures (see table 4.2), since this enzyme was shown to efficiently degrade TRC under *in vitro* conditions (Cabana *et al.*, 2007).

4.4.3. In vitro degradation of individual EDCs with purified laccase and MnP from *P. tigrinus*

In order to determine the impact of LMEs secreted during the *in vivo* degradation of EDCs, purified laccase and MnP from *P. tigrinus* have been used *in vitro* for the treatment of these target chemicals. The results of both mediated and non-mediated reactions are summarized in the Tables 4.3-4.6.

The amount of EE2 initially added to the reaction tubes (5 mg l^{-1}) was quantitatively degraded by laccase and MnP within 9 hours under the condition tested, at a rate of $0.56 \text{ mg l}^{-1} \text{ h}^{-1}$ (Table 4.3).

Table 4.3. Time course of EE2 degradation by laccase and MnP from *P. tigrinus*.

EDC	Time (h)	Residual concentration (% of time 0 h)			
		Laccase (1 U ml^{-1})		MnP (1 U ml^{-1})	
		-HBT	+HBT ^{a)}	-GSH	+GSH ^{b)}
EE2	3	28.3±8.5	0.9±1.9	55.4±6.3	14.3±7.6
	6	12.0±6.3	0.0±0.0	21.6±3.6	0.0±0.0
	9	1.2±1.7	0.0±0.0	3.6±2.2	0.0±0.0
	24	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

a) and b) in mediated reaction HBT and GSH were added at a final concentration of 1 and 5 mM, respectively.

The use of mediators, HBT and GSH, improved the reaction rate of EE2 oxidation. The laccase-HBT system was the most efficient reaction mixture among those tested, promoting the complete oxidation (>98%) of the synthetic estrogen within 3 h (at a rate of $1.67 \text{ mg l}^{-1} \text{ h}^{-1}$). MnP-GSH system led to a 2.56-fold increase in the EE2 oxidation with respect to that achieved by MnP alone,

yielding a conversion rate of approx. $1.43 \text{ mg l}^{-1} \text{ h}^{-1}$. In a previous work (Suzuki *et al.*, 2003) faster EE2-degradation rates were reported with *T. versicolor* laccase-HBT system and *P. chrysosporium* MnP system (approx. $3 \text{ mg l}^{-1} \text{ h}^{-1}$) using lower amounts of enzyme (10 nkat , $\approx 0.6 \text{ U ml}^{-1}$). In addition, *T. versicolor* commercial laccase (20 U ml^{-1}) was recently used to degrade 100 ng l^{-1} of EE2 in both wastewater samples and synthetic solutions (Auriol *et al.*, 2008); the catalytic activity of the enzyme was improved by HBT (Auriol *et al.*, 2007).

BPA (initial concentration 5 mg l^{-1}) was efficiently degraded by both laccase and MnP from *P. tigrinus* after 9 hours under non-mediated conditions (Table 4.4).

Table 4.4. Time course of BPA degradation by laccase and MnP from *P. tigrinus*.

EDC	Time (h)	Residual concentration (% of time 0 h)			
		Laccase (1 U ml^{-1})		MnP (1 U ml^{-1})	
		-HBT	+HBT ^{a)}	-GSH	+GSH ^{b)}
BPA	3	31.3 ± 6.2	4.4 ± 2.8	67.0 ± 8.2	76.9 ± 7.6
	6	13.1 ± 4.8	0.0 ± 0.0	23.5 ± 7.5	33.5 ± 0.0
	9	1.6 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.4
	24	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

a) and b) in mediated reaction HBT and GSH were added at a final concentration of 1 and 5 mM, respectively.

The removal rate for both the enzymatic reaction mixtures was about $0.56 \text{ mg l}^{-1} \text{ h}^{-1}$. Interestingly, the presence of HBT improved significantly (approx. 2.8-fold) the laccase catalytic efficiency towards BPA. The resulting removal rate under the latter condition was about $1.6 \text{ mg l}^{-1} \text{ h}^{-1}$, whereas GSH did not enhance BPA oxidation by *P. tigrinus* MnP under the condition tested.

The *in vitro* oxidation of BPA with free LMEs has been extensively studied (Hirano *et al.*, 2000; Tsutsumi *et al.*, 2001; Uchida *et al.*, 2001; Fukuda *et al.*, 2001; Saito *et al.*, 2004; Kim and Nicell, 2006; Cabana *et al.*, 2007). Reaction conditions for the degradation of BPA by *C. polyzona* laccase were optimized by Cabana and colleagues (2007): the enzyme (10 U l^{-1}) catalyzed the complete oxidation of 5 mg l^{-1} of the EDC in 4 hours at $50 \text{ }^{\circ}\text{C}$ and pH 5. Moreover, the laccase efficiency in BPA degradation was enhanced by $10 \text{ }\mu\text{M}$ of ABTS (2 fold-increase in the reaction rate) and, to a lesser extent by the same amount of HBT. Similarly, Kim and Nicell (2006) reported that BPA oxidation by commercial laccase from *T. versicolor* was best at $45 \text{ }^{\circ}\text{C}$ and pH 5 and that the BPA conversion was enhanced by ABTS and negatively affected by HBT. Purified laccase from a soil

fungus (50 U ml⁻¹) was able to degrade 5 mM of BPA within 6 hours of incubation, and the degradation rate was not enhanced by the addition of HBT (Saito *et al.*, 2003).

The treatment of BPA was indeed achieved *in vitro* using the MnP secreted by the WRF *P. ostreatus* O-48. BPA was eliminated from a 0.4 mM solution using 10 U ml⁻¹ of MnP, 2.0 mM MnSO₄ and 2.0 mM H₂O₂ at a pH of 4.5 and at room temperature (Hirano *et al.*, 2000). Remarkably, the complete removal of this EDC was highlighted by the modification of the UV spectra of the BPA-containing solution after a 1-hour treatment.

Partially purified MnP (100 U l⁻¹) from *P. chrysosporium* ME-446 was used for the removal of BPA from a 0.22 mM solution (Tsutsumi *et al.*, 2001). The EDC was completely removed from the medium in 1 hour, at a rate of 50 mg l⁻¹ h⁻¹.

In vitro treatment of NP with purified LMEs from *P. tigrinus* is summarized in Table 4.5.

Table 4.5. Time course of NP degradation by laccase and MnP from *P. tigrinus*.

EDC	Time (h)	Residual concentration (% of time 0 h)			
		Laccase (1 U ml ⁻¹)		MnP (1 U ml ⁻¹)	
		-HBT	+HBT ^{a)}	-GSH	+GSH ^{b)}
NP	3	97.2±4.5	80.1±6.2	31.6±7.6	59.7±7.8
	6	93.3±2.9	68.1±7.2	8.2±3.2	15.8±6.2
	9	90.3±3.2	55.9±5.6	0.0±0.0	0.0±0.0
	24	71.1±2.6	36.1±5.3	0.0±0.0	0.0±0.0
	48	50.7±4.3	8.3±4.9	0.0±0.0	0.0±0.0
	72	32.2±5.8	0.0±0.0	0.0±0.0	0.0±0.0
	96	16.9±5.7	0.0±0.0	0.0±0.0	0.0±0.0
	120	2.3±1.2	0.0±0.0	0.0±0.0	0.0±0.0
	144	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

a) and b) in mediated reaction HBT and GSH were added at a final concentration of 1 and 5 mM, respectively.

The results show that MnP was more efficient than laccase, under both mediated and non-mediated conditions. The overall reaction rate for MnP catalyzed oxidation of NP was 0.77 mg l⁻¹ h⁻¹ and the redox mediator GSH did not improve the conversion of the phenolic substrate. Much slower reaction rate were achieved when laccase was the catalyst (1 mg l⁻¹ d⁻¹), although the addition of HBT led to a 2.3-fold increase in the rate of NP degradation.

Laccase in free form has been used for the treatment of NP present in aqueous solutions at concentrations ranging from 0.023 to 0.32 mM. The laccase used originated from the WRF *T. versicolor*, I-4 strain (fam. *Chetomiaceae*) isolated from soil, *C. polyzona* and the aquatic fungal strains UHH 1-6-18-4 and *C. aquatica* (Tsutsumi *et al.*, 2001; Saito *et al.*, 2003; Dubroca *et al.*, 2005; Moeder *et al.*, 2006; Cabana *et al.*, 2007). In regard to the laccase activity used, the highest conversion of NP was achieved with culture supernatant from the WRF *C. polyzona*. Complete removal was achieved after a contact time of less than 4 hours using a laccase activity of 1 U l⁻¹ (Cabana *et al.*, 2007).

As for the *in vitro* oxidation of NP by MnPs, faster reaction rates than those achieved in the present work were reported by Tsutsumi and co-workers (2001). NP (50 mg l⁻¹) was completely removed by 0.1 U ml⁻¹ of the enzyme (*P. chrysosporium* MnP) at a rate of 50 mg l⁻¹ h⁻¹ and, consistently with our results, the laccase mediated reaction was proved to be less efficient.

The oxidation of TRC by purified laccase and MnP from *P. tigrinus* is reported in Table 4.6.

Table 4.6. Time course of TRC degradation by laccase and MnP from *P. tigrinus*.

EDC	Time (h)	Residual concentration (% of time 0 h)			
		Laccase (1 U ml ⁻¹)		MnP (1 U ml ⁻¹)	
		-HBT	+HBT ^{a)}	-GSH	+GSH ^{b)}
TRC	3	95.0±3.5	88.7±3.5	95.1±2.7	96.3±3.8
	6	90.2±2.9	77.8±4.2	87.9±3.3	89.6±2.1
	9	83.3±3.1	58.3±2.8	83.0±2.9	85.7±3.7
	24	59.2±6.5	8.3±3.9	45.5±4.8	50.0±5.4
	48	41.3±5.7	0.5±0.5	27.3±3.6	32.5±6.1
	72	25.5±8.6	0.0±0.0	8.7±3.9	11.9±4.6
	96	16.5±4.9	0.0±0.0	0.3±0.1	2.9±1.0
	120	9.4±5.2	0.0±0.0	0.0±0.0	0.0±0.0
	144	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

a) and b) in mediated reaction HBT and GSH were added at a final concentration of 1 and 5 mM, respectively.

In non-mediated reactions, as already observed for NP, MnP reaction mixture led to faster TRC-degradation rates than those observed for the laccase catalyzed reaction. However, the fastest TRC-oxidation was achieved by the lacase-HBT system, in which 5 mg l⁻¹ of the substrate were

completely degraded in 48 hours ($2.5 \text{ mg l}^{-1} \text{ d}^{-1}$). On the contrary, the addition of GSH did not improve the catalytic efficiency of *P. tigrinus* MnP.

TRC was also found to be removed using laccase from the previously described *C. polyzona* (Cabana *et al.*, 2007), although its conversion appeared to be less effective than those of BPA and NP under the same condition. Cabana and colleagues (2007), in fact, reported that 65% of 5 mg l^{-1} solution of TRC was removed by 100 U l^{-1} of laccase after 8 hours (rate of $0.4 \text{ mg l}^{-1} \text{ h}^{-1}$). Moreover, Kim and Nicell (2006) reported the complete removal of $20 \text{ }\mu\text{M}$ of TRC (approx. 5.5 mg l^{-1}) by *T. versicolor* laccase in 2 hours treatment. In addition, the authors reported a substantial improvement of treatment efficiency and reaction rates at high temperatures and pH 5; enhancement in TRC conversion was supported by the use of a protective additive, poly(ethylene glycol) (PEG-35000), and a mediator ABTS. In contrast with our findings, HBT did not improve the catalytic activity of *T. versicolor* laccase.

4.4.4. Estrogenic activity following *in vitro* treatment of EDC with laccase and MnP

In order to complete this survey of the enzyme-catalyzed removal of selected EDCs, it is important to determine residual estrogenic activity following MnP and laccase treatments; data, expressed as percentages with respect to incubation controls with the parent ED at the same set concentration (0.001, 0.01, 1 and 1 ppm for EE2, BPA, NP and TRC, respectively). The statistical significance between treatments were then tested by the Tukey test ($P \leq 0.05$).

All the enzyme treatments investigated were capable of decreasing the estrogenic activity of the parent EDCs. Concerning EE2, both enzymatic treatments decreased the estrogenic activity to 38-50% of that measured with the incubation controls. No statistically significant differences, however, were detected among the residual estrogenic activities in laccase and MnP reaction mixtures. Although the end-point was set when EE2 had been degraded below the HPLC detection limits (0.44 ppm), it is noteworthy that enzyme-treated reaction mixtures still maintained residual estrogenic activity. These results are consistent with findings of Suzuki and colleagues (2003) that observed that the complete removal of EE2 by laccase (1 h treatment) did not completely suppress the estrogenic activity.

Regardless of the enzyme type and the presence or the absence of mediators, the estrogenic activity associated to BPA was removed up to 20-35% of that of the incubation controls although no significant differences were observed between enzyme treatments. Tsutsumi *et al.* (2001) also showed that the elimination of the estrogenic activity of a 0.88 mM solution of BPA by MnP was

not directly related to the degradation of the EDC and suggested that the degradation intermediates produced still had estrogenic activity.

With regard to NP and TRC, laccase was more efficient than MnP in removing the estrogenic activity. At the end of the incubation, in fact, the activities of both compounds were completely removed by laccase, under both mediated and non-mediated conditions. Estrogenic activities of 23-26% and 24-32%, with respect to the relative incubation controls, were instead observed in MnP reaction mixtures with NP and TRC, respectively.

In this respect, it is noteworthy that the catalytic action of laccase and MnP on EDCs promotes the formation of phenoxy radicals, which are then prone to oxidative coupling reactions yielding oligomers and polymers (Huang and Weber, 2005). Such coupling products show very low structural similarities with estrogens and therefore their formation results in the elimination of the xeno-estrogenic character of the parent compounds (Cabana *et al.*, 2007).

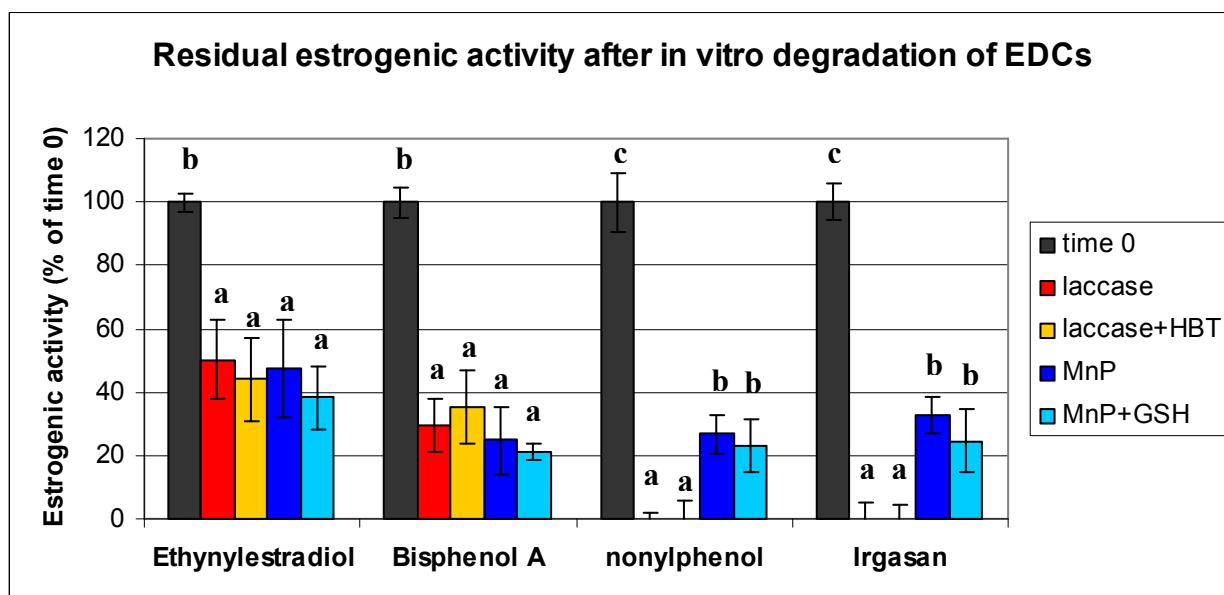


Figure 4.3. Residual estrogenic activity (% of time 0) in the ethyl acetate extracts of laccase and MnP reaction mixtures at the end of incubation with individual EDC. Standard (time 0) concentrations of incubation controls were: 0.001 mg l⁻¹ EE2, 0.01 mg l⁻¹ BPA, and 1 mg l⁻¹ for NP and TRC (Irgasan), respectively. Multiple pair-wise comparisons were performed by the Tukey test ($P < 0.05$); same letters above bars indicate that differences between samples within the same culture condition were not significant.

4.5. Conclusions

P. tigrinus liquid cultures proved to be able to effectively remove EE2, BPA and NP, the degradations of which were not significantly affected by both the type of medium as well as the presence or the absence of shaking conditions. By contrast, TRC was more recalcitrant to degradation and its concentration did not significantly change in shaken cultures conducted on LNKM, probably due to its inherent toxicity towards the fungus that was amplified by the combined association of stirring conditions promoting a high fungal surface area and low N concentration. Accordingly, a high residual estrogenic activity was found in ethyl acetate extracts of TRC-spiked cultures which was best in LNKM shaken cultures.

Under non-mediated conditions, laccase was more efficient than MnP in the oxidation of EE2 and BPA while the latter enzyme oxidized NP and TRC at a faster rate than the former. Under mediated conditions, best degradation performances towards EE2 and BPA and TRC were observed with the laccase/HBT system while NP was more susceptible to oxidation by MnP-regardless of the presence or the absence of GSH. Although the estrogenic activities of EE2 and BPA were significantly removed by in *all vitro* treatments, their removal extents were not significantly affected by the type of treatment; a high impact of the treatment typology, conversely, was observed for NP and TRC the residual estrogenic activities of which were best removed by the laccase and laccase/HBT systems.

In summary, these results clearly feature the capacity of the strain under study to degrade 4 representative compounds of another class of ubiquitous pollutants thus extending the range of application of this fungus for environmental clean-up purposes.

5. LAB-SCALE MYCOREMEDIATION TRIALS

The previous Chapters of the present PhD thesis dealt with the investigation of *P. tigrinus* capabilities to degrade different classes of ubiquitous pollutants in liquid media. Fungal liquid cultures, in fact, constitute appropriate model systems to explore the biotransformation of a wide variety of compounds (Singh, 2006). However, during the last decade, fungi have been used in the treatment of soils and sediments from a variety of polluted sites and their role in the bioremediation of various hazardous and toxic compounds has been established (Singh, 2006). In this respect, owing to the complex nature of environmental samples, several conditions and factors which can stimulate or inhibit fungal degradation of pollutants have to be considered. Bench-scale treatability studies, such as those reported in the present Chapter, are therefore needed in order to develop successful field-scale application of mycoremediation technologies (Lamar and White, 2001; Singh, 2006).

5.1. Mycoaugmentation of PAH-contaminated solid matrices from a wood preservation plant: impact of inoculum carrier and contaminants bioavailability on degradation performances of representative white rot strains.

5.1.1. Introduction

Physico-chemical and toxicological properties of PAHs, as well as their ubiquitous presence in the environment, have been widely described in Chapter 2 of the present PhD thesis.

Bioremediation is still thought to be an environmentally-sustainable approach to the clean-up of PAH-contaminated soils with respect to physico-chemical treatments. In addition to the reported PAH-degrading ability of several bacteria and actinomycetes in soil (Cerniglia, 1992), bioaugmentation with white rot fungi has been shown to be rather promising for this specific purpose (Boyle, 1995; Bhatt *et al.*, 2002; Šašek, 2003). This is due to the fact that these fungi possess a non-specific and radical-based ligninolytic machinery operating in the extracellular environment that enables them to degrade a wide array of contaminants, including high molecular mass PAHs which are generally scarcely susceptible to bacterial attack (Bhatt *et al.*, 2002; Šašek, 2003). The hyphal growth mode of WRFs makes them able to extensively penetrate into soil and to serve, at the same time, as dispersion vectors of indigenous pollutant-degrading bacteria (Kohlmeier *et al.*, 2005).

Nonetheless, competition with the indigenous microflora and adverse physicochemical characteristics of the solid contaminated matrix negatively affect the colonization ability of white-rot fungi (Lange *et al.*, 1998) and, in several cases, the overall depletion of contaminants (Tucker *et*

al., 1995; Šásek, 2003; in der Wiesche *et al.*, 2003). These negative factors might be mitigated by the use of lignocellulosic residues either as amendants or inoculum carriers (Boyle *et al.*, 1995; Leonardi *et al.*, 2008); in this respect, the positive impact of lignocellulosic residues has been ascribed to their either nutritional or protective effects (Sampedro *et al.*, 2009). However, the large variability of reported results, mainly highlighting substrate- and species-dependence effects, suggests the need for further investigations.

Consequently, objective of the present study was to assess the effect of three lignocellulosic inoculum carriers (*i.e.*, wheat straw, corn cobs and straw-based commercial pellets) on both growth and PAH degradation performances of three fungal strains (*i.e.*, *Dichomitus squalens* DSM 9615, *Pleurotus ostreatus* CCBAS 278, *Coprinus comatus* CCBAS 700). The three strains were selected since they all belong to species with PAH-degrading ability (Gramms *et al.*, 1999; Leonardi *et al.*, 2007; in der Wiesche *et al.*, 2003) but are reported to differ in their capacities to compete with the indigenous microflora and to colonize the soil (Lang *et al.*, 1998; Gramms *et al.*, 1999; Martens and Zadrazil, 1998). A historically contaminated soil (HCS) and creosote-treated shavings (CTS), characterized by different abundances of the PAH bioavailability, were used in order to assess the effect of the contaminated matrix and its interaction with both inoculum carrier and fungal strain in terms of fungal growth and PAH degradation.

Furthermore, following the same experimental set-up used for the above-cited WRF, the experiment was repeated in order to assess the impact of the three lignocellulosic carriers on both growth and PAH degradation performances of *P. tigrinus* CBS 577.79. To better elucidate the effect of the contaminated matrix and its interaction with both inoculum carrier and fungus, data obtained with *P. tigrinus* were compared with those of *Irpex lacteus* CCBAS 238, the PAH-degrading capacity of which had been extensively demonstrated (Novotny *et al.*, 2000; Cajthaml *et al.*, 2002; 2006; Baborova *et al.*, 2006).

This study represents an original contribution in the field of mycoaugmentation since, despite the general recognition of the role exerted by bioavailability on degradation performances, in the large majority of augmentation studies with basidiomycetes the clean-up efficiency was not related to the actual contaminants bioavailability of the matrices under study.

5.1.2. Materials and methods

5.1.2.1. Materials

Three different lignocellulosic substrates were employed in this study as carriers for fungal inocula: (i) chopped wheat straw (CWS), (ii) ground corncobs (GC) and (iii) commercial pellets (0.8 mm Ø) (CP, *ATEA Praha*, Prague, Czech Republic). The last one is a wheat straw-based composite material produced for domestic-heating purposes (pellet stoves), having a density of 1.34 kg dm⁻³, a moisture of 6.4 % (w/w) and a C/N ratio of 45:1.

The HCS, with a sandy loamy texture, was collected from the wood treatment plant in Soběslav (southern Bohemia, CZ), where sleepers were impregnated. The HCS air-dried for 7 days at room temperature and sieved (2 mm). Its main properties were as follows: organic C 0.5%, total organics 1.0%, pH 5.1, water-holding capacity 20%. It contained several metals (mg kg⁻¹): As 17.5, Cd 0.4, Co 1.74, Cr 9.41, Cu 6.8, Fe 322, Hg 5.7, Pb 4.5 and Zn 75.6. CTS from oak wood sleepers were provided by the company Eko-Bio Vysočina, Ltd., Czech Republic. CTS were air-dried for 7 days at room temperature and sieved (2 mm). The sandy-loamy soil (MBU), collected from the garden of the Academy of Sciences of the Czech Republic, was used as the non-contaminated control. All solvents of p.a. quality, trace analysis quality or gradient-grade were purchased from Merck (Darmstadt, Germany).

5.1.2.2 Microorganisms and inocula preparation

Dichomitus squalens DSM 9615, *Coprinus comatus* CCBAS 700, *Pleurotus ostreatus* CCBAS 278 and *Irpex lacteus* CCBAS 238 were from the Culture Collection of Basidiomycetes (Institute of Microbiology AS CR, Prague, CZ). *P. tigrinus* CBS 577.79 was from CBS culture collection (Baarn, The Netherlands). During the study the strains were maintained at 4°C and sub-cultured every 21 days on MEG agar plates (per liter: malt extract 5 g, glucose 10 g, agar 15 g, pH 5.0).

Fungal pre-inocula were grown in MEG liquid medium (20 ml in 250 ml Erlenmeyer flasks). After 7 d of incubation at 28°C, the stationary shallow cultures were homogenized with the Ultraturrax-T25 (IKA-Labortechnik, Staufen, Germany) and 1.0 ml aliquots of the mycelial suspension used to inoculate either CWS (2 g) or GCC (6 g) or CP (10 g), the moisture contents of which were adjusted to 70% (w/w). The carriers were transferred to test-tubes (16 x 3.5 cm) which were covered with cotton-wool stoppers and subsequently sterilized by autoclaving (121°C, 45 min). After inoculation, cultures were grown for 2 weeks at 28 °C under stationary conditions.

5.1.2.3 Fungal treatment of polluted matrices

A layer of either PAH-polluted matrices (25.0 and 5.0 g of HCS and CTS, respectively) or MBU control soil (25 g) was added to the test-tubes containing supported inocula (see above). The moisture contents of the two soils (*i.e.*, HCS and MBU) and of the CTS were adjusted to 15% and 25% (w/w) respectively, with sterile deionized water. Cultures grown on the lignocellulosic materials for 2 weeks and killed by autoclaving (121°C, 20 min) before addition of the polluted matrices were referred to as incubation controls. Each microcosm, namely the combination of inoculum carrier, fungal strain and target-matrix, was prepared in triplicate (controls included) and incubated for 60 days at 28 °C. The moisture content of each test-tube was kept constant for the whole period of incubation with periodical additions of sterile deionized water.

5.1.2.4 Extraction and analyses of ergosterol and aromatic pollutants

Total ergosterol was extracted and analyzed as described by Šnajdr *et al.* (2008). Samples (0.5 g) were sonicated with 3 mL of 10% KOH in methanol at 70 °C for 90 min. Distilled water (1 ml) was added and the samples were extracted 3 times with 2 ml of cyclohexane, evaporated under nitrogen, redissolved in methanol and analyzed isocratically using a Waters Alliance HPLC system (Waters Milford, MA) with methanol as a mobile phase at a flow rate of 1 ml min⁻¹. Ergosterol was detected by UV detection at 282 nm.

Extraction of aromatic pollutants was performed with ASE 200 System (Dionex, Voisins-le-Bretonneux, France) packing the extraction cell with either 10 g of HCS or 2 g of CTS. The cell (11 ml) was then loaded into the oven and extracted with hexane-acetone (3:1 v/v). Static heating was applied to the vessel (150°C, 5 min) and subsequent extraction was performed at 150°C under 103.4 bar for 7 min. The cell was then flushed with fresh solvent (60% of total cell volume) and finally the solvent was purged from the cell by nitrogen for 60 s. For each sample the extraction cycle was performed twice. The resulting organic extracts collected in 40 ml vials, air-dried under vacuum at room temperature and finally dissolved in acetonitrile (5 and 20 ml for HCS and CTS, respectively) are referred from here onwards to as contaminants extract (CE). RP-HPLC analyses were performed using a system consisting of a 2695 Separations Module (Waters, Milford, MA) equipped with a LichroCart-PAH column (250 × 5 mm, particle Ø 5 µm; Merck, Darmstadt, Germany), a 2996 diode-array detector and 2475 fluorescent detector (Waters). Separation of the PAHs was achieved with a gradient programme, using (A) a mixture of methanol:acetonitrile (1:1, v/v) and (B) Milli-Q

water. After 5 minutes of isocratic elution with 70% A, the ratio was raised to 100% A in 15 minutes and kept constant for the subsequent 20 min. PAHs were identified on the basis of both UV spectra and match of retention times with commercially available standards. (Dr. Ehrenstorfer, Augsburg, Germany). Concentrations of 13 PAHs out of the 16 compounds according to the U.S. EPA method 610 were determined. Naphthalene, acenaphthylene, and acenaphthene were below detection limits probably due to volatilization. The detected compounds were quantified with the fluorescent detector under following excitation/emission conditions: phenanthrene (PHE), anthracene (ANT) indeno[1,2,3-cd]pyrene (IPY) – 250/390 nm; fluorene (FLU), fluoranthene (FLT), pyrene (PYR), benz[a]anthracene (BaA), chrysene (CHR) 280/340 nm; benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBA), benzo[g,h,i]perylene (BghiP) – 305/430 nm. Calibration curves with the standards were determined over a linear range from 0.1. to 10 µg/ml for each compound.

5.1.2.5 Estimation of PAH bioavailability

Bioavailable fractions of PAHs were estimated using sequential supercritical fluid extraction (SFE) (Leonardi et al, 2007). The extraction was performed with a PrepMaster extractor (Suprex, Pittsburgh, PA) equipped with VaryFlow restrictor operating at 40 °C and with a downward stream of carbon dioxide (5.5 SFE/SFC, Messer Technogas, Prague, CZ). The samples (1 g of either HCS or 0,5 g of CTS) were extracted at 50 °C and 200 bar. Each extraction was carried out in triplicate and the compounds were collected after 5, 10, 20, 40, 60, 80, 120, 160 and 200 min. Sequential supercritical fluid extraction represents a desorption model presuming generally that the extraction is controlled by the two rate constants differing by orders of magnitude (Williamson *et al.*, 1998). The chemical release data can be modelled by an empirical two-site model, consisting of the two first-order equations (1):

$$(1) \quad S_t = F \cdot S_o \cdot e^{-k_1 t} + (1 - F) \cdot S_o \cdot e^{-k_2 t}$$

where S_t is the pollutant concentration remaining in the soil after time t ; F is the fraction of chemical rapidly released; S_o is the original concentration of the pollutant in soil; k_1 and k_2 are the first order rate constants. The so-called “ F fraction” is usually assumed to be representative of equilibrium release conditions, and the remaining, slowly released portion is considered to be kinetically rate limited. Therefore, F fraction represents the portion of the target chemical that is bioavailable in soil (Hawthorne *et al*, 2001, Cajthaml and Šásek, 2005). The fractions were

analyzed separately to complete desorption-kinetic profiles. Prism version 4.0 (GraphPad, La Jolla, CA) was used for calculating the F values. To relate the degradation performances of each microcosm to the actual PAH bioavailability, the degradation fold with respect to the bioavailable fraction (DFBF) was calculated for each contaminant by the following expression:

$$(2) \quad \text{DFBF} = \frac{(C_{\text{ic}} - C_{\text{r}})}{\text{BF}}$$

where C_{ic} and C_{r} are the residual concentrations in the incubation control and fungal-treated material, respectively, and BF the mass amount of the bioavailable fraction per 1 gram of the contaminated material. The relative percent abundances of the bioavailable fraction for each PAH compound detected in the matrices under study are reported in Table 1 along with their respective water solubility and ionization potential.

5.1.2.6. Phytotoxicity assay

Static-type germinability assays with barley (*Hordeum vulgare* L.) seeds were conducted for 3 days at room temperature in 90-mm Petri dishes containing Whatman GF/C filters soaked with 2.0 ml of CE derived from both fungal-treated matrices and relative incubation controls. After CE addition, the filters were held at room temperature for 12 h to allow solvent evaporation; then 2.0 ml distilled water were added on a daily basis. A randomized complete blocks experimental design with 3 replicates and 50 seeds per Petri dish was used. Germinability tests conducted in the presence of distilled water were also run in parallel and served as the control. Percent inhibition (I%) of germinability was calculated from equation (3).

$$(3) \quad \text{I}(\%) = \left(1 - \frac{G}{G_{\text{c}}}\right) \cdot 100$$

where G is the number of germinated seeds in the presence of CE from either fungal-treated matrices or their incubation controls and G_{c} the same parameter in the absence of the CE.

5.1.2.7. Statistical analysis

Two-way analysis of variance (ANOVA) was conducted on both growth, PAH degradation and phytotoxicity data for each matrix by selecting type of inoculum carrier as the first variable (X_1), fungal species as the second one (X_2). Percent data were converted by arcsin of the square root transformation.

5.1.3. Mycoaugmentation of PAH-contaminated solid matrices from a wood preservation plant: impact of inoculum carrier and contaminants bioavailability on degradation performances of *D. squalens*, *P. ostreatus* and *C. comatus*. Results and discussion

5.1.3.1. Fungal growth

Figure 5.1-A shows that the highest growth on MBU was observed with CP-immobilized *D. squalens* ($4.6 \mu\text{g g}^{-1}$ soil). On the HCS, and regardless of the species, growth was best with CP as the carrier (Figure 5.1-B). On the other hand, with the notable exception of GCC-immobilized *D. squalens*, similar growth was observed on CTS (Figure 5.1-C).

Regardless of the species, CWS- and GCC-immobilized fungi grew better on CTS than on the remaining matrices (Figure 5.1-C).

Two-way ANOVA as a function of both inoculation carrier (X_1) and fungal strain (X_2) showed that only the main effect of the former was significant ($P < 0.001$), while a significant interaction between the two variables was only observed for MBU and CTS ($P < 0.001$ and 0.014 , respectively). The highly significant main effect of the inoculum carrier highlights the importance of inoculum formulation on both growth and survival of fungi. Unlike bacteria, in fact, basidiomycetes do not utilize organopollutants as C and N sources and, therefore, an external nutritional supply is required (Boyle *et al.*, 1995). With this regard, natural carriers, such as lignocellulosic materials, provide mycelia with nutrients thus conferring them an initial competitive advantage over free inocula (Leštan and Lamar, 1996; Ford *et al.*, 2007). In addition, immobilized mycelia have been proven to be less prone to growth inhibition by contaminants than free mycelia (Leštan *et al.*, 1996).

The better growth of the strains under study on CTS than on HCS, despite an approx. 20-fold higher PAH concentration in the former matrix, might be due to the possible trophic contribution of CTS, which, being made of oak wood, has a high content of potential growth substrates such as cellulose and hemicellulose (Vane *et al.*, 2006).

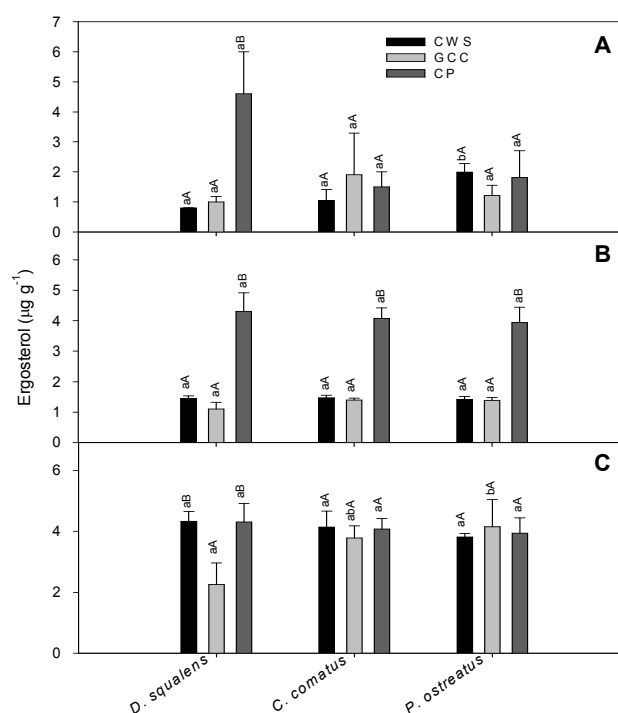


Figure 5.1. Ergosterol concentrations in non-contaminated MBU soil (A), historically-contaminated Soběslav soil (B) and creosote-treated shavings (C) incubated for 60 d at 28 °C with *D. squalens*, *C. comatus* and *P. ostreatus* previously supported on either chopped wheat straw (CWS), ground corn cobs (GCC) or commercial pellets (CP). Data are the mean $\pm$ standard deviation of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P < 0.05$). Same lowercase letters above bars indicate that differences between fungi within the same inoculum carrier were not significant. Same uppercase letters indicate lack of statistically significant difference within each fungus at different inoculation supports.

5.1.3.2. PAH removal from contaminated matrices

The strains under study were tested for their ability to degrade PAHs in both a historically creosote contaminated soil from a wood treatment plant where sleepers were produced and in sleepers treated also with creosote. For each matrix, no significant differences were found between the PAH residual contents of incubation controls carried out with the three carriers; thus, Table 5.1 reports their averaged contents, irrespective of the inoculation carrier.

Table 5.1. Residual concentrations observed in the averaged incubation controls and after incubation with *Dichomitus squalens*, *Coprinus comatus* and *Pleurotus ostreatus* on historically contaminated soil (HCS) and creosote-treated shavings CTS using either chopped wheat straw (CWS) or ground corn cobs (GCC) or commercial pellets (CP) as the inoculum carrier.

Experimental condition	Residual concentrations ($\mu\text{g g}^{-1}$ soil)*										
	FLU	PHE	ANT	FLT	PYR	BaA	CHR	BbF	BKF	BaP	DBA
Historically contaminated soil											
Control†	278.0±18.1	359.9±36.1	104.6±11.2	659.0±51.8	429.0±42.2	77.0±6.9	353.9±18.7	12.7±1.2	6.4±0.7	6.1±0.5	0.6±0.1
<i>D. squalens</i>											
-CWS	64.6±8.9 ^b	84.1±18.2 ^b	17.3±3.0 ^a	471.7±30.6 ^a	331.3±26.1 ^a	70.6±5.6 ^a	346.1±13.0 ^a	12.3±1.0 ^a	5.6±0.6 ^a	5.7±0.1 ^a	0.19±0.0 ^a
-GCC	7.7±0.7 ^a	10.5±0.3 ^a	11.2±1.3 ^a	409.6±61.7 ^a	302.7±6.5 ^a	62.5±1.8 ^a	316.1±24.5 ^a	9.6±0.4 ^a	5.3±0.1 ^a	5.2±0.3 ^a	0.55±0.1 ^b
-CP	3.1±0.6 ^a	6.2±1.9 ^a	11.1±1.2 ^a	403.6±58.5 ^a	351.7±49.8 ^a	77.8±12.5 ^a	324.4±2.1 ^a	10.8±2.1 ^a	5.2±0.4 ^a	5.8±1.3 ^a	0.54±0.0 ^b
<i>C. comatus</i>											
-CWS	49.6±3.5 ^b	94.0±6.5 ^b	16.9±0.4 ^a	408.8±16.1 ^a	298.9±7.0 ^a	61.4±2.9 ^a	321.9±15.1 ^a	10.6±1.1 ^a	4.6±0.4 ^a	4.8±0.3 ^a	0.18±0.0 ^a
-GCC	55.5±0.4 ^b	76.2±2.8 ^b	23.7±1.5 ^b	498.0±1.1 ^b	361.4±9.0 ^b	81.6±4.0 ^c	335.6±4.2 ^a	9.2±0.8 ^a	6.0±0.0 ^b	6.1±0.8 ^a	0.61±0.1 ^b
-CP	5.0±2.7 ^a	7.5±1.3 ^a	13.9±2.4 ^a	452.4±4.8 ^{ab}	334.7±3.6 ^b	72.7±1.4 ^b	338.1±0.1 ^a	10.2±0.1 ^a	4.8±0.3 ^a	5.6±0.7 ^a	0.58±0.0 ^b
<i>P. ostreatus</i>											
-CWS	29.9±3.0 ^c	58.1±12.9 ^c	15.9±0.9 ^b	434.3±22.3 ^b	313.6±17.8 ^b	69.3±1.7 ^b	339.1±12.2 ^c	10.7±0.3 ^b	5.2±0.3 ^b	6.2±0.6 ^c	0.20±0.1 ^a
-GCC	23.5±2.3 ^b	31.7±0.7 ^b	15.5±0.9 ^b	429.7±19.5 ^b	287.5±24.5 ^b	67.6±2.6 ^b	336.1±9.0 ^b	9.4±1.1 ^{ab}	5.7±0.1 ^b	4.8±0.5 ^b	0.28±0.1 ^a
-CP	1.8±0.8 ^a	2.3±0.4 ^b	2.3±0.2 ^a	102.8±13.3 ^a	66.6±11.6 ^a	23.8±0.9 ^a	248.8±7.7 ^a	7.7±0.2 ^a	4.3±0.4 ^a	0.9±0.3 ^a	0.35±0.1 ^a

<Continued in the next page>

Experimental condition	Residual concentrations ($\mu\text{g g}^{-1}$ soil)*												
	FLU	PHE	ANT	FLT	PYR	BaA	CHR	BbF	BKF	BaP	DBA	BghiP	IPY
Creosote-treated shavings													
Control†	2647±163	6698±400	19502±131	8510±529	5308±372	1923±119	1324±105	651±30	385±67	481±111	55.1±4.5	87.7±19.2	155.9±19.1
D.squalens													
- CWS	1484±157 ^{ab}	2584±167 ^c	16155±490 ^b	6743±609 ^b	4474±352 ^b	1266±115 ^b	1312±21 ^b	577±61 ^b	317±32 ^b	467±49 ^b	23.0±2.3 ^b	85.2±17.9 ^{ab}	135.7±15.4 ^b
- GCC	1177±139 ^a	2136±145 ^b	9379±301 ^a	5368±177 ^a	3659±115 ^a	1033±32 ^a	1085±47 ^a	445±9 ^a	223±5 ^a	341±13 ^a	18.2±0.4 ^a	65.2±8.3 ^a	104.7±4.0 ^a
- CP	1976±276 ^b	1681±194 ^a	8996±1382 ^a	5770±277 ^{ab}	3911±113 ^a	1244±78 ^b	1315±89 ^b	554±24 ^b	304±17 ^b	419±19 ^{ab}	23.7±1.5 ^b	110.5±13.6 ^b	126.9±13.9 ^{ab}
C. comatus													
- CWS	1598±37 ^b	2542±42 ^b	13989±1701 ^a	6034±548 ^{ab}	4081±321 ^{ab}	1022±36 ^a	1074±89 ^a	494±38 ^{ab}	276±25 ^b	393±31 ^b	19.8±1.7 ^{ab}	89.9±14.5 ^a	121.0±8.2 ^a
- GCC	973±172 ^a	1843±79 ^a	9792±1364 ^a	5308±16 ^a	3619±33 ^a	955±16 ^a	1366±51 ^b	455±34 ^a	222±7 ^a	333±5 ^a	18.4±1.0 ^a	73.7±15.3 ^a	115.3±19.2 ^a
- CP	1655±187 ^b	2563±277 ^b	11127±1929 ^a	6213±217 ^b	4194±148 ^b	1173±24 ^b	1297±19 ^b	544±7 ^b	277±5 ^b	410±10 ^b	22.6±0.6 ^b	98.2±8.3 ^a	117.9±5.4 ^a
P. ostreatus													
- CWS	1600±181 ^{ab}	2535±179 ^a	15780±922 ^b	7162±423 ^b	4803±245 ^b	1299±108 ^b	1294±108 ^a	592±45 ^b	335±24 ^b	474±34 ^b	66±7.3 ^b	72±6.7 ^{ab}	145±13.7 ^b
- GCC	1560±115 ^a	2216±233 ^a	13476±1471 ^{ab}	5859±140 ^a	3977±99 ^a	1072±35 ^a	1195±4 ^a	482±8 ^a	248±9 ^a	383±16 ^a	19±0.5 ^a	64±2.4 ^a	102±9.2 ^a
- CP	1957±132 ^b	2782±343 ^a	10397±1644 ^a	6229±391 ^a	4292±291 ^{ab}	1226±59 ^{ab}	1374±59 ^a	572±13 ^b	277±4 ^a	412±21 ^{ab}	23±0.9 ^a	93±17.1 ^b	126±3.2 ^b

*Data are the mean ± standard deviation of three independent experiments. Statistical multiple pair-wise comparison was carried out within column means pertaining to each strain by the Tukey test: means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

In the Soběslav soil, 13 PAHs were detected with FLT, PYR, PHE, CHR and FLU being the most abundant compounds (28.8, 18.8, 15.8, 15.5 and 12.2%, respectively, with respect to the total PAH content) (Table 2). By contrast, high molecular mass PAH compounds, such as DBA, BghiP and IPY were detected in trace amounts (0.03, 0.04 and 0.08%, respectively). Overall, and on the basis of their susceptibility to fungal degradation, the most representative PAHs in the HCS might be grouped into three categories: highly degraded (*i.e.*, FLU, PHE and ANT) with removal extents ranging from 73 to 99, moderately degradable (*i.e.*, FLT and PYR) with 16-35 percent removal range and, finally, scarcely or non-degradable (*i.e.*, BaA, CHR, BbF, BkF, BaP) (Table 5.1). CP-immobilized *P. ostreatus* was by far the most effective fungal inoculant in degrading 3-ring PAHs and the moderately degradable 4-ring PAH members, namely FLT and PYR. In addition, it was able to deplete even members of the third category with BaA, CHR, BbF, BkF and BaP being removed by 69.1, 29.7, 39.7, 32.8 and 85.2%, respectively.

FLU and PHE degradation by CWS-immobilized *D. squalens* was lower than with the remaining carriers (Table 5.1). The low susceptibility of BaP to fungal degradation was unexpected since this compound, due to its low ionization potential (Table 5.2), has been shown to be rather prone to oxidative attack by both fungi (Novotný *et al.*, 1999) and purified preparations of lignin-modifying enzymes (Johannes and Majcherczyk, 2000). One likely explanation for this result might be the aging of the HCS that led to a very low bioavailability of this compound amounting to 21.9% of its total content (Table 2). Among four-ring compounds, CHR was the least degraded thus confirming the results of other studies conducted with other fungi (Valentín *et al.*, 2006; Sack *et al.*, 2006; Leonardi *et al.*, 2008); this might be due to both its lower solubility and low bioavailability in this matrix (Table 5.2).

Although PAHs detected in CTS were identical to those found in HCS, their respective concentrations and relative abundances changed markedly (Table 5.1). The most abundant compounds, in fact, were ANT (40.9%), FLT (17.8%), PHE (14%) and PYR (11.1%). As opposed to the HCS soil, it was not possible to devise the same categorization of PAHs on the basis of their susceptibility to degradation; this was due to the wide removal ranges that were found for each contaminant in dependence on both the fungus and the inoculum carrier. In particular, ANT, FLT and PHE degradations widely ranged from 13 to 62%, from 15 to 40% and from 55 to 93%, respectively. GCC and CP were better supports than CWS for *D. squalens* leading to improved removal performances of ANT (51.9 and 53.9, respectively, *vs.* 17.1) and PHE (68.1 and 74.9%, respectively, *vs.* 61.2%); similar results were also observed for *P. ostreatus* (Table 5.1). The differences observed between the two matrices might be also ascribed to the different extents of the

bioavailable fractions of contaminants which regarded both low- and high-molecular mass PAHs (Table 5.2).

Table 5.2. Water solubility, ionization potential (IP) and percent abundances of the bioavailable fraction of each PAHs detected in the historically contaminated soil (HCS) and creosote-treated shavings (CTS) incubation controls.

Contaminant	Abbreviation	Water solubility [‡] (mg l ⁻¹)	IP [‡] (eV)	Percent abundance of the bioavailable fraction in:†	
				HCS	CTS
Fluorene	FLU	0.039	7.88	96.0±4.5	97.8±5.5
Phenanthrene	PHE	1.29	8.03	96.6±12.3	40.3±4.7
Anthracene	ANT	0.07	7.43	94.1±14.8	59.6±5.4
Fluoranthene	FLT	0.26	7.90	92.5±6.8	96.7±7.2
Pyrene	PYR	0.135	7.53	88.3±5.8	93.1±7.0
Benz[a]anthracene	BaA	0.009	7.56	37.7±9.1	90.0±9.0
Chrysene	CHR	0.002	7.59	41.1±12.6	73.4±5.8
Benzo[b]fluoranthene	BbF	0.0015	7.65	33.7±8.6	67.2±2.3
Benzo[k]fluoranthene	BkF	0.0008	7.48	21.7±14.1	69.0±6.4
Benzo[a]pyrene	BaP	0.0038	7.12	21.9±9.9	54.4±2.4
Dibenzo[a,h]anthracene	DBA	0.0010	7.38	n.r. [‡]	n.r. [‡]
Benzo[g,h,i]perylene	BghiP	0.0003	7.15	n.r. [‡]	n.r. [‡]
Indeno[1,2,3-cd]pyrene	IPY	0.0002	8.02*	n.r. [‡]	n.r. [‡]

† Estimated from the *F*-fraction of supercritical fluid extraction. Data are the mean ± standard deviation of three extractions.‡ From either Bogan and Lamar (1995) or Zheng and Obbard (2002). ‡ n.r., not reported: desorption data of these compounds upon SFE were not fitted by Equation (1); thus it was not possible to gain a reliable estimate of their bioavailability.

In this respect, for instance, the bioavailability of PHE and ANT in HCS was markedly higher than in CTS (96.6 and 94.1%, respectively, vs. 40.3 and 59.6%, respectively). Similarly, large differences were found in the bioavailability of high molecular mass PAHs between the two matrices; in this case, however, the relative abundances of compounds such as BaP, BkF and BghiP were markedly higher in CTS than in HCS (Table 5.1).

To provide an overview of the degradation performances in incubation controls and fungal treated matrices, residual PAH sums were calculated for each condition, regardless of the identity of contaminants. Figure 5.2 shows that non significant PAH reductions were observed in the

incubation controls of both HCS and CTS. By contrast, and regardless of both species and inoculum carrier, residual PAH sum amounted to less than 60% of that observed in the relative HCS incubation controls (Figure 5.2-A); as already stated, the most effective inoculant for the HCS was CP-immobilized *P. ostreatus*, the use of which gave rise to a 80% PAH removal with respect to the incubation control (460 vs. 2290 $\mu\text{g g}^{-1}$ dry soil, respectively) (Figure 5.2-B).

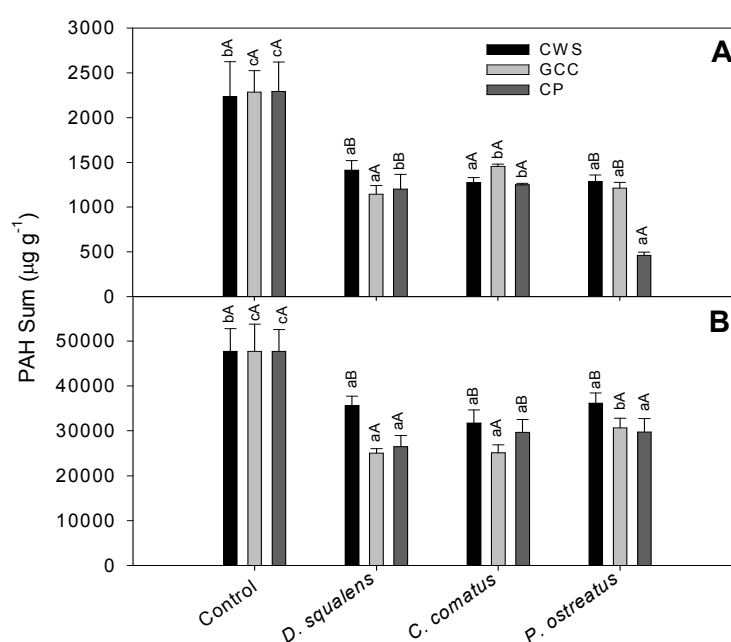


Figure 5.2. Residual PAH sums in historically-contaminated Soběslav soil and creosote-treated shavings incubation controls (A and B, respectively) and after incubation for 60 d at 28 °C with *D. squalens*, *C. comatus* and *P. ostreatus* previously supported on either chopped wheat straw (CWS), ground corn cobs (GCC) or commercial pellets (CP). Data are the mean $\pm$ standard deviation of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P < 0.05$). Same lowercase letters above bars indicate that differences between fungi within the same inoculum carrier were not significant. Same uppercase letters indicate lack of statistically significant difference within each fungus at different inoculation supports.

Two-way ANOVA conducted on the residual PAH sum in HCS showed that both main effects and interaction of variables X_1 and X_2 were highly significant ($P < 0.001$). In agreement with findings obtained for the HCS, CP was more adequate than CWS for *P. ostreatus* to support PAH degradation in CTS; this result was also observed with *D. squalens* (Figure 5.2-B). Besides the already mentioned stimulating effects on growth, an additional reason for the dependence of fungal degradation on the type of inoculum carrier might derive from their ability to slowly release phenolic compounds able to act as mediators in PAH degradation (Cañas *et al.*, 2007) and to stimulate the fungal ligninolytic system (Crestini *et al.*, 1996).

Regardless of the matrix, a good correlation between mycelial biomass and PAH depletion was only found for *P. ostreatus* (Figures 5.3-C and 5.3-F); in *C. comatus* microcosms, instead, the same parameters were significantly correlated ($R^2_{adj} = 0.82$; $P < 0.01$) only on CTS (Figure 5.3-E).

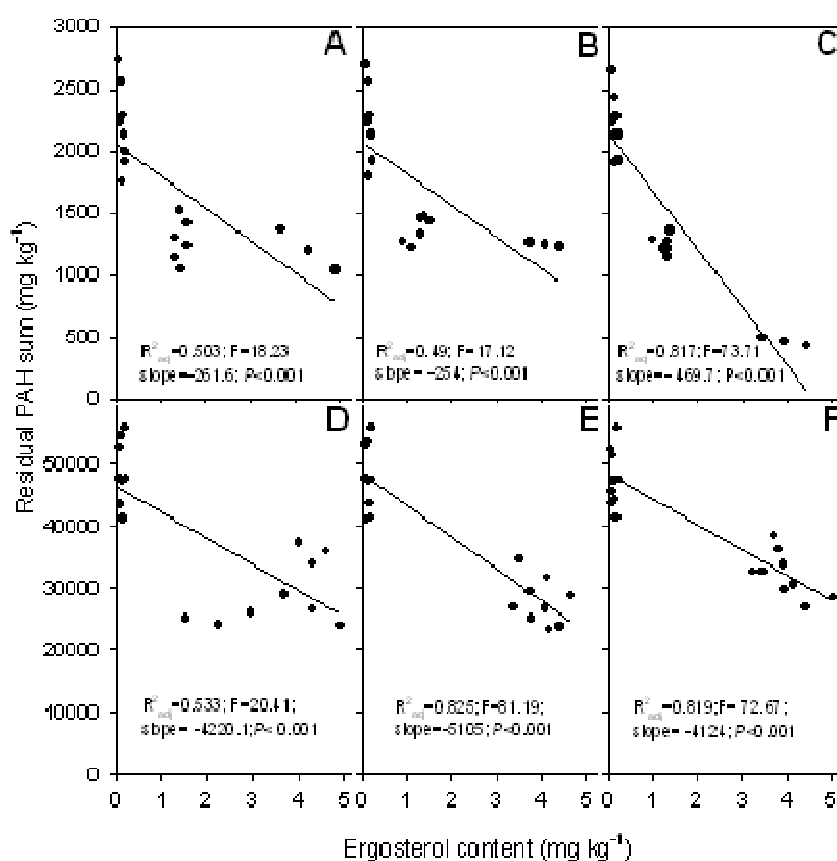


Figure 5.3. Linear regression between amounts of residual PAH and ergosterol contents in incubation controls and fungal microcosms of both historically contaminated soil and creosote-contaminated shavings with *D. squalens* (A and D, respectively), *C. comatus* (B and E, respectively), *P. ostreatus* (C and F, respectively). Each plot reports the following parameters of regression analysis: squared correlation coefficient adjusted by the degrees of freedom (R^2_{adj}), F value and alpha level (P) of the model and slope of the regression line.

With the exception of few studies (Martens and Zadrazil, 1998; Andersson *et al.*, 2003), the relationship between fungal growth and PAH-degradation ability in mycoaugmentation has not been either explored or shown to be directly absent. For instance, in a soil spiked with radio-labeled PAH that had been placed in contact with wheat straw pre-colonized by different white-rot species, the amount of $^{14}\text{CO}_2$ evolution was higher with fungi unable to efficiently colonize the soil (Martens and Zadrazil, 1998); this unexpected result was explained by assuming that water from the colonized straw transported hydrolysis products and enzymes that, in turn, boosted the degradation activity of the indigenous microbial community in the underlying soil (Martens and Zadrazil, 1998).

A high correlation between mycelial biomass and PAH degradation does not necessarily imply that the clean-up process be solely due to the fungus. This fact could be supported by the absence of accumulation of dead-end degradation products of PAHs in *P. ostreatus* microcosms (corresponding quinones were not detected – data not shown), where this correlation had been observed (present study). The use of this species, in fact, in several augmentation studies led to the accumulation of dead-end products, such as 9,10-anthracenedione and benzo[*a*]-anthracene-7,12-dione (Andersson and Henrysson, 1996; Andersson *et al.*, 2003); the absence of these intermediates suggests their ready conversion by the resident community (present study). In this respect, PAH degradation in soil and in lignocellulose-based matrices, such as litter, is thought to be a synergic process where fungi initially convert parent compounds into more polar metabolites with increased bioavailability to bacteria (Meulenberg *et al.*, 1997; Kotterman *et al.*, 1998).

To provide further insights into the impact of PAHs bioavailability on fungal degradation, degradation fold with respect to the bioavailable fraction (DFBF) was calculated for each contaminant in both HCS and CTS (Figure 5.4) with the exceptions of DBA, BghiP and IPY. Desorption data of these compounds, in fact, did not fit the model described by the Equation (1) (Williamson *et al.*, 1998) and, therefore, it was not possible to calculate F fraction representing the bioavailable portions.

Some *C. comatus* and *P. ostreatus*-based inoculants were able to degrade BbF and BkF beyond their respective bioavailable fractions (Figures. 5.4-B and 5.4-C, respectively); the highest DFBF value (about 4.0) was found for BaP with CP-immobilized *P. ostreatus*. On the other hand, on CTS only PHE degradation slightly exceeded its respective bioavailability with DFBF values ranging from 1.42 to 1.86 (Figure 5.4-D, 5.4-E and 5.4-F).

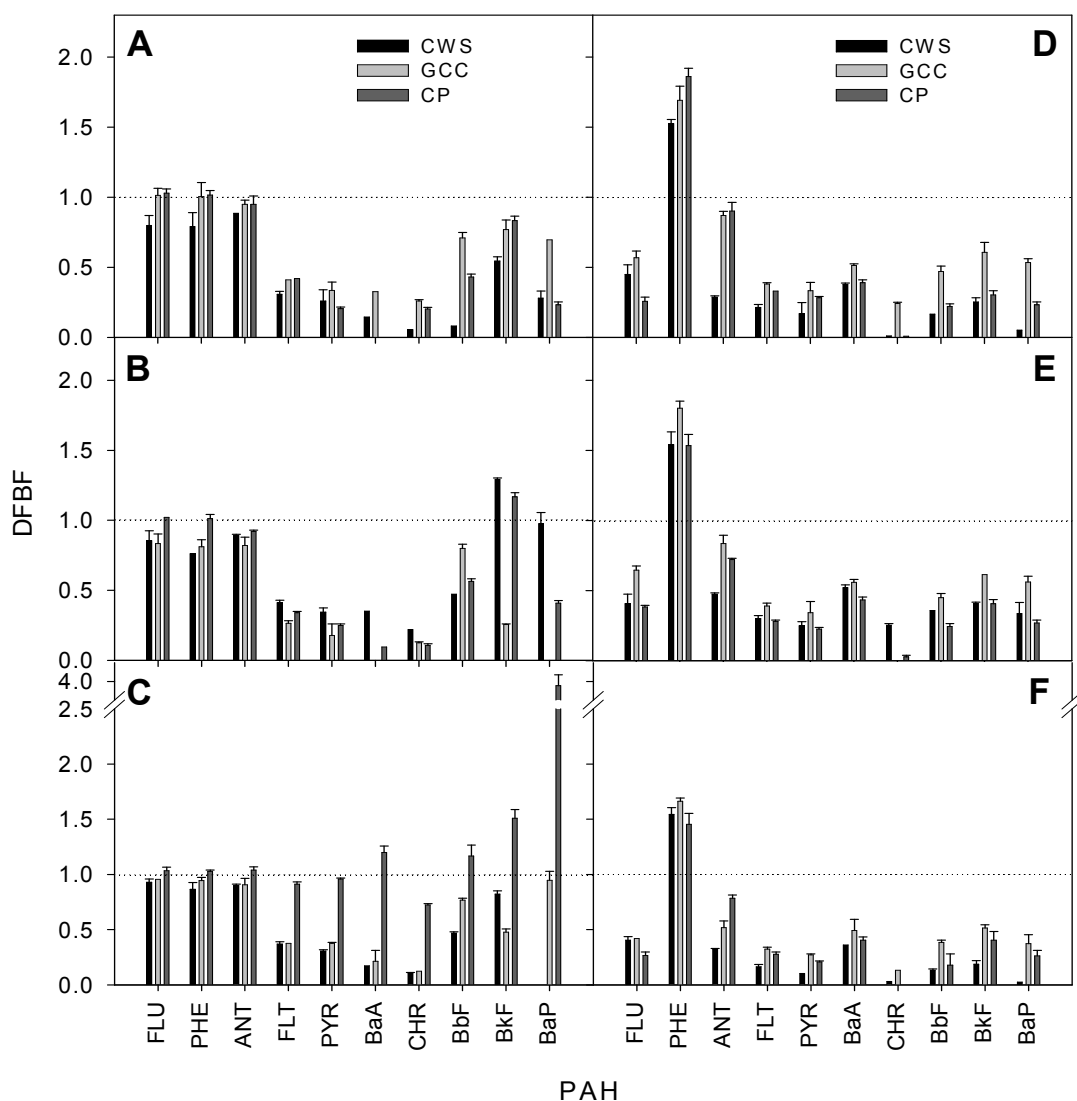


Figure 5.4. Degradation fold with respect to the bioavailable fraction (DFBF) of fluorene (FLU), phenanthrene (PHE), anthracene (ANT), Fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF) and benzo[a]pyrene (BaP) in the historically contaminated Soběslav soil and creosote-treated shavings after incubation with for 60 d at 28 °C with *D. squalens* (A and D, respectively), *C. comatus* (B and E, respectively) and *P. ostreatus* (C and F, respectively) previously supported on either chopped wheat straw (CWS), ground corn cobs (GCC) or commercial pellets (CP). Data are the mean $\pm$ standard deviation of three replicates.

In this respect, fungi have been suggested to be less dependent on bioavailability than bacteria; the intracellular location of the PAH degradation machinery in the latter ones, in fact, makes them largely dependent on the mass transfer rate of these contaminants from the solid to the liquid phase and cell uptake becomes a rate-limiting step in degradation. In addition to an intra-cellular degradation system, mainly based on the cytochrome P450-epoxide hydrolase complex, basidiomycetes are able to act *via* non-specific radical-based degradation mechanisms occurring in the extracellular environment (Chung *et al.*, 2000). Moreover, the ability of the basidiomycetes *P. ostreatus* to produce an emulsifying agent able to pseudo-solubilise PAHs was recently reported (Nikiforova *et al.*, 2009). Few investigations have dealt with the impact of the actual PAH bioavailability in the matrices under study on the degradation performances of basidiomycetes (Leonardi *et al.*, 2007); the present study shows the ability of the investigated strains to degrade certain PAHs beyond their respective bioavailable amount in dependence on the solid contaminated matrix.

5.1.3.3. Phytotoxicity removal

Germinability of barley seeds was inhibited in the presence of CEs from both HCS and CTS incubation controls (Fig. 5.5-A and 5.5-B). In the former matrix, and with the exception of GCC-immobilized *C. comatus* and CWS-immobilized *D. squalens*, phytotoxicity was significantly reduced; best results were observed with CP-immobilized *P. ostreatus* were I% accounted to about 10%. (Fig. 5.5-A).

Although I% was rather high in the incubation controls of CTS (62-65%), a generalized and significant decrease of phytotoxicity was observed in the matrix that had been incubated with fungi; best results, however, were generally obtained with GCC- and CP-immobilized fungi (Figure 5.5-B). Regardless of the matrix, the regression analysis showed that germinability was strongly correlated with the absolute mass of added PAH ($P < 0.001$) and indicated a linear dose-response relationship (see insets of Figures 5.5-A and 5.5-B). The IC_{50} values of CEs from HCS and CTS, calculated from the regression equations, however, were found to differ (10250 vs. 7205 μg , respectively) (insets of Figures 5.5-A and 5.5-B, respectively).

Two-way ANOVA, applied to the arcsin of the square-root-transformed I% data, showed that the main effects of variables X_1 and X_2 (*i.e.*, inoculation carrier and fungal species, respectively) were highly significant for both matrices ($P < 0.001$) for both the HCS and CTS; their interaction, however, was significant only in the former one.

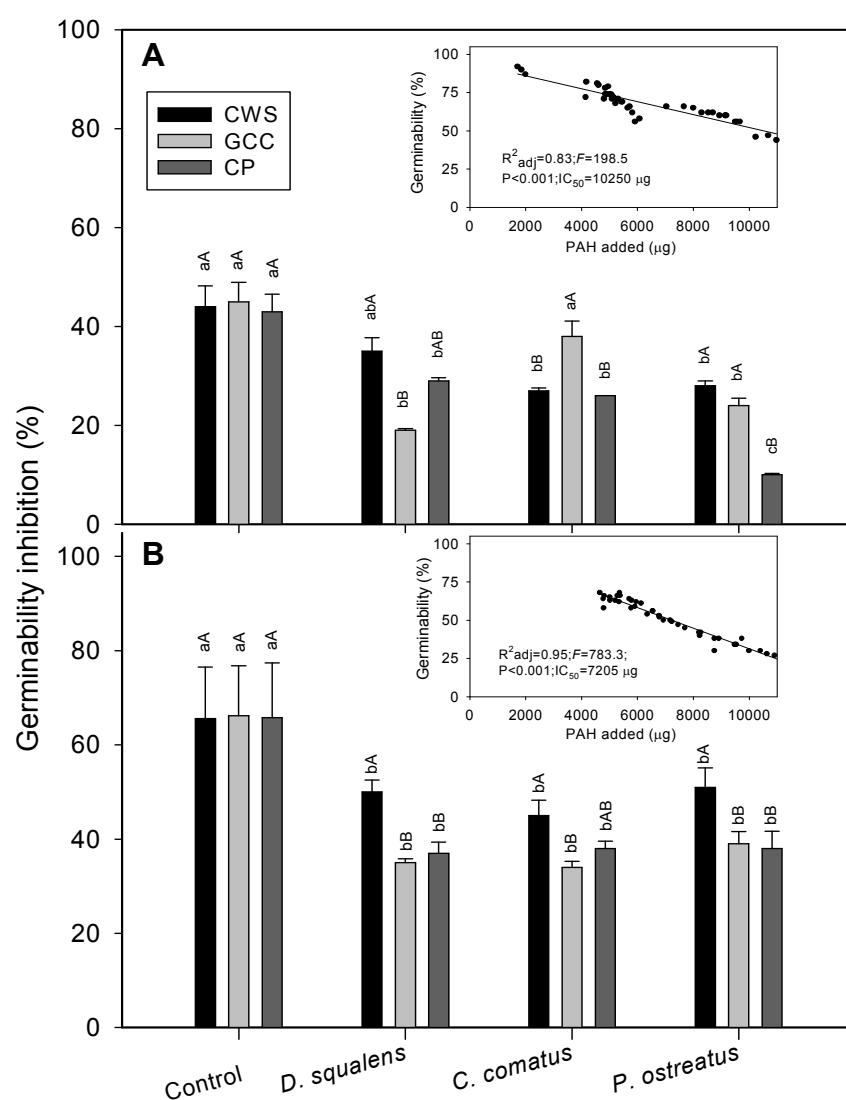


Figure 5.5. Germinability inhibition of barley seeds after incubation for 72 h at room temperature with contaminants extract (see subsection 2.4) derived from the historically contaminated Soběslav soil and creosote-treated shavings incubation controls (A and B, respectively) and the same matrices incubated for 60 d at 28 °C with *D. squalens*, *C. comatus* and *P. ostreatus* previously supported on either chopped wheat straw (CWS), ground corn cobs (GCC) or commercial pellets (CP). Data are the mean $\pm$ standard deviation of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P < 0.05$). Same lowercase letters above bars indicate that differences between fungi within the same inoculum carrier were not significant. Same uppercase letters indicate lack of statistically significant difference within each fungus at different inoculation supports. The insets show the regression lines of percent germinability vs. amounts of PAHs added and respective squared correlation coefficient adjusted by the degrees of freedom (R^2_{adj}), F value, alpha level (P) of the model and IC_{50} values calculated from the regression equation.

5.1.3.4. Conclusions

This study shows the importance of the inoculum carrier on both growth and degradation performances of fungal microcosms and provides firm evidence on the ability of the strains under study to degrade certain PAHs even partially beyond their respective bioavailable amounts. The best PAH degrader appeared to be the strain *P. ostreatus* CCBAS 278 cultivated on commercial pellets that was able to remove 80% of total PAH sum from the historically contaminated soil. The lack of correlation between ergosterol levels and PAH depletion observed in the majority of mycoaugmented microcosms indicates that the degradation process arises from the cooperative action between the inoculated fungus and the indigenous microbial community; the notable exception to this general trend of *P. ostreatus* microcosms might indicate the need of adequate colonisation to provide a first oxidative attack of PAHs leading to quinone derivatives. The non-accumulation of these degradation intermediates that had been reported to be dead-end products for this species provides an additional, albeit indirect, proof of a cooperation of this fungus with the resident microbiota.

Fungal microcosms partially removed phytotoxicity, which, in turn, was significantly correlated with residual PAH contents of both matrices, thus confirming that no toxic dead-end degradation products had been accumulated.

5.1.4. Impact of inoculum carrier and contaminants bioavailability on PAH degradation performances of *Panus tigrinus* and *Irpex lacteus* on contaminated solid matrices from a wood preservation plant. Results and discussions.

5.1.4.1. Fungal growth

Table 5.3 summarizes growth responses of *I. lacteus* and *P. tigrinus* after 60 d incubation on the MBU Sobeslav soils and on CTS. Both CP-immobilized best grew on the MBU soil (12.2 and 13.7 $\mu\text{g g}^{-1}$, respectively). The type of the inoculum carrier did not significantly affect *P. tigrinus* growth on HCS where *I. lacteus*, instead, exhibited best growth (4.01 $\mu\text{g g}^{-1}$) when immobilized on CP.

The situation was reversed when determining fungal growth on CTS where the type of carrier did not affect *I. lacteus* growth and CP significantly stimulated that of *P. tigrinus* with respect to WS and CC (9.32 vs. 4.96 and 5.3, respectively).

Table 5.3. Ergosterol concentrations in non-contaminated MBU soil, historically-contaminated Soběslav soil and creosote-treated shavings incubated for 60 d at 28 °C with *I. lacteus* 617/93 and *P. tigrinus* CBS 577.79 previously supported on either wheat straw (WS), corn cobs (CC) or commercial pellets (CP).

Experimental condition	Ergosterol content ($\mu\text{g g}^{-1}$)*		
	MBU	Soběslav	Shavings
<i>I. lacteus</i>			
- WS	1.92±0.25 ^{aA}	1.46±0.06 ^{aA}	4.25±0.18 ^{aB}
- CC	3.25±1.15 ^{aA}	1.41±0.10 ^{aA}	3.15±0.20 ^{aA}
- CP	12.20±4.10 ^{aB}	4.01±0.90 ^{bB}	4.01±0.90 ^{aB}
<i>P. tigrinus</i>			
- WS	1.15±0.29 ^{aA}	1.88±0.30 ^{aA}	4.96±0.07 ^{bA}
- CC	5.81±0.66 ^{bB}	2.11±0.44 ^{bA}	5.30±0.09 ^{bA}
- CP	13.71±2.26 ^{aC}	1.91±0.11 ^{aA}	9.32±0.99 ^{bB}

*Data are the mean $\pm$ standard deviation of three independent experiments. Statistical multiple pair-wise comparison was carried out within column means by the Tukey test. Same superscript lowercase letters indicate that differences between fungi within the same inoculum carrier were not significant. Same superscript uppercase letters indicate lack of statistically significant difference within each fungus at different inoculation supports.

Regardless of the carrier, *P. tigrinus* invariably exhibited better growth than *I. lacteus* on CTS (Table 1). Both fungi grew better on CTS than on the Sobeslav soil, despite the former had an approx. 20-fold higher PAH concentration than the latter; with this regard, the high content of potential growth substrates (*i.e.*, cellulose and hemicelluloses) in oak wood which is the predominant component of CTS, might explain this effect (Vane *et al.*, 2006).

5.1.4.2. Fungal PAH degradation

The PAH degradation ability of *P. tigrinus* and *I. lacteus* was comparatively examined on both a historically-contaminated soil taken from the Sobeslav wood treatment plant and on CTS that had been produced there. For both matrices, the PAH residual contents of non-inoculated incubation controls carried out in the presence of the three carriers did not significantly differ each

one another ($P < 0.05$). Consequently, Tables 5.4 and 5.5 show for each matrix a single incubation control, which, irrespective of the added inoculation carrier, report the averaged PAH contents of all incubation controls.

Table 5.4 shows that the most abundant contaminants in the Soběslav soil were FLT, PYR, PHE, CHR and FLU the relative contents of which amounted to 28.8, 18.8, 15.8, 15.5 and 12.2%, respectively, with respect to the total PAH content. On the one hand, and regardless of both the fungus and the inoculation carrier, the most degraded contaminants were FLU, PHE and ANT the removal extents of which ranged from 75 to 91% with respect to the incubation control. On the other hand, CHR was the least degraded compound with percent removals ranging from 1 to 9% . thus confirming the results of other studies conducted with different fungi (Valentín *et al.*, 2006; Sack *et al.*, 2006; Leonardi *et al.*, 2008); this might be due to both the lower water-solubility of CHR than other 4-ring PAHs and to its low amount of the relative bioavailable fraction (41.1%) in this matrix. The degradation activity of *P. tigrinus* towards FLT, PYR and BaP was significantly ($P \leq 0.05$) higher than that of *I. lacteus* with the largest differences observed for the last compound. Although degradation performances of *P. tigrinus* were not significantly affected by the type of the support, they were invariably better than those of *I. lacteus*. The worst inoculant was WS-immobilized *I. lacteus*, the use of which led to the highest residual PAH sum (1408 mg kg⁻¹) corresponding to an overall removal of 38.4%.

Although the same PAHs were detected on CTS incubation controls, an approx 21-fold higher concentration than that of the HCS was found in incubation controls of this matrix (Table 5.5). Moreover, the relative abundances of single contaminants greatly differed from that found in the HCS. In particular, the most abundant PAHs on CTS were ANT (41%), FLT (18%), PHE (14%) and PYR (11%).

Although PHE, FLU and ANT exhibited the highest susceptibility to fungal degradation, *P. tigrinus* proved to be much more efficient than *I. lacteus* in the degradation of these compounds. Only exception was CP-immobilized *I. lacteus* the PHE degradation capacity of which did not differ from that of *P. tigrinus*-based inoculants. Regardless of the type of inoculation support, the largest differences between the two fungi were observed for the degradation of FLU, and, to a lesser extent, for PHE and ANT. As observed for the Sobeslav soil, CHR was poorly degraded. No differences in BaP degradation were observed between the inoculants under study. On an overall basis, best degradation results were observed with CP-immobilized *P. tigrinus* leading to a 68% reduction in the PAH sum with respect to the incubation control (15494 vs. 47731, respectively).

Table 5.4. PAH residual concentrations observed in averaged incubation control and after incubation for 60 d at 28 °C with *Irpex lacteus* 617/93 and *P. tigrinus* CBS 577.79 on historically-contaminated Soběslav soil using either wheat straw (WS), corn cobs (CC) and commercial pellets (CP) as the inoculums carrier.

PAH	Control	Residual concentrations ($\mu\text{g g}^{-1}$ soil)*					
		<i>I. lacteus</i>			<i>P. tigrinus</i>		
		WS	CC	CP	WS	CC	CP
FLU	278.0±18.1	48.3±6.7 ^b	41.3±0.4 ^b	31.9±1.3 ^a	33.2±0.2 ^a	30.0±2.2 ^a	29.1±3.3 ^a
PHE	353.9±36.1	87.1±7.7 ^b	48.1±1.9 ^a	56.5±5.1 ^a	69.0±1.6 ^{ab}	67.2±9.5 ^{ab}	48.9±13.2 ^a
ANT	104.6±11.2	20.6±2.2 ^b	19.0±1.2 ^b	16.5±0.6 ^b	9.7±1.9 ^a	12.9±1.3 ^{ab}	10.4±1.5 ^a
FLT	659.0±51.8	466.8±1.5 ^b	426.5±32.8 ^b	496.9±49.0 ^b	373.5±12.4 ^a	368.8±45.3 ^a	388.6±36.5 ^{ab}
PYR	429.0±42.2	345.1±31.3 ^b	309.2±56.9 ^b	283.9±20.4 ^b	252.5±4.4 ^a	254.4±29.4 ^a	267.3±2.5 ^a
BaA	77.0±6.9	75.9±9.7 ^b	66.7±12.0 ^{ab}	60.4±0.6 ^{ab}	55.9±0.7 ^a	56.8±6.7 ^a	59.0±0.2 ^{ab}
CHR	353.9±18.7	337.7±14.2 ^a	314.4±10.7 ^a	349.9±3.3 ^a	322.0±11.6 ^a	319.5±31.4 ^a	322.3±9.2 ^a
BbF	12.7±1.2	11.8±1.7 ^b	9.7±1.6 ^{ab}	10.9±0.1 ^{ab}	8.6±0.1 ^a	8.6±0.9 ^a	9.0±0.2 ^{ab}
BkF	6.4±0.7	6.1±0.8 ^a	4.8±0.8 ^a	5.0±0.3 ^a	4.3±0.1 ^a	4.4±0.5 ^a	4.5±0.1 ^a
BaP	6.1±0.5	5.8±1.0 ^b	5.4±0.8 ^b	5.1±0.5 ^b	2.8±0.2 ^a	3.0±0.3 ^a	3.1±0.3 ^a
DBA	0.6±0.1	0.16±0.0 ^c	0.51±0.0 ^b	0.58±0.1 ^b	0.40±0.1 ^a	0.31±0.1 ^a	0.32±0.1 ^a
BghiP	1.0±0.2	1.0±0.3 ^a	0.6±0.1 ^a	0.8±0.1 ^a	0.8±0.2 ^a	0.8±0.1 ^a	0.8±0.2 ^a
IPY	1.9±0.3	1.6±0.2 ^b	0.1±0.0 ^a	0.1±0.0 ^a	1.1±0.2 ^b	0.6±0.1 ^{ab}	0.4±0.1 ^a
PAH Sum	2284.1±188.1	1408.2±77.3 ^{ab}	1245.8±119.1 ^a	1318.1±98.2 ^a	1133.8±33.7 ^a	1127.1±128.7 ^a	1143.7±67.5 ^a

*Data are the mean ± standard deviation of three independent experiments. Statistical multiple pair-wise comparison was carried out within row means pertaining to each strain by the Tukey test: means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

Table 5.5. PAH residual concentrations observed in averaged incubation control and after incubation for 60 d at 28 °C with *Irpex lacteus* 617/93 and *P. tigrinus* CBS 577.79 on creosote-treated shavings using either wheat straw (WS), corn cobs (CC) and commercial pellets (CP) as the inoculums carrier.

PAH	Control	Residual concentrations ($\mu\text{g g}^{-1}$ soil)*					
		<i>I. lacteus</i>			<i>P. tigrinus</i>		
		WS	CC	CP	WS	CC	CP
FLU	2647±163	1562±365 ^a	1193±92 ^a	1228±151 ^a	36±9 ^a	26±1 ^a	31±0 ^a
PHE	6698±400	3021±406 ^b	2388±185 ^b	424±121 ^a	1218±215 ^a	916±161 ^a	847±100 ^a
ANT	19502±131	16869±903 ^b	7445±1756 ^a	8388±1792 ^a	3855±545 ^a	4042±184 ^a	4663±610 ^a
FLT	8510±529	6525±331 ^a	5090±460 ^a	5159±1384 ^a	4533±193 ^{ab}	5125±662 ^b	3268±747 ^a
PYR	5308±372	4439±189 ^a	3495±284 ^a	3823±646 ^a	4544±265 ^b	3346±249 ^a	3374±174 ^a
BaA	1923±119	895±623 ^a	970±60 ^a	1060±142 ^a	1106±15 ^a	1212±132 ^a	953±159 ^a
CHR	1324±105	1327±225 ^a	1022±73 ^a	1222±189 ^a	1046±35 ^{ab}	1078±70 ^b	930±54 ^a
BbF	651±30	551±14 ^b	435±32 ^a	494±22 ^{ab}	623±21 ^a	587±47 ^a	560±11 ^a
BkF	385±67	288±12 ^b	215±14 ^a	276±23 ^b	346±9 ^b	319±15 ^{ab}	292±11 ^a
BaP	481±111	438±47 ^a	335±44 ^a	414±74 ^a	451±41 ^a	367±30 ^a	363±47 ^a
DBA	55±4	23±1 ^a	17±1 ^a	21±4 ^a	48±3 ^b	20±6 ^a	19±5 ^a
BghiP	88±19	83±10 ^b	52±5 ^a	76±9 ^b	84±3 ^a	75±2 ^a	75±7 ^a
IPY	156±19	124±6 ^b	102±6 ^a	125±10 ^b	141±13 ^a	131±6 ^a	119±9 ^a
PAH Sum	47731±2075	36144±3135 ^b	22761±3015 ^a	22859±4574 ^a	18034±1373 ^a	17246±1568 ^a	15497±1938 ^a

*Data are the mean ± standard deviation of three independent experiments. Statistical multiple pair-wise comparison was carried out within line means pertaining to each strain by the Tukey test: means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

To provide further insights into the impact of PAHs bioavailability on fungal degradation, degradation fold with respect to the bioavailable fraction (DFBF) was calculated for each contaminant in both HCS and CTS (Figures 5.6 and 5.7) with the exceptions of DBA, BghiP and IPY. Desorption data of these compounds, in fact, did not fit the model described by the Equation (1) (Williamson *et al.*, 1998) and, therefore, it was not possible to calculate F fraction representing the bioavailable portions.

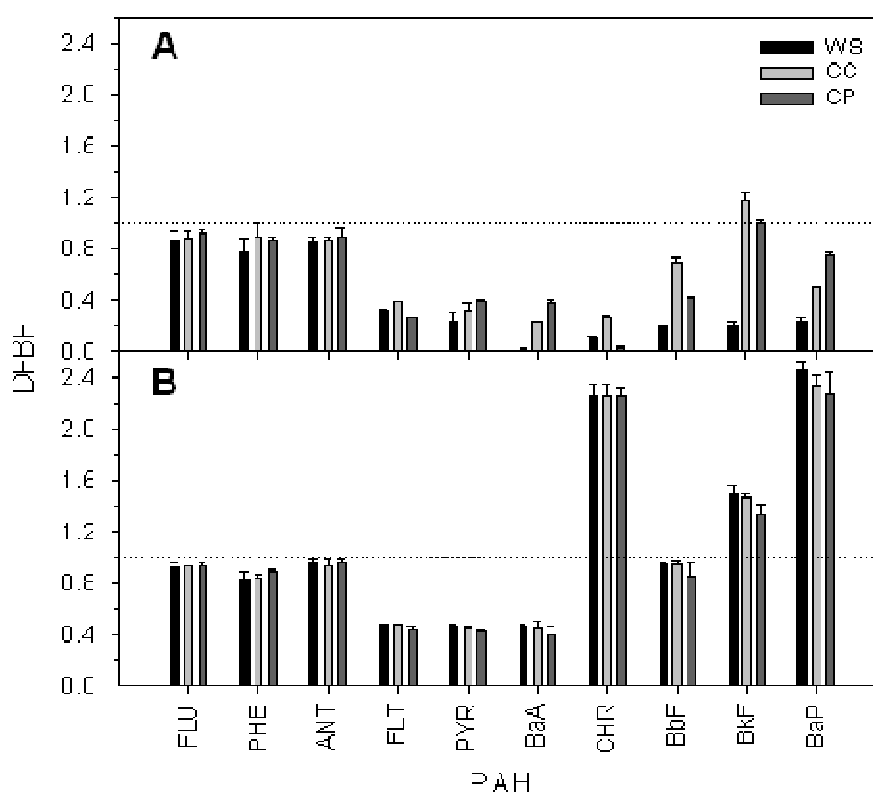


Figure 5.6. Degradation fold with respect to the bioavailable fraction (DFBF) of FLU, PHE, ANT, FLT, PYR, BaA, CHR, BbF, BkF and BaP in historically contaminated soil (HCS) after incubation for 60 d at 28 °C with *I. lacteus* (A) and *P. tigrinus* (B) previously supported on either chopped wheat straw (CWS), ground corn cobs (GCC) or commercial pellets (CP). Data are the mean $\pm$ standard deviation of three replicates.

Regardless of the inoculum carrier, FLU, PHE and ANT in Sobeslav soil were degraded to the limit of their respective bioavailable portion by both *P. tigrinus* and *I. lacteus* (Figure 5.6). By contrast, *P. tigrinus*-based inoculants degraded CHR, BkF and BaP far beyond their respective bioavailable fraction; in particular, regardless of the growth support used DFBF values for the former and the latter compound were the highest (approx. 2.25 and 2.35, respectively). Conversely,

I. lacteus was able to degrade only BkF beyond its respective bioavailable portion only when pre-grown on CC and CP (DFBF values of 1.18 and 1.05, respectively).

On an overall basis, on CTS (Figure 5.7) the highest DFBF values were obtained for PHE with both fungi.

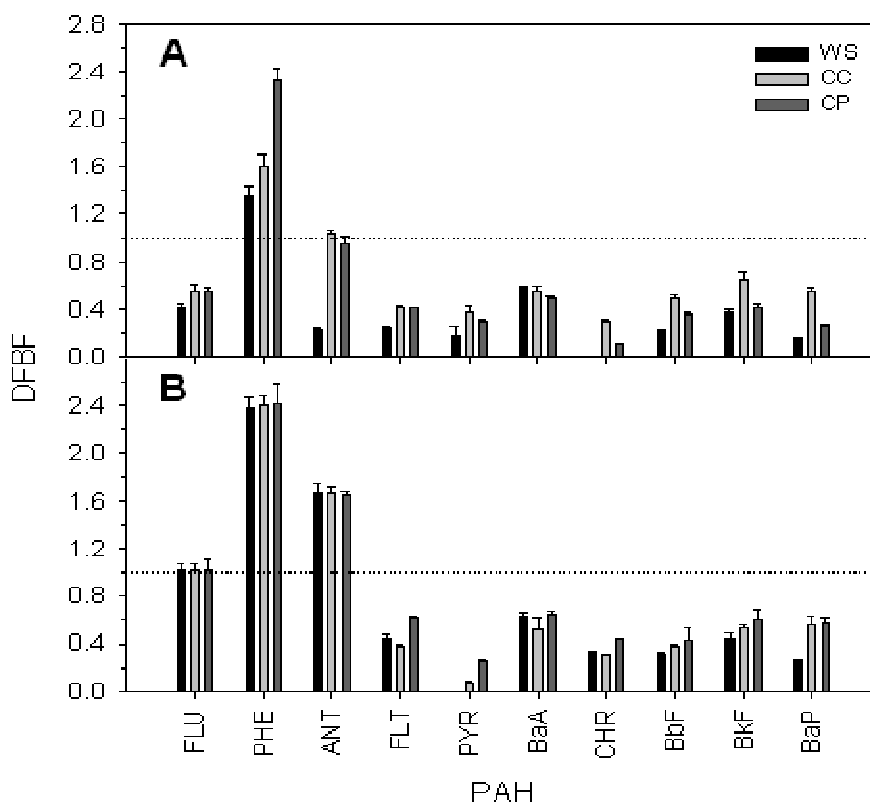


Figure 5.7. Degradation fold with respect to the bioavailable fraction (DFBF) of FLU, PHE, ANT, FLT, PYR, BaA, CHR, BbF, BkF and BaP in creosote-treated shavings (CTS) after incubation for 60 d at 28 °C with *I. lacteus* (A) and *P. tigrinus* (B) previously supported on either chopped wheat straw (CWS), ground corn cobs (GCC) or commercial pellets (CP). Data are the mean $\pm$ standard deviation of three replicates.

In this respect, all *P. tigrinus* inoculants degraded PHE far beyond its bioavailable portion (DFBF values of 2.38, 2.39, 2.4 for WS-, CC-, and CP-immobilized fungus), while PHE degradation by *I. lacteus* was significantly enhanced by CP as growth support (DFBF values of 2.36). As for the other PAHs investigated, ANT degradation by *P. tigrinus* slightly exceeded its respective bioavailable portion, thus yielding DFBF values of 1.35, 1.33 and 1.27 when pre-grown on WS, CC and CP, respectively (Figure 5.7).

5.1.4.3. Phytotoxicity removal

Germinability of barley seeds was inhibited in the presence of CEs from both HCS and CTS incubation controls (Table 5.6).

Table 5.6. Germinability inhibition of barley seeds after incubation for 72 h at room temperature with contaminants extract (see subsection 2.4) derived from the historically contaminated Soběslav soil and creosote-treated shavings (CTS) incubated for 60 d at 28 °C with *P. tigrinus* and *I. lacteus* previously supported on either chopped wheat straw (WS), ground corn cobs (CC) or commercial pellets (CP) and from their relative incubation controls.

Sample	Germinability inhibition† in:	
	Soběslav	CTS
<i>Incubation controls</i>		
WS	44.2±4.2 ^b	65.6±10.9 ^b
CC	45.0±3.9 ^b	66.2±10.6 ^b
CP	43.0±3.5 ^b	65.8±11.5 ^b
<i>P. tigrinus</i>		
WS	20.7±4.6 ^a	29.3±3.1 ^a
CC	23.3±2.3 ^a	32.0±4.2 ^a
CP	20.7±4.2 ^a	20.0±6.0 ^a
<i>I. lacteus</i>		
WS	34.0±5.6 ^b	51.0±5.1 ^b
CC	24.0±2.8 ^a	29.0±4.2 ^a
CP	30.0±2.8 ^{ab}	30.0±2.8 ^a

†Data are the mean ± standard deviation of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P < 0.05$). Same uppercase letters indicate lack of statistically significant difference within column means.

As for the Sobeslav soil, phytotoxicity was significantly removed by all *P. tigrinus* inoculants, the I% values of which were 20.7, 23.3 and 20.7% for WS-, CC- and CP-immobilized fungus, respectively. On the contrary, best phytotoxicity reduction by *I. lacteus*-based inoculants was obtained with the CC-immobilized fungus (I = 24.0%).

Although I% was rather high in the incubation controls of CTS (62-65%), a generalized and significant decrease of phytotoxicity was observed in the matrix that had been incubated with *P. tigrinus* and *I. lacteus*. Sole exception was the latter fungus immobilized on WS (I = 51.0%).

5.1.4.4. Conclusions

The present study shows the ability of *P. tigrinus* to colonize and detoxify solid PAH-contaminated matrices derived from a wood treatment facility under non-sterile conditions. Thus, the positive degradation results observed with both axenic stationary and shaken cultures of the strain were confirmed by the present investigation. In particular, *P. tigrinus* CBS 577.79 growth was invariably higher than that of *I. lacteus* CCBAS 238, a reference strain that had been particularly efficient in mycoremediation; it also showed higher ability than *I. lacteus* to degrade FLU, ANT and PHE which were abundant components in both matrices. Accordingly, the extent of detoxification achieved with the former was significantly higher than with the latter. In addition, the degradation ability of *P. tigrinus* towards certain contaminants (*i.e.*, CHR, BkF and BaP on HCS and PHE and ANT on CTS) largely exceeded their respective bioavailable fractions thus featuring a lower dependence than *I. lacteus* on bioavailability.

5.2. Effect of mobilizing agents on mycoremediation of an artificially contaminated soil and impact on the indigenous microflora

5.2.1. Introduction

The concomitantly low water solubility and bioavailability of PAHs make them rather recalcitrant to microbial attack (Tiehm *et al.*, 1994). Consequently, the biological degradation of PAHs has been shown to be promoted by the use of certain additives able to mobilize them from the soil organic phase to the aqueous one (Bramwell and Laha, 2000).

For the above reasons, the combined use of surfactants (*i.e.*, mobilizing agents, MA) and WRF in soil remediation has received some attention leading to largely variable results in terms of decontamination efficiency (Chung *et al.*, 2000; Marquez-Rocha *et al.* 2000; Zheng and Obbard, 2001), impact on the indigenous microflora (Zheng and Obbard, 2002; Leonardi *et al.*, 2008) and release of PAH-degrading enzymes (Novotny *et al.*, 1999; Zheng and Obbard, 2002). Most of studies on this specific topic point out on the importance of finding appropriate WRF/MA combinations (Bogan and Lamar, 1999; Marquez-Rocha *et al.*, 2000; Leonardi *et al.*, 2007). Another recurring point related to the use of MAs in soil remediation concerns their concentrations which have to be generally well above their critical micelle concentrations due to known reasons that have been extensively reviewed by Haigh (1996). This emphasizes the utmost importance of

using cost-effective MAs, such as plant oils, as a valuable alternative to synthetic ones (Bogan and Lamar, 1999; Bogan *et al.*, 2003; Pizzul *et al.*, 2007). Moreover, there is an ever increasing interest in the use of fungi isolated from historically contaminated sites in bioaugmentation due to their presumed forced adaptation to either efficiently degrade or tolerate high concentrations of contaminants (van der Gast *et al.*, 2003; Atagana *et al.*, 2004; Potin *et al.*, 2004; D'Annibale *et al.*, 2006).

Consequently, the general objective of this study was to comparatively assess the impact of soybean oil and two commonly used MAs (*i.e.* Tween 20 and 80) on the fungal remediation of a simplified model system where a sandy-loamy soil was artificially spiked with a mixture of PAHs. To this aim, mycoaugmentation made use of two strains, namely the WRF *Phlebia* sp. DABAC 9 and *Allescheriella* sp. DABAC1, the anamorph of *Botryobasidium* sp. (Benade *et al.*, 1998), that had been previously isolated from an historically contaminated site and were found to efficiently degrade aromatic hydrocarbons (D'Annibale *et al.*, 2006). Soil moisture conditions lower than the water-holding capacity (WHC) were selected in order to investigate the effect of MAs on (i) fungal growth (ii) activity levels of enzymes putatively involved in both PAH degradation and hydrolysis of the inoculum carrier (*i.e.* maize stalks), (iii) contaminant degradation and detoxification, (iv) microbial density of cultivable heterotrophic bacteria and (v) diversity of the indigenous bacterial population.

5.2.2. Materials and Methods

5.2.2.1. Materials

The soil employed in this study was collected from Pian Ciliano (VT), air-dried for 7 days at room temperature, then sieved (2 mm) and stored at 4 °C until used. Its main properties were as follows: sandy-loamy texture (sand 65%, silt 23%, clay 13%), water-holding capacity 25.5%, real and potential acidity (pH 6.4 and 5.1, respectively), organic carbon 1.5%, total nitrogen 0.15%, available phosphorous (as P₂O₅) 16 mg kg⁻¹. PAHs standards (phenanthrene, PHE; Anthracene, ANT; fluoranthene, FLT; pyrene, PYR; chrysene, CHRY; benzo[*k*]fluoranthene, BkFLT; benzo[*a*]pyrene, BaPYR), the purity degree of which was ≥ 98%, were purchased from either Fluka (Milan, Italy) or Aldrich (Milan, Italy). Soybean oil, Tween 20 (polyoxyethylenesorbitan-monolaurate) and Tween 80 (polyoxyethylenesorbitan-monooleate) were purchased from Fluka.

5.2.2.2. *Microorganisms*

The strains *Allescheriella* sp. DABAC 1 and *Phlebia* sp. DABAC 9 used in the present study were previously isolated from a historically contaminated site (D'Annibale *et al.*, 2006); during the research work, the isolates were maintained at 4 °C and sub-cultured every month on potato dextrose agar (Difco, Detroit, MI, USA) slants. The preparation of fungal inocula was described elsewhere (D'Annibale *et al.*, 2005).

5.2.2.3. *Soil contamination and MAs addition*

Stock solution containing 1.6 mg ml⁻¹ of each PAH in analytical grade acetone was mixed with 2 g sterile quartz sand, air-dried for 24 h at room temperature to allow solvent evaporation and subsequently mixed with non sterile soil to reach a final concentration of 50 µg g⁻¹ soil (Löser *et al.*, 1999). Previously sterilized (121 °C for 15 min) MAs were added to soil by spraying and subsequent mixing to reach a final concentration of 2.5% (w/w). Random sampling and subsequent PAH analysis were performed to assess mixing homogeneity and recovery yield.

5.2.2.4. *Fungal treatment*

Maize stalks (5.0 g dry mass), previously colonized by the fungus for 2 weeks at 24° C, was covered with 15.0 g (dry mass) of contaminated soil, either in the presence or the absence of MA. The soil moisture content was adjusted to 15% (w/w) with sterile deionized water. The flasks were incubated at 24 °C for 6 weeks in the dark and the moisture content was kept constant by periodical additions of deionized water. All experiments were carried out in three parallel replicates under non-sterile conditions. For each condition, specific, non-inoculated controls were prepared, incubated as above and from here onwards referred to as incubation controls.

5.2.2.5. *Extraction and analysis of organic contaminants*

PAH contaminated soil as such and fungal-treated and relative incubation control soils were extracted for 12 h by Soxhlet using an hexane:acetone mixture (3:1, v/v) (Novotny *et al.*, 1999). The extracts were passed through anhydrous Na₂SO₄, evaporated at 30 °C under reduced pressure in Rotavapor (Büchi, Switzerland), dissolved in acetonitrile and analyzed by reversed-phase HPLC. The analyses were performed with a Waters (USA) chromatographic system consisting of a

gradient pump module mod. 1525 and 2487 UV detector module equipped with Envirosep PP column (125 x 3.2 mm i.d., Phenomenex USA). Elution was performed with Milli-Q water (A) and CH₃CN (B) using the following program: isocratic elution 48:52 (A/B) for 8 min, CH₃CN gradient up to 30:70 (A/B) in 12 min and, finally, isocratic elution at 30:70 (A/B) for 15 min. The elution process was monitored at 254 nm. Overall degradation activities were also evaluated by total weight of organic contaminants (TWOC) obtained by summing the concentrations of each single contaminant.

5.2.2.6. Biochemical determinations

The content of ergosterol, a specific indicator of fungal growth, was determined in soil by the method of Novotny *et al.* (1999). Extracellular ligninolytic enzymes were extracted from soil samples and subsequently assayed as previously reported (D'Annibale *et al.*, 2005). Endo- β -1,4-glucanase, cellobiohydrolase and endo- β -1,4-xylanase activities were determined as reported by Sampedro *et al.* (2005). All activities were expressed in international units (IU), defined as the amount of enzyme producing 1 μ mol of product per minute under the assay conditions. Extracellular soluble protein was determined by the Bradford's method (1976).

5.2.2.7. Microbial counts and ecotoxicity tests

Bacterial counts were performed in pristine soil, incubation controls and fungal-treated soils by the procedure described by Fava and Bertin (1999) with the exception that cycloheximide (0.5 g l⁻¹) was added to Plate Count Agar (Oxoid) to inhibit fungal growth. The acute toxicity test with *Folsomia candida* (Willem) was performed as reported elsewhere (D'Annibale *et al.*, 2005).

5.2.2.8. DNA Extraction and PCR amplification

Total community DNA was extracted from 250 mg of soil using the PowerSoil DNA Extraction Kit (MoBio Laboratories, USA) following the manufacturer's instruction. The variable V3 region of 16S rDNA was amplified by PCR using primers targeted to conserved regions of the 16S rRNA genes: 341F (ATTACCGCGGCTGCTGG) and 534R (ATTACCGCGGCTGCTGG) (Muyzer *et al.*, 1993). Primer 341F had at its 5' end an additional 40-nucleotide GC-rich sequence (GC clamp) to facilitate separation by DGGE. The 16S rRNA gene was amplified from 10 ng of DNA in a PCR reaction with 0.4 μ M of each primer, using the illustraTM HotStart Master Mix (GE Healthcare,

UK). PCR amplification was performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA) as follows: denaturation at 95 °C for 2 min followed by a touchdown procedure. In particular, the annealing temperature was initially set at 65 °C and then decreased by 0.5 °C every cycle until it reached 55 °C; then 10 additional cycles were carried out at 55 °C followed by primer extension for 5 min at 72 °C. PCR products from three parallel amplifications were pooled, concentrated with a Microcon filter concentrator (Millipore, Bedford, MA), separated in 1.5% (w/v) agarose gel and then stained with ethidium bromide.

5.2.2.9. DGGE analysis

The INGENYphorU-2 system for DGGE (Ingeny International BV, Goes, NL) was used. Samples were loaded onto 6% polyacrylamide-bisacrylamide (37.5:1) gels with denaturing gradients from 40% to 60% (where 100% corresponds to 7 M urea and 40% [v/v] deionized formamide). Gels were run at 100 V in 0.5x TAE (20 mM Tris acetate, 10 mM sodium acetate, 0.5 mM Na-EDTA at pH 7.4) at 60 °C for 16 h, then stained with SBYR Gold (Invitrogen, Carlsbad, CA) in 1x TAE for 45 min at room temperature and visualized under UV illumination. DGGE banding patterns were digitized and subsequently processed using the Quantity-one image analysis software (version 4.2.5; Bio-Rad Laboratories, Hercules, CA) and manually corrected for further analyses. Bacterial diversity indices for each sample were also calculated: richness (S) was determined from the number of bands in each lane, and the Shannon-Weaver index (H) was calculated from $H = -\sum (n_i/N) \log (n_i/N)$, where n_i is the peak height of a band and N is the sum of all peak heights in a lane. An unweighed pair group method with arithmetic means (UPGMA) dendrogram was generated from a similarity matrix based on common band positions between lanes and calculated using the Dice's coefficient (Li and Moe, 2004).

5.2.3. Results and Discussion

5.2.3.1. Effect of MAs on fungal growth and extracellular enzyme production

Although the use of MAs in mycoremediation has been widely reported, very limited information is currently available on the impact of these additives on mycelial growth at soil's moisture contents close or lower than the WHC. These humidity values, in fact, reflect real field situation and, therefore, it should be advisable to assess growth under these conditions.

In present study, where the soil's initial moisture content was set to 59% of the WHC, colonization by *Phlebia* sp. was more rapid and clearly visible than that of *Allescheriella* sp. These visual observations were confirmed by the soil content in ergosterol, a specific indicator of fungal growth (Table 5.7).

Table 5.7.. Ergosterol content and soluble protein concentration in soil spiked with PAHs and incubated with either *Phlebia* sp. DABAC 9 or *Allescheriella* sp. DABAC 1 for 6 weeks at 28 °C in the absence (None) and in the presence of mobilizing agents.

Mobilizing agent	<i>Phlebia</i> sp.		<i>Allescheriella</i> sp.	
	Ergosterol ($\mu\text{g g}^{-1}$ soil)	Soluble protein ($\mu\text{g g}^{-1}$ soil)	Ergosterol ($\mu\text{g g}^{-1}$ soil)	Soluble protein ($\mu\text{g g}^{-1}$ soil)
None	3.0 $\pm$ 0.1 ^{ab}	0.17 $\pm$ 0.002 ^a	0.1 $\pm$ 0.0 ^a	0.16 $\pm$ 0.001 ^a
Soybean oil	5.2 $\pm$ 1.8 ^a	0.18 $\pm$ 0.04 ^a	3.5 $\pm$ 0.8 ^b	0.21 $\pm$ 0.1 ^a
Tween 20	4.2 $\pm$ 0.1 ^a	0.25 $\pm$ 0.05 ^a	0.2 $\pm$ 1.3 ^a	0.25 $\pm$ 0.1 ^a
Tween 80	3.2 $\pm$ 1.8 ^a	0.33 $\pm$ 0.1 ^a	1.3 $\pm$ 1.1 ^{ab}	0.28 $\pm$ 0.02 ^a

Data are the mean $\pm$ standard deviation of three independent experiments. Statistical pairwise multiple comparison of homogeneous data was carried out by the Tukey test: column means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

None of the MAs exerted any inhibitory action on fungal growth, the amount of which, however, was not affected by the type of additive in *Phlebia* sp. By contrast, soybean oil led to a phenomenal increase in biomass of *Allescheriella* sp. In this respect, this plant oil was reported to exert a strong stimulatory effect on *Pleurotus ostreatus* and *Irpex lacteus* growth in soil (Leonardi *et al.*, 2008) and the same effect was also reported for *Grifola frondosa* (Hsieh *et al.*, 2008) and *Agaricus bisporus* (Wardle and Schisler, 1969) liquid cultures. As previously observed for growth, the concentration of soil's soluble protein, which being undetectable in incubation controls, might be an indirect indicator of fungal activity, was not negatively affected by these additives in both fungal microcosms (Table 5.7). It is known that lignocellulosic materials are generally used in mycoremediation either as inoculum carriers (Leštan and Lamar, 1996) or as additives to boost fungal growth (Boyle, 1995). Whichever their role, their enzymatic hydrolysis provides easily assimilable compounds that are used by the fungus. Consequently, it is important to assess whether MAs might negatively affect the fungal production of glycosyl-hydrolases. In the present study, the additives under study either stimulated or did not significantly affect these enzyme activities. In fact, Figure 5.8 shows that, both soybean oil and Tween 20 had a stimulatory effect on endo- β -1,4-xylanase, cellobiohydrolase and endo- β -1,4-glucanase activities in *Phlebia* sp.

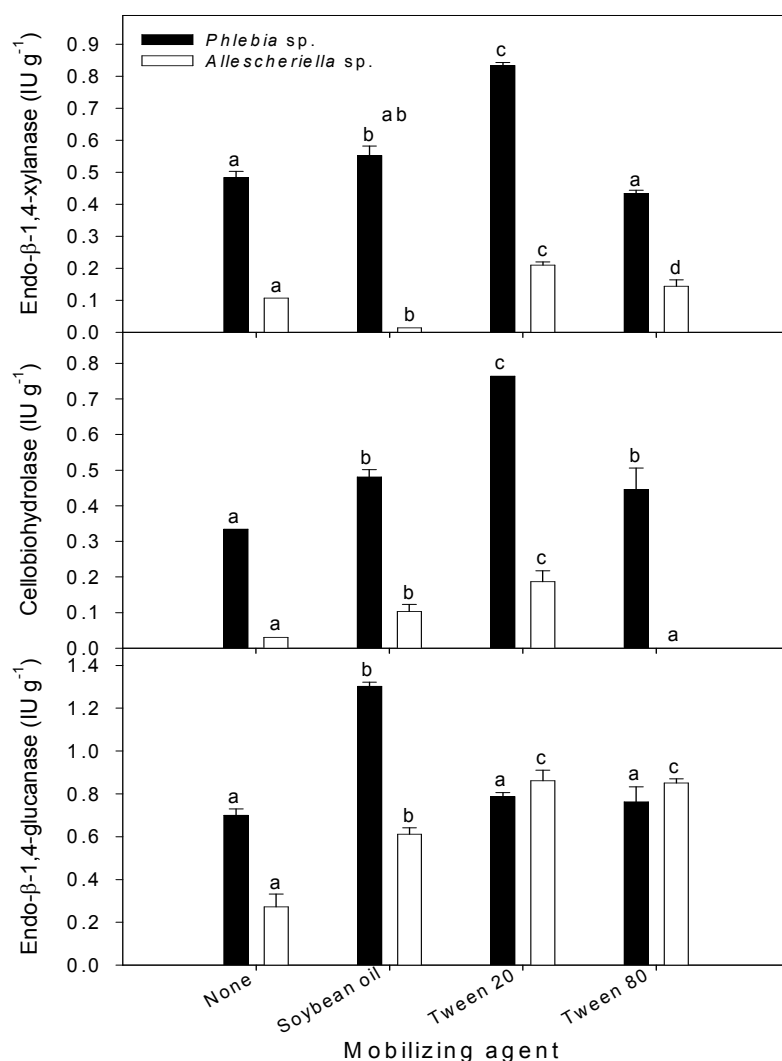


Figure 5.8. Glycosyl-hydrolase activities in the sandy-loamy soil artificially spiked with PAHs and incubated (42 days at 28 °C) with either *Phlebia* sp. DABAC 9 or *Allescheriella* sp. DABAC 1 in the absence (None) and in the presence of mobilizing agents (MAs). Values are the means of three independent experiments and error bars indicate the standard deviations. For each fungus, multiple pairwise comparisons were performed between treatments (*i.e.*, absence or presence and typology of MA) by the Tukey test. Same letters above bars indicate lack of statistical significance ($P \leq 0.05$).

With the exception of endo-β-1,4-xylanase activity, similar effects were observed also with *Allescheriella* sp. Lignocellulosic amendants have been also suggested to provide natural mediators for contaminants oxidation (Johannes and Majcherczyk, 2000; Cañas *et al.*, 2007) and to exert a potentially eliciting/inducing effect on lignin-modifying enzymes (Novotny *et al.*, 1999), some of which involved in PAH oxidation (Moen and Hammel, 1994; Bohmer *et al.*, 1998).

In this respect, maize stalks have been shown to contain water-soluble aromatic compounds able to play both roles (Crestini *et al.*, 1996).

Figure 5.9 shows that the presence of MAs did not affect the extracellular laccase activity of both fungi, while a significant increase in Mn-peroxidase (MnP) activity was observed in the presence of Tween 20 in both fungal microcosms.

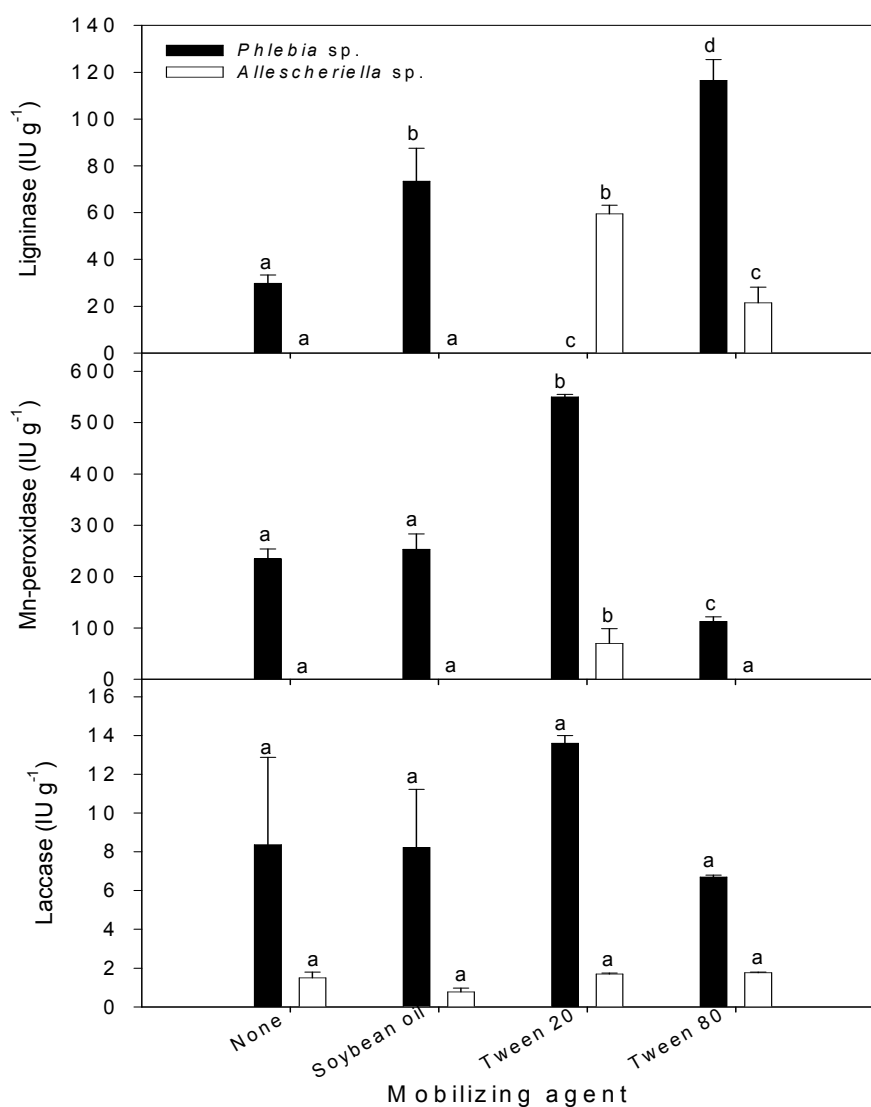


Figure 5.9. Lignin-modifying enzyme activities in the sandy-loamy soil artificially spiked with PAHs and incubated (42 days at 28 °C) with either *Phlebia* sp. DABAC 9 or *Allescheriella* sp. DABAC 1 in the absence (None) and in the presence of mobilizing agents (MAs). Values are the means of three independent experiments and error bars indicate the standard deviations. For each fungus, multiple pairwise comparisons were performed between treatments (*i.e.*, absence or presence and typology of MA) by the Tukey test. Same letters above bars indicate lack of statistical significance ($P \leq 0.05$).

In *Allescheriella* sp.-augmented soil, ligninase (LiP) activity was significantly increased by the addition of both Tweens; on the other hand, the same activity was stimulated in the presence of both soybean oil and Tween 80 in *Phlebia* sp. (Figure 5.9). In this respect, it has to be pointed out that the response of LMEs production to MAs appears to be both species- and enzyme-specific. For instance, some surfactants either repressed or strongly reduced the production of ligninolytic peroxidases by *Bjerkandera* sp. BOS55 (Kotterman *et al.*, 1998), *Phanerochaete chrysosporium* (Zheng and Obbard, 2001) and *Irpex lacteus* (Leonardi *et al.*, 2008); on the other hand, *Pleurotus ostreatus* laccase activity was stimulated in soil by the presence of Tween 80 and the present study shows that the MAs under study even stimulated ligninolytic peroxidases in *Phlebia* sp. and *Allescheriella* sp.

5.2.3.2. Effect of mobilizing agents on PAHs degradation and soil detoxification

Fungal microcosms conducted in the presence and in the absence of MAs were comparatively investigated for their PAH-degrading ability. However, in order to assess the cumulative action of both indigenous microflora and abiotic factors, the same investigations were performed on the incubation controls. In this respect, Table 2 shows that low molecular weight PAHs, such as PHE and ANT, were depleted by about 61 and 41%, respectively, in incubation controls conducted in the absence of MAs; on the other hand, four- and five-rings components were scarcely or not depleted at all. The presence of soybean oil in incubation controls positively affected the removals of ANT, PYR, BkFLT and BaPYR while Tween 20 either exerted a negative or a void effect on PAH degradation (Table 5.8).

Soybean oil was the sole MA which significantly enhanced the degradation efficiency by *Phlebia* sp. Table 5.9 shows that the removal extents of PYR, CHRY, BkFLT and BaPYR in the presence of this additive were significantly larger than those in fungal microcosms without MAs.

In *Allescheriella* sp., a different degradation pattern was observed as a function of the type of MA. In particular, Tween 20 addition enhanced degradation only in the case of three- and four-rings PAHs, such as ANT, FLT, PYR, and CHRY (Table 5.10). By contrast, soybean oil markedly improved the degradation of CHRY, BkFLT and BaPYR. In the present study, irrespective of the presence or the absence of MAs, in both fungal microcosms ANT was degraded more efficiently than PYR although these compounds are characterized by very similar ionization potentials (IP 7.43 vs. 7.44 eV, respectively) and water-solubilities (0.073 vs. 0.153 mg l⁻¹ at 25 °C, respectively). The fungal degradation of PAHs has been suggested to be inversely correlated with their ionization potential (IP) values (Bogan and Lamar, 1995; Zheng and Obbard, 2002).

Table 5.8. Residual concentrations of individual components of PAH mixture in incubation controls (6 weeks at 28°C) in the absence and in the presence of mobilizing agents. Each PAH was added to soil at a concentration of 50 $\mu\text{g g}^{-1}$ soil. The components were as follows: phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYP), chrysene (CHRY), benzo[k]fluoranthene (BKFLT) and benzo[a]pyrene (BaPYR).

Mobilizing agent	Residual concentrations ($\mu\text{g g}^{-1}$ soil)						
	PHE	ANTR	FLT	PYP	CHRY	BKFLT	BaPYR
None	19.4±0.8 ^a	29.4±2.0 ^a	37.9±2.4 ^a	38.8±1.7 ^a	45.4±2.8 ^a	42.1±2.8 ^a	39.8±1.6 ^a
Soybean oil	37.5±3.5 ^{bd}	10.4±1.3 ^b	39.6±1.7 ^a	23.3±1.1 ^b	44.5±3.1 ^a	23.8±1.8 ^c	26.4±0.8 ^b
Tween 20	34.6±0.8 ^b	28.8±1.1 ^a	45.8±2.4 ^b	41.7±1.9 ^a	48.7±2.9 ^a	39.2±2.5 ^a	38.8±1.3 ^a
Tween 80	36.9±0.6 ^{bd}	12.9±1.2 ^b	44.8±1.9 ^b	42.1±2.4 ^a	42.4±2.6 ^a	32.4±1.2 ^b	26.1±0.6 ^b

Data are the mean ± standard deviation of three independent experiments. Statistical pair-wise multiple comparison of homogeneous data was carried out by the Tukey test: column means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

Table 5.9.. Residual concentrations of individual components of PAH mixture in soil incubated with *Phlebia* sp. DABAC 9 for 6 weeks at 28°C in the absence and in the presence of mobilizing agents. Each PAH (for acronyms see Table 2) was added to soil at a concentration of 50 $\mu\text{g g}^{-1}$ soil. Values in square brackets represent the percent reduction of each contaminant calculated with respect to data of corresponding incubation controls.

Mobilizing agent	Residual concentrations ($\mu\text{g g}^{-1}$ soil)						
	PHE	ANTR	FLT	PYR	CHRY	BkFLT	BaPYR
None	4.5±0.5 ^a	12.0±1.0 ^a	15.4±1.5 ^a	25.9±0.4 ^a	43.5±2.6 ^a	34.3±2.4 ^a	30.3±1.5 ^a
Soybean oil	4.4±0.6 ^a	10.1±0.0 ^a	24.3±0.6 ^b	19.6±1.1 ^b	25.9±2.0 ^b	21.6±0.4 ^b	7.9±0.3 ^b
Tween 20	2.1±0.1 ^b	10.7±0.0 ^a	23.6±5.7 ^b	24.1±3.2 ^a	38.7±1.3 ^{ac}	30.5±0.4 ^a	26.4±1.8 ^{ac}
Tween 80	3.1±0.1 ^{ab}	10.6±0.9 ^a	24.5±3.9 ^b	24.1±0.3 ^a	41.6±0.4 ^a	30.6±1.3 ^a	24.2±2.0 ^c

Data are the mean ± standard deviation of three independent experiments. Statistical pairwise multiple comparison of homogeneous data was carried out by the Tukey test: column means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

Table 5.10 Residual concentrations of individual components of PAH mixture in soil incubated with *Allescheriella* sp. DABAC 1 for 6 weeks at 28°C in the absence and in the presence of mobilizing agents. Each PAH (for acronyms see Table 2) was added to soil at a concentration of 50 µg g⁻¹ soil. Values in square brackets represent the percent reduction of each contaminant calculated with respect to data of corresponding incubation controls.

Mobilizing agent	Residual concentrations (µg g ⁻¹ soil)						
	PHE	ANTR	FLT	PYR	CHRY	BkFLT	BaPYR
None	6.2±1.9 ^{ac}	11.3±0.9 ^a	17.0±3.2 ^a	20.2±0.2 ^a	47.1±5.9 ^a	34.7±0.7 ^{ac}	33.7±2.4 ^{ac}
Soybean oil	28.4±1.9 ^b	8.3±0.2 ^b	29.7±1.1 ^b	19.8±1.0 ^a	31.6±2.1 ^b	22.3±0.6 ^b	9.0±0.3 ^b
Tween 20	3.9±0.3 ^a	9.0±1.5 ^b	9.7±2.1 ^c	13.5±1.4 ^b	33.8±4.8 ^b	32.7±2.9 ^a	30.9±1.8 ^a
Tween 80	5.4±1.5 ^{ac}	9.6±0.8 ^{ab}	12.0±0.8 ^{cd}	15.1±1.3 ^b	36.7±0.4 ^{bc}	33.6±1.8 ^a	30.5±0.4 ^a

Data are the mean ± standard deviation of three independent experiments. Statistical pairwise multiple comparison of homogeneous data was carried out by the Tukey test: column means followed by the same superscript letters were not significantly different ($P \leq 0.05$).

The present study, however, shows that those PAHs characterized by the highest IP values, namely PHE and FLT (8.03 and 7.95 eV, respectively), were efficiently removed even in the absence of MAs. The original belief that IP might be the most important determinant in PAH biotransformation has been retrenched by several *in vivo* (Leonardi *et al.*, 2008; Wu *et al.*, 2008) and *in vitro* (Majcherczyk *et al.*, 1998; Cañas *et al.*, 2007) studies. Undoubtedly, PAH degradation extents in soil is governed by the interaction of several factors, such as IP, availability of natural redox mediators (Cañas *et al.*, 2007) and organic matter content, which along with intrinsic solubility, determines their bioavailability (Juhasz and Naidu, 2000). With regard to the intrinsic water-solubility, the present study shows that, regardless of the fungus employed, the enhancing effect due to the use of soybean oil mainly regarded the least soluble components of the mixture, namely CHRY, BkFLT and BaP. In this respect, the molar solubility ratios for PAHs of plant seed oils, including soybean oil, have been shown to be very similar to those of synthetic MAs (Pannu *et al.*, 2004; Pizzul *et al.*, 2007). Consequently, the use of plant oils enables an effective desorption of these contaminants from soil organic colloids (Bogan and Lamar, 1999; Bogan *et al.*, 2003). An overall view on the degradation results is provided by Figure 5.10, showing the TWOC values in incubation controls and fungal cultures.

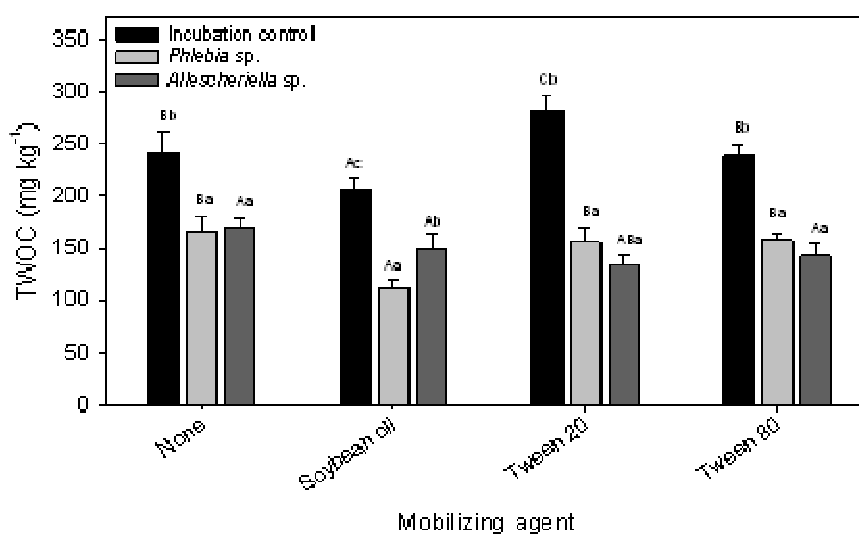


Figure 5.10. Total weight of organic contaminants (TWOC) in the incubation controls of soil spiked with PAHs in the absence (None) and in the presence of mobilizing agents (MAs) and after incubation with either *Phlebia* sp. DABAC 9 or *Allescheriella* sp. DABAC 1. Initial TWOC value of the spiked soil was 350 mg kg⁻¹ soil. Values represent the means of three independent experiments and error bars indicate the standard deviations. Multiple pair-wise comparisons were performed between groups and within each group by the Tukey test. Same uppercase and lowercase letters above bars indicate lack of statistical significance ($P \leq 0.05$) between and within groups, respectively.

First of all, soybean oil was the sole MA leading to an increased overall PAH depletion in the incubation controls. With *Phlebia* sp., the best MA was soybean oil leading to a 31.4% decrease in the TWOC value with respect to fungal cultures without MAs (113.8 vs. 165.9 mg kg⁻¹, respectively); on the other hand, with *Allescheriella* sp., best performances were observed with Tween 20 leading to a TWOC decrease by 21.6%.

The addition of the MAs exerted a negative effect on the toxicity in the incubation controls of the PAHs-contaminated soil leading to a significant increase in mortality of the *Collembola Folsomia candida* (Willem), as shown in Figure 5.11.

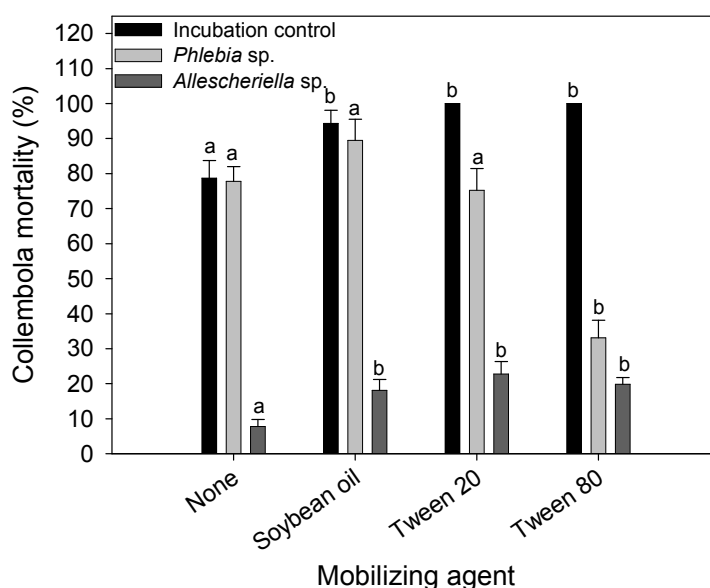


Figure 5.11. Collembola mortality percentages in the incubation controls of soil spiked with PAHs in the absence (None) and in the presence of mobilizing agents (MAs) and after incubation with either *Phlebia* sp. DABAC 9 or *Allescheriella* sp. DABAC 1. Values are the means of three independent experiments and error bars indicate the standard deviations.

High levels of mortality were also found in the *Phlebia* sp.-augmented soil both in absence of MA and, unexpectedly, in the presence of soybean oil; a statistically significant detoxification was only observed in the case of Tween 80 (33.1% mortality).

By contrast, *Allescheriella* sp. led to a remarkable reduction in toxicity that was maximum when no MA was used (7.8% mortality) and increased to a 18.1-22.8% range depending on the MA. Therefore, *Allescheriella* sp. proved to be much more effective than *Phlebia* sp. in the detoxification of the spiked soil although the PAH was far from being quantitative. In this respect, although this strain was less effective than other fungal isolates in degrading aromatic hydrocarbons

of a soil from a decommissioned industrial site, its use led to similar detoxification levels (D'Annibale *et al.*, 2006). This variability in detoxification has been suggested to depend on the balance between PAH depletion and formation of degradation intermediates with different degree of toxicity (Holt *et al.*, 2005). However, RP-HPLC analyses did not show the presence of additional UV-absorbing peaks ascribable to either hydroxylated or PAH quinone derivatives (present study).

5.2.3.3. Impact of MAs on heterotrophic cultivable bacteria and on diversity of the indigenous bacterial community

Fungal degradation of PAHs soils often resulted in the accumulation of dead-end metabolites, such as PAH diones (Andersson and Henrysson, 1996; Vyas *et al.*, 1994), or did not lead to mineralization of contaminants (Andersson *et al.*, 2003), thus requiring the action of the indigenous microflora to complete the degradation process (in der Wiesche *et al.*, 1996; Meulenberg *et al.*, 1997; Kotterman *et al.*, 1998). Therefore, the impact of either mycoremediation and other associated interventions on the resident microflora might not be ignored in order to understand their failure or success. In the present study, the addition of MAs to the pristine soil did not result in a reduction of cultivable bacteria (Figure 5.12).

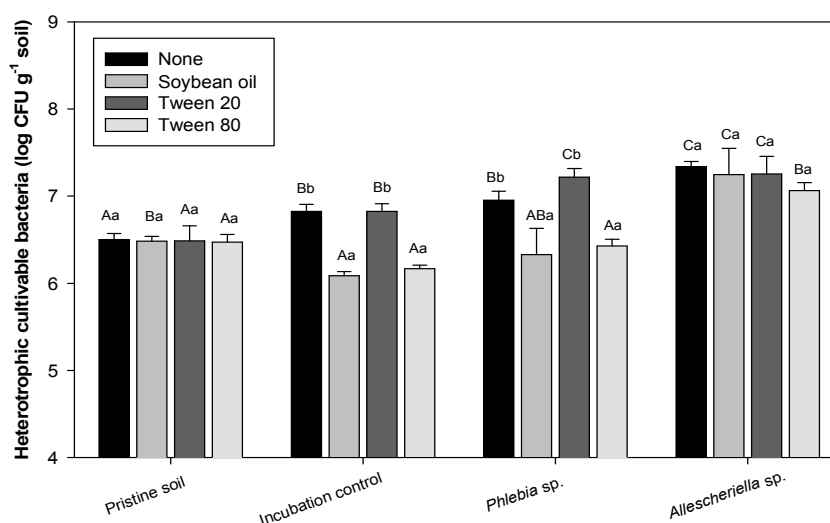


Figure 5.12. Concentration of indigenous cultivable bacteria in soil spiked with PAHs (Pristine soil) in the absence (None) and in the presence of mobilizing agents (MAs), in the related incubation controls and after incubation with either *Phlebia* sp. DABAC 9 or *Allescheriella* sp. DABAC 1. Values are the means of three independent experiments and error bars indicate the standard deviations. Multiple pair-wise comparisons were performed between groups and within

each group by the Tukey test. Same uppercase and lowercase letters above bars indicate lack of statistical significance ($P \leq 0.05$) between and within groups, respectively.

On the other hand, microbial densities of incubation controls and *Phlebia*-augmented soil in the presence of both soybean oil and Tween 80 were significantly lower than without MAs. The negative effect of soybean oil on cultivable bacteria was also claimed by other studies (Pizzul *et al.*, 2007; Leonardi *et al.*, 2008) and has been suggested to be due to either an imbalance between the added carbon and soil nutrients (Pizzul *et al.*, 2007) or to the likely occurrence of a physical barrier impairing gaseous exchanges (Borden, 2007). In *Allescheriella*-augmented soils, microbial densities did not significantly differ each one another, irrespective of the presence or the absence of MAs; however, they were significantly higher than in the related incubation controls (Figure 5.12). Two-way ANOVA performed on bacterial counts as a function of type and presence of mobilizing agent as the first variable (X_1) and presence or absence of fungal inoculum as the second one (X_2) showed that both the effects of each variable and their interaction were highly significant ($P < 0.001$).

A cultivation-independent approach was used in the present study to gain further insights into the impact of both fungal augmentation and MAs on aerobic bacteria. Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rRNA genes showed that in incubation control without any MAs, richness (S) and Shannon-Weaver index (H) did not significantly differ from the pristine soil (Figure 5.13).

Both parameters generally increased in the incubation controls added with MAs. In *Phlebia* sp.-augmented soil, both S and H increased in the presence of Tweens and were negatively affected by soybean oil. By contrast, the same parameters were generally improved in soil augmented with *Allescheriella* sp., regardless of the presence or the absence of MAs. It is noteworthy that both fungi increased bacterial diversity in the absence of MAs. With this regard, exogenously added fungi might have variable impact on the indigenous bacterial community ranging from evident inhibition (Andersson *et al.*, 2003) to stimulation (Leonardi *et al.*, 2008; Wu *et al.*, 2008).

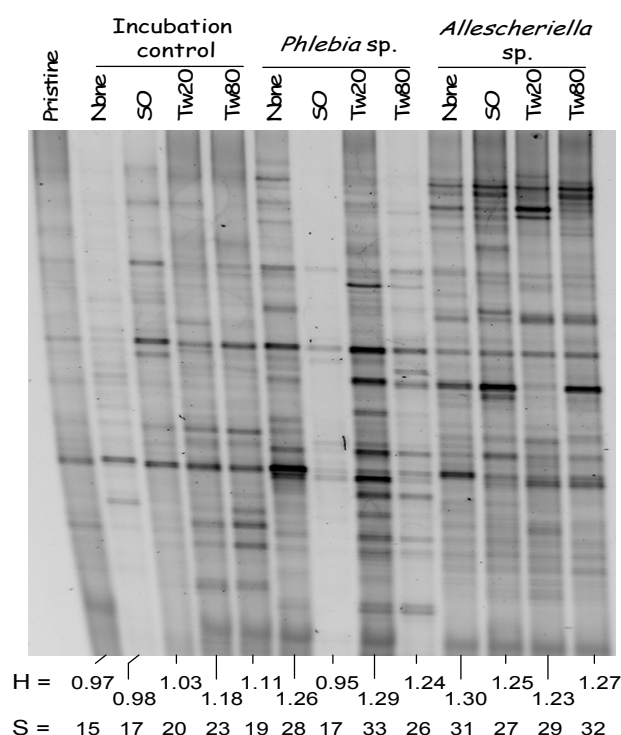


Figure 5.13. DGGE analysis of the bacterial communities in soil spiked with PAHs (Pristine soil) in the absence (None) and in the presence of mobilizing agents (MAs), in the related incubation controls and after incubation with either *Phlebia* sp. DABAC 9 or *Allescheriella* sp. DABAC 1. Shannon–Weaver index (H) and richness (S) are reported for each lane at the bottom of the gel. Abbreviations: SO, soybean oil; Tw20, Tween 20; Tw80, Tween 80.

Figure 5.14 shows the cluster analysis of DNA banding patterns, based on the averaged similarity matrix, in samples derived from pristine soil, its related incubation controls and fungal-augmented soils with or without MAs. Interestingly, incubation controls and fungal-augmented soils clustered separately with a similarity value of 0.47 with the sole exception of the incubation control without MAs that clustered with fungal treatments. The remaining incubation controls added with MAs clustered together with the pristine soil, thus indicating limited modifications due to both the incubation and the presence of additives. Interestingly, within these three main clusters (*i.e.*, incubation control, *Phlebia* sp. and *Allescheriella* sp.) the soil samples added with Tween 20 and 80 always clustered together (0.71, 0.75 and 0.82 similarity, respectively). These results indicate that the degree of similarity was mainly governed by the treatment typology rather than by the type of MA employed. Similar results were obtained in another study with *P. ostreatus* 3004 (Leonardi *et al.*, 2008).

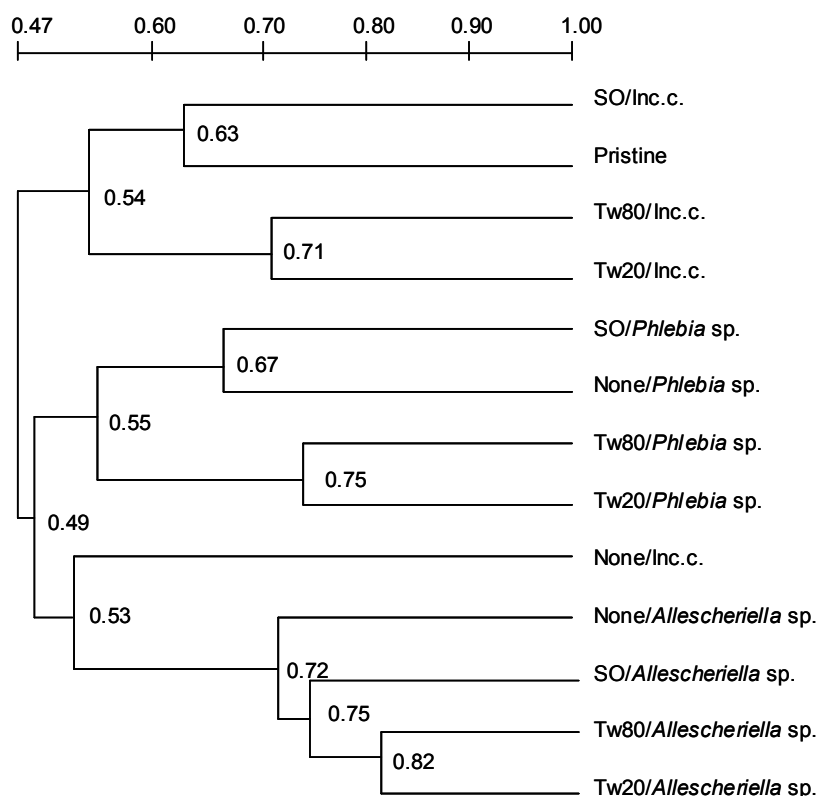


Figure 5.14. Cluster analysis of bacterial communities obtained from the DGGE profiles of pristine soil, incubation controls and *Phlebia* sp.- or *Allescheriella* sp.-augmented soils. The dendrograms were generated from a similarity matrix based on common band positions between lanes using an unweighed pair group method with arithmetic means (UPGMA). Abbreviations as in Figure 5.13.

5.2.4. Conclusions

With reference to the main aims of the study, the following main points can be drawn: (i) growth of both fungi was not negatively affected by the MAs employed; (ii) as opposed to other studies ligninolytic peroxidase activities, potentially involved in PAH oxidation, were stimulated by the presence of MAs; (iii) the enhancing effect of soybean oil on PAHs mainly regarded the least water-soluble components; (iv) the biodiversity of indigenous bacteria was markedly stimulated by *Allescheriella* sp. regardless of the presence or the absence of MAs.

6. CONCLUDING REMARKS

Due to the promising characteristics of the strain CBS 577.79 of *P. tigrinus*, the main aim of the present Ph.D project was to investigate its degradation capabilities in defined liquid media towards different classes of ubiquitous aromatic contaminants: polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), chlorobenzoic acids (CBAs) and endocrine disrupting compounds (EDCs). Furthermore, in order to gain more information on the degradation machinery of the strain CBS 577.79, the kinetic and redox properties of its major MnP isoenzyme (MnP II) were characterized. The isoenzyme in question along with a purified laccase from the same strain were exploited for *in vitro* degradation experiments of the above-cited classes of pollutants both in the presence and in the absence of redox mediators.

It is noteworthy that fungal liquid cultures constitute appropriate model systems to explore the biotransformation of a wide variety of compounds. However, it is of paramount importance to investigate the use of fungal remediation under non-sterile conditions and with soils from real contaminated sites, thus making mycoremediation technology potentially transferable to the field scale. Therefore, additional goal of the present Ph.D thesis was to assess the effectiveness of *P. tigrinus* and other representative white rot fungi in lab-scale mycoremediation trials of PAH-polluted environmental matrices (soils and lignocellulosic materials).

The results achieved in the frame of the present PhD project are listed below:

- ***Kinetic and redox properties of MnP II, a major MnP isoenzyme from P. tigrinus CBS 577.79.*** A MnP isoenzyme was produced from *P. tigrinus* liquid culture and purified to apparent homogeneity. The enzyme turned out to be a monomeric protein with molecular mass of 50.5 kDa, *pI* of 4.07 and an extent of *N*-glycosylation by about 5.3% of the high-mannose type. Temperature and pH optima for manganic chelates formation were 45 °C and 5.5, respectively. MnP proved to be poorly thermostable at 50 and 60 °C with half-lives of 11 min and 105 s, respectively. K_m values for H_2O_2 and Mn^{2+} were 16 and 124 μM , respectively. Although MnP exhibited both Mn^{2+} -dependent and Mn^{2+} -independent oxidation of several phenolic substrates, reaction rates in the absence of Mn^{2+} were markedly lower than those observed in the presence of Mn^{2+} . Opposite to other versatile fungal peroxidases, veratryl alcohol oxidation by *P. tigrinus* MnP CBS 577.79 required the presence of both H_2O_2 and Mn^{2+} . Kinetic properties and substrate specificity of the isoenzyme purified in this study markedly differ from those reported for a versatile MnP isolated from liquid cultures of the reference strain *P. tigrinus* 8/18. This work reports for

the first time a thorough electrochemical characterization of a MnP from this fungus. Voltammetric measurements clearly showed that the electrode-immobilized enzyme retained its catalytic activity and was able to perform direct electron transfer: this might open the door to its use in the possible development of electron-transfer based biosensors.

- **PAH degradation by *P. tigrinus* CBS 577.79.** The ability of the fungus to degrade two representative PAHs was assessed in liquid media, which were conducive to the preferential production of laccase and MnP, respectively. Direct micellar systems were used in order to increase the solubility of the aromatic contaminants in aqueous phase. ANT and BaP were degraded to a larger extent in low-N medium, where the activity of MnP was stimulated with respect to the biotic control lacking of PAHs.

Subsequently, an in-depth analysis of the PAH-degrading capability of *P. tigrinus* was carried out in N-rich and low-N standard media (*i.e.*, MEG and NKM) spiked with a mixture of 7 representative PAHs. The highest removal of the PAHs were achieved under shaken conditions, in both media, after 4 weeks of incubation. *In vitro* PAH degradation studies with purified laccase and MnP clearly showed that the latter had wider substrate PAH range and higher oxidation ability than the former. This might suggest a predominant role of MnP under *in vivo* conditions with important implications in practical remediation cases where both N-limiting conditions and absence of mediators are common scenarios. The structural identification of PAH degradation products clearly showed the combined action of both the extracellular and the intracellular enzyme systems of *P. tigrinus* CBS 577.79.

In a successive work, the ability of *P. tigrinus* CBS 577.79 to colonize and detoxify solid PAH-contaminated matrices (soil and hardwoods) derived from a wood treatment facility under non-sterile conditions was shown. Thus, the positive degradation results observed with both axenic stationary and shaken cultures of the strain were confirmed. In particular, *P. tigrinus* growth was invariably higher than that of *I. lacteus* CCBAS 238, a reference strain that had been particularly efficient in mycoremediation; it also showed higher ability than *I. lacteus* to degrade the most abundant pollutants present in both matrices. Accordingly, the extent of detoxification achieved with the former was significantly higher than with the latter. In addition, the degradation ability of *P. tigrinus* towards certain contaminants largely exceeded their respective bioavailable fractions thus featuring a lower dependence than *I. lacteus* on bioavailability.

- Degradation of PCBs and CBAs by *P. tigrinus* CBS 577.79.** The white rot fungus *P. tigrinus* was unable to remove PCB congeners in liquid cultures. On the contrary, the majority of the CBAs were successfully degraded in the early phases of incubation in standard liquid media, under both stationary and shaken conditions. Although the CBAM stimulated laccase production by the fungus, *in vitro* experiments with purified laccase and MnP showed that these enzymes are unable to degrade individual CBAs, thus indicating that other enzymatic systems are involved in the initial attack to chlorobenzoates under *in vivo* conditions. The structural identification of CBA degradation products allowed us to hypothesize that the degradation of these compounds by *P. tigrinus* was the result of a combined action of both extracellular and intracellular enzyme systems. Results from an ecotoxicological test (inhibition of *Vibrio fischeri* light emission) revealed that partial removal of the toxicity associated to the CBAM was achieved in LNKM shaken cultures and, transiently, in the early phases of static incubation in MEG medium. In this respect, it is not possible to exclude that metabolites produced during the *in vivo* treatment of the CBA mixture might have similar or even higher toxicity than the parent compounds.
- EDC degradation by *P. tigrinus* CBS 577.79.** *P. tigrinus* liquid cultures proved to be able to effectively remove EE2, BPA and NP and their relative estrogenic activity. The degradation of these compounds was not significantly affected by both the type of medium as well as the presence or the absence of shaking conditions. By contrast, TRC was more recalcitrant to degradation and its concentration did not significantly change in shaken cultures conducted on LNKM. Accordingly, a high residual estrogenic activity was found in ethyl acetate extracts of TRC-spiked cultures which was best in LNKM shaken cultures. *In vitro* experiments with purified enzymes revealed that, under non-mediated conditions, laccase was more efficient than MnP in the oxidation of EE2 and BPA, while the latter enzyme oxidized NP and TRC at a faster rate than the former. Under mediated conditions, best degradation performances towards EE2 and BPA and TRC were observed with the laccase/HBT system while NP was more susceptible to oxidation by MnP-regardless of the presence or the absence of GSH. Although the estrogenic activities of EE2 and BPA were significantly removed by in *all vitro* treatments, their removal extents were not significantly affected by the type of treatment; a high impact of the treatment typology, conversely, was observed for NP and TRC the residual estrogenic activities of which were best removed by the laccase and laccase/HBT systems.

- **Mycoremediation of PAH-polluted matrices.** The importance of the inoculum carrier on both growth and PAH-degradation performances of fungal microcosms was shown during lab-scale mycoremediation of PAH-polluted matrices from a wood treatment plant. The ability of the strains under study to degrade certain PAHs even partially beyond their respective bioavailable amounts, was also evidenced. The best PAH degrader appeared to be the strain *P. ostreatus* CCBAS 278 cultivated on commercial (straw-based) pellets that was able to remove 80% of total PAH sum from the historically contaminated soil. The lack of correlation between fungal growth and PAH depletion observed in the majority of mycoaugmented microcosms indicates that the degradation process arises from the cooperative action between the inoculated fungus and the indigenous microbial community; the notable exception to this general trend of *P. ostreatus* microcosms might indicate the need of adequate colonisation to provide a first oxidative attack of PAHs leading to quinone derivatives. The non-accumulation of these degradation intermediates that had been reported to be dead-end products for this species provides an additional, albeit indirect, proof of a cooperation of this fungus with the resident microbiota. All fungal microcosms partially removed phytotoxicity, which, in turn, was significantly correlated with residual PAH contents of both matrices, thus confirming that no toxic dead-end degradation products had been accumulated.

In a previous work, moreover, the effect of mobilizing agents (MAs) on the mycoremediation of a PAH-contaminated soil was studied. The presence of MAs did not negatively affected the growth of the two white rot strains (*Allescheriella* sp. and *Phlebia* sp.) and stimulated the production of peroxidases. Soybean oil led to a 35-fold increase of *Allescheriella* sp. growth and enhanced the degradation of the least water-soluble PAHs. Enumeration of cultivable bacteria and a molecular approach (DGGE) showed that microbial density and biodiversity were positively affected by the mycoremediation especially with *Allescheriella* sp., the use of which led to an evident detoxification. The different response of the two fungi to MAs addition, however, confirms the need for a preliminary lab-scale assessment of fungus/MA combinations prior to application.

In conclusion, the results presented clearly feature the capacity of *P. tigrinus* (strain CBS 577.79) to degrade representative compounds belonging to different classes of ubiquitous pollutants, thus extending the range of application of this fungus for environmental clean-up purposes.

7. BIBLIOGRAPHY

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APPENDIX

1. Papers published on International journals:

Giubilei M.A., Leonardi V., Federici E., Covino S., Šašek, V., Novotný, C., Federici F., D'Annibale A. and M. Petruccioli (2009). Effect of mobilizing agents on mycoremediation and impact on the indigenous microbiota. *J. Chem. Technol. Biotechnol.* 84: 836–844

Petruccioli M., Frascioni M., Quarantino D, Covino S., Favero G., Mazzei F., Federici F., D'Annibale A., (2009). Kinetic and redox properties of MnP II, a major manganese peroxidase isoenzyme from *Panus tigrinus* CBS 577.59. *J. Biol. Inorg. Chem.* 14: 1153-1163.

Covino S., Svobodová K., Křesinova Z, Petruccioli M, Federici F, D'Annibale A, Čvančarova M., Cajthaml T (2010). *In vivo* and *in vitro* polycyclic aromatic hydrocarbons degradation by *Lentinus (Panus) tigrinus* CBS 577.79. *Biores. Technol.* 101: 3004-3014.

2. Submitted manuscripts

Covino S., Svobodová K., Čvančarova M., D'Annibale A., Petruccioli M., Federici F., Křesinova Z., Galli E., Cajthaml T. Mycoaugmentation of PAH-contaminated solid matrices from a wood preservation plant: impact of inoculum carrier and contaminants bioavailability on degradation performances. *Chemosphere*

3. Manuscripts in preparation

Covino S., Čvančarova M., D'Annibale A., Petruccioli M, Federici F, Svobodová K., Křesinova Z., Cajthaml T. Impact of lignocellulosic materials and contaminants bioavailability on PAH degradation performances of *P. tigrinus* and *I. lacteus* during mycoremediation of contaminated solid matrices from a wood treatment facility.

Covino S., Křesinova Z., D'Annibale A., Petruccioli M, Federici F, Muzikař M. Svobodová K., Cajthaml T. Biotransformation of chlorobenzoic acids by the white rot fungus *P. tigrinus*.

Covino S., Křesinová Z., D'Annibale A., Petruccioli M., Federici F., Muzikař M., Svobodová K., Cajthaml T. *P. tigrinus* and its oxidases efficiently remove endocrine disrupting compounds and estrogenic activity from liquid media.

4. Poster and oral communications:

Giubilei M.A., Leonardi V., Federici E., Covino S., D'Annibale A. and Petruccioli M. (2008). Mobilizing agents affect soil mycoremediation and indigenous bacterial population. Atti X Convegno FISV, Riva del Garda (TN), 24-27 Settembre 2008, p. D09.07.

Svobodová K., Covino S., Křesinová Z., Cajthaml T. (2009). Comparison of in vitro tests for measurement of estrogenicity of chlorinated compounds and their use in the evaluation of biodegradation of chlorbenzoates by the fungus *Panus tigrinus* (translated from the original Czech language). Odpadové fórum 2009 - Milovy, Czech Republic (22.4.2009-24.4.2009), Conference Proceedings [ISBN 978-80-02-02108-7, pages 3619-3622].

Covino S., Svobodová K., Křesinová Z., Federici F., Cajthaml T. (2009). Study of PAH degradation by the white rot fungus *Panus tigrinus* CBS 577.79. Proceedings of SIMGBM 28th Meeting, Spoleto, June 11-13, 2009: P039, page 119.

Covino S., Galli E., Petruccioli M., D'Annibale A., Křesinová Z., Erbanová P., Novotný, C., Cajthaml T., (2009). Effect of inoculum carrier on fungal remediation of polycyclic aromatic hydrocarbons-contaminated matrices. Proceedings of FISV Congress 2009, Riva del Garda (Italy), 23-25 September 2009: D 07.02.

