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Evaluation of *Trametes versicolor* ability to bioremediate Polycyclic Aromatic Hydrocarbons (PAHs) in different matrices

PhD Thesis

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Certifiquen:

Que el llicenciat en Ciències Ambientals **Eduard Borràs Camps** ha realitzat sota la nostra direcció, en els laboratoris del Departament d'Enginyeria Química, el treball **"Evaluation of *Trametes versicolor* ability to bioremediate Polycyclic Aromatic Hydrocarbons (PAHs) in different matrices"**, el qual presenta en aquesta memòria i constitueix la seva Tesi per a optar al Grau de Doctor en Ciències Ambientals per la Universitat Autònoma de Barcelona.

I perquè es prengui coneixement i consti als efectes oportuns, presentem a l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona l'esmentada tesi, signant el present certificat a

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Dra. Glòria Caminal i Saperas

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'Les idees no duren molt.
S'ha de fer alguna cosa amb elles'

Santiago Ramón y Cajal

'Perseguim la veritat, sigui la que sigui,
però per a trobar-la necessitem imaginació i escepticisme'

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Summary

As a result of human activities pollution aroused as a global concern due to improper release of chemicals into the environment. Contamination represents a real threat to humans and can affect any ecosystem compartment. Petroleum hydrocarbons affect typically soil, including polycyclic aromatic hydrocarbons (PAHs). Several conventional clean-up techniques are available for site restoration, including bioremediation. Mycoremediation, attained interest in the last decades as it is assumed to be an environmental-friendly technique.

This work shows the results of the basic research previous to the development of a polycyclic aromatic hydrocarbons polluted soil bioremediation treatment by means of the white rot fungus *Trametes versicolor*. The results of the mentioned research are presented in three differentiated sections.

The first section focuses on the fungal biomass production for posterior applications in bioremediation processes. It is divided into two subsections:

- The first part focuses on biomass production of in submerged cultures. The experiments were aimed at formulating a low-cost defined medium to obtain high amounts of biomass, in the preferred morphology (pellets). The air-pulsed fluidized bioreactors equipped with pH control were the most appropriate. The production was scaled-up to a 10 liters bioreactor.
- The second part analyzes the fungal colonization of lignocellulosic supports for further application in soil. The selection of the optimal substrate for colonization based on active biomass amounts, laccase production and the capacity to degrade naproxene in 24 hours. It was demonstrated that the fungus was capable of colonizing soil both under sterile and non-sterile conditions maintaining, in any case, the degradative capacity.

The second section focuses on the fungal ability to degrade polycyclic aromatic hydrocarbons. It is ddivided into three subsections:

- The first part focuses on the selection of an optimal surfactant for PAHs degradation in liquid medium, obtaining the best results with the non-ionic surfactant Tween 80. The degradation of several PAHs by *T. versicolor* in liquid medium was demonstrated; both in individual-PAHs experiment as

well as in PAHs-mixtures. Likewise, it was also demonstrated that under culture conditions laccase might degrade some of the studied compounds.

- The second section includes the identification of PAHs metabolites arising from degradation. The fungal degradation capacity of these intermediates was also examined.
- In the third section different degradation systems of were tested for soil treatment, the bioslurry resulted as the most effective in terms of degradation efficiency. It was checked out that in aerated biopiles, the fungus had problems derived from attaining termophilic ranges.

The last section deals with the creosote-PAHs degradation. It is divided into two subsections:

- The first part focuses on the fungal PAHs-degradation of the creosote aromatic fraction. Different systems were studied and it was determined that the fungus was more efficient at degrading PAHs in biopiles approach. In submerged cultures, liquid medium and slurry, the fungus was effective at degrading low-molecular-weight PAHs but not those of high-molecular-weight. It was also observed inhibitory effects on *Trametes* due to the additional compounds present in creosote as well as when increasing the total PAHs concentration.
- The second section is a result of the collaboration with the Laboratory of Environmental Biotechnology in the Institute of Microbiology, which belongs to the Academy of Sciences of the Czech Republic (Prague). The effect on PAHs removal during the interaction between soil microbial population and white-rot fungi during soil bioremediation processes was studied.

The experiments have been carried out in the "Group of degradation of industrial pollutants and valuation of waste" from the Department of Chemical Engineering in the UAB. The main research motivation of the group is to develop specific biotechnological processes to degrade xenobiotic compounds that are scarcely degraded by conventional treatments.

Resum

Com a resultat de les activitats humanes, la contaminació deguda a productes químics alliberats al medi s'ha convertit en un problema global, essent una amenaça real per a l'activitat humana. La contaminació pot afectar qualsevol compartiment de l'ecosistema. Els hidrocarburs derivats del petroli, on s'inclouen els hidrocarburs policíclics aromàtics (HAPs), són contaminants que afecten de manera particular el sòl. Actualment existeixen diverses tècniques per a la restauració d'emplaçaments contaminats, inclosa la bioremediació. La micorremediació, àrea de coneixement en què s'emmarca la tesi, ha guanyat atenció en els últims anys ja que és una tècnica ambientalment respectuosa.

Aquest treball presenta els resultats de les investigacions prèvies al desenvolupament d'un tractament de bioremediació de sols contaminats per hidrocarburs policíclics aromàtics mitjançant el fong ligninolític *Trametes versicolor*. Els resultats de l'esmentada investigació es presenten en tres apartats diferenciats.

El primer es centra en la producció de biomassa del fong per a posteriors aplicacions en processos de bioremediació. Es divideix en dues seccions:

- La primera es basa en la producció de biomassa en cultiu submergit. Els experiments es van centrar en formular un medi definit de cultiu de baix cost que permetés obtenir nivells elevats de biomassa, en la morfologia desitjada (pellets). El reactor fluïditzat per polsos d'aire amb control de pH va resultar ser el més adequat. La producció s'escalà a un bioreactor de 10 litres.
- La segona secció analitza la colonització del fong sobre suports lignocel·lulòsics provinents de residus agrícoles per a posterior aplicació en el sòl. La selecció dels millors substrats per a la colonització es va basar en el nivell de biomassa (ergosterol), la producció de lacasa i la capacitat de degradar naproxè en 24 hores. Es va demostrar que el fong era capaç de colonitzar el sòl tant en condicions estèrils com en no estèrils mantenint, en tot cas, la capacitat degradativa.

El segon apartat es centra en la degradació d'hidrocarburs policíclics aromàtics pel fong. Es divideix en tres seccions:

- La primera es basa en la selecció d'un surfactant per a la degradació de HAPs en medi líquid, essent el millor el surfactant no iònic Tween 80. Es va poder demostrar la capacitat degradativa de diversos HAPs en medi líquid; tant en experiment per separat com en mescles. Així mateix, també es va demostrar que en les condicions de cultiu l'enzim lacasa podia degradar alguns dels compostos.
- La segona secció fa referència a la identificació de productes intermediàris de degradació d'HAPs. També es va estudiar la capacitat de degradació d'aquests intermediaris per part del fong.
- En la tercera secció es van provar diferents sistemes de degradació en sòl, on el bioslurry es presenta com el més efectiu en termes d'eficàcia de degradació. Es va poder comprovar que en les biopiles airejades el fong va tenir problemes derivats del rang termofílic assolit.

El darrer apartat es basa en la degradació dels HAPs de la creosota. Es divideix en dues seccions:

- La primera es centra en la degradació dels HAPs de la fracció aromàtica de la creosota. Es van estudiar diferents sistemes i es va determinar que el fong era més eficient en degradació en biopiles. En cultius submergits, medi líquid i slurry, el fong era efectiu en la degradació d'HAPs de baix pes molecular però no en aquells d'alt pes molecular. Es va observar l'efecte inhibitori sobre *Trametes* tant dels compostos addicionals afegits junt amb la creosota com en augmentar la concentració de HAPs.
- La segona secció és fruit de la col·laboració amb el Laboratori de Biotecnologia Ambiental de l'Institut de Microbiologia de l'Acadèmia de les Ciències de la República Txeca de Praga. S'estudià l'efecte de la interacció del fong amb la població microbiana del sòl durant processos de bioremediació d'HAPs en sòl.

El treball experimental s'ha dut a terme en el "Grup de degradació de contaminants industrials i valorització de residus" del Departament d'Enginyeria Química de la UAB. L'objectiu general de recerca del grup és el desenvolupament de processos biotecnològics per a degradar compostos xenobiòtics difícilment degradables per tractaments convencionals.

Resumen

Como consecuencia de las actividades humanas, la contaminación debida a productos químicos liberados en el medio se ha convertido en un problema global, siendo una amenaza real para la actividad y la salud de los seres vivos. La contaminación puede afectar a cualquier compartimento del ecosistema. Concretamente, los hidrocarburos derivados del petróleo son contaminantes que afectan de manera particular al suelo, entre los cuales destacan los hidrocarburos policíclicos aromáticos (HPAs). Actualmente existen diversas técnicas para la restauración de emplazamientos contaminados por estos compuestos. Cabe destacar que en los últimos años la comunidad científica ha centrado especialmente los esfuerzos en el campo de la micorremediación (área de conocimiento donde se enmarca la presente tesis) dado que se trata de una técnica ambientalmente respetuosa.

El trabajo presenta los resultados de las investigaciones previas al desarrollo de un tratamiento de bioremediación de suelos contaminados por hidrocarburos policíclicos aromáticos mediante el hongo ligninolítico *Trametes versicolor*. Los resultados de la mencionada investigación se presentan en tres apartados diferenciados.

El primero se centra en la producción de biomasa del hongo para posteriores aplicaciones en procesos de bioremediación. Se divide en dos secciones:

- La primera se basa en la producción de biomasa en cultivo sumergido. Los experimentos se centraron en formular un medio de cultivo definido de bajo coste que permitiera obtener niveles elevados de biomasa, en la morfología deseada (pellets). El reactor fluidizado por pulsos de aire con control de pH resultó ser el más adecuado. La producción se escaló a un bioreactor de 10 litros.
- La segunda sección analiza la colonización del hongo sobre soportes lignocelulósicos provenientes de residuos agrícolas para posterior aplicación en el suelo. La selección de los mejores sustratos para la colonización se basó en el nivel de biomasa (ergosterol), la producción de lacasa y la capacidad de degradar naproxeno en 24 horas. Se demostró que el hongo era capaz de colonizar el suelo tanto en condiciones

estériles como no estériles manteniendo, en todo caso, la capacidad degradativa.

El segundo apartado se centra en la degradación de hidrocarburos policíclicos aromáticos por el hongo. Se divide en tres secciones:

- La primera se basa en la selección de un surfactante para la degradación de HPAs en medio líquido, siendo el surfactante no iónico Tween 80 aquel que dio mejores resultados. Se pudo demostrar la capacidad degradativa de diversos HPAs en medio líquido; tanto en experimentos por separado como en mezclas. Asimismo, también se demostró que en las condiciones de cultivo la enzima lacasa podía degradar algunos de los compuestos.
- La segunda sección hace referencia a la identificación de productos intermediarios de degradación de HPAs. También se estudió la capacidad de degradación de estos intermediarios por parte del hongo.
- En la tercera sección se probaron diferentes sistemas de degradación en suelos, donde el bioslurry resultó el más efectivo en términos de eficacia de degradación. Se pudo comprobar que en las biopilas aireadas el hongo tuvo problemas derivados del rango termofílico alcanzado.

El último apartado se basa en la degradación de los HPAs de la creosota. Se divide en dos secciones:

- La primera se centra en la degradación de los HPAs de la fracción aromática de la creosota. Se estudiaron diferentes sistemas y se determinó que el hongo era más eficiente en degradación en las biopilas. En cultivos sumergidos, medio líquido y slurry, el hongo era efectivo en la degradación de HPAs de bajo peso molecular pero no en aquellos de alto peso molecular. Se observó el efecto inhibitorio sobre *Trametes versicolo*r tanto de los compuestos adicionales añadidos junto con la creosota como al aumentar la concentración de HPAs.
- La segunda sección es fruto de la colaboración con el Laboratorio de Biotecnología Ambiental del Instituto de Microbiología de la Academia de las Ciencias de la República Checa en Praga. Se estudió el efecto de la interacción del hongo con la población microbiana del suelo durante procesos de bioremediación de HPAs en suelos.

El trabajo experimental se ha llevado a cabo en el "Grupo de degradación de contaminantes industriales y valorización de residuos" del Departamento de Ingeniería Química de la UAB. El objetivo general de investigación del grupo es el desarrollo de procesos biotecnológicos para degradar compuestos xenobióticos difícilmente degradables mediante tratamientos convencionales.

Chapter 1

General Introduction

1.1 Soil pollution

The progress of humanity since the industrial revolution has proportioned countless advances in our lifestyle. Environmental pollution aroused as a new risk due to a wide range of industrial and agricultural activities. A high number of contaminants were and still are released into the environment. Their introduction can come from leaking tanks, incomplete combustions, industrial processes, improper disposals and other origins. It is estimated that there are between 8 and 16 million molecular species of natural or anthropogenic origin present in the biosphere; form which 40,000 are predominant in our daily lives (Hou *et al.*, 2003).

Contamination can affect any compartment of the ecosystem. These products accumulate in the ecosystem (air, water and soil) and bioaccumulate in living organisms. At the same time, the pollutants undergo physico-chemical and biological processes that affect their final fate. Recently, together with the increase in environmental conscientiousness, this problem has been faced.

Soil pollution is due to several types of contaminants (Singh *et al.*, 2009). The organics include alkenes, monoaromatics, chlorinated hydrocarbons, monocyclic and polycyclic aromatic compounds. These are usually present in the environment as complex mixtures. Organic contaminants occur in petroleum refineries, petrochemical plants, gas stations and wood preservative industries. Halogenated pollutants are potentially found in chemical manufacturing plants, pesticide/herbicide treated areas, marine sediments and landfills. Explosive contaminants such as TNT, DNT, RDX are found in military areas, marine sediments and landfills. Soils polluted by heavy metals include battery disposal areas, chemical disposal areas, mining sites and marine/river sediments. The release of xenobiotics into the soil environment can affect its properties, the biodiversity and alter its ecological function. The recovery to non-contaminated soils is tough and sometimes not achieved (Kostelnik and Clark, 2008).

The European Environmental Agency recently reported approximately 250,000 sites that are in need of remediation due to risks for humans and ecosystem health (EEA, 2007). The National Plan for Polluted Soils Recuperation in Spain (1995-2005) illustrated the magnitude of soil contamination in Spain. The Royal Decree RD 9/2005 listed the activities susceptible of causing soil pollution, provided a methodology to characterize polluted sites as well as established the reference levels in soil for most pollutants. At least, 455 sites were classified as polluted in

Catalonia (ARC, 2005). At present, hydrocarbons derived from petroleum are the most common soil contaminants in Catalonia (Solanas *et al.*, 2009). These are mostly released in soil due to combustion, non-proper disposals and/or petroleum transformation processes. They occur in mixtures particularly at high concentrations in industrial sites associated to gas and petroleum production as well as wood-preserving industry. By definition, these compounds are associated to areas near industrial activities. Particularly, polycyclic aromatic hydrocarbons (PAHs) are a unique class of recalcitrant organic pollutants. PAHs present two or more benzene-fused rings. They can be formed when organic materials are burned and during incomplete combustion of petroleum and/or coal. PAHs present affinity for organic fractions of sediments, soil and biota. They accumulate along the food chain. Taking into account their carcinogenic and toxic properties, are listed as priority pollutant by the Environmental Protection Agency (EPA) of the United States as well as the European Environmental Agency (EEA).

1.2 Soil remediation treatments

When soil pollution occurs, the contaminant is distributed in its different components: solid, water, gas and biota. The final fate of pollutants depends on its physico-chemical properties as well as soil characteristics (Alexander, 1994). At the same time several processes can take place such as: evaporation, dissolution, dispersion, bioaccumulation, adsorption, chemical reaction, biodegradation and photooxidation (Solanas *et al.*, 2009). These aspects along with the quantity of pollutant, aging status and environmental conditions have to be taken into account when selecting the optimal remediation treatment.

The international remediation market is estimated to be around US$ 25-30 billion (Singh *et al.*, 2009). Soil remediation processes may be implemented using a variety of different engineered configuration, most of them solid-phase technologies. Traditional treatment of polluted sites focused on pollutant removal and consisted essentially in removing the affected layer by direct excavation and further disposal in barrier-isolated sites. Current treatments focus on pollutant removal or transformation to less toxic compounds (USEPA, 2004). They can be divided in two groups:

- *Ex situ*: the soil is withdrawn from the site to a treatment plant. If this is located near the site it is named *on site* and if this is elsewhere is called *off site*. Once it has been decontaminated it is returned to the site.

- *In situ*: the soil is treated directly in the site without any excavation. It allows the recovery and the support of activities on surface. These are usually low-cost techniques that depend mainly on physico-chemical and geologic characteristics of the soil. Also, a wider range of technologies has been developed for this type of treatments; all of them allow short recovery periods and homogenous remediation.

Before selecting the optimal technique for remediation, it is necessary to determine the soil properties as well as the concentration and properties of the pollutant, as listed in table 1.1. This information will facilitate the selection of a proper treatment.

Moreover, it is necessary to determine the microbiological activity in soil if bioremediation is willing to be applied. It is important to know whether degradation can occur naturally. Identification and quantification of indigenous microorganisms give an idea of bioremediation feasibility. The general assumption stands that the microbial diversity is proportional to the catabolic potential.

Table 1.1 Parameters affecting availability and transport of the contaminnats in soil (adapted from Solanas *et al.*, 2009)

Pollutant properties	Soil parameters
Solubility	Holding capacity
Vapor pressure	Compactness
Functional groups	Clay and organic matter content
Polarity	Granulometry
Density	Structure
Partitioning coefficients (K_{ow} and K_{oc})	Porosity, density and permeability
Electronegativity	pH

According with this, a *decision tree* was purposed by Solanas and co-workers (2009). As presented in figure 1.1, it may help to select the most efficient technology among the different available ones. Additionally, native soil population degrading capacity can be enhanced by means of adding nutrients; also adding external microorganisms. The limits in biodisponibility can also be overcome adding surfactants.

Successful treatment of a contaminated site depends on proper selection, design and adjustment of remediation technology. Its operation must be based on soil and pollutant properties to achieve acceptable performance (Khan *et al.*, 2004). A

site may require a combination of several procedures to allow the optimal remediation to achieve regulation levels; so biological, physical and chemical technologies may be used in conjunction (Reddy *et al.*, 1999). In addition, an economical overall evaluation is required to ensure that the technique is competitive respect other alternatives. Recently, life cycle assessment (LCA) has been employed for selecting optimal site remediation technique (Suer *et al.*, 2004; Cadotte *et al.*, 2007).

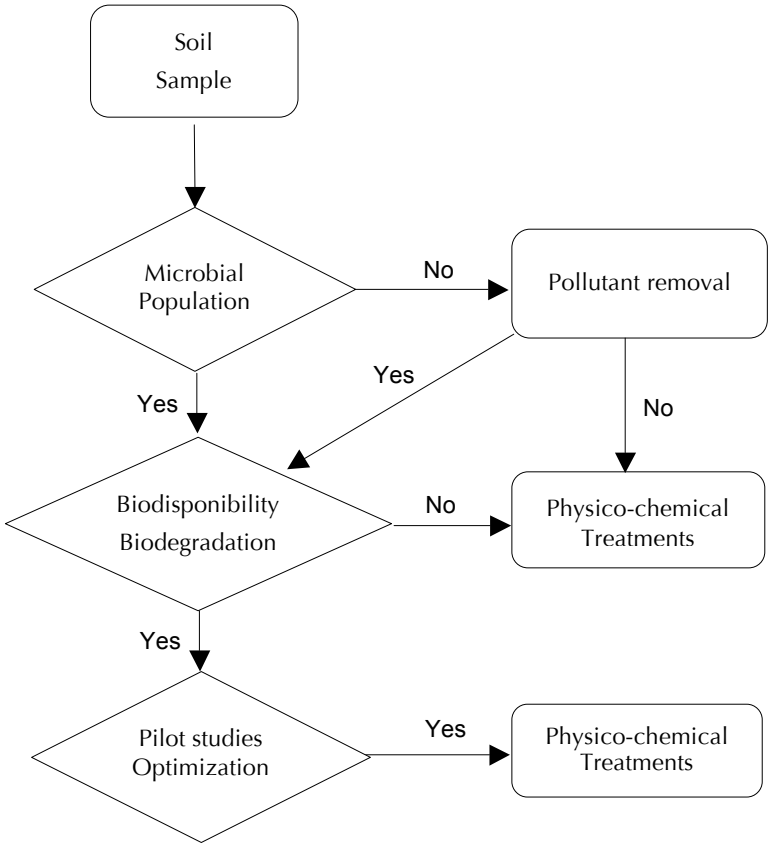


Figure 1.1 Decision tree for the selection of the optimal soil remediation treatment depening on site particularities (adapted from Solanas *et al.*, 2009).

1.2.1 Thermal treatments

Thermal treatments use heat to separate, destroy or immobilize soil contaminants. Generally, they offer rapid cleanup times but are greatly costly due to energy consumption, soil excavation, transport and equipment.

1.2.1.1 Soil Vapor extraction (SVE)

Soil vapor extraction (SVE), also known as soil venting or vacuum extraction is an accepted and cost-effective *in situ* technology for removing volatile (VOCs) and semivolatile (SVOCs) contaminants from unsaturated soils (Halmemies *et al.*, 2003). SVE requires the installation of vertical and/or horizontal wells in the polluted site. Air blowers are used to aid the evaporation process. The volatile constituents are extracted from soil though an extraction well (see figure 1.2). The extracted vapors are then treated properly before discharging them into the atmosphere (USEPA, 1998a). SVE is applicable in relatively permeable and homogeneous soils, and is a successful technology to treat light fraction-petroleum products such as gasoline for sites with a groundwater table located less than 1 meter below the land surface. Ideally, the site should be covered to minimize air infiltration.

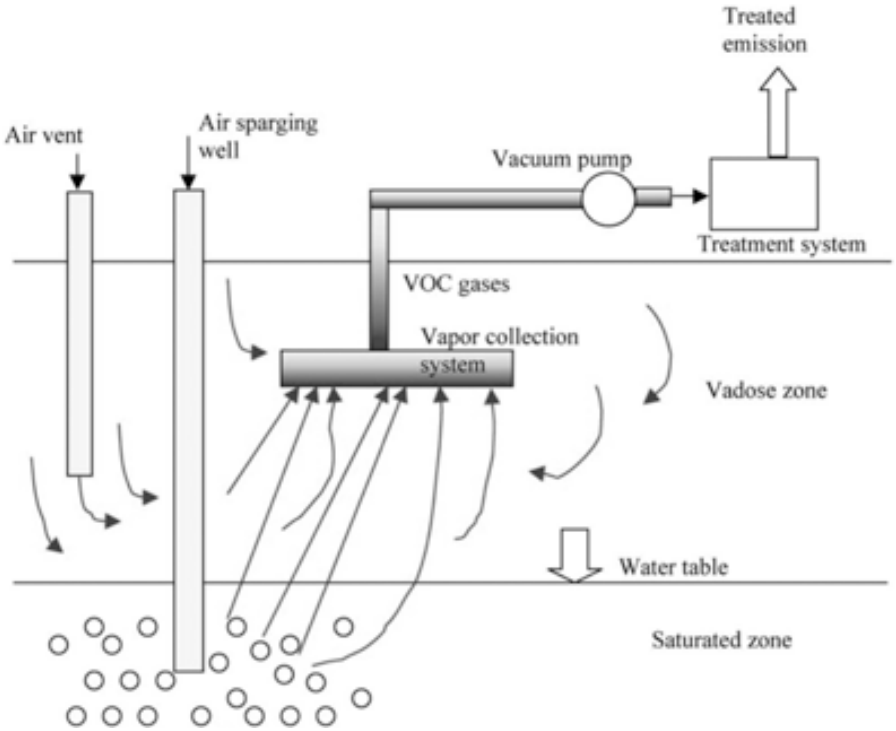


Figure 1.2 Solid Vapor extraction process diagram (Khan *et al.*, 2004).

Radio frequency heating (RFH) is an *in situ* process employed to enhance SVE. It uses electromagnetic energy to heat soil. RFH technique heats a discrete volume of soil using vertical electrodes "implanted" in soil. This procedure can heat soils to over 300°C, favoring contaminant desorption.

1.2.1.2 Solidification/Stabilization

Solidification/stabilization refers to waste fixation in order to confine and to reduce mobility of pollutants through soil. Thus encapsulating the pollutant in a solid structure and reducing its possible toxics effects (FRTR, 1999a). This technology is applied mostly to soils contaminated with heavy metals and inorganic compounds (Khan *et al.*, 2004); although it is feasible for volatile organic compounds (Druss, 2003).

Asphalt batching is a method for treating hydrocarbons-contaminated sites. It consists in mixing the soil with hot asphalt mixtures. This mixture is used for paving and reduces mobility of water-soluble pollutants. The initial heating can lead to high volatile hydrocarbons loss (Asante-Duah, 1996).

Vitrification uses elevated amounts of energy to "melt" soil at 1,600-2,000ºC, immobilizing most inorganic and destroying organic pollutants (Hamby, 1996; FRTR, 1999a). The final product is a glass and crystalline material similar to obsidian or basalt rock, which retains the pollutant in its crystalline structure. The high temperatures can lead to volatile products losses and formation of by-products. Three different types of vitrification exist: electrical processes (Acar and Alshawabkeh, 1993), thermal processes (Wait and Thomas, 2003) and plasma processes (Suthersan, 1997).

1.2.1.3 Thermal desorption

Thermal desorption is an *ex situ* treatment in which soil is excavated and heated to remove pollutants (USEPA, 1995). Soil is heated (100-600ºC) so that contaminants are evaporated, separated from soil and then collected with an special equipment to be treated by proper means (USEPA, 1996a). It is important to highlight that this treatment does not aim to destroy the pollutant but rather change its form into a treatable one. This technology is suitable for hydrocarbon-contaminated sites, allowing treating also PAHs (FRTR, 1999b). The process effectiveness can be affected due to water excess and soil composition.

1.2.1.4 Incineration and Pyrolisis

Incineration is a technology focused on pollutant removal by burning soil in presence of oxygen; and can also be applied to other liquid and solid matrixes (FRTR, 1999c). The process aims to destroy the pollutant at temperatures that range from 870-1,200ºC (Hamby, 1996). Non-proper incinerated wastes can arise

from incomplete combustions leading to the formation of dioxins, furans or other like-products. Tuning the operation can minimize these setbacks. Commonly, incinerated soils cannot be placed back on site.

Pyrolysis consists on heating a soil, at operating temperatures above 430ºC, in absence of oxygen and under pressure. Like in the former case, the formed gases may need further treatment; they can be used as combustible in a later step of combustion to recover the accumulated energy. 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 or any waste rich in carbonous material.

1.2.2 Physico-chemical treatments

These technologies are based on the pollutant extraction, as a result of a physico-chemical or washing treatment. Pollutants are transferred from soil to gas or liquid phase and then appropriately treated. Alternatively, pollutants may be confined to avoid mobility through soil.

1.2.2.1 Soil venting/aeration

Soil venting/aeration is an *in situ* technology that evaporates volatile components in the vadose zone of soil into the air (Khan, 2004). VOCs, especially volatile compounds of petroleum, are target compounds (FRTR, 1999d). The collected gases require further treatment.

1.2.2.2 Soil washing

Soil washing is an *ex situ* technology that uses liquids and mechanical process to scrub polluted soils (Urum *et al.*, 2003); and is mostly considered as a pre-treatment as it is combined with other technologies. It separates fine soil from coarse soil. The effectiveness of the process can be enhanced by means of surfactants and co-solvents (Chu, 2003; Rodríguez, 2010). Petroleum hydrocarbons, fuel residuals and PAHs are target compounds for this technology (FRTR, 1999e).

1.2.2.3 Soil flushing

In situ soil flushing extracts the contaminants with water or other aqueous solutions to an area where they can be removed (Di Palma *et al.*, 2003). This is accomplished by means of passing an extraction fluid through soil using injection or infiltration process (see figure 1.3). Extracted water with the desorbed contaminants must be treated, achieving discharge standards before being recycled or released (Son *et al.*, 2003). As soil washing, this technology is usually employed as pre-treatment technology. Since it is conducted in situ, it reduces the total operating costs. The target contaminants are VOCs, SVOCs, fuels and pesticides (FRTR, 1999f).

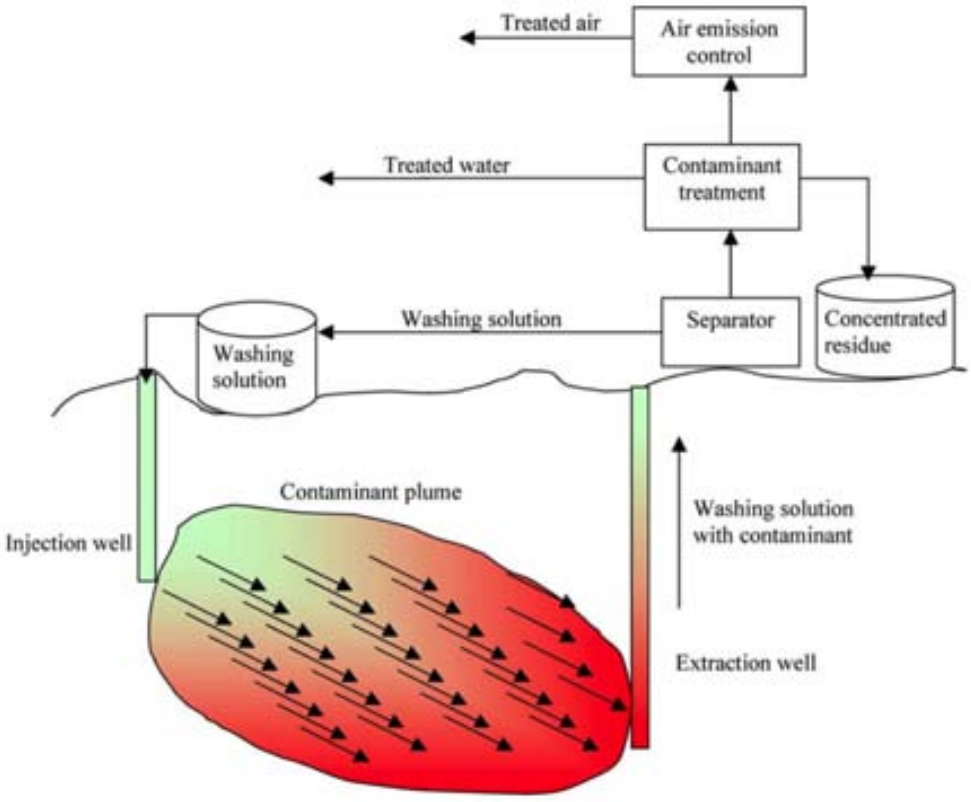


Figure 1.3 *In situ* soil flushing process scheme (Khan *et al.*, 2004).

1.2.2.4 Encapsulation

Encapsulation is a technology based on the physical isolation and containment of the polluted soil. In this technique soils are isolated with low permeability or cut-off walls (Khan *et al.*, 2004). The contaminated area is also covered with low permeability materials, thus preventing leaching or migration of contaminants into groundwater (Robertson *et al.*, 2003).

1.2.2.5 Passive/reactive treatment walls

The passive/reactive treatment walls comprise a sort of *in situ* technologies for site remediation. Treatment walls are structures installed underground to treat groundwater (Suthersan, 1997; Baciocchi *et al.*, 2003). As contaminated groundwater passes through the treatment wall remediation occurs (USEPA, 1996b). The specific filling for the wall is based on the site contaminant. Three main types of treatment walls are employed (Kahn *et al.*, 2004) as described next. *Sorption barriers* remove the pollutant by adsorption to active surfaces, usually zeolite or activated carbon (Woinarski *et al.*, 2003). *Precipitation barriers* contain filings that turn pollutants into insoluble products and trap them (Birke *et al.*, 2003). *Degradation barriers,* filled with nutrients, which stimulate the degrading capacity of the nearby microorganisms (Guerin *et al.*, 2002). This technology is suitable to treat VOCs, SVOCs and even some petroleum hydrocarbons (Guerin *et al.*, 2002).

1.2.3 Biological treatments: Bioremediation

Bioremediation includes all technologies that rely on microorganism and plant activity to degrade environmental contaminants to appropriate concentrations or transform them into less toxic forms. In some cases, microorganisms are capable to transform pollutants into CO_2 and H_2O. Several technologies have been developed to remove soil pollutants successfully since 1970s in the USA; and more recently in several European countries. The Catalonian Residues Agency promoted the use of this sort of techniques in recent years (Solanas *et al.*, 2009).

Most technologies focus on enhancing the existing slow biodegradation processes. Soil bacteria and fungi are responsible for detoxification. These may be indigenous or can be isolated somewhere else and brought to the site. Pollutants are transformed by means of metabolic processes of single species or as result of synergistic effects among multiple organisms. Most techniques run under aerobic conditions, but also anoxic/anaerobic conditions permit degradation.

It is important to consider several factors before choosing the most appropriate bioremediation technology for treating a site. Environmental factors as presence of electron acceptors, moisture, temperature, pH and nutrients content influences biodegradation rate capacity of soil microorganisms (Van Hamme *et al.*, 2003). Also the concentration, aging and physicol-chemical properties of the

contaminant affect the biodisponibility. In addition, soil characteristics, like texture, permeability and structure can affect the process effectiveness.

When a bioremediation project has been designed and executed, it is imperative to assess that the toxicity has been lowered and that the site has recuperated its initial biological activity and productivity (Andreoni and Gianfreda, 2007).

Bioremediation is considered a good alternative to thermal and physico-chemical clean-up techniques. The major reason is its lower cost, as it is less energy consuming. Apparently, it is a versatile technology applicable to a broad variety of pollutants. As it can be carried out *on site*, it does not interrupt human activities, which makes this positively perceived by the public. The most common technologies are listed next.

1.2.3.1 Natural attenuation (NA)

Natural Attenuation (NA) is also known as passive remediation, bioattenuation or intrinsic bioremediation. This technology uses natural processes to reduce pollutant content in sites. NA processes are often categorized as destructive and non-destructive depending on the contaminant reduction way (USEPA, 1996c). Traditionally, NA is known as "do nothing" approach to site clean-up; but on the contrary, NA is a proactive approach that focuses on the verification and monitoring of natural remediation processes rather than relying on engineered processes (Khan and Husain, 2003). Before deciding to apply this technique it is necessary to estimate effectiveness of natural processes. NA can reduce pollutant concentration by means of binding processes, biodegradation processes or simple dilution. The compounds suitable for NA treatment include petroleum hydrocarbons, wood treating wastes, chlorinated aromatic and aliphatic compounds (Boulding, 1996).

1.2.3.2 Bioventing

The bioventing process infuses air inside soil polluted-matrix at a specific rate in order to maximize *in situ* biodegradation and reduce the transfer of volatile pollutants to the atmosphere. This technique is applicable to the non-saturated soil layers (see figure 1.4). Extracted gases may require treatment as they contain removed volatile compounds. Compared to SVE, bioventing requires less amounts of oxygen to achieve successful removal.

Aerobic bioventing is designed to degrade less volatile compounds from petroleum as VOCs and SVOCs (FRTR, 1999g; USEPA, 1998b). It is only effective in unsaturated soils and can be coupled to other technologies.

Anaerobic bioventing injects nitrogen and an electron donor into soil, instead of air, to produce reducing conditions. These facilitate microbial dechlorination of chlorinated pollutants.

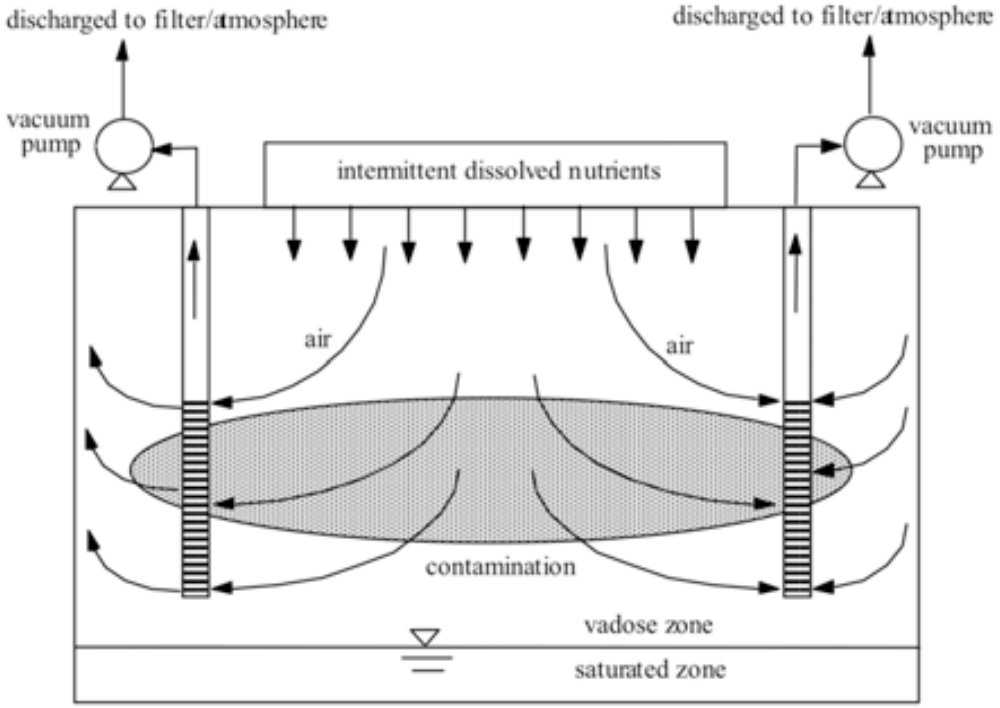


Figure 1.4 Bioventing process scheme (Irvine and Frost, 2003).

1.2.3.3 Biosparging

Biosparging involves the injection of atmospheric gas, under pressure, into the saturated zone of soil to volatilize groundwater pollutants and to promote biodegradation. The air replaces pore water, volatilizes contaminants, and exits the saturated zone into the unsaturated one, creating channels through the pollution plume as it goes upwards (see figure 1.5). This way, the contaminants are transported to the non-saturated zone where they can be degraded or extracted for further treatment. (Kirtland and Aelion, 2000). This technology permits groundwater clean up without the need of pumping; and is often coupled with soil vapor extraction to avoid losses. The target contaminants of this technology include gasoline, fuel components, BTEX and chlorinated solvents (GWRTAC, 1996; USEPA, 2004).

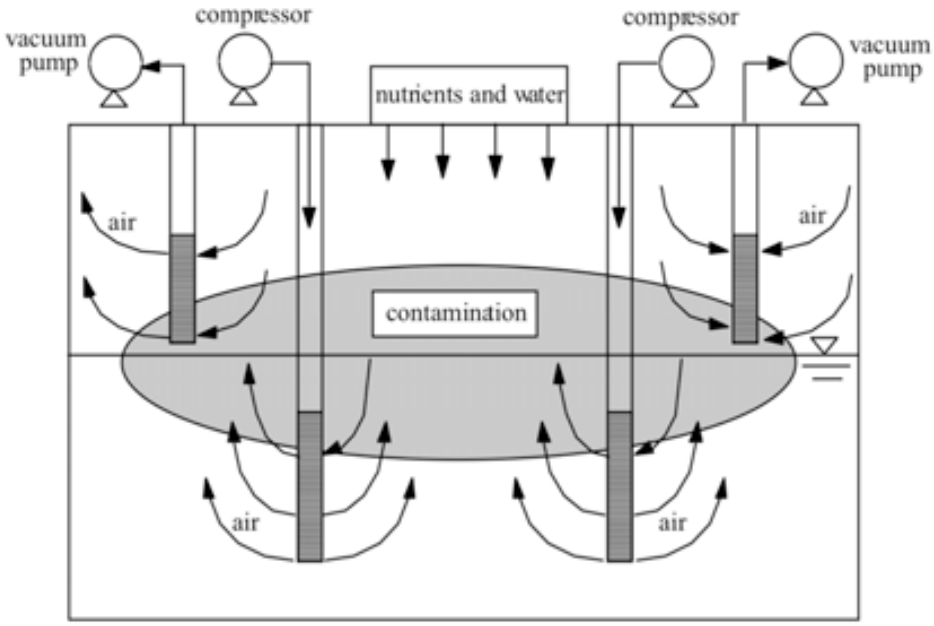


Figure 1.5 Biosparging process scheme (Irvine and Frost, 2003).

1.2.3.4 Bioslurping

Bioslurping is a relatively new technology for *in situ* remediation that combines elements of bioventing and vacuum-enhanced pumping to recover free product from groundwater and soil while promoting aerobic remediation (FRTR, 1999h). The system consists of wells into which an adjustable length 'slurp tube' is installed. This tube is lowered into the light non-aqueous phase liquid (LNAPL) layer, connected to a vacuum pump. It removes all free products along with groundwater (Khan *et al.*, 2004). This technology is limited to 7-8 meters below ground surface. The LNAPL and vapors extracted must be properly treated. It is a technique that enhances intrinsic in situ natural bioremediation in the non-saturated soil layer. It is not suitable for low porosity soils.

1.2.3.5 Phytoremediation

Phytoremediation involves the use of higher plants that, directly or indirectly, result in the clean-up of contaminated sites (USEPA, 1996d; Rai and Singhal, 2003). This technology includes process that may involve uptake or accumulation of the contaminant by the plant and biodegradation by microorganisms colonizing the root or the soil immediately (Cunningham *et al.*, 1996). A major advantage of this technique is its low cost compared to many other biological treatments. However, phytoremediation is a slow technique. It is believed to offer efficient removal for sites where the contamination is near the surface, between 1

to 2 meters. It is assumed that this technology is not suitable for aged and/or sorved pollutants. The basic phytoremediation techniques are: rhizofiltration, phytoextraction, phytotransformation, phytostimulation and phytostabilization (Barter, 1999). The types of compounds that are likely to be removed by phytoremediation are broad: heavy metals, chlorinated compounds, petroleum hydrocarbons, PAHs, insecticides, explosives and surfactants (Nedunuri *et al.*, 2000).

1.2.3.6 Landfarming

Landfarming is an aboveground technology that reduces the concentration of petroleum aerobically degradable constituents presents in soils favoring intrinsic bioremediation (Kahn *et al.*, 2004). This technique involves the spreading of excavated soil in a thin layer (less than 1.5 meters) in the site ground surface (see figure 1.6). Subsequent stimulating of aerobic microbial activity is achieved by means of aeration and/or nutrients addition (USEPA, 1998c).

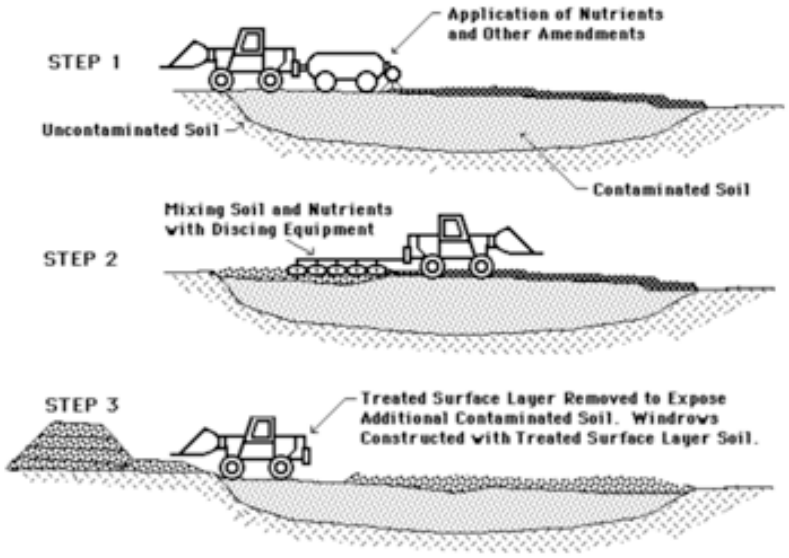


Figure 1.6 Landfarming process scheme (adapted from Irvine and Frost, 2003).

The soils required being periodically tilled, and moisture is added when needed (figure 1.6). The performance of landfarming differs upon the contaminants to be treated. This technology is useful for treating lighter petroleum hydrocarbons (Riser-Roberts, 1998; FRTR, 1999i); and thus it has been employed by the petroleum industry to treat polluted sites. However, contaminants that are difficult to degrade, such as heavier petroleum hydrocarbons, PAHs, pesticides, or chlorinated organic compounds, are still subjects of research.

1.2.3.7 Biopiles

Biopile is the name of the technology that involves the pilling of contaminated soils into piles or heaps and then stimulating aerobic microbial activity by means of adding nutrients, minerals and moisture (USEPA, 1998d; Jorgensen *et al.*, 2000). This technique can also be named biocell, bioheaps, biomounds, compost cells, heap pile bioremediation and static-pile composting (USEPA, 1998d; FRTR, 1999j). Biopiles are typically 2–3 meters high and might be covered to prevent runoff, evaporation, volatilization and promote solar heating (Kahn *et al.*, 2004). Heat and pH can also be controlled to enhance biodegradation. The air supply structure is covered by the soil as the biopile is assembled (Li *et al.*, 2002). Oxygen exchange can be accomplished utilizing vacuum or forced air (see figure 1.7). Low air-flow rates are required to minimize pollutant loss by volatilization. Target pollutants of this technology include: most petroleum products, non-halogenated VOCs, halogenated VOCs and pesticides (FRTR; 1999j). Light petroleum compounds tend to evaporate and the heavier to be biodegraded (Chaineau *et al.*, 2003).

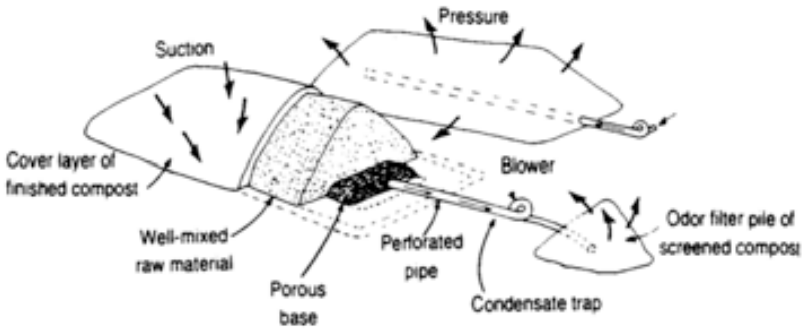


Figure 1.7 Biopiles process scheme (adapted from Crawford and Crawford, 1996).

1.2.3.8 Composting

In composting technology soil is dug out from site and mixed with bulking agents and organic amendments. The major difference with respect to biopiles is that it operates under thermophilic conditions (40–50°C) due to microbial activity. Oxygen content, humidity level and temperature are monitored during the process and can be changed to optimize pollutant removal. Pollutants treated by such technique include: petroleum hydrocarbons, persticides, PAHs, herbicides and explosives (USEPA, 1997; USEPA, 2004).

1.2.3.9 Slurry bioreactors

Slurry bioreactors are an *ex situ* technology employed for soil, sediments, sludge, and other solid or semi-solid wastes pollutant remediation. This technique requires excavation of contaminated soil from site that is treated in a controlled bioreactor where the slurry is mixed to keep solids suspended (Kahn *et al.*, 2004). Nutrients, neutralizing agents, surfactants, and co-metabolites may be added to increase microbial degradation rates. Indigenous soil-microbes may be used or microorganisms may be added at the outset to seed the bioreactor or may be added continuously to preserve proper biomass levels. Generally, degradation occurs at a rapid rate compared to the former technologies, ranging from 1 to 6 months (RAAG, 2000). Once the process is completed, the slurry is dewatered and soil is replaced (FRTR, 1999k). This type of reactors is successful at treating: non-halogenated VOCs and SVOCs, pesticides, petroleum hydrocarbons and PCBs (FRTR, 1999k; Kuyukina *et al.*, 2003; Machín-Ramírez *et al.*, 2008). In addition, soils can also be treated in bioreactors that are either fixed film or stirred tank types (Wilson and Jones, 1993), operating at continuous or batch mode.

1.2.3.10 Enhanced biodegradation

Enhanced biodegradation is an *in situ* technology that increases the rate of pollutant removal by means of soil microorganisms (FRTR, 1999l). When removal is achieved by means of nutrients addition and electron acceptors into soil that improve degrading-microbial populations ability, this approach is called *biostimulation*. Oxygen is the main electron acceptor under aerobic conditions, while nitrate acts as electron acceptor under anoxic conditions. When native soil microorganisms are not capable to carry out degradation and external pre-grown degradative-microbial populations have to be added into soil, this approach is named *bioaugmentation*. As with other *in situ* biodegradation processes, the success of this technology is highly dependent upon soil properties and biodegradability of pollutants. Target contaminants for this technique include: non-halogenated VOCs, non-halogenated SVOCs and fuels (FRTR, 1999l).

1.3 Mycoremediation by white-rot fungi

Most studies on bioremediation have focused on bacteria as degraders because of their rapid growth, even employing the pollutant as unique substrate. The capacity of fungi to transform and mineralize a wide range of pollutants without a pre-conditioning period via co-metabolic pathways makes them interesting for the degradation of recalcitrant xenobiotics. The term mycoremediation (Singh, 2006) encloses the branch of science studying fungi as xenobiotic degraders.

Fungi play an important role in the environment as decomposers of cellulose, hemicelluloses and lignin (Kirk and Fenn, 1982). Among them, the group of white-rot fungi comprises the most active lignin degraders (Hammel, 1997). Kirk and Farrel (1987) referred to lignin degradation by white-rot fungi as "enzymatic combustion". In a seminal paper, Bumpus *et al.* (1985) described the white-rot fungus *Phanerochaete chrysosporium* as responsible for the degradation of several groups of pollutants. Ever since, numerous fungi have been used for the degradation of a broad spectrum of contaminants, both in liquid and solid state.

The principal relevance of ligninolytic fungi for bioremediation purposes lies in their ability to degrade aromatic and derivative compounds such as PAHs, BTEX, synthetic dyes, PCPs, nitroaromatics, pesticides and chlorinated aromatic compounds (Gadd *et al.*, 2001; Pointing, 2001; Singh, 2006; Gao *et al.*, 2010). The mode of attack on pollutants is normally oxidative, leading to biodegradation. It can also occur complete mineralization to CO_2 or polymerization producing conjugates.

The white-rot basidiomycetes present an extracellular oxidative system employed in the primary attack of lignin and posterior mineralization (Martinez *et al.*, 2005). Lignin degradation involves non-specific and non-selective mechanism. This system includes the enzymes manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase, described in detail in next section. The reactions that they can catalyze include lignin polymerization and dimethoxylation, decarboxilation, hydroxylation and aromatic ring opening. The extracellular enzymes generate diffusible oxidizing agents that attack the molecules, many of these being activated oxygen species (Bumpus *et al.*, 1985; Collins *et al.*, 1996). This capacity represents an advantage respect bacteria as it prevents the need to internalize the pollutant inside the cell, thus avoiding toxicity problems. Moreover, it permits to attack low-soluble compounds. Their hyphae enter the polluted matrix and secrete the ligninolytic enzymes. Whilst mobility of bacteria in soil is limited.

Likewise, most ligninolytic fungi incorporate also an intracellular degradative system involving cytochrome P450 monooxigenase-epoxide hydrolase. This pathway is present in eukaryotic organisms. It regulates normally the conversion of hormones. In white-rot fungi it has been revealed that it can cooperate with the ligninolytic system in the degradation of PAHs (Bezael *et al.*, 1996), TCE/PCE (Marco-Urrea *et al.*, 2008) and other xenobiotics.

One of the key points in bioremediation by white rot fungi is to put the active organism in contact with the pollutant. Although white-rot fungi are ubiquitous in nature, it should be considered their ecological niche as wood degraders. Accordingly, soil is not a favorable environment for them. Before introducing them into soil, some lignocellulosic co-substrate is needed to guarantee their proper colonization and degrading capacity.

The capacity of fungi to grow on soil can be affected by its interaction with native microorganisms (Baldrian, 2008). Depending on the specie it can affect negatively, as for *P. chrysosporium* (Radtke *et al.*, 1994), or have synergic effects, as the case of *P. ostreatus* (Grams *et al.*, 1999). Other factors affecting the growth of fungi into soil are the lack of nutrients or unfavorable pH and temperature (Tekere *et al.*, 2001).

Singh (2006) foresaw three phases for the successful implementation of mycoremediation in polluted matrices. First of all, appropriate techniques for the proper inoculum production, inoculation and growth of the fungi into soil should be established. The second phase includes the determination of protocols for monitoring the process in soil; studying the degradation capacity of the organism under sterile conditions both in liquid and solid culture and the identification of possible metabolites. The later phase consist in studying the interaction or competition with native microorganisms during the bioremediation process and determine the treatment effectiveness.

1.4 Lignin modifiying enzymes (LMEs)

White rot fungi secrete mainly two different groups of ligninolytic enzymes, laccases and peroxidases, named MnP and LiP. The main difference is the electron acceptor; being O_2 for laccases and H_2O_2 in the case of peroxidases. Laccases are a homogeneous group in terms of specificity for substrate and function, while other peroxidases present a major variability. During lignin degradation they act synergistically.

Lignin modifying enzymes produced by white-rot fungi can be classified in three groups (Leonowicz *et al.*, 2001). The first one includes enzymes acting on cellulose, hemicellulose and lignin, which are known as ligninolytic modifying enzymes (LMEs). The second group comprises enzymes that cooperate in the attack of lignin but cannot degrade it directly. The last group is the so-called feed back type enzymes that combine metabolic chains during biodeterioration of wood. All these enzymes can occur together during both biodeterioration and biodegradation processes.

Different white-rot species produce various combinations of the main lignin degrading enzymes: LiP, MnP and laccase. According to its production, white-rot fungi can be grouped in three different groups (Canet *et al.*, 1999): LiP-MnP group, like *P. crysosporium*; MnP-laccase group, including *Dichomitus squalens*, *Ceriporiopsis subvermispora*, *Pleurotus ostreatus, Lentinus erodes* and *Panus tigrinus*; Lip-laccase group, like *Phlebia ochraceofulva*. Hatakka (1994) purposed an additional group, the laccase-aryl alcoholoxidase group.

The production of ligninolytic enzymes takes place during secondary metabolism. The lignin degrading system is induced when starvation of C or N occurs. The secretion pattern is specie-dependent. Moreover, agitation and temperature can significantly affect the levels of these enzymes.

1.4.1 Laccase (Lac)

Fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper blue phenoloxidases. It is a glycosilated protein expressed in multiple forms and shows high molecular weight variability, ranging from 59 to 110 kDa. Nearly all white-rot fungi produce it. Laccase is expressed as multiple isoenzymes being both constitutive and inductive. It can be intracellular and/or extracellular. In some fungi are associated with fungal cell walls (Zhu *et al.*, 2001; Svobodová *et al.*, 2008).

The enzyme contains four copper atoms (Cu), in different states of oxidation (I, II, III), implied in the catalytic cycle (Thurston, 1994). These ions play an important role in the catalytic mechanism. Laccase oxidizes different compounds while reducing O_2 to H_2O, a total reduction of four electrons. It presents low specificity to electron-donating substrates.

The catalytic cycle of laccase (see figure 1.8) comprises several one-electron transfers between the copper atoms while O_2 is bound to the active enzyme site, similar to that of ascorbate oxidase (Messerschmidt *et al.*, 1989). With this mechanism, laccases generate phenoxy radicals that undergo non-enzymatic reactions (Thurston, 1994). Multiple reactions lead finally to polymerization, alkyl-aryl cleavage, quinone formation, C_α-oxidation or demethoxylation of the phenolic reductant (Fakoussa and Hofrichter, 1999).

T. versicolor laccase may oxidize non-phenolic aromatic substances in the presence of appropriate mediator substances (Johannes and Majcherczyk, 2000a), thus similar to LiP activity. These mediators can be natural or synthetic. *Trametes* laccase also produces Mn(II) chelates in presence of Mn(II) and appropriate phenolic substances (Roy and Archibald, 1992). The ability to oxidize Mn(II) to Mn(III) indirectly has been proposed to be a partly autocatalytic process involving the enzyme-catalyzed formation of phenoxyl radicals and spontaneous reactions to form superoxide ($O_2^{\cdot-}$) (Archibald and Fridovich, 1982).

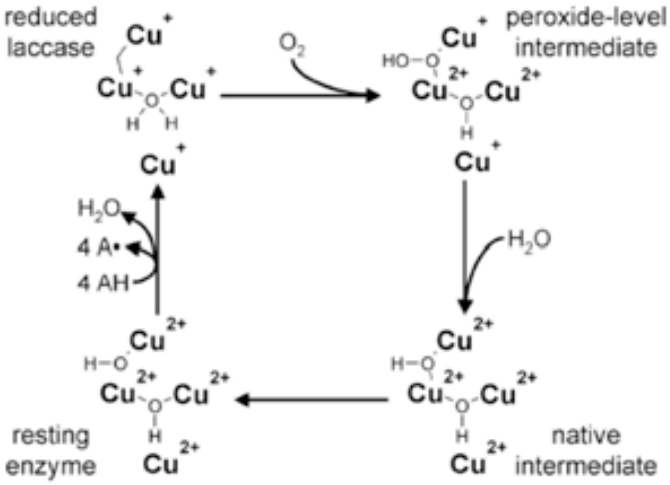


Figure 1.8 Catalytic cycle of laccase (Wesenberg *et al.*, 2003).

The yellow laccases were discovered in solid-state fermentations of *Panus tigrinus* and *Panus radiata* (Leonievsky *et al.*, 1997). This type of laccase is able to oxidize non-phenolic compounds even in absence of mediators due to a change in copper oxidation state. Fakoussa and Hofrichter (1999) firstly reported the yellow laccase in solid-state cultures of *Trametes versicolor*.

The exact mechanism of laccase in PAHs degradation is not completely understood. Majcherczyk *et al.* (1998) reported that laccase of *T. versicolor* is capable to oxidize 14 PAHs leading to the formation of quinone products.

Moreover, laccases are involved in other fungal processes such as development of fruit bodies, pigmentation and interaction with pathogens (Thurston, 1994; Baldrian, 2004,2006).

1.4.2 Manganese peroxidase (MnP)

The enzyme manganese peroxidase (MnP, E.C. 1.11.1.13) was discovered in *P. chrysosporium* (Kuwahara *et al.*, 1984) and has been described in several basidiomycetes (Fakoussa and Hofrichter, 1999). It is an extracellular, glycosylated heme-containing peroxidase (Paszcynsky *et al.*, 1985). The enzyme presents multiple isoforms ranging from 38 to 50 kDa.

MnP catalyses an H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}, similar to that described for LiP. However, significant differences appear in the reductive reactions. Mn^{3+} are stabilized via chelation with organic acids like oxalate, malonate, malate, tartrate or lactate (Wariishi *et al.*, 1992). Chelated Mn^{3+} operates as diffusible redox mediator that oxidizes phenols, methoxylated aromatics and chloroaromatics (Fakoussa and Hofrichter, 1999). MnP is sensitive to elevated concentrations of H_2O_2 that can cause inactivation of the enzyme by forming compound III. The reaction cycle is shown in figure 1.9.

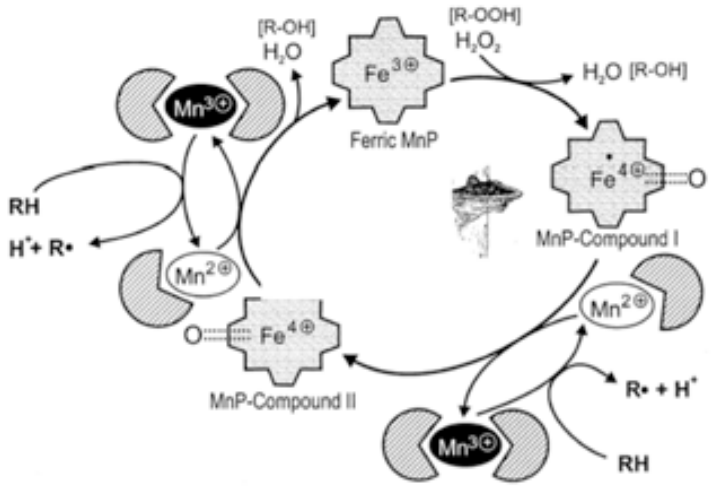


Figure 1.9 Catalytic cycle of manganese peroxidase (Hofrichter, 2002).

The advantage of MnP system compared to that of Lac is its action via the real redox mediator couple Mn^{2+}/Mn^{3+} that is a diffusible oxidative agent. The oxidative strength of the MnP system can be considerably enhanced in the presence of additional co-oxidants such as thiols or lipids (Leonowicz *et al.*, 2001).

1.4.3 Lignin peroxidase (Lip)

Lignin peroxidase (LiP, E.C. 1.11.1.14) was the first ligninolytic enzyme discovered in the white-rot fungus *P. chrysosporium* (Glenn *et al.*, 1983; Tien and Kirk, 1983). Later it was also found in other basidiomycetes. LiP is a glycoprotein that contains iron protoporphyrin IX (heme) as a prostetic group and requires H_2O_2 for catalytic activity. It is expressed in multiple isoenzymes with molecular weights ranging from 38 to 47 kDa. It is capable of oxidizing recalcitrant non-phenolic lignin model substrates by one-electron abstraction to form reactive aryl cation radicals, cleaving C-C and C-O bonds (Kersten *et al.*, 1985). It is also capable of cleaving aromatic rings via initial one-electron abstraction and subsequent incorporation of oxygen.

LiP is secreted during secondary metabolism and stimulated under nitrogen limitation and after veratryl alcohol addition (Leonowicz *et al.*, 2001). It requires high oxygen levels but is suppressed by agitation. It is not present in the ligninolytic system of all white-rot fungi (Hatakka, 1994).

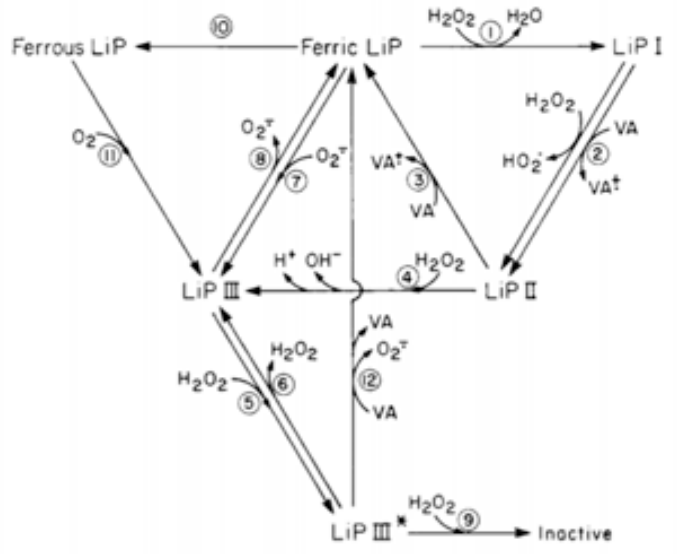


Figure 1.10 Catalytic cycle of lignin peroxidase (Wariishi and Gold, 1990).

The catalytic cycle is started when H_2O_2 oxidizes LiP to form a two-electron intermediate (LiP-compound I). This oxidizes substrates by removing one electron leading to the formation of another intermediate (LiP-compound II). The later can oxidize substrates by one electron completing the cycle. However, LiP-compound II can react with H_2O_2 with low substrate concentration, leading to the formation of an inactive form of the enzyme (LiP-compound III). A scheme of the complete cycle can be observed in figure 1.10.

1.4.3 Versatil peroxidase (VP)

Versatil peroxidase (VP, E.C. 1.11.1.46) was discovered in liquid cultures of *Pleurotus eryngii* growing on peptone as nitrogen source (Martínez *et al.*, 1996; Camarero *et al.*, 1999) and *Bjerkandera* sp. (Mester and Field, 1998). VP is able to oxidize both LiP and MnP substrates (Heinfling *et al.*, 1998). As its name indicates, this enzyme has a wider catalytic versatility for electron donors as compared to LiP and MnP. On the one hand, it can oxidize aromatic-phenolic compounds and non-phenolic, like LiP. On the other hand, it oxidizes Mn^{2+}, like MnP, even increasing the catalytic efficiency (Heinfling *et al.*, 1998). Thus, its catalytic cycle is constituted by the sum of the catalytic cycles of both LiP and MnP (Gómez-Toribio, 2006). The optimal pH for oxidation of Mn^{+2} (pH 5) and aromatic compounds or dyes (pH 3) differs, being similar to those of optimal MnP and LiP activity (Ruiz-Dueñas *et al.*, 2001).

1.4.4 Coupling enzymes

White rot fungi also possess different enzymes that are capable of reducing aromatic aldehydes and quinones (Muheim *et al.*, 1991; Roy *et al.*, 1996; Cameron and Aust, 2001; Gómez-Toribio *et al.*, 2001). The reduction of aromatic aldehydes is catalyzed by an intracellular enzyme called aryl-aldehyde reductase (aryl-alcohol:$NADP^+$ oxidorreductase, EC 1.1.1.91). In the reduction of quinones an extracellular enzyme can participate, named cellobiose dehidrogenase (celelobiose:acceptor 1-oxidorreductasa, EC 1.1.99.18) or an intracellular one, named quinone reductase (NAD(P)H:quinone-acceptor oxidorreductase, E.C. 1.6.99.2). Additionally to these enzymes, it has also been described the existence of trans-membrane redox systems that can reduce different compounds (Stahl and Aust, 1993). Finally, also cytochrome P450-mediated conversion steps are suggested to be a part of many specific fungal biotransformation processes; resembling metabolic pattern observed in mammalian cytochrome systems (Van den Brink *et al.*, 1998).

1.5 The white-rot fungus *Trametes versicolor*

The mushroom *Trametes versicolor* belongs to the family polyporaceae. Many different names have been used in the literature including *Agaricus versicolor, Boletus versicolor, Polyporus versicolor, Polystictus versicolor, Poria versicolor*, Yun-Zhi (Chinese), and Kawaratake (Japanese). It is commonly known as ''turkey tail'' mushroom or "bracketed-rot fungus".

Morphological characteristics of *T. versicolor* fruiting body (annual) have been described as follows: 3–5 cm across semicircular bracket, compacted, thin, and tough. Young brackets are flexible. Brackets usually occur in tiers and spread along branches. The upper surface is velvety and attractively marked with concentric zones of diverse colours: brown, yellow, gray, greenish, or black. The margin is usually wavy. Algae usually cover it, which confers green color. The mushroom has white spores that are oblong and cylindrical, which are not produced under laboratory conditions (Gerdhart, 2000).

T. versicolor is an obligate aerobic fungus commonly found year-round on dead logs, stumps, tree trunks, and branches. The fungus occurs all over the wooded temperate zones of Europe, Asia, and North America. It is supposed to be the most common shelf fungus in the northern hemisphere. It plays an important role as wood degrader and a minor as tree parasite.

Figure 1.11 *Trametes versicolor* fruit bodies (left) and grown in pelleted morphology (right).

In agitated submerged culture the fungus grows as dispersed or pelleted mycelium, but neither fruiting body nor spores are formed. More than 120 strains of *T. versicolor* have been accounted.

Commercial polysaccharopeptides PSK and PSP are obtained from the mycelium of the fungus to be employed as antitumor and immunomodulatory agents (Ünyayar *et al.*, 2006). In fact, this fungus was historically used in tradicional oriental therapies of cancer in Japan, China and Korea.

The ligninolytic system of the fungus is stimulated by nitrogen limitation. This is capable to produce lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (lac) (Fiechter *et al.*, 1995; Wesenberg *et al.*, 2003). Acebes (2008) studied the enzymatic expression of LMEs of the strain ATCC#42530 and determined that it is capable to secrete the three of them, but its expression depends on the medium composition. Laccase is the most characteristic enzyme and can be produced either constitutive or induced, maintaining a similar catalytic activity (Sariaslani, 1989). Additionally, the fungus enzymatic machinery includes cytochrome P450 that can be implied in degradation mechanisms (Ichinose *et al.*, 2002).

The strain ATCC 42530 is capable to degrade several pollutants such as: synthetic dyes (Blánquez *et al.*, 2004), TCE and PCE (Marco-Urrea *et al.*, 2006), endocrine disruptors (Blánquez and Guinesse, 2008), PCPs (Rodríguez-Rodríguez *et al.*, 2010a) and PAHs (Borràs *et al.*, 2010). There was no available information on the capacity of this strain to degrade PAHs before starting the thesis work.

Chapter 2

Objectives

The main objective of the present thesis is to **evaluate *Trametes versicolor* ability to biodegrade Polycyclic Aromatic Hydrocarbons (PAHs) in different approaches in order to develop a process to bioremediate creosote-polluted soils**. This general objective is divided in more specific goals:

- To develop a low-cost methodology to produce large amounts of *T. versicolor* biomass in submerged cultures: media formulation, bioreactor selection and scale-up.

- To select an optimal lignocellulosic substrate for soil bioaugmentation of *T. versicolor* in terms of viable biomass, enzymatic activity and degrading capacity.

- To determine the capacity of the fungus to degrade individual PAHs in liquid media, identify degradation metabolites and determine some enzymes involved in such biotransformations.

- To determine the ability of *T versicolor* to degrade individual PAHs spiked in soil using different approaches (biopile microcosms, slurry and aerated biopiles).

- To assess the capacity of *T. versicolor* to degrade PAH-mixtures from creosote in liquid and solid matrices.

- To study the effect of soil indigenous microflora in the PAHs removal ability of the fungus.

Chapter 3

Materials and Methods

3.1 Microorganisms

3.1.1 Fungi

3.1.1.1 Fungal strains

The fungus *Trametes versicolor* ATCC#42530 was acquired from the American Type Culture Collection, and the fungus *Irpex lacteus* 617/93 was provided by the culture collection service of the Institute of Microbiology from the Academy of Sciences of the Czech Republic. Both strains were maintained by subculturing every 30 days on 2% malt extract (w/v) agar plates (pH 4.5) at 25°C.

3.1.1.2 Mycelial suspension

For the production of mycelial suspension, four agar plugs were cutted from the fungal growing area on Petri dishes. These cubes were used to inoculate 500 ml flask containing 150 ml of malt extract medium previously sterilized at 121ºC during 30 minutes. Flasks were maintained in orbital agitation (135 rpm) at 25ºC for 5-6 days. After this period, a dense mycelia layer around the cubes appeared. Mycelia was separated from the broth using a sieve and resuspended in a solution of 0.8% (w/v) NaCl in a ratio of 1:1 (v/v). Then, it was disrupted with an X10/20 (Ystral GmbH) homogenizer to obtain a mycelial suspension (5-6 days).

3.1.1.3 Pellets

For pellets production, 1ml of mycelial suspension was used to inoculate 250 ml of malt extract medium in 1 liter Erlenmeyer flask. These were maintained in orbital agitation (135 rpm) at 25ºC for 5-6 days. The resulting pellets were separated from the broth using a sieve, resuspended in a 0.8% (w/v) NaCl solution and stored at 4ºC until use. A diagram of the process, including the obtention of mycelial suspension, can be observed in figure 3.1.

3.1.1.4 Fungus pre-grown on lignocellulosic substrate as solid-state innoculum

Test tubes, 100 mm and 20 mm ID (Àfora, Spain), containing 3 g dry weight (DW) of lignocelulosic were stoppered with cotton plugs and autoclaved at 121°C for 30 minutes; after two days the tubes were autoclaved again. The substrate was then inoculated with 1 ml of mycelial suspension. Humidity content was adjusted to 60% of the water holding capacity. Cultures were incubated statically at 25°C

until the mycelium colonized completely the substrate after 1-2 weeks. Humidity was maintained by adding water once per week if necessary.

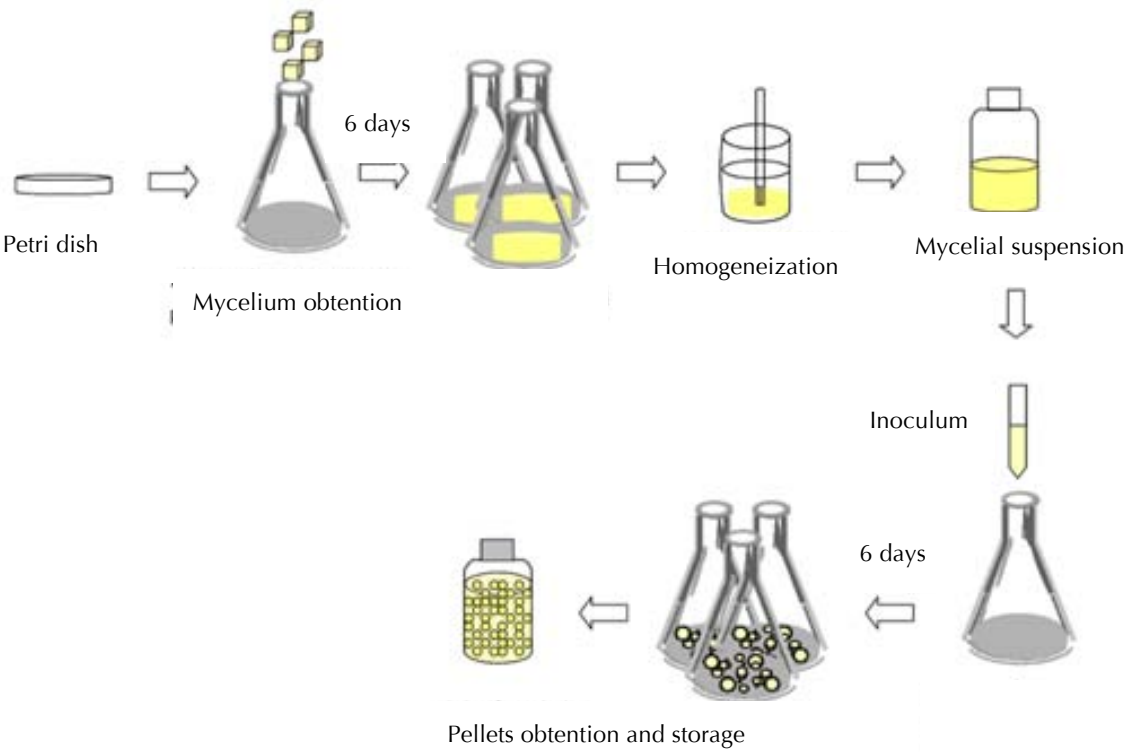


Figure 3.1 Scheme of the methodology to obtain pellets of *T. versicolor* (Blánquez, 2005).

3.1.2 Bacteria

Pseudomonas aeruginosa CCM 1960 and *Rhodococus erythropolis* CCM 2595 (both Czech Collection of Microorganisms, Masaryk University, Brno) were kindly provided by K. Malachová (University of Ostrava, Ostrava) and M. Pátek (Institute of Microbiology of the ASCR, v.v.i., Prague), respectively.

Both strains were maintained by monthly subcultivation on tryptone agar plates containing ($g \cdot l^{-1}$) 8 tryptone, 0.5 yeast agar, 0.25 NaCl, pH 7.0 at 25ºC. A bacterial aliquot was used to inoculate 150 ml of tryptone medium in a 250 ml Erlenmeyer flask, previously autoclaved at 121ºC for 30 minutes. Cultures grown overnight at 25ºC under orbital agitation (135 rpm) were used for soil inoculation.

3.2 Media, substrates and soil

3.2.1 Liquid media

Two different growth media were employed in fungal submerged cultures:

- *Malt extract* (ME): This medium contained 20 $g \cdot l^{-1}$ of malt extract (Scharlau, Spain), pH was adjusted to 4.5 with HCl (1M) and sterilized in the autoclave at 121ºC for 30 minutes.
- *Defined medium* (DM): The medium composition is listed in table 3.1. Its pH was adjusted to 4.5 and sterilized in the autoclave at 121ºC for 30 minutes. Tables 3.2 and 3.3 show the composition of the macronutrients and micronutrients employed (Kirk *et al.*, 1978). Thiamine was sterilized by filtration after sterilization. For bioreactor experiments, 2,2-dimethylsuccinic acid was not required and 2 drops per liter of antifoam 204 (Sigma-Aldrich, Spain) was added.

Table 3.1 DM medium composition.

Nutrients	Concentration
Glucose*	7-17 $g \cdot l^{-1}$
Ammonium chloride	2.1 $g \cdot l^{-1}$
Macronutrients	100 $ml \cdot l^{-1}$
Micronutrients	10 $ml \cdot l^{-1}$
Thiamine	0.1 $g \cdot l^{-1}$
2,2-dimethylsuccinic acid	1.168 $g \cdot l^{-1}$

* Glucose content changed depending on the experiment

Tables 3.2 and 3.3 Composition of macronutrients and micronutrients solutions

Micronutrients	Concentration ($g \cdot l^{-1}$)
Nitril-tri-acetic acid	1.5
$MgSO_4 \cdot 7H_2O$	3.0
$MnSO_4 \cdot H_2O$	0.5
NaCl	1.0
$FeSO_4 \cdot 7H_2O$	0.1
$CoSO_4$	0.1
$ZnSO_4 \cdot 7H_2O$	0.1
$CaCl_2 \cdot 2H_2O$	0.1
$CuSO_4 \cdot 5H_2O$	0.01
$AlK(SO_4)_2 \cdot 12H_2O$	0.01
H_3BO_3	0.01
$NaMoO_4$	0.01

Macronutrients	Concentration ($g \cdot l^{-1}$)
KH_2PO_4	20
$MgSO_4 \cdot 7H_2O$	5
$CaCl_2$	1

3.2.2 Lignocellulosic substrates

Several substrates were used for growing *T. versicolor* in solid-state culture for further use as soil innoculum. All of them are lignocellulosic materials from agricultural subproducts. They can be classified in two different types as listed next.

Agricultural wastes:

- Pn: maize stalks
- Pa: wheat straw
- Se: pine sawdust
- Ar: rice husks

Processed Agricultural wastes for animal feeding:

- C1: wheat straw pellets (ATEA Praha s.r.o.)
- C2: rabbit feedstock pellets (Suprem®)
- C3: rabbit feedstock pellets (Figueres®)

3.2.3 Soil

Soil was collected from an agricultural site at Prades (Catalunya, Spain), sieved through a 5 mm steel mesh and stored in the dark at 4ºC. The soil had the following physical characteristics: 5.1 pH_{H2O}, 4.5 pH_{KCl}, 2% organic matter, 77.35% sand, 12.10% silt, 10.55% clay, 1.28% C_{org}, 0.12% N_{tot} and 12.1% water holding capacity (all w/w).

3.2.4 Chemicals and reagents

Solvents: dichloromethane (DCM) was purchased from SDS (Spain). Cyclohexane, chloroform and ethyl acetate were purchased from Sigma-Aldrich (Spain). Acetone was purchased from Panreac (Spain). All of them were of analytical grade.

Polycyclic aromatic hydrocarbons (PAHs) and metabolites: acenaphthene (APE), acenaphthylene (APY), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fuoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaA), benzo[k]fluoranthene (BkF), benzo[b]fluoranthene (BbF), benzo[a]pyrene (BaP), 9-hydroxyfluorenone, 9-fluorenone, anthrone, anthraquinone, alpha-tetralone, 1,4-naphthaquinone and benzo[a]anthracene-7,12-dione were purchased from Sigma-Aldrich (Spain). Chrysene (CRY) was purchased from Acros Organics (Belgium).

Creosote was purchased from Chem Service (Sugelabor, Spain).

Surfactants: Tween 80 (T80), Tween 20 (T20) and Triton X-100 were purchased from Sigma-Aldrich (Spain) and BS-400 was purchased from IEP-sorbents (Spain).

Mediators: hydrate-1-hydroxybenzotriazole (HOBT), violuric acid (VA), 3,5.dimethoxy-4-hydroxyacetophenon (DMHAP) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diamonium salt (ABTS) were purchased from Sigma-Aldrich.

Enzymes: purified laccase of *T. versicolor* was purchased from Fluka (reference 53739). The enzyme was lyophilized and kept at -20ºC.

Cytochrome P-450 inhibitor: 1-aminobenzotriazol (ABT) was purchased from Sigma-Aldrich (Spain).

3.3 Reactors

3.3.1 Submerged cultures

3.3.1.1 Bottles

Biodegradation experiments were performed in bottles of 500 ml (Schott-Duran) with Teflon stoppers. Cultures were maintained in orbital agitation (135 rpm) at 25ºC.

Enzymatic degradation experiments were performed in 161 ml serum bottles (Wheaton) closed with Teflon lined butyl stoppers (Wheaton) and aluminum rings (Baxter Scientific Products). These were maintained in orbital agitation (135 rpm) for 24 hours at 25ºC.

3.3.1.2 Mechanically stirred bioreactor

For growth experiments a mechanically stirred tank reactor (Biolab, Braun) was used. The bioreactor was equipped with pH control and dissolved oxygen probe (figure 3.2 shows a picture of the bioreactor). Air enters through a metal tube with six holes located under the system of agitation at the bottom of the reactor vessel. A 6-blade turbine at 130-140 rpm agitated the broth.

Figures 3.2 Picture of the mechanically stirred bioreactor.

3.3.1.3 Air-pulsed fluidized bioreactor

For growth experiments two different sizes of air-pulsed fluidized bioreactors were used. The common bioreactor features that can be highlighted are:

- The central body is a cylindrical vertical column in which fluidization occurs. The air is introduced at the bottom by crossing a porous plate, generating small bubbles rising though the liquid phase.
- The head or top of the reactor has a wither diameter than the central body, reducing the liquid up-flow velocity and achieving good separation of the solid/liquid/gas phase.

Figures 3.3 and 3.4 present the two bioreactor outlines used. Both are glass-made and have a working volume of 1.5 and 10 liters, respectively. Tables 3.4 and 3.5 show the main dimensions of both bioreactors. Their heads feature several ports that are used for the pH probe, dissolved oxygen probe, air exit system, scum collection system, glucose feeding, acid and base entry and sampling.

The homogenization in the bioreactor is achieved by entering air in pulses. The pulsating flow is generated by a pneumatic transmission disturbance in the air pulse form to the culture broth contained in the bioreactor. The equipment used to generate the pulse consisted on an air needle valve and the pulse control working as a cycling timer which open and close a solenoid according the fixed times (Font, 1997). In present work, the closing time is 5 seconds and the opening time is 1 second.

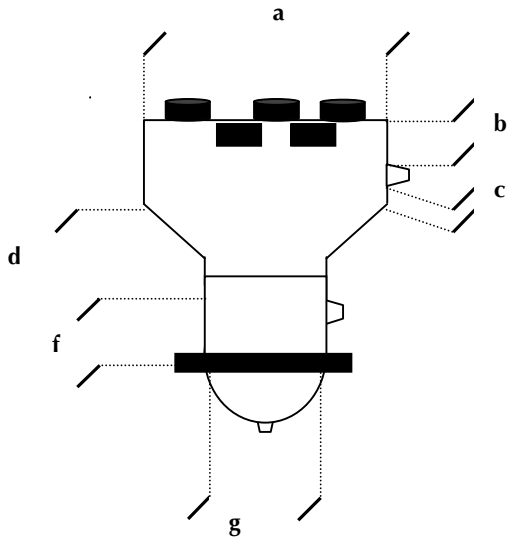


Table 3.4 Geometric characteristics of the 1.5 liters air fluidized pulsed bioreactors.

Piece	Dimensions (mm)
a	105
b	40
c	25
d	40
f	40
g	72

Figure 3.3 Scheme of the 1.5 liters air pulsed fluidized bioreactor.

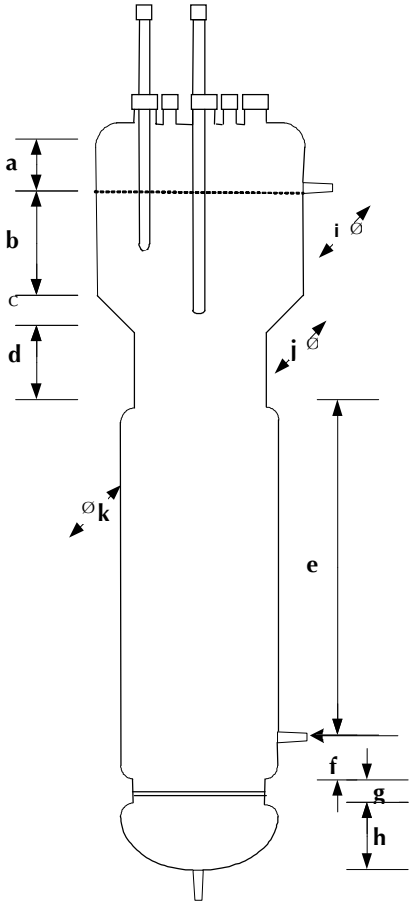


Table 3.5 Geometric characteristics of the 10 liters air fluidized pulsed bioreactors.

Piece	Dimensions (mm)
a	70
b	90
c	35
d	95
e	330
f	30
g	90
h	60
Φi (int)	180
Φj (int)	100
Φk (int)	140

Figure 3.3 Scheme of the 10 liters air pulsed fluidized bioreactor.

3.3.2 Solid-state cultures

3.3.2.1 Biopile microcosms

Biopiles microcosm experiments for soil colonization and PAHs degradation in soil were performed in tubes described in section 3.1.1.4.

3.3.2.2 Aerated biopiles

For composting experiments, Dewar® vessels with a capacity of 4.5 liters were used. The vessels were modified and conditioned to operate as batch reactors for composting purpose. Temperature was monitored by Pt-100 sensors (Sensotran, Spain) connected to a data acquisition system (DAS-8000, Desin, Spain). Aeration was provided periodically to the reactors according to the process performance, where oxygen concentration was maintained between 15–18% to ensure aerobic conditions. Oxygen concentration was measured by means of an oxygen sensor (Crowcon's Xgard, United Kingdom).

3.3.2.3 Serum bottles

For substrate colonization experiments 160 ml Wheaton-bottle type were used for CO_2 measurements. In each bottle, 3 g of lignocellulosic substrate were placed and moistened to adjust the holding capacity. After that, bottles were autoclaved and inoculated with mycelial suspension under sterile conditions. Afterwards, they were hermetically closed with Teflon septum. The cultures were incubated during 14 days in static conditions at 25ºC. CO_2 was measured, as described later, every two days.

3.4 Biodegradation experiments

3.4.1 Submerged cultures

3.4.1.1 *In vivo* degradation of PAHs and PAHs-metabolites

Biodegradation experiments were carried out in 500 ml bottles (Schott-Duran). PAHs were added from an stock solution (10 $mg \cdot ml^{-1}$) to achieve the desired concentration in each experiment. Bottles were left open until the DCM was completely evaporated at room temperature in a flow hood. Then, 50 ml of DM medium and 3 $gDW \cdot l^{-1}$ of fungus in pellet morphology were added. Three types of

bottles were prepared: experimental (containing active fungus), abiotic control and heat-killed control. Unitary samples were prepared by triplicate and sacrificed at each sampling time. Bottles were maintained in orbital shaking (135 rpm) at 25ºC. Samples were taken at days 4, 8 and 12.

3.4.1.2 *In vivo* degradation of creosote

Biodegradation experiments were carried out in 500 ml bottles (Schott-Duran) as described in section 3.4.1.1 with minor modifications. Creosote was added from an stock solution, obtained as described later in section 3.6.10.1, to achieve the desired concentration in each experiment.

3.4.2. Spiked soils

3.4.2.1 Soil spiking

Soil was artificially contaminated in the experiments by adding the pollutant from a stock solution to 9 g of dry soil and air-dried for 24 hours at room temperature in a flow cabine. To obtain an homogeneous distribution of PAHs, the soil was mixed with a metal stick. The initial PAH concentration was determined 24 hours after spiking the soil. The soil samples were stored in the freezer for less than 24 hours before being analyzed.

3.4.2.2 Bioslurries

Slurry experiments were performed in 500 ml bottles (Schott-Duran) with Teflon stoppers. To obtain the slurry conditions, 5 grams of spiked soil were added per 50 ml of DM medium in each bottle to get a final mixture of 10% (w/v). Experimental procedure was identical to that described in section 3.4.1.1.

3.4.2.3 Biopile microcosms

Biopile microcosms experiments were performed in tubes of 100 mm and 20 mm ID (Àfora, Spain) with the fungus grown on a lignocelulosic substrate as described in section 3.1.1.4. An amount of 9 g of spiked soil was added to the tube. In order to obtain an homogeneous mixture of substrate and soil, a metal stick was used to mix them. The tubes were maintained at 25ºC in static conditions until sampling.

3.4.2.4 Aerated biopiles

PAHs were mixed together in a stock solution and then spiked into soil, where the applied concentration was set on 1 $g \cdot kg^{-1}$ (dry matter). Percentages of total PAHs were as follows: 30% fluorene, 28% phenanthrene, 9% anthracene, 20% fluoranthene, 3.5% pyrene, 3% benzo[a]anthracene and 6.5% chrysene, respectively. Afterwards, the contaminated soil was manually mixed with the proposed organic co-substrates at a ratio 1: 0.25 (soil: co-substrate, dry weight). In treatments where bioaugmentation was to be evaluated, mycelial suspension of *T. versicolor* was introduced (1ml:3g of co-substrate, dry weight) within the mixture. The porosity of the obtained mixture was modified to ensure aerobic conditions by introducing wood chips at a ratio of (1:1, v/v) as bulking agent. In all treatments, tap water was added during the preparation of the mixture to modify the water content according to the recommended values for composting process (40-60%). All composting experiments were carried out in duplicates during 30 days of incubation. Samples from the reactors were drawn at different periods (5, 10, 20 and 30 days) in order to follow up the contaminants degradation through out the different stages of the process. The reactor content was manually mixed to obtain homogenous and representative samples about 30-40 grams used for carrying out further analysis. If necessary, humidity was modified during sampling time.

3.4.2.5 Soil in presence of bacteria

The fungus was pre-grown on a lignocellulosic substrate as described in section 3.1.1.4. Once the fungus had completely colonized the substrate a layer of spiked soil (9 g) was added on the surface of each solid-state culture. At this point, in the treatments of soil amended with bacteria, 10^8 bacterial $CFU \cdot g^{-1}$ dry soil were added and humidity content was adjusted to 60% of the water holding capacity. Humidity was maintained by adding deionised water once per week. The tubes were maintained at 25ºC in static conditions until sampling.

Unitary samples were prepared by triplicate and sacrificed when sampling. Three different types of treatments were performed: with sterile soil (S), with non-sterile soil (NS), and with sterile soil inoculated with *P. aeruginosa* and *R. erythropolis* (S+Bac).

3.5 Degradation experiments with purified enzymes and inhibitors

3.5.1 Laccase (*in vitro*) experiments

Experiments were carried out in serum bottles. PAHs were added from a stock solution. Bottles were left open until the DCM was completely evaporated at room temperature in a flow box. In these experiments the total volume was 10 ml, being 9 ml miliQ water buffered at pH 4.5 with 2,2-dimethylsuccinic acid and 1 ml of laccase dissolved in miliQ water. Unitary samples were prepared by triplicate. The initial laccase activity in the bottles was about 3000 $AU \cdot l^{-1}$. After adding the enzyme, bottles were immediately closed with butyl Teflon-covered stoppers and aluminum rings. Bottles were incubated at 25°C in an orbital shaker at 135 rpm during 24 hours. Abiotic control bottles containing only water MilliQ were performed. Control bottles containing deactivated laccase were also included.

Some mediators were used to enhance the enzymatic activity of commercial laccase: VA, HOBT, DMHAP and ABTS. The concentrations used for each mediator were 1mM HOBT and 0.8 mM in the case of AV, DMHAP and ABTS.

3.5.2 Cytochrome P450 inhibition experiments

Submerged biodegradation experiments as described in section 3.4.1.1 in presence of 1-aminobenzotriazol (ABT, 1 mM) were performed to determine the implication of cytochrome P450 in the degradation of PAHs.

3.6 Analytical methods and sample preparation

3.6.1. Biomass measurements

3.6.1.1. Dry cell weight

Samples were filtered with pre-weight vacuum filters Whatman GF/C and washed with 10 ml of distilled water. The biomass filter was dried at 100ºC to constant weight. The difference between the tare and the filter containing the biomass was calculated as measure of dry cell weight.

3.6.1.2 Pellet measurements

Pellet size and morphology were followed using two optical instruments as described:

Optical microscope (Zeiss, Aixoskop) coupled with a digital camera (Zeiss JAI 2060) was used for routine observations to assess breaking-up and shaving off pellet surface.

Magnifying glass (Meiji Labax model SKT to|in (40x)) was used for the measurement of pellets diameter. The average diameter estimation was determined over a total 40 random measures of a pellets aliquot. The average value was estimated statistically using the *t*-student distribution test.

3.6.1.3 Ergosterol extraction and analysis

Fungal biomass in solid-state cultures was assessed via ergosterol content in the solid matrix. Ergosterol was analyzed in homogeneously mixed samples of the soil or substrate employing a modified method used by Novotný *et al.* (1999). A sample of 0.5-0.8 g soil removed from the soil culture was placed in a test tube and extracted with 1 ml cyclohexane and 3 ml KOH-methanol mixture (10% w/v) for 90 min at 70°C. Ultrasonication was applied for the first 15 min (Selecta, Spain); then 1 ml distilled water and 2 ml cyclohexane were added and the tube was vortexed for 30 seconds and centrifuged at 3,500 rpm for 5 minutes. The organic phase was recovered and the aqueous phase was washed twice with 2 ml cyclohexane. The organic phases were pooled and evaporated to dryness under N_2. The dry sterol residue was dissolved in 1 ml methanol (15 min, 40 °C), vortexed for 30 s and centrifuged in eppendorf vials (6,000 rpm, 3 min). Finally the solution was transferred to amber vials and analyzed in a Dionex 3000 Ultimate HPLC equipped with a UV detector at 282 nm (reverse phase Grace Smart RP18 column, 250 mm × 4 mm, particle size 5 µm). Methanol was isocratically supplied at 1 $ml \cdot min^{-1}$. Retention time was 7.9 minutes. The ergosterol content was expressed in micrograms per gram of solid dry weight ($\mu g \cdot g\ DW^{-1}$).

3.6.2 CO_2 analysis

CO_2 analysis was performed using a HP5890 gas chromatograph equipped with an TCD detector (180ºC). Chromatographic separation was done by injection of

100 µl of gas sample (injector at 130ºC) in a Porapak-Q column (3 m 1/8″ column) at 70ºC (isotherm conditions). Helium was used as carrier gas (340 KPa). The retention time was approximately 1.8 minutes.

3.6.3 Glucose

Glucose was measured with an enzymatic glucose and lactate analyzer YSI Model 2700 (Yellow Springs Instruments & Co., USA). The allowable range for this glucose concentration analyzer is 0 to 20 $g \cdot l^{-1}$ with an accuracy of ± 2% or 0.04 $g \cdot l^{-1}$. The analysis is based on the glucose enzymatic oxidation to peroxide using glucose oxidase immobilized on the membrane and the subsequent reduction of peroxide in a platinum anode.

3.6.4 Laccase and MnP extraction and analysis

Aliquots from liquid culture (1.5 ml) were transferred to an eppendorf vial and centrifuged at 6,000 rpm. Laccase and MnP activity of the supernatant were then analyzed as described next.

The enzymes extraction from soil was carried out using the modified method of Lang *et al*. (1998) where 30 ml sodium acetate buffer (0.16 M, pH 5) were added to 3 grams of a homogenized soil sample and shaken for 30 min at 4°C. Aliquots (1.5 ml) of the extracts were transferred to eppendorf vials and centrifuged (15,000 g, 15 minutes); laccase and MnP activity of the supernatant were then determined.

Laccase activity was measured using the first step of the method for determination of MnP (Wariishi *et al*., 1992) where 2,6-dimethoxy phenol (DMP) was oxidized by laccase in the absence of Mn^{2+}. One activity unit (AU) was defined as the number of micromoles of DMP oxidized per minute. The DMP extinction coefficient was 24,800 $M^{-1} \cdot cm^{-1}$. MnP was measured with the same method in the presence of Mn^{2+} (Wariishi *et al*., 1992).

3.6.5 Elementary composition of lignoceluosic substrates

These analyses were performed in the Chemical Analysis Service of the Universitat Autònoma de Barcelona (Bellaterra, Spain). The determination of carbon, nitrogen and hydrogen is made by burning the samples at 1,200°C in oxygen atmosphere and subsequent quantification by gas chromatography.

Meanwhile, sulfur was determined using a CHNS elemental analyzer Eurovector 3011. Results were expressed as mass percentage.

3.6.6 ND24 test: spiking, extraction and quantification of naproxene

The test based on naproxene degradation in 24 hours (ND24) was used as defined by Rodríguez-Rodríguez *et al.* (2010b). Tubes described in section 3.1.1.4 were filled with 0.5 g of substrate and inoculated with the fungus (7 days). Then 1.5 g of soil were added and the content was mixed to obtain a homogeneous matrix. Unitary samples were prepared by triplicate. Naproxene was added to obtain a final concentration about 0.1 $mg \cdot gDW^{-1}$. In addition, three tubes were autoclaved and used as heat controls at each sampling time. After 24 h, the whole content was lyophilized (freeze-dryers team VirTis Sentry, Gardiner, NY) and then subjected to soxhlet extraction (Buchi 811, Switzerland) using methanol as solvent and with the following parameters of operation.

- *Phase 1*: consisted in 20 cycles at temperature level 14.
- *Phase 2*: 10 min, at temperature level 14 and inert gas (N_2).
- *Phase 3*: 30 min, at temperature level 5 and inert gas (N_2).

Sample volume was adjusted to 10 ml. Aliquots (1.5 ml) were centrifuged at 10,000 rpm during 5 minutes, the supernatant was transferred to amber HPLC vials for later analysis. The naproxene extraction efficiency was estimated to be over 95%.

Naproxene was analyzed by HPLC using a Dionex Ultimate 3000 (Sunnyvale, CA) equipped with a UV detector at 230 nm. The chromatographic separation was carried out by injection of 20 µl samples in a Grace Smart RP18 column (250 x 4 mm, 5 µm particle size, Deerfield, IL) and a mobile phase of 65% glacial acetic acid (6.9 $mmol \cdot l^{-1}$) and 35% of acetonitril pumped isocratically at $1ml \cdot min^{-1}$ (Stafiej *et al.*, 2007). The retention time was approximately 1.14 min.

3.6.7 Holding capacity determination

Field capacity determination of soil and different substrates was performed in a cylinder specially prepared for such purpose. The cylinder was then deposited in a tray with water, which covers the height of the substrate without exceeding it, for 2 hours. After that, it was left on a filter paper for 30 min to remove gravimetric water (wet weight) and then were dried at 105°C for 24 hours (dry weight). Field capacity of substrates was calculated as the difference between wet

and final dry weight. It was expressed as $gH_2O \cdot gDW^{-1}$. Each sample was performed by triplicate.

3.6.8 pH measurements

Both pH_{aq} and pH_{KCl} were measured from lignocellulosic substrates and soil. For the case of lignocellulosic substrates, 5 g were soaked in 25 ml of solution, distilled water and KCl, respectively, and they were agitated for 15 minutes at 120 rpm in orbital agitation. Afterwards, the suspension was settled for 1 hour and the pH of the supernatant was measured. Soil samples (10 g) were processed likewise. pH was measured with a pH meter GLP-22 (Crison) equipped with a Pt 100 temperature probe.

3.6.9 Polycyclic Aromatic Hydrocarbons extraction and analysis

3.6.9.1 PAHs extraction: liquid cultures and solid-state cultures

The extraction of PAHs in liquid cultures was carried out with DCM (1:1, v/v) with 1.5 hours contact time at 150 rpm in orbital agitation.

PAHs and creosote-PAHs were extracted from soil samples (9 g) for 4 hours by Soxhlet (Buchi B-811, Switzerland) using DCM:acetone mixture (200 ml, 1:1, v/v). Extraction consists in the following steps: an initial period of 2.5 hours at heating 10; 1.5 hours at heating 10 under N_2 flow and a final period of 0.5 hours at heating 3 under N_2 flow. The extracts were then dissolved in 10 ml of DCM and then analyzed by gas chromatography as described next.

3.6.9.2 PAHs analysis: GC/FID

PAHs concentration was determined by gas chromatography (Agilent 6890N) equipped with a flame ionization detector (FID using a DB-5 column (30 m lenght, ID 0.25 mm, thickness 0.25 µm) (Agilent). The 1.5 ml vials containing the sample were placed in the autosampler carousel (Agilent 7683B Sampler) and maintained at 4ºC with a cooling bath (Heto CBN 8-30). Then 1 µl of sample was injected in splitless mode. The chromatograph operating conditions are as follows: injector (splitless 1 minute) 300°C, oven temperature, 50°C (1 minute), ramp $7°C \cdot min^{-1}$, final temperature 320ºC; detector (FID) 320°C, carrier gas, helium at 4 $ml \cdot min^{-1}$ and nitrogen as make-up gas.

Data was acquired and quantified using the software Millennium 32 (Waters Corp.). Identification and quantification of PAHs were based on matching their retention times with a mixture of PAHs standards. The standard curves were linear in the concentration range of 1-50 $mg \cdot l^{-1}$. The system was calibrated routinely.

3.6.10. Creosote preparation, extraction and analysis

The extraction of creosote-PAHs in liquid and solid experiments was identical to that described in section 3.6.9.1.

3.6.10.1. Creosote preparation: clean-up

The creosote-aromatic fraction was obtained according to the method 3611B (EPA) by cleaning up 5 grams of creosote with alumina to obtain the base-neutral aromatic compounds fraction. The procedure to obtain cleaned-up creosote was as follows.

A chromatographic column (300mm x 10 ID, Pyrex® glass with polytetrafluoro-ethylene stopcock; Àfora, Spain) was packed with neutral alumina (Mercks, Germany) retained by glass wool on the base of the column. All glass material used in the procedure was previously washed with an alkaline detergent, then distilled water and finally acetone or DCM. On the top of the alumina, 1 cm anhydrous Na_2SO_4 (purified by heating at 400ºC for 4 hours) was placed. The column was pre-cleaned with the elution solvent. Then, a maximum amount of 0.3 g of creosote (to avoid overloading) was transferred to the top of the column, which was eluted with 100 ml of DCM subsequently collected in a 250 ml flask. The volume obtained after cleaning up creosote was concentrated under N_2 and adjusted to a final volume of 150 ml to obtain an stock solution. The procedure was performed under a hood. The stock solution was analyzed as described in next section to determine PAHs concentration.

3.6.10.2 Creosote-aromatic fraction analysis: GC/FID

PAHs concentration from creosote-aromatic fraction was determined by gas chromatography (Agilent 6890N) equipped with a flame ionization detector (FID using a ZB-5HT INFERNO capillary column (Phenomenex) (30 m length, 0.25 mm ID, thickness 0.25 µm) (Agilent). The 1.5 ml vials containing the sample were placed in the autosampler carousel (Agilent 7683B Sampler) and maintained at 4ºC with a cooling bath (Heto CBN 8-30). Then 1 µl of sample was injected in splitlles mode. The operating conditions of chromatograph are as follows: injector

(splitless 1 minute) 300°C, oven temperature, 50°C (1 minute), ramp 7°C·min^{-1}, final temperature 400ºC that was maintained for 10 minutes; detector (FID) 320°C, carrier gas, helium at 4 ml·min^{-1} and nitrogen as make-up gas.

The data was acquired and quantified using the software Millennium 32 (Waters Corp.). Identification and quantification of PAHs were based on matching their retention times with a mixture of PAHs standards. The standard curves were linear in the concentration range of 1-50 mg·l^{-1}. The system was calibrated routinely.

3.6.11 PAHs-metabolites identification: GC/MS

For experiments aimed at identifying the PAHs degradation products, three different extracting agents were used independently: DCM, ethyl acetate and chloroform. The extraction procedure was similar to that described in section 3.6.9.1 but decreasing pH to 2.0 prior to extraction. All glass material used for this procedure was previously washed with an alkaline detergent; then rinsed with distilled water and finally acetone to remove any organic residue.

The Chemical Analysis Service of the Universitat Autònoma de Barcelona (Bellaterra, Spain) performed these analyses. For this purpose, aliquots of 10 ml from the organic-extraction-solvent fraction were dried under vacuum and dissolved in 1 ml of the corresponding solvent.

Samples were analyzed using gas chromatography (Agilent HP 6890 Series II) coupled to a mass selective detector by electronic impact ionization (Agilent HP 5973) using a HP5-MS (30 m x 0.25 mm x 0.25 µm) (Agilent). The operating conditions of the chromatograph are as follows: injector (splitless 1 minute) 320ºC, injection volume 1-3 µl (depending on the sample), oven temperature, 50°C (1 min), ramp 7°C·min^{-1}, final temperature 320ºC, carrier gas helium at 0,7 ml·min^{-1}. The detector worked at solvent delay mode (3.2 minutes) and the mass range measured was 40-400 (m/z). Products detected were identified by comparing the mass spectra with data in the Wiley 7® library.

3.7 Statistical analyses

ANOVA and principal component analysis (PCA) were performed using Statistica 7.0 software package (StatSoft, USA).

CHAPTER 4

Production of *T. versicolor* biomass for soil bioaugmentation applications

The present chapter deals with the production of Trametes versicolor biomass for bioremediation purposes. It is divided in two sections. The first one is focused on the production of large amounts of fungal biomass at low cost in pelleted morphology. A defined medium for growth was formulated and the production was scaled-up from Erlenmeyer flask to a 10 liters bioreactor. The other section deals with the inoculation of the fungus in soil environment for further implementation in bioaugmentation of polluted soils. For such purpose, different lignocellulosic substrates were tested to guarantee optimal soil colonization in terms of biomass, enzymatic activity and degrading capacity; under both sterile and non-sterile conditions

A slightly modified part of this chapter has been published as:

Borràs, E., Blánquez., P., Sarrà, M., Caminal, G., Vicent, T., 2008. *Trametes versicolor* pellets production: Low-cost medium and scale-up. Biochemical Engineering Journal 42, 61-66.

Borràs, E., Llorens, G., Rodríguez-Rodríguez. C-E., Caminal, G., Sarrà, M., 2010. Soil colonization and naproxen degradation by *Trametes versicolor* grown on lignocellulosic materials: in the search of optimal substrates for bioaugmentation strategies (in preparation).

4.1 Culture media formulation and scale-up production of *T. versicolor* in pellets

The research group in which this thesis has been carried out has developed a process for the treatment of textile industry wastewater using the fungus *T. versicolor* in pellets morphology (Romero *et al.*, 2006; Casas *et al.*, 2009; Blánquez *et al.*, 2004). Degradation of other recalcitrant pollutants has been carried out using the fungus in this morphology (Vilaplana *et al.*, 2008; Blánquez *et al.*, 2008; Marco-Urrea *et al.*, 2009). One of the bottlenecks for biomass production at large scale is the high cost due to the culture medium. In this section, experiments are focused to the formulation a low-cost medium to produce fungal biomass in pellets morphology. This biomass could be later employed for experiments in submerged culture or even to inoculate soils.

4.1.1 Fungal pellets production in submerged cultures

4.1.1.1 Fungal growth mechanisms and morphology

Filamentous fungi are organisms of complex morphology that may present a great variety of structures along its vital cycle, from spores to mycelia. The germination of spores origins a tube (hypha), which grows due to the consumption of stored products in the spore. Each hypha starts from a spore that upon germination produces one or several germinal tubes. The development of hyphae contributes in obtaining nutrients from the environment. Most fungi are composed of vegetative structures termed mycelia, which grow from their tips as binary branching trees (Viniegra-Gonzalez *et al.*, 1993).

When grown in submerged cultures, fungi present diverse morphologies ranging from disperse mycelia, forming clumps, to some dense interwoven hyphae, known as pellets. The fungal existing morphology comes determined by the species genotype, but is also affected by the inoculum nature, as well as by chemical conditions and physical culture factors (Kossen, 2000).

Growth morphology can have a significant effect in the rheology of the culture and in the behavior of the bioreactor. Cultures with disperse growth are generally highly viscous with a clearly non-Newtonian behavior of pseudoplastic type (Sarrà *et al.*, 1996). The high viscosity has a negative effect

on the mass transfer, especially regarding to gas-liquid transfer (Papagianni, 2004). On the contrary, the growth in the form of pellets presents low viscosities and behaves closer to the Newtonian model.

However, pellet morphology can generate nutrients and oxygen limitations, which cause the phenomena of cell lysis in the pellet central part of (Sarrà *et al.*, 1997). For a suitable metabolite production and an optimal bioreactor operation it is necessary to know growth characteristics and fungal physiology. The morphology control of the microorganism is a key point that needs great attention to optimize the productive capacities.

4.1.1.2 Fungal growth in liquid medium

The growth of filamentous organisms in submerged cultures can differ between the dispersed form and the pelleted one, depending on the culture conditions and the strain of organism employed.

Dispersed growth

Disperse mycelium refers to a homogeneous suspension made of ramified hyphae that does not present difusional limitations to exchange substrate and metabolites with the broth. In this sense, the disperse mycelium is similar to ideal unicellular cultures. These present many analogous characteristics to this type of growth.

Oxygen limitation is a critical restriction of this type of cultures due to their rheologic properties. Cultures with disperse mycelium have a non-newtonian behavior and the apparent viscosity augments on increasing agitation. Due to complex morphologies in submerged cultures, the agitation conditions require great attention: the total power input, the choice of the proper impeller geometry, position and number determines the mechanical forces that may affect fermenter cultivation, influencing growth or metabolite production. Potential damage to microorganisms can limit the impeller speed or power input and consequently the oxygen and nutrient transfer capability of a bioreactor (Makagiansar *et al.*, 1993; Jüsten *et al.*, 1996).

Pellets

Pellets are spherical aggregates of hyphae that can present smooth or hairy surface. This morphology is usually employed in fungal submerged cultures, as it is equal to having immobilized the microorganism inside the reactor. The viscosity is low, which favors mass transfer, resisting higher agitation conditions (Braun and Vecht-Lifshitz, 1991). The biomass separation from the broth is also simple. At industrial level, mycelium has been used in the form of pellets for the production of different compounds such as antibiotics, citric acid and fungal biomass (Papagianni, 2004).

The internal structure of the pellets is variable. It can differ from strongly compacted to loose hyphae. A detailed image of the pellets interior of denotes an internal structure of superimposed layers, generally three, constituted by heterogeneous biomass (Sarrà *et al.*, 1997). The central core presents partially lised biomass. The intermediate layer is responsible of the secondary metabolites production. Finally, the external layer is where the growth takes place for the accessibility to the nutrients.

Dense and compact pellets cause diffusional problems of nutrients and oxygen in the inner biomass. This phenomenon can be the cause of both the decrease of secondary metabolites production and the fungal growth. Moreover, it can induce the autolysis of the biomass located at the pellet core (Prosser and Tought, 1991). During secondary metabolism, the rate of substrate utilization is limited by the rate of oxygen transfer into the pellets (Michel *et al.*, 1992). A less dense degree of pellet compactness avoids diffusional problems, but at the same time augments the risk of fragmentation.

Type of pellets formation

The diverse types of pellets formation can be attributed to interaction mechanisms among the hyphae itself, solid particles with hyphae and, at last, spores with hyphas (Prosser and Tough, 1991). Two main types of pellets formation from spores are described:

- *Coagulative*: aggregation of non-germinated spores occurs to form the nucleus of the initial pellets. These small particles of germinated spores can aggregate with other agglomerates and form pellets. A clear example of this type of growth is described by *Aspergillus niger* (Kossen, 2000).

- *Non-coagulative*: a pellet is formed from each spore inoculated in the medium. A clear example of this growth is reported for *Penicillium chrysogenum* (Pazouki and Panda, 2000). *P. chrysosporium* appears to involve surface polysaccharide binding during the initial aggregation, but it is not clear whether this mechanism is applicable to all fungi (Gerin *et al.*, 1993)

Another type of pellets formation can take place without the need to use spores as an initial inoculum for the culture. Minced mycelium can be used as inoculum. Pellets may also result from the agglomeration of hyphal aggregates and fragments, which is called formation from *arthrospores* (Braun and Vecht-Lifshitz, 1991).

Factors affecting the pellets formation

The morphology of the pellets is not only influenced by biological factors. Physical and chemical factors in the growth medium also play an important role. It is difficult to describe a unique mechanism for the formation of the pellets since it normally depends on more than one variable (Gibbs *et al.*, 2000), some of which are listed next:

- *Growth medium composition*: pellets formation occurs in conditions that prevent the fast and abounding growth of mycelium. It has been determined for *Aspergillus niger* that a high carbon and nitrogen ratio (C/N) favors the growth of disperse mycelium (Prosser and Tough, 1991). High concentrations of nitrogen can stimulate the formation of spongy pellets (Sarrà *et al.*, 1997; Pazouki and Panda, 2000).

- *Viscosity*: fungi excrete polysaccharides and proteins, which can both facilitate or disfavor the fungus hyphae aggregation by modifying the viscosity of the culture broth. On one hand, several carbohydrate and protein substrates have been used to avoid biomass aggregation (Prosser and Tough, 1991). On the other hand, Trinci (1983) demonstrated that the addition of poliacrilic acids in cultures of basidiomicets can cause disperse growth under conditions of pelletization.

- *Surfactants*: the addition of non-ionic surfactants to growth media reduces the aggregation of spores, so that disperse growth is favored. Addition of anionic surfactants favors the formation of pellets of minor diameter.

• *Agitation*: the morphology of the fungi in submerged cultures depends, to a large extent, on the agitation. Pellets of basidiomicets like *T. versicolor* are only formed with little agitation. Pellets size is inversely proportional to agitation rate; as for high agitation breaking of the pellets can occur. The action of the turbines blades in the agitated tanks can cause damages to the hyphae of the pellets surface or directly to cause the break as it shows the figure 4.1. It has also been described that the type of agitation can affect the degree of compacting of the obtained pellets. Hansson and Seifert (1987) observed that, using identical culture media, *Armillaria mellea* forms very compact pellets in turbine-agitated bioreactors, whereas non-compact pellets predominate in fluidized bioreactors. In addition, agitation variations during growth can affect the size distribution and pellet structures (Gómez *et al.*, 1988).

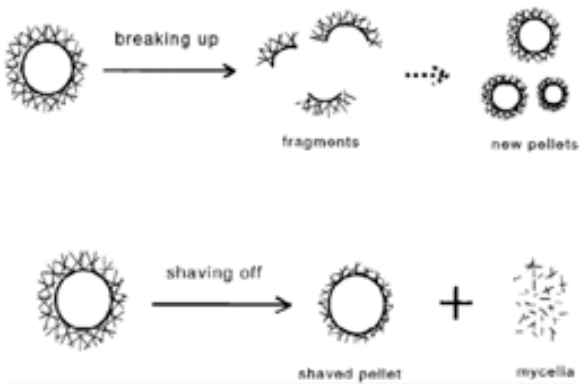


Figure 4.1. Breaking up and shaving off of pellets (Cui *et al.*, 1997).

• *pH*: the effect of pH on the cellular aggregation is not well understood. On the one hand, Metz and Van Suijdam (1981) did not elucidate a clear relationship among the morphology of *A. niger* and the variation of the pH during its growth. On the other hand, Galbraith and Smith (1969) determined for *A. niger*, that pH plays a crucial role in the process of pelletization, specially in the coagulation of spores.

• *Inoculum*: high inoculum concentration favors growth of disperse biomass while the formation of pellets is favored at lower innoculum level (Pazouki and Panda, 2000). In general terms, bigger size pellets are produced at low inoculum level (Prosser and Tought, 1991).

• *Solids in suspension*: the presence of solids in the broth culture favors pelletization (Prosser and Tough, 1991).

- *Dissolved oxygen*: oxygen limiting conditions can favor disperse growth, while a sufficient oxygenation level seems to favor pellets formation (Pazouki and Panda, 2000). A reduction in oxygen concentration in the pellet core due to an inadequate transfer can cause autolysis problems to the fungus. This limitation occurs especially in pellets of a size bigger than a critical value.
- *Temperature*: it influences the pellets formation because it affects the oxygen solubility in the culture medium (Braun and Vecht-Lifshitz, 1991).

Pellets growth

The growth of pellet cultures follow cubic root kinetic which was described after an extensive research with a great number of fungi (Marshall and Alexander, 1960). Growth is restricted to an outer layer of the pellet, the distance that nutrients can penetrate the pellet determines its extent. Therefore, the kinetics of growth is not exponential but of cubic root, with part of the biomass of the fungus not growing. The nutrient diffusion to the pellet core is limited when a critical radius is attained. This is determined by the compacting degree of hyphae.

Other models can serve to describe the pellets growth but they have certain limitations. The effects of substrate transfer limitation neither the heterogeneity of the culture are not considered, assuming that all pellets present both equal size and density and that all the biomass of the culture presents pelleted morphology.

Typology of pellets

Pellets can be classified in three main groups according to their pilosity and their degree of compacting (Pazouki and Panda, 2000).

- *Spongy*: these pellets have a compact core and an outer zone of small, fuzzy and spongy hyphae.
- *Compact*: the whole pellet is compact and its superficial layer is smooth.
- *Smooth and empty*: the superficial layer of the pellet it is soft but the core is empty because of the fungal autolysis phenomena.

Methods to assess pellet morphology

The mycelium morphology is important since the production of several products is related to it (Braun and Vecht-Lifshitz, 1991). A clear example is the production of citric acid with *A. niger,* which requires a specific pellet size (Gómez *et al.*, 1988). The characterization of the morphology is usually carried out by ocular inspection of the culture broth. The biomass is already observed directly on the reactor, qualitatively, or taking aliquots from the reactor and characterizing them with the help of a microscope or a magnifying glass.

Early quantitative studies of biomass morphology consisted in pictures of mycelial particles projected on a digitalizing table coupled to a computer (Metz *et al.*, 1981). This first method was costly in terms of time, difficult to automate and had a hunted accuracy. A variety of methods are now recognized for the analysis of fungal morphology (see table 4.1). Image analysis methods keep on improving and have been applied to several fields: studies of growth, dynamics of hyphae aggregation, fragmentation, rheology and physiology (Papagianni, 2004).

Table 4.1 Methods for analysis of fungal morphology (adapted from Singh 2006).

Method	Reference
Autoradiography	Pitt and Bull, 1982
Image analysis	Cox *et al.*, 1998
Automated image analysis and software	Packer and Thomas, 1990; Dorge *et al.*, 2000; Nelson *et al.*, 2000
Automated image analysis and fluorescent staining	Morgan *et al.*, 1991a

4.1.1.3 Aim of this section

The aim of this section was to formulate a low-cost medium for the production of large biomass amounts of *T. versicolor* in pelleted morphology. The first phase was the formulation of an economic and defined medium for growing the fungus. The next step was to produce pellets employing a 1.5 L bioreactor. Finally, the later step was to scale-up the production to a 10 L bioreactor.

4.1.2 Results and discussion

4.1.2.1 Optimization of growth medium for biomass production: screening for alternative growth media

The treatment process of colored wastewater using *T. versicolor* was evaluated economically (Blánquez, 2005). Its objective was to eliminate Grey Lanaset G up to 85% from a synthetic effluent containing an initial concentration of 150 $mg \cdot l^{-1}$ of this textile dye. The results of this work showed that near 94% of the total cost per cubic meter was due to the raw materials, especially malt extract employed for the production of fungal pellets. Thus, there is a need to search for a less expensive culture medium in order to reduce the pellets production cost.

The medium used typically for the growth of *T. versicolor* contains malt extract ($20\ g \cdot l^{-1}$) and therefore, it is used as a reference. After a 5 days growth period in such medium, the biomass level reached 3 $gDCW \cdot l^{-1}$ in the form of pellets. These presented a size, degree of compacting and morphology suitable for the biodegradation applications in which they are used.

The main disadvantage of using the mentioned medium is its high cost (42.06 $€ \cdot kg^{-1}$); that represents $0.28€ \cdot gDCW^{-1}$ for pellet formation. Another inconvenience is that during the growth it is not possible to measure any nutrient as indicator of biomass development. Merely, pellets growth is assessed visually.

The first step for the formulation of an alternative and low-priced medium was carried out qualitatively at Erlenmeyer scale. In this section the capacity of *T. versicolor* to grow as pellets is evaluated in different media. The fungal growth in each medium was tested by triplicate in 1 liter Erlenmeyer flask with 250 ml of medium. Glucose concentration and pH were determined from time-course samples. The biomass level was measured at the end of the culture.

The alternative medium used for this initial screening was a defined medium (DM) with an entirely known and easily reproducible composition as described in section 3.2.1. Glucose and NH_4Cl were added to the medium as carbon and nitrogen source, respectively. Macronutrients and micronutrients were added as done by Marco-Urrea *et al.* (2006). Thiamine was also added to the medium ($10\ mg \cdot l^{-1}$). Thiamine is a vitamin commonly used in growth media for *P. chrysosporium* (Kirk *et al.*, 1978; Tien and Kirk, 1988) and *T. versicolor* (Roy and Archibald, 1993; Gill and Aros, 2003; Tavares *et al.*, 2005a and b).

As it can be observed in figure 4.2, pH was maintained around 4.5 in malt extract medium but it decreased below 3.0 in defined medium during growth, even though dimethylsuccinic acid was added as buffer. This indicated that the buffer was not capable to maintain the pH or it was uptaken by the fungus. Evidences in our research group suggested that the fungus is capable to consume the buffer as assessed by HPLC analysis. Tavares *et al.* (2005b) reported an analogous behavior, and linked it to the synthesis of organic acids, such as oxalic and glicoxalic acid, which are associated to the primary metabolism of the fungus in the glucose uptake (Roy and Archibald, 1993). Similar performance has also been described in medium containing ammonium salts and weak buffer; in which pH tends to drop during fungal growth (Papagianni, 2004).

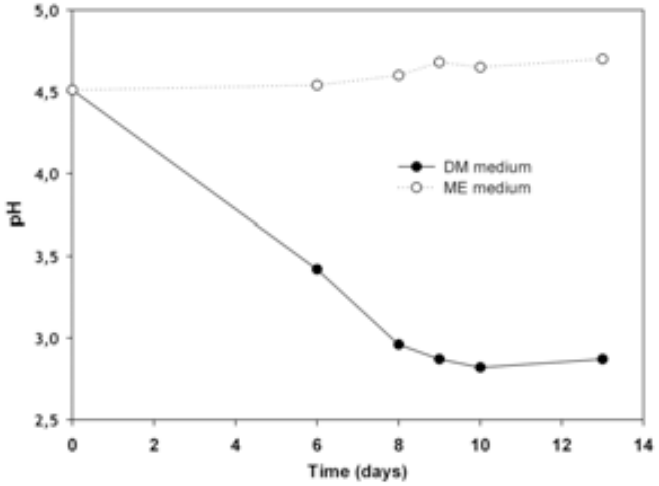


Figure 4.2 pH during growth in DM and ME media in Erlenmeyer.

After 12 days of culture, 1.1 gDCW·l^{-1} were obtained as pelleted fungal biomass. Growth arrest was evidentiated by the stabilization of glucose levels in 12 g·l^{-1} at the end of the experiment. On the contrary, when growing in ME medium 3 gDCW·l^{-1} of pellets were obtained. The possibility of further experimentation at Erlenmeyer scale was ruled out due to problems for pH control.

4.1.2.2 Pellets production at bioreactor scale

Stirred tank bioreactor

A stirred tank (model Biolab) was used as bioreactor as described in section 3.3.1.2. The medium had the composition previously described. In addition, antifoam 204 was added to avoid foam formation. The system incorporated a

pH controller that maintained it at 4.5 by adding automatically HCl or NaOH (1M).

Glucose was completely depleted after 5 days of culture as depicted in figure 4.3. In terms of biomass production, 5.47 gDCW·l^{-1} were attained as dispersed biomass and pellets. This value is superior to that obtained with malt extract. The disperse growth favored the adherence of biomass to the bioreactor surfaces. Thus, it could not be properly considered when measuring dry weight. Nevertheless, these results confirmed that pH decrease inhibited the growth and that the formulated medium was suitable for biomass production.

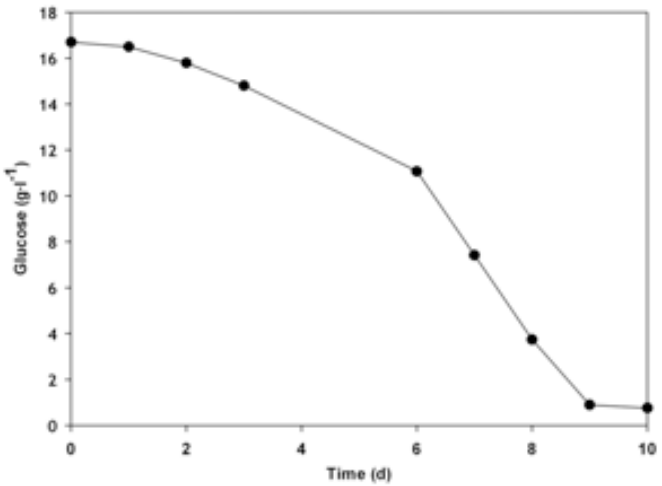


Figure 4.3 Glucose profile along *Trametes* growth in DM medium in stirred tank bioreactor.

However, the morphology of the obtained biomass was not the one expected, as illustrated in figure 4.4. Most of the biomass, at the end of the culture, presented dispersed morphology and only a small fraction was present as pellets. Its final size was 2.07 ± 0.67 mm.

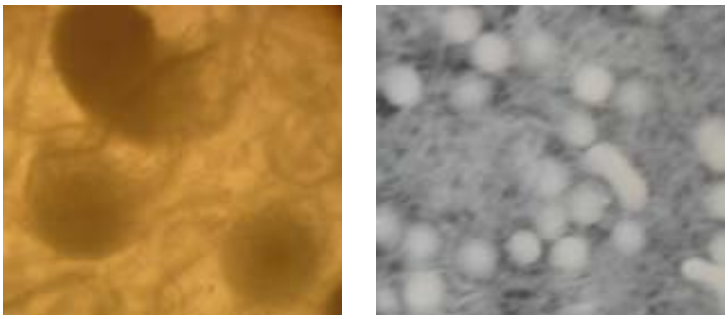


Figure 4.4 Biomass of *T. versicolor* after culture (right) and detail of pellets (left), obtained in stirred tank bioreactor in DM medium.

The observed dispersed growth can be caused by three factors: agitation, low oxygen transfer and/or insufficient nitrogen concentration in the medium. The agitation in a stirred tank bioreactor with turbine creates mechanical strengths that can erode the hyphae growing on the pellets surface (Cui et al., 1998). The growth of free filaments is favored respect to that of pellets since disperse biomass have less oxygen and/or other nutrients diffusion limitation, as for pellets. In addition, the viscosity of the broth increases with the dispersed growth. The disperse mycelia fraction in the broth is augmented increasing the agitation (Cui *et al.*, 1998). Thus, increasing the impellers agitation would not contribute to increase the percentage of biomass in the form of pellets.

Another reason that can explain the morphology of disperse fungal growth is an insufficient nitrogen concentration source (Braun and Vecht-Lifshitz, 1991). The ammonium concentration at the end of the experiment was 0.33 $g \cdot l^{-1}$, which represented 23.5% respect the initial. This demonstrates that the culture was not limited by nitrogen source.

The limitation or lack of oxygen is, also, one of the factors that can influence the morphology of the obtained biomass. The oxygen dissolved along the experiment was not measured, but given these results, it was followed in next experiments.

Pellet production using stirred tank bioreactor

An experiment aimed at reducing the content of dispersed mycelia was performed in stirred tank bioreactor. As formerly mentioned, the biomass obtained in the previous experiment was overly high. The carbon and nitrogen sources were reduced to 7 and 2.1 $g \cdot l^{-1}$ respectively; maintaing the C/N ratio, reducing problems of oxygen supply due to biomass excess. Dissolved oxygen during the culture was measured and was attempted to sustain it above 20% (pO_2) increasing the aeration flow manually.

In figure 4.5 is presented the glucose concentration profile along the experiment. It followed a similar pattern to that in the former experiment. A two days latency phase was followed by an exponential growth phase when glucose was rapidly consumed.

Figure 4.5 shows the air inflow and dissolved oxygen along the experiment. The initial air in-flow was setted to 85 $l \cdot h^{-1}$ and was manually controlled. The observed high oxygen consumption can be related to the fungal growth phase. The highest oxygen consumption can be observed at the end of the fungal

growth phase. This behaviour is correlated with the trends presented in figure 4.5. It can be appreciated that when glucose was fully consumed (at day 4), growth stopped and the broth became oxygen-saturated.

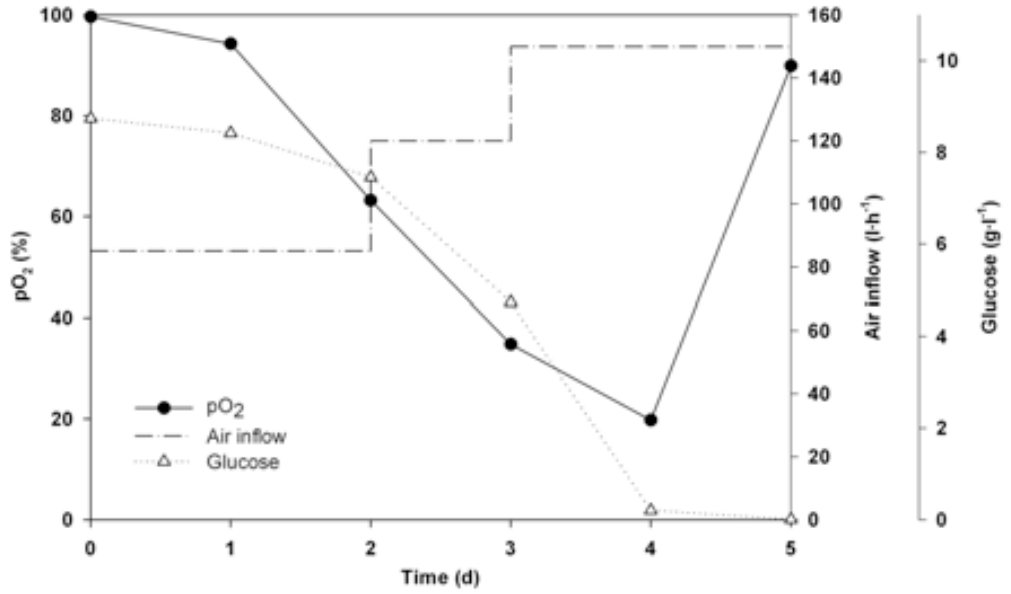


Figure 4.5 Glucose, dissolved oxygen and airflow during pellet production using the stirred bioreactor in stirred tank bioreactor

From the third day of growth on, the air inflow had to be increased to the maximum supplying capacity (150 $l \cdot h^{-1}$), which represented 1.6 vvm. Despite this, the airflow was insufficient to sustain the oxygen concentration in the liquid phase over 20%. The agitation increase to enhance oxygen transfer from was an option, but it was ruled out to avoid biomass dispersion and favor dispersed mycelial growth.

The pelleted biomass in the broth culture represented 1.95 $gDCW \cdot l^{-1}$. However, this value was not completely significant respect to the total biomass grown in the bioreactor. Even though a major part of the biomass grew in the form of pellets, as illustrated in figure 4.6. A significant portion grew adhered as clumps to the surface of the bioreactor and probes. This was not taken into account since the aliquots were only taken from the suspended biomass fraction from the broth culture.

Unlike the former experiment, biomass grew from the beginning in the morphology of fluffy pellets mostly. The disperse mycelia represented a minimum fraction, but these were slightly hairy and favored the biomass adhesion to bioreactor surfaces.

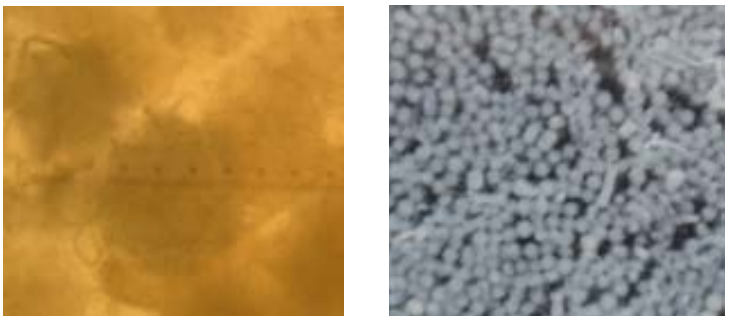


Figure 4.6 *T. versicolor* biomass obtained (right) and detail of pellets (left) growing in an stirred tank bioreactor.

Pellet size and shape was studied along the growth period. At the third day of growth, pellets size was 1.46 ± 0.48 mm. Many of them presented a great diversity in non-round ways, mostly oval, and were hairy. Some pellet surface hyphae presented a length superior to the pellet diameter. At the end of the culture, the size was 1.66 ± 0.54 mm. This value is inferior to the obtained in the former experiment, with more glucose content.

Another characteristic of the obtained pellets is the increased fluffiness or more porosity compared to those obtained from malt extract medium. Fluffiness of pellets is directly related to their porosity. This is directly related with the oxygen dissolved in the culture medium, a low porosity is presented when there is a high level of oxygen (Cui *et al.*, 1997).

In the stirred tank reactors, low dissolved oxygen levels are correlated to poor transfer of this gas. The supply of oxygen in the bioreactor is carried out through the entry of air by a tube with six holes placed on the lower agitator part. The agitator blades break the air bubbles and a good oxygen transfer to the broth is achieved at high agitation speeds. However, this aeration system was clearly insufficient to cover the requirements for oxygen of *T. versicolor* growth at low agitation speed. This could not be increased without breaking excessively the mycelia and, consequently, difficulting the pellets formation. In summary if pH is controlled, the defined medium is suitable to obtain enough biomass level but the morphology is not suitable due to the oxygen transfer limitation.

Pellets production using an air pulsed fluidized bioreactor

In order to improve oxygen transfer while minimizing mechanical stress, an air pulsed fluidized bed reactor was tested for *T. versicolor* pellet production. This type of bioreactor had been used with excellent results for fungal

biodegradation of several pollutants (Blánquez *et al.*, 2004 and 2008) with preformed pellets but under growth limiting conditions. Pellets were obtained in Erlenmeyer flasks and then inoculated to the bioreactor to carry out the degradation experiment.

In air pulsed bioreactors the air is introduced at high pressure by the bottom of the bioreactor through a micro porous plate (Roca *et al.*, 1996). Small bubbles are formed and cross the liquid column allowing to increase oxygen transfer and favoring the mixing. The air surface/liquid volume ratio is very high and therefore oxygen transfer is better than mechanical stirrer reactor even at lower flow rates. Additionally, the damages by mechanical stress on biomass are reduced; consequently, disperse growth is avoided.

For these experiments, the same defined medium described for stirred tank was employed. The initial air inflow was 40 $l \cdot h^{-1}$ (0.44 vvm). This value was significantly lower with respect the inlet necessary in the mechanically stirred reactor. The minimum pO_2 was fixed to 20%. No increase in air inflow rate was necessary during culture.

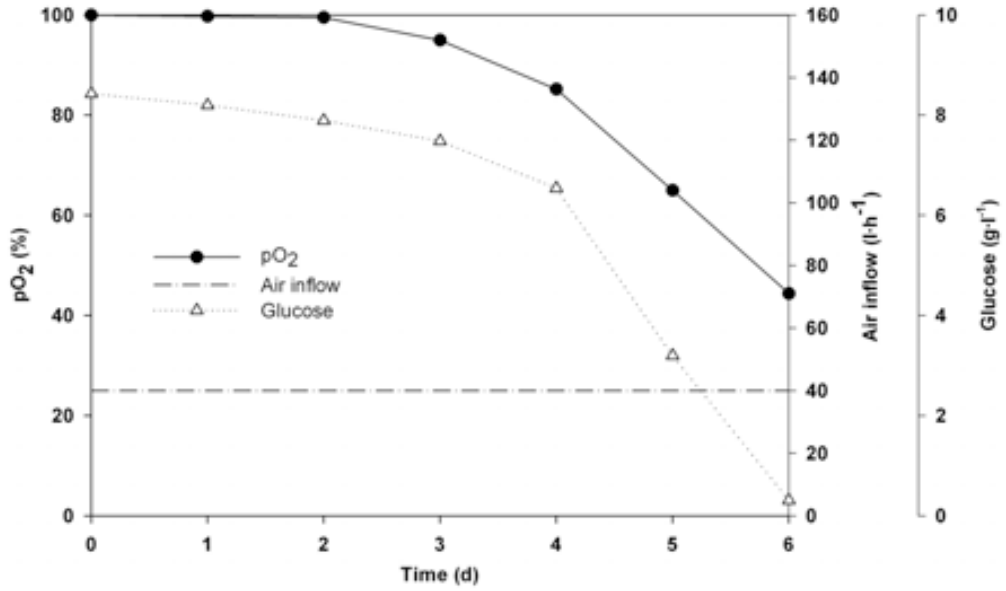


Figure 4.7 Glucose, dissolved oxygen and airflow during pellet production using the air pulsed-fluidized bioreactor.

The pattern of glucose consumption (see figure 4.7) was very similar to that of the former experiments. Presenting an initial lag phase followed by an exponential growth. Figure 4.7 shows the pO_2 profile and the air inflow inlet along the experiment. The flow was enough to maintain the required dissolved oxygen levels even during exponential growth. It is important to highlight that air inflow represented barely 26% of the maximum inflow necessary in the

stirrer reactor, allowing savings in terms of air and energy consumption for pellets production.

In terms of biomass, 2.96 gDCW·l^{-1} were obtained. The biomass yield ($Y_{f/g}$), was 0.423 g·g^{-1}. Unlike former experiments there was not disperse mycelia in the final broth. No clumps were formed and there was no attached biomass on the bioreactor surfaces.

Table 4.2 presents mean pellet size values along the experiment. After 3 days of culture, pellets were formed and presented a size similar to the final value. The compacting degree of pellets, however, did augment with time in a noticeable way. This fact seems to point out that pellets achieved their final size at early cultivation stages, whereas their compactness increased during exponential phase. Figure 4.8 shows how pellets pilosity reduced compared to those obtained in the stirred tank bioreactor. Hence, it can be concluded that this type of bioreactor was suitable for the production of fungal biomass with the preferred morphology.

Table 4.2 Pellets size along time-course of the experiment.

Time (days)	Size (mm)
3	2.80 ± 0.22
4	3.31 ± 0.30
6	3.09 ± 0.20

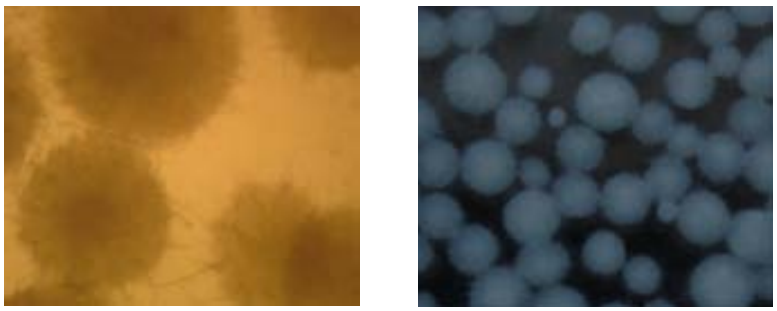


Figure 4.8 Obtained biomass of *T. versicolor* (right) and retail of pellets (left) in the air pulsed-fluidized bioreactor.

Scale-up production: 10 liters bioreactor

The different scale-up methodologies aim to anticipate the scale change effects and to introduce the variations required in the equipment design as their size is increased. In any case, it has to be understood that the scale change is a

complicated process due to the combination of multiple factors (Solà and Gòdia, 1994).

One of the most common criteria to maintain in the scale-up of a growth process is the air volumetric flow per volume of reactor unit, in the case of air fluidized bed reactor. For mechanically agitated bioreactors K_{La} is the most employed criteria to sustain oxygen supply. However, in this case, the air pulsed reactor evidenced a good oxygen transfer. Since no issues regarding oxygen transfer were observed, it was decided to impose the criterion of geometric similarity.

Using a bigger bioreactor allowed taking out larger samples (50 ml) without affecting the total volume of culture broth (10 liters) during the experiment. Consequently, biomass and pellet diameter were more representative.

Figure 4.9 shows the glucose profile and the biomass increase along the experiment. The lag phase lasted two days. After that, exponential growth started, with a specific growth rate of 0.075 h^{-1}. This value is similar to the values for certain fungi like *Aspergillus* sp. and *Penicillium* sp. The biomass obtained was 3.89 ± 0.39 gDCW·l^{-1}, most of it as pellets. The final size of these was 3.05 ± 0.21 mm, very similar to that obtained in the 1.5 liters bioreactor.

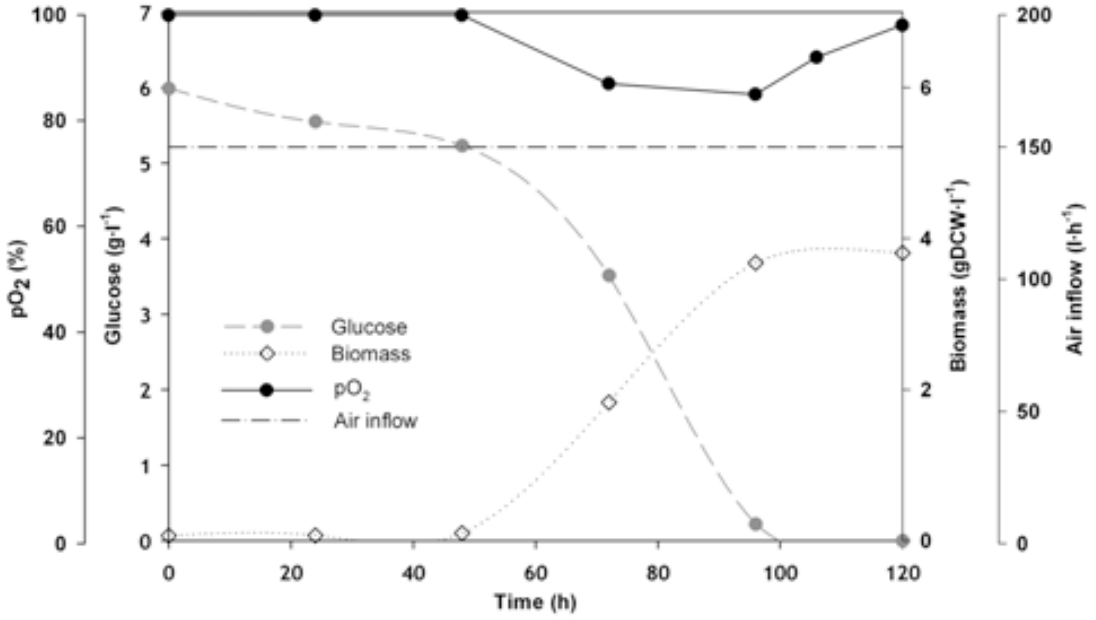


Figure 4.9 Biomass, glucose, dissolved oxygen and air flow during pellet production using the 10 liters air pulsed-fluidized bioreactor.

Figure 4.9 also shows dissolved oxygen as well as the air inflow profiles along the experiment. The air inflow was kept constant, and was sufficient to

guarantee the dissolved oxygen levels required for the fungal growth even during exponential growth. The air-flow was 150 $l \cdot h^{-1}$, equivalent to 0.25 vvm, a value inferior to the one used in the case of 1.5 liters air pulsed bioreactor. This supported that scaling the process enhanced the gas-liquid transfer.

4.1.2.4 Cost evaluation

Blánquez (2005) compared a process of physico-chemical decolourisation using commercial active carbon with a biotechnological decolourisation process using pellets of *T. versicolor*, concluding that the biological treatment was 20% more expensive respect to the physico-chemical. The cost was owed mainly to raw materials for pellets production. Malt extract cost represented 97% of the total cost. Thus, an important goal of the present study was to minimize the cost of the medium for pellets growth.

Cost estimation for defined media is shown in table 4.3. values are compared with the cost of malt extract medium, presented in table 4.4. Products prices are based on industrial quality products.

Table 4.3 Defined medium cost (prices correspond to 2008).

Nutrients	Concentration	Cost	€·l^{-1} of media
Glucose	7 g·l^{-1}	0.9 (€·Kg^{-1})	0.0063
NH_4Cl	2.1 g·l^{-1}	0.5 (€·Kg^{-1})	0.00105
Macronutrients	100 ml·l^{-1}	8.97 (€·m^{-3})	0.00897
Micronutrients	10 ml·l^{-1}	2.01 (€·m^{-3})	0.00201
Thiamine	10 mg·l^{-1}	305.50 (€·Kg^{-1})	0.00306
Total cost			0.02139

Table 4.4 Malt extract medium cost (prices correspond to 2008).

Nutrient	Concentration	Cost (€·Kg^{-1})	€·l^{-1} of media
Malta Extract	20 g·l^{-1}	42.06	0.84

Table 4.5 Cost of pellets production in defined and malt extract media.

Media	Biomass (g·l^{-1})	Media cost (€·l^{-1})	Biomass cost (€·Kg^{-1})
Defined	3.58	0.02139	5.98
Malta extract	3	0.84	280

The defined medium permitted a noteworthy reduction on the biomass production cost (98%). This reduction could be even higher since 14% of the cost of the medium is owed to thiamine and concentration of this vitamin could be probably optimized. Further experiments could be performed to

determine the minimum requirement of this component. In this sense, one of the main goals of the work was attained.

The cost per unit of treated effluent was recalculated respect to the biotechnological process proposed by Blánquez (2005). Table 4.6 presents costs associated to each media. It was taken into account that pellets in defined medium were produced in an air pulsed bioreactor, demanding less flow and requiring a smaller energy amounts for agitation. This reduced the process energetic consumption by 20%. Consequently, the media change for pellet production resulted in a great reduction in terms of cost, saving 92.4% with respect to the previous biotechnological treatment.

Table 4.6 Biotechnological decoulorisation process cost employing different media for pellet production.

	Biological ME	**Biological DM**
Raw material Cost (€·m^{-3})	34.95	1.72
Energy cost (€·m^{-3})	1.10	0.88
Final disposition cost (€·m^{-3})	0.15	0.15
Total cost (€·m^{-3})	36.21	2.75

4.1.3 Conclusions

- A defined medium that allows *T. versicolor* growth in the form of pellets was formulated in replacement of malt extract medium.
- The cost of the growth media has been reduced in 94%.
- It was elucidated that pH is a key parameter and can only be sustained using a bioreactor with a pH controller.
- The use of a stirred reactor produces the breaking of hyphae and favors disperse growth; then causing operational problems, even at low agitation.
- The use of a stirred reactor does not allow a good oxygen transfer and this is a key parameter in the fungal morphology. The limitation of dissolved oxygen favors the pellets pilosity and their posterior breaking.
- The air pulsed fluidized permits a less aggressive agitation as well as improves the oxygen transfer. It also allows the obtention of pellets with suitable size and morphology.
- Pellets production has been scaled-up from a 1.5 liters bioreactor to a 10 liters bioreactor satisfactorily.

4.2 Substrate colonization for soil bioaugmentation with *T. versicolor*

Once the methodology to produce large amounts of biomass was established, experiments focused into the soil colonization were carried out. Different lignocellulosic substrates were screened as possible innoculum carriers to support the growth of *T. versicolor* in soil. Three of them were selected for soil colonization experiments. At the same time, effectiveness of the process was measured by means of ergosterol increase (active biomass), laccase (enzymatic activity) and ND24 test (degradative capacity).

4.2.1 Soil colonization strategies using white rot fungi

The ability of white rot fungi to degrade soil pollutants has been broadly studied (Redy, 1995; Pointing, 2001; Gao *et al.*, 2010), even though such contaminants are seldom present in wood, the natural habitat of this fungal family. It is important to investigate ways to improve the survival of white rot fungi in polluted soils, an unusual habitat to such organisms. This research is especially difficult due to problems associated to fungal biomass measurement in solid state and the detection of ligninolitic enzymes. Moreover, overcoming competition with soil native microflora maintaining the fungal degradative capacity remains challenging.

Soil is diverse from wood environment in many aspects. It contains a reduced amount of nutrients, in different forms than wood and presents an heterogeneous spatial distribution (Baldrian, 2008). The growth of white rot fungi in soils is mainly limited due to the reduced nutrients accessibility, particularly carbon and nitrogen (Boyle, 1995). The majority of protocols to employ white rot fungi for soil bioaugmentation have been adopted from mushroom growers (Gadd, 2001). These consist of producing fungal innoculum from lignocellulosic wastes and are known as solid-state fermentation (SSF). Generally, white-rot fungi are introduced into soil pre-grown on lignocellulosic substrate used as innoculum carrier. Consequently, white rot fungi have been grown on inexpensive substrates such as corn-cobs, sawdust, wood chips and/or wheat straw (see table 4.7). Then, these subtrates colonized by fungi are mixed with the polluted soil (Barr and Aust, 1994).

Generally, the larger the inoculum biomass, the faster and more successful is the establishment of the fungus in the soil (Lestan *et al.*, 1996). Special care is required when balancing the carbon and nitrogen ratio in the substrates, which have a significant influence on the degradative performance of white-rot fungi. The selection of a suitable innoculum carrier can easily overcome nutrient lacks and allow soil-colonization under sterile conditions (Lang *et al.*, 1997; Mougin *et al.*, 1997).

Table 4.7 Studies employing lignocellulosic substrates for both bioremediation and enzyme production purposes (adapted from Llorens 2010).

Degraded Pollutant Synthetized product	Fungi	Substrates	Authors
Polichlorophenols (PCP)	*P. chrysosporium* and *T.versicolor*	Sawdust, starch and maize flour	Leštan *et al.* (1996)
Benzo[a]pyrene, chlorobenzene, chrysene, naphthalene, phenols and heavy metals	*P. chrysosporium* and *Marasmiellus troyanus*	Sawdust and maize stalks	Childress *et al.* (1998)
PAHs	*Pleurotus ostreatus*, *P. chrysosporium* and *T.versicolor*	Sawdust and wheat straw	Novotný *et al.* (1999)
Enzyme production	*Phanerochaete chrysosporium*	Malt	Rodríguez Couto *et al.* (2001)
Red phenol	*T.versicolor*	Seeds and stems of vine and barley	Lorenzo *et al.* (2002)
Enzyme production	*T.versicolor*	Malt	Rodríguez Couto *et al.* (2003)
Polichlorophenols (PCP)	*T.versicolor* and *Trametes sp.*	Sawdust of *Pinus radiata*, *Pseudotsuga menziesii* and *Populus deltoids*.	Christopher *et al.* (2007)
Molasses distillery wastewater	*P. ostreatus*	Wheat straw and maize stalks	Pant and Adholeya (2007)
Enzyme production	*P. ostreatus* and *Pleurotus sajor-caju*	vineyard wastes, sawdust, rise husks, sesame husks and wheat husks	Kurt and Buyukalaca (2010)
Enzyme production	*P. chrysosporium*	Apple pulp, beer brewery residues, fisherie residues and papermill industry residues	Gassara *et al.* (2010)

Another strategy for bioaugmentation fungi in soils is known as encapsulation technology. This employs fungal inocula coated with alginate, gelatin, agarose and/or chitosan (Lamar *et al.*, 1990; Leštan *et al.*, 1996). It provides more viability of the inoculum and sustains the degradation capacity for longer periods of time. Although some promising results have been obtained, the inoculum quantity

required per unit of polluted soil limit the commercial viability of this process (Lamar *et al.*, 1994, 2002).

In sterile soils, colonization ability is affected due to factors such as soil texture, pH and temperature and presence of toxic compounds (Baldrian, 2008). The major issue affecting the soil colonization ability by white rot fungi is the presence of indigenous microorganisms. Liang and McFarland (1994) demonstrated the striking difference at colonizing sterile and non-sterile soil. Under non-sterile conditions fungi have "to pay" the interactions costs with soil microorganisms; resulting in a higher demand on nutrients from the innoculum substrate for an optimal colonization (Gramss, 1979). Different species of white rot fungi can be grouped in weak (*Ganoderma* sp., *Dichomitus* sp.) or strong competitors (*Pleurotus* sp., *Phanerochaete* sp., *Trametes* sp.) depending on their ability to colonize non-sterile soil (Baldrian, 2008). However, even colonizing abilities are variable within single specie and depend on the soil type (Tornberg *et al.*, 2003; Šnajdr and Baldrian, 2006). Once stablished in soil, fungi can survive for extended periods, to more than 200 days (Axtell *et al.*, 2000; Walter *et al.*, 2005).

Soil colonization goes together with the increase of ligninolytic enzymes activity and coupling enzymes (Lang *et al.*, 1998; D'Anniballe *et al.*, 2005; Šnajdr and Baldrian, 2006). This fact is interesting for applications in bioremediation, since these enzymes are responsible for some xenobiotic transformations. Organic compounds and humic substances present in the lignocellulosic substrate can act as natural mediators increasing the degradation capacity of fungi in soil (Zavarzina *et al.*, 2004). Additionally, laccase activity can augment several folds due to interactions with soil microorganisms as it also acts as a stress-response enzyme (Velazquez-Cedeno *et al.*, 2004; Baldrian, 2004).

The monitoring of fungi in bioremediation and/or soil colonization processes is based on the estimation of fungal biomass, enzymatic activity and biodegradation capacity. Ergosterol measurements indicate the active fungal biomass in solid matrix (Stahl and Parkin, 1996; Ruzicka *et al.*, 2000; Bååth, 2001; Barajas-Aceves *et al.*, 2002). Laccase is the enzyme secreted in highest amounts by *T. versicolor* and is involved in biodegradation mechanisms as well as interactions with other microorganisms (Baldrian, 2004 and 2006). Additionaly, it serves as a measure of the fungus enzymatic machinery state. ND24 is a novel biodegradation test that permits to monitor the degradation capacity status of the fungus (Rodríguez-Rodríguez *et al.*, 2010b).

In the present study, processed and not processed low-cost lignocellulosic substrates were employed for *T. versicolor* growth in solid-state prior to application into soil. Similar studies with relative success have been reported for *Phanerochaete chrysosporium* (Leštan *et al.*, 1996), but they are ambiguous for *T. versicolor* (Katagiri *et al.*, 1995; Novotný *et al.*, 1999; Rama *et al.*, 2001; Schmidt *et al.*, 2005; Ford *et al.*, 2007). The substrates employed can be aggrupated in two different types, as listed next.

- *Agricultural low-cost lignocellulosic substrates*: maize stalks (Pn); wheat straw (Pa); pine stardust (Se); rice husks (Ar).
- *Processed Agricultural low-cost lignocellulosic substrates for animal feeding*: wheat straw pellets (ATEA Praha s.r.o., Czech Republic) (C1); rabbit feedstock (Suprem®) (C2); rabbit feedstock (Figueres®) (C3).

4.2.1.1 Aims of this section

This section presents the results obtained in the experiments focused on the selection of an optimal lignocellulosic substrate for employing it in further biodegradation experiments in solid-state culture. Experiments were divided into three phases. The first one is presented as the characterization of all substrates and the selection of the most appropriate substrates for further experiments measured as CO_2 accumulation, biomass (ergosterol content) and laccase activity of the fungus. The second experimental phase evaluated the fungal ability to colonize soils, under both sterile and non-sterile conditions unsing biopile-like systems, amended with the selected substrates in the previous phase. The last phase focused on the evaluation of the fungal degradative competence when colonizing the soil using the ND24 test.

4.2.2 Results and discussion

4.2.2.1 Substrates characterization

The selection of an adequate support to perform soil bioremediation with WRF is essential, since the process success depends on guaranteeing both the colonization of the solid matrix and the fungal degrading ability throughout the treatment period.

Table 4.8 presents major physical and chemical characteristics of the various substrates employed in the study as well as its elemental composition. In general, fungi growth is favored at pH values in the acidic range. Maximum laccase activity occurs at pH around 4.5-5 for *T. versicolor* (Tavares *et al.*, 2006; Stoilova *et al.*, 2010), while values over 7.4 have shown inactivation of WRF enzymes (Lu-Chau *et al.*, 2004). In this respect, all substrates seemed suitable for both growth and laccase production, although the pH values of Pn and Se were slightly acid.

Table 4.8 Physico-chemical properties and elemental composition of the different substrates.

Substrate	Field capacity ($gH_2O \cdot gDW^{-1}$)	C (% w)	H (%w)	N (%w)	O (%w)	S (%w)	C/N (w/w)	pH_{H2O}	pH_{KCl}
C1	3.13	41.51	5.89	1.03	51.6	< 1%	40.15	5.65	5.66
C2	3.33	40.65	6.10	3.46	49.8	< 1%	11.75	5.35	5.15
C3	3.37	39.93	6.21	2.50	51.4	< 1%	15.98	5.31	5.13
Pn	1.03	45.15	6.48	0.57	47.8	< 1%	79.67	3.99	3.44
Pa	3.66	42.69	5.99	0.83	50.5	< 1%	51.39	6.96	6.75
Se	2.3	40.80	5.38	0.33	53.5	< 1%	121.32	3.96	3.47
Ar	7.2	46.60	6.11	0.04	47.2	< 1%	1075.32	5.33	4.79

Although production of ligninolytic enzymes has been typically associated with a limitation of N, the relation C/N has been considered as a better indicator of ligninolytic activity rather than the absolute values of C and N. Reports of optimal C/N ratio are quite dissimilar for production of ligninolytic enzymes in *T. versicolor*. Studies in liquid media suggest optimal ratios ranging from 10 to 46 (Fakoussa and Frost, 1999; Rodríguez Couto *et al.*, 2002; Lorenzo *et al.*, 2002). However, in solid substrates ratios between 120 and 200 produced higher laccase activity than ratios above 200 in solid substrate mixtures (Schmidt *et al.*, 2005). The C/N ratios of substrates are around the range described to produce laccase. Nevertheless, it has to be pointed out that the accessibility of both C and N by the fungus is not clear. So, these ratios are not directly comparable. Yadav and Tripathi (1991) used wheat bran and wheat straw to culture *T. versicolor* in solid-state fermentation. The optimal fermentation conditions were 55% moisture and

pH 5.5 after 21 days of culture. These results are in accordance with those obtained in this study.

Field capacity measures the substrate apparent porosity and gives an idea of the ability to retain water by capillarity and the permissiveness of oxygen diffusion. The latter is necessary for inner colonization of substrates by hyphae. Meanwhile, the maximum field capacity is given by Ar substrate, but is quite similar to substrates C1, C2, C3 and Pa. It has to be taken into account that the fungus requires the presence of water in the environment. Therefore, it is necessary to select those substrates that allow a good supply of water for growth, but at the same time it is important to prevent an excess of water that favors other microorganisms to compete with the fungus.

4.2.2.2 Initial Screening of substrates: CO_2 accumulation measurements

An initial screening in sterile conditions was performed in order to recognize the most suitable substrates for fungal growth. Monitoring CO_2 accumulation in such closed system during colonization of substrates permits a semi quantitative assessment of fungal respiration proportional to biomass. Also, visual observations were performed. As depicted in figure 4.10, CO_2 production and subsequent accumulation was different for each substrate, but can be grouped into two main groups based on the trends and ranges of CO_2 concentration. On one hand, there is the group formed by C2 and C3 substrates. These presented a maximum accumulation of 40 percentage units above the others. Although the lag phase was similar to other substrates, the growth phase was prolonged and the CO_2 accumulation was not well stabilized at the end of the experiment. Another group is formed by substrates C1, Pa, Pn, and Ar. These also presented a lag phase during the first 24 hours and an exponential growth phase until fifth day of culture. Then CO_2 values stabilized.

Regarding to water content, CO_2 accumulation profiles showed slight dissimilarity for different water contents between the same substrate except for C1, C2 and C3. In general terms, the higher moisture contents the higher concentration of CO_2; except above 60% of the holding capacity. This fact can be explained by a different behavior towards the substrate moisture. The substrates C1, C2 and C3 show a higher apparent porosity with respect to the substrates Pa, Pn, Se and Ar. Substrate volume increased while increasing moisture content, resulting in larger and spongy materials favoring oxygen transfer to hyphae.

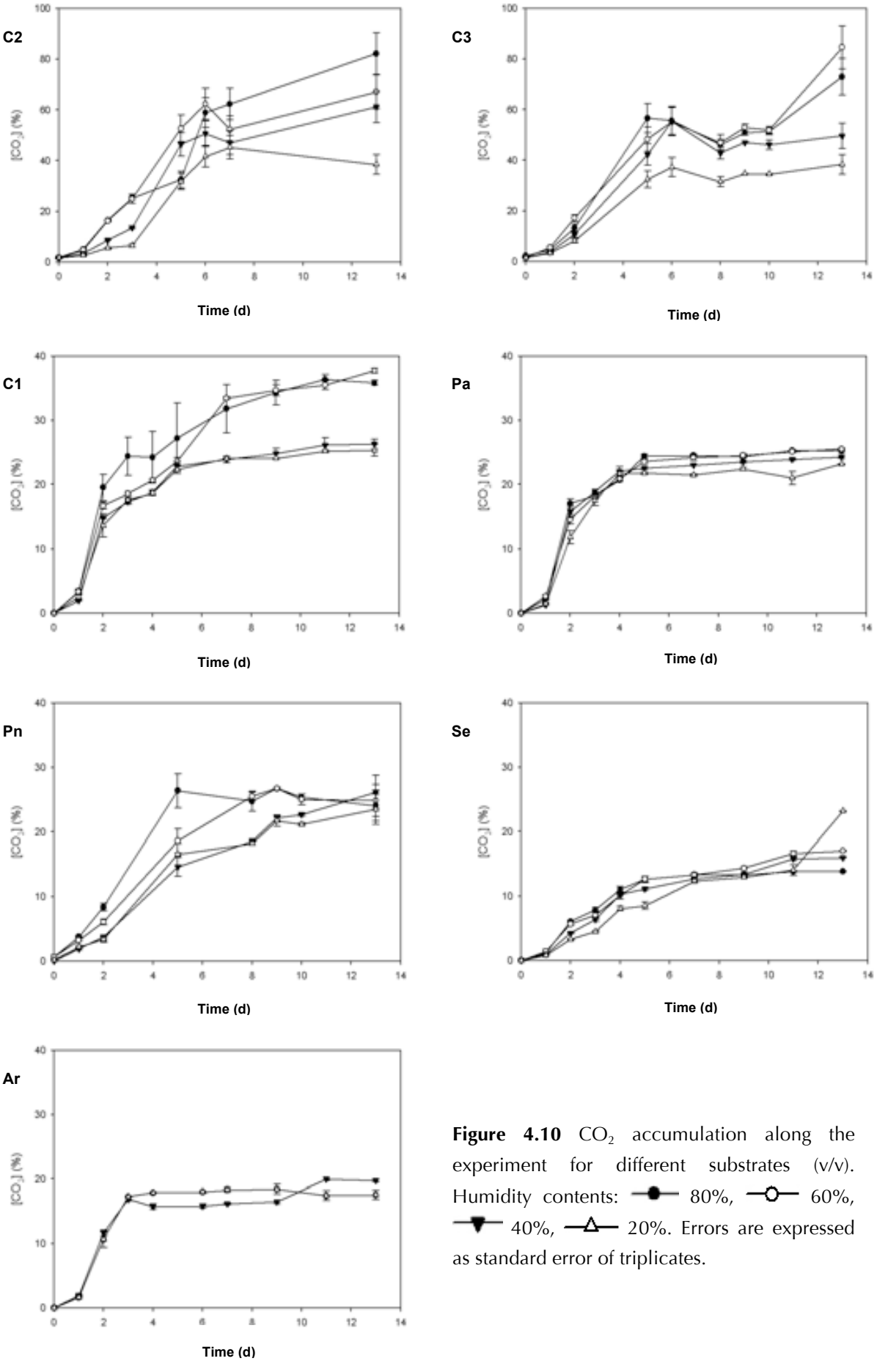


Figure 4.10 CO_2 accumulation along the experiment for different substrates (v/v). Humidity contents: ●— 80%, —○— 60%, —▼— 40%, —△— 20%. Errors are expressed as standard error of triplicates.

It is noteworthy that substrates that present higher N content (C1, C2, C3) supported the best growth in terms of CO_2 accumulation. Also Pa and Pn permitted relevant growth although N levels are lower.

Visual observations determined that the fungus colonized rapidly the substrate during the two first days. A tiny layer of mycelia covered the substrate initially, turning later into a dense and white interwoven mycelia. Only for the case of Se and Ar poor colonization was observed.

According to the results, this experiment could be used as an initial screening of substrates depending on the CO_2 produced, assuming that this is related to the ability of *T. versicolor* to assimilate each substrate for growth purposes. It was decided not to use Se and Ar for next experiments because these substrates did not permit proper fungal growth. Otherwise, we decided to use 60% of field capacity as humidity content for further experiments. This allowed obtaining high concentrations of CO_2 while lower water contents decreased the chances of contamination by other microorganisms. That is, minor amount of water to greater effect by selective fungal growth towards other organisms.

4.2.2.3 Quantitative colonization of substrates

In this second part, the soil colonization by fungus grown on substrate under sterile conditions was studied. Growth was measured by ergosterol quantification in order to track viable fungal biomass. Laccase activity was monitored to follow the fungal extracellular enzymatic system.

Figure 4.11 presents ergosterol values on sterile substrates inoculated with *T. versicolor*. As observed, there is great variability over time and between different substrates. However, certain trends among groups of substrates can be discussed.

On the first sampling point, after three days, ergosterol content augmented considerably in C2 and C3, being 0.13 $mg \cdot gDW^{-1}$ and 0.20 $mg \cdot gDW^{-1}$, respectively; but decreased in the next sampling days. After 7 days, biomass increased significantly with substrates C2 and C3, yielding high amounts of biomass with maximum values of 0.33 $mg \cdot gDW^{-1}$ and 0.25 $mg \cdot gDW^{-1}$ after 17 days of culture. These surpassed clearly the values from the other substrates. These concentrations represented an increase in active fungal biomass respect the inoculum of 210% and 160%, respectively.

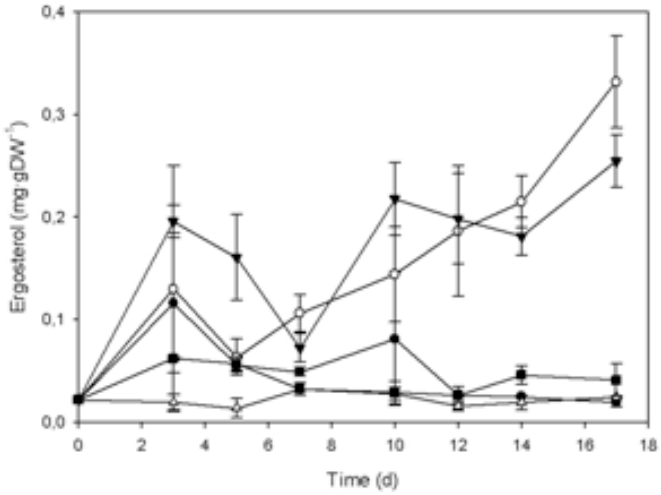


Figure 4.11 Ergosterol content in substrates colonized by *T. versicolor*. Substrates: ***C1*** —●—, ***C2*** —○—, ***C3*** —▼—, ***Pn*** —△— i ***Pa*** —■—. Error bars represent the standard error of triplicates.

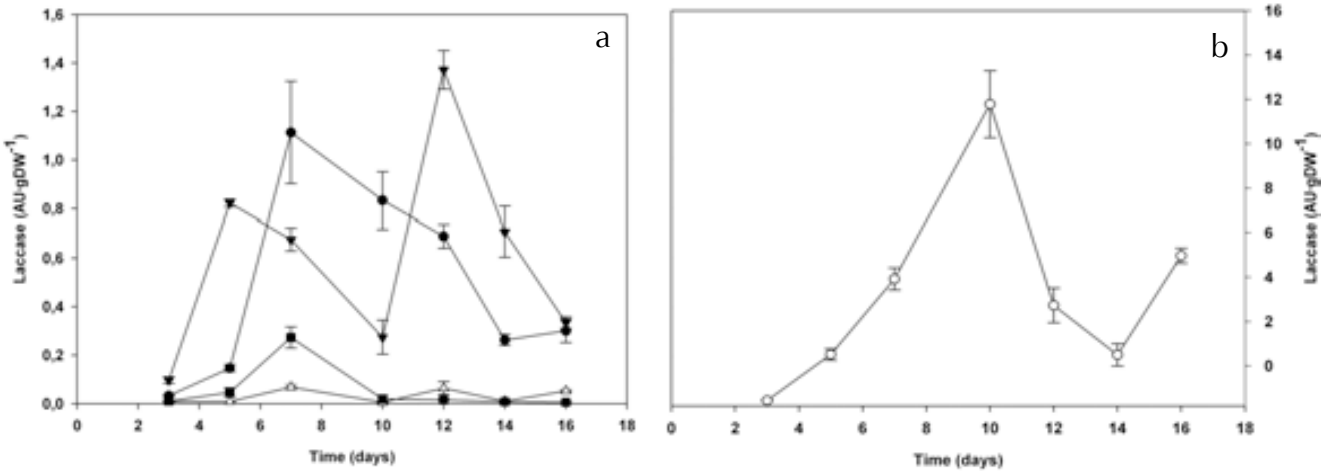


Figure 4.12a and 4.12b Laccase activity in substrates colonized by *T. versicolor* . Substrates: ***C1*** —●—, ***C2*** —○—, ***C3*** —▼—, ***Pn*** —△— i ***Pa*** —■—. Laccase activity is expressed as unit of activity (AU) per gram of dry weight in solid matrix. Error bars represent the standard error of triplicates.

In the case of C1, ergosterol increased after 3 days of culture (0.12 mg·gDW^{-1}), then decreased gradually up to the end of the culture period, reaching values similar to the initial inoculated biomass. These results contradicted visual observations supporting the right colonization by the fungus. Substrate was completely covered with white and dense hyphae. Laccase values also correlated with these observations, as will be described later, suggesting problems in extraction procedure for those samples. Finally, for substrates Pn and Pa were detected at very low levels after 3 days, 0.06 mg·gDW^{-1} and 0.02 mg·gDW^{-1}, respectively. Little variation over time occurred and no increase in viable biomass with respect the initially inoculated was obtained.

Visually, it was determined that C2 and C3 substrates were allowing the highest fungal growth, followed closely by C1. For all three substrates the growth was relatively homogeneous among the different cultures throughout the experiment. Substrate Pa permitted scarce growth and growth on Pn was rather poor and could not be observed until day 5.

Low laccase activity was detected in substrates C1, C2 and C3 (figure 4.12a), within the same range and with different trends, reaching maximums of 1.11 $AU \cdot gDW^{-1}$ and 1.37 $AU \cdot gDW^{-1}$ after 7 and 12 days, respectively. For substrate C1, the laccase excretion pattern was oscillating and reached its maximum value at day 7. The measured activity did not correlate with the ergosterol values for this substrate, confirming there had been some problem related to ergosterol extraction. Substrate C3 also presented an oscillating behavior, reaching the maximum activity after 13 days of culture. Substrates Pn and Pa showed the lowest values, with maximal of 0.27 $AU \cdot gDW^{-1}$ and 0.07 $AU \cdot gDW^{-1}$, respectively after 7 days of culture. Before and after the laccase level was negligible for any of them.

As depicted in figure 4.12b, substrate C2 presented the highest laccase activity, 12.21 $AU \cdot gDW^{-1}$ at day 10 of culture, approximately 10-fold higher respect to other substrates. This substrate allowed a sustained increase in laccase activity, reaching a peak after 10 days of culture. Similar behavior has been recently described for *T. versicolor* growing in an analogous solid-state system by Rodríguez-Rodríguez *et al.* (2010a).

For soil bioremediation purposes, colonization and activity should be guaranteed. For this reason, and based on the obtained results, substrates Pn and Pa were discarded from subsequent experimentation.

4.2.2.4 Colonization of sterile soil

In this section, the capacity of *T. versicolor* pre-grown on C1, C2 and C3 substrates to colonize soil under sterile conditions was studied. Colonization was assessed in terms of ergosterol content, laccase activity and degradative capacity by means of the ND24 test. Variation in pH of the mixture soil-substrate (table 4.9) was less than 5% respect to the values shown in table 4.7 for the substrates alone. Thus, acidic conditions, favorable to fungi, were maintained.

Table 4.9 pH values of soil amended with different substrates.

Mixture	pH_{H2O}	pH_{KCl}
Soil + *C1*	5.81	5.47
Soil + *C2*	5.26	5.01
Soil + *C3*	5.21	4.91

Visual observations confirmed that the fungus was capable to colonize soil employing any substrate. The day after mixing some hypae was already extending through soil. These augmented gradually, conferring a white coverage to the matrix (see figure 4.13). An increase in soil hydrophobicity was observed at expanding fungal biomass as previously reported by White and co-workers (2000).

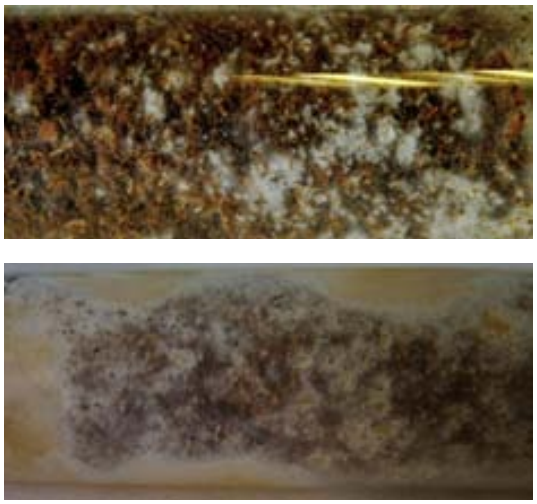


Figure 4.13 Detail of soil colonized by *T. versicolor* after 4 days (a) and 15 days (b) of solid-state culture.

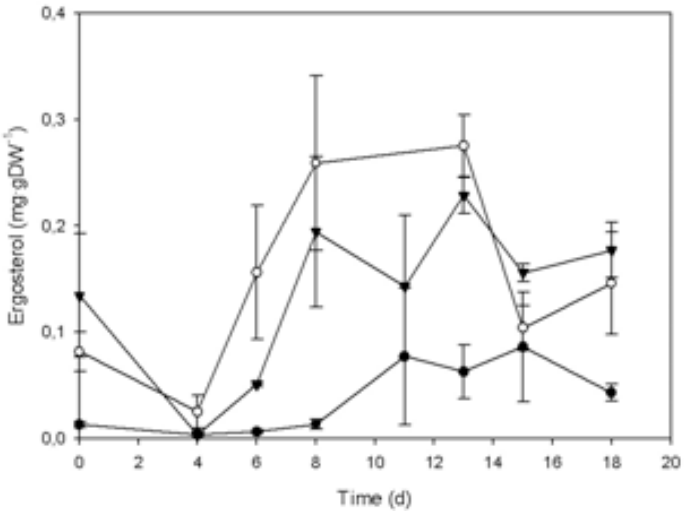


Figure 4.14 Ergosterol content in soil-substrates mixture colonized by *T. versicolor* under sterile conditions. Substrates: ***C1*** —●—, ***C2*** —○—, ***C3*** —▼—. Error bars represent the standard error of triplicates.

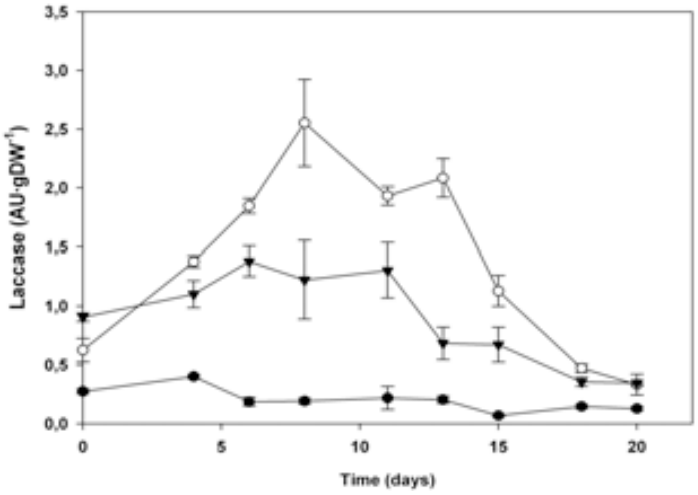


Figure 4.15 Laccase activity in soil-substrates mixture colonized by *T. versicolor* under sterile conditions. Substrates: ***C1*** —●—, ***C2*** —○—, ***C3*** —▼—. Error bars represent the standard error of triplicates.

In terms of ergosterol, it decreased sharply as a result of the addition and homogenization of the substrate-soil matrix (see figure 4.14). For substrates C2 and C3 a lag phase lasted until day 4 whereas for C1 it did until day 6. As for substrate colonization, ergosterol trends oscillated but followed similar patterns.

Growth over substrate C1 was the slowest in terms of ergosterol per dry mater along the experiment. Moreover, it allowed the highest increase in terms of viable biomass. A maximum of 0.09 mg·gDW^{-1} was achieved (day 15), representing a 760% increase in terms of viable biomass. The final ergosterol content was 0.04 mg·gDW^{-1}, a 330% augment of viable biomass.

As regards to substrate C2, it allowed a continuous increase of viable biomass until day 13 (0.28 mg·gDW^{-1}), and then it decreases slowly at the end of the experiment (0.17 mg·gDW^{-1}). In previous experiments of substrate colonization, C2 allowed similar biomass production in terms of ergosterol content. The viable biomass increased along the experiment, reaching a maximum increase of 206% (day 13) and a final value of 83% with respect to the initial biomass.

Substrate C3 achieved its maximum of ergosterol content at day 13 (0.23 mg·gDW^{-1}), which represented an increase of 70.9% in viable biomass. From that on it decreased until the end of the experiment (0.18 mg·gDW^{-1}). Thus, the total augment in viable biomass was only 32%.

Similar oscillatory ergosterol profiles to those were obtained in colonization of sewage sludge by analogous solid-phase systems with *T. versicolor* (Rodríguez-Rodríguez *et al.*, 2010a). Meanwhile, very low levels (up to 0.002 mg·gDW^{-1})

were accomplished in soil colonization with *T. versicolor* exploratory mycelium (Novotný *et al.*, 1999).

For colonization of sterile soil, lowest laccase activity values were detected with C1 (figure 4.15). Activity remains constant around a range of 0.20 AU·gDW^{-1} and decreases up to 0.13 AU·gDW^{-1} at the end of the experiment. These values are not negligible in terms of absolute activity, but when normalizing by dry weight are lower respect the values obtained with the other substrates. The values obtained are quite close to the activity range observed for C1 at figure 4.11a, when colonizing substrate.

Substrate C2 exhibited the highest laccase activity values, reaching a maximum of 2.55 AU·gDW^{-1} after 8 days. But unlike the substrates colonization experiment, this presents a lower laccase activity (4.8-fold). Activity reaches a final value of 0.33 AU·gDW^{-1}.

Finally, substrate C3 presented the major initial laccase activity (0.91 AU·gDW^{-1}), but from there on its activity was lower compared to C2. Laccase activity reached a steady value and then decreased to 0.35 AU·gDW^{-1} at the end of the experiment.

The maximal activity was relatively similar to those obtained in the substrates colonization. Peak values ranging from 4 to 7 AU·gDW^{-1} were reported by Rodríguez-Rodríguez *et al.* (2010) during sludge colonization by *T. versicolor*.

Figure 4.16 shows the results obtained in testing for ND24 *T. versicolor* in sterile soil. C1 is the substrate that presented a higher percentage of degradation capacity, reaching values of 46.7% on day 11 and 48.3% on day 20. C2 reaches maximum degradation on day 11, with 24.4%, and decreased to 11.9% on day 20. In contrast, C3 has a lower degradation percentage on day 11 (11.5%) and increases on day 20, with a value of 21.4%. The results demonstrated that although C1 did not permit the maximum biomass and enzymatic activities; it allowed the maximum degradative capacity when colonizing soil. These observations are in line with the fact that C1 allowed the maximum increase in viable biomass.

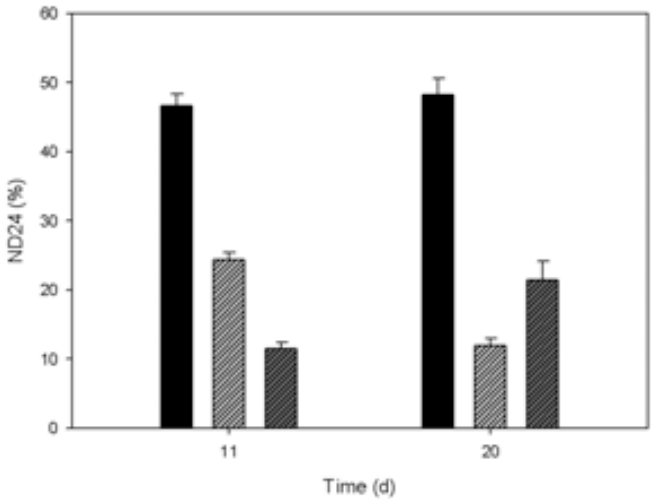


Figure 4.16 Naproxene degradation (ND24) by *T.versicolor* grown on soil-substrate mixtures under sterile conditions. Substrates: ***C1*** ■, ***C2*** ▨ and ***C3*** ▨. The error bars represent the standard deviation of triplicates.

4.2.2.5 Colonization of non-sterile soil

The next research step aimed to evaluate the substrates performance to support soil colonization by *T. versicolor* under non-sterile conditions, as a more real approach towards a bioremediation process conditions. Experiments were analogous to those described in previous section, but using non-sterile soil. Visual observations showed a similar colonization pattern to sterile soil. In most experimental tubes, colonies of other microorganisms grew, thus competing with *T. versicolor*.

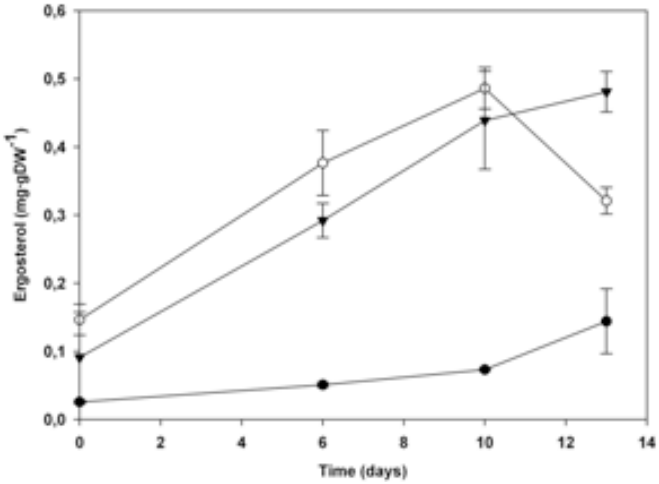


Figure 4.17 Ergosterol content in soil-substrates matrix colonized by *T. versicolor* under non-sterile conditions. Substrates: ***C1*** —●—, ***C2*** —○—, ***C3*** —▼—. Error bars represent the standard error of triplicates.

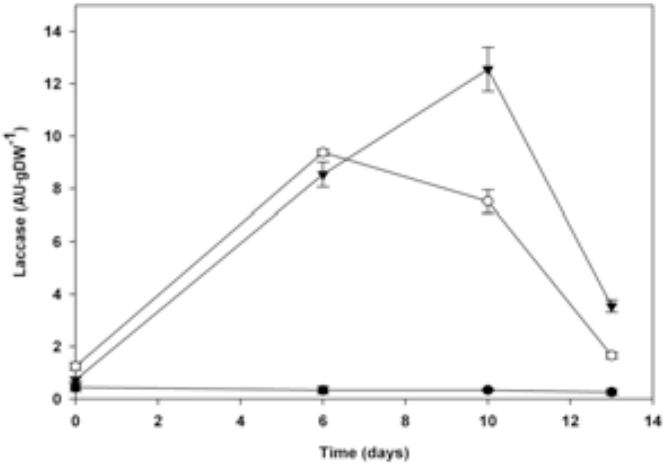


Figure 4.18 Laccase activity in soil-substrates mixture colonized by *T. versicolor* under non-sterile conditions. Substrates: ***C1*** —●—, ***C2*** —○—, ***C3*** —▼—. Error bars represent the standard error of triplicates.

Ergosterol monitoring on non-sterile soil can be observed in Figure 4.17. In general terms, ergosterol contents were similar to those obtained in sterile soil. Otherwise, maximum values of viable biomass were reached earlier respect to sterile conditions, showing no-negative effects due to competition with soil microorganisms. Nonetheless, colonization was accomplished after 3 days, as it was supported by visual observation.

As observed in earlier tests, substrate C1 presented the lowest concentration of ergosterol in the experimental time course. The results obtained in non-sterile soil are quite similar to the trend observed in sterile soil, showing a constant increase in ergosterol content. The initial viable biomass was quite low respect the other substrates. It rose gradually to reach a final value of 0.11 mg·gDW^{-1} that supposed an increase of 3-fold in terms of viable biomass.

Substrates C2 and C3 also showed a similar trend as for sterile soil. Both initial ergosterol values are theoretician since extraction problems did not permit samples correct quantification. C2 showed an oscillating pattern. Ergosterol increased to reach a maximum at day 10, 0.49 mg·gDW^{-1}, which represents an increase of 53.3-fold in viable biomass. The final value of 0.32 mg·gDW^{-1} corresponded to an increase of 35.2-fold respect the inoculated biomass. In contrast, C3 reached its maximum (0.48 mg·gDW^{-1}) at the end of the experiment, which represented an increase in viable biomass content of 22.6-fold.

Figure 4.18 presents laccase activity measurements in non-sterile soil colonization experiment. As shown, results for C2 and C3 surpassed those obtained in sterile soil. Both substrates allowed laccase expression during the early days. Both

substrates presented the same pattern, reaching a maximum value followed by a decrease in activity. Increments of 4-fold (9.38 $AU \cdot gDW^{-1}$) and 9-fold (12.55 $AU \cdot DWg^{-1}$) were obtained for the peak values with C2 and C3, respectively. This suggests an important fungal activity during colonization. Substrate C1 allowed less laccase activity per unit of soil respect to the other substrates. Laccase values were near 1 $AU \cdot gDW^{-1}$ along the colonization. Therefore the enzyme levels detected are much lower than the two other substrates but in a similar range if compared to colonization in sterile soil amended with substrate C1.

Leštan and Lamar (1996) demonstrated the capacity of *T. versicolor* to colonize unsterilized soil when inoculated with sawdust pellets. White *et al.* (2000) suggested that both *T. versicolor* and *P. chrysosporium* produce hydrophobic compounds when grow in soil. These substances enhance their survival because they prevent bacterial growth. Tuomela *et al.* (2002) demonstrated that *T. versicolor* was better at soil colonization respect *P. ostreatus* and *P. chrysosporium* using wheat straw as lignocellulosic substrate.

It has been demonstrated that interaction between *T. versicolor* and microorganisms in non-sterile soil caused an increase in laccase activity (Baldrian, 2008). White-rot fungi in soil environment have to compete with other organisms to colonize it and obtain the required resources. This type of fungus uses lignocellulosic substrates via its LME system. Additionally, this extracellular enzymatic system can be used to prevent and to compete against microbial populations present in soils. Therefore, the increase of laccase excretion when colonizing non-sterile soils may be partially understood as a response of *T. versicolor* to interactions with other soil microorganisms (Thorn and Tsuneda, 1992; Baldrian, 2004). Results obtained in sterile and non-sterile soil confirm this fact, with values of laccase activity one order of magnitude higher than in non-sterile soil.

Figure 4.19 shows the results obtained in testing for ND24 *T. versicolor* in non-sterile soil. Substrate C1 permits the maximum degradation: 20.9% on day 10 and 28.2% on day 13. Substrate C2 allows a degradation of 6.9% on day 10 and 25.1% on day 13. Finally, substrate C3 gives values of 5.1% degradation on day 10 and 9.9% on day 13.

ND24 test suggested that substrate C1 is more suitable for possible mycoremediation approaches under non-sterile treatments. It must be considered that this degradation is evaluated using a specific compound. Therefore,

degradation capacity of *T. versicolor* against other pollutants should be assessed when trying to apply it in specific bioremediation purposes.

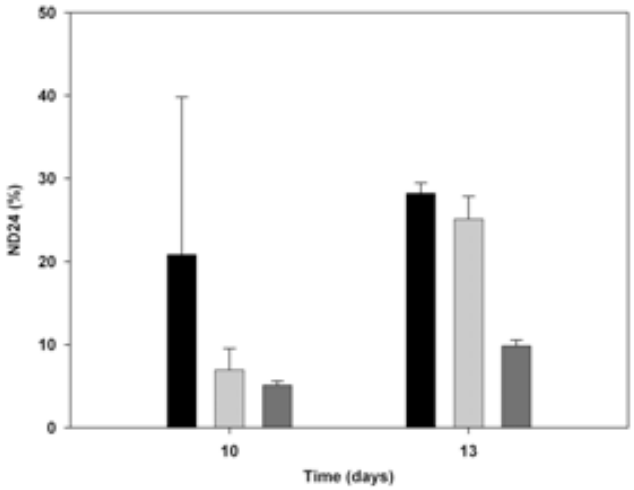


Figure 4.19 Naproxene degradation (ND24) by *T.versicolor* grown on soil-substrate mixtures under non-sterile conditions. Substrates: ***C1*** , ***C2*** and ***C3*** . The error bars represent the standard deviation of triplicates.

4.2.3 Conclusions

- The use of CO_2 accumulation coupled to visual observations was a useful technique for an initial screening of suitable substrates to grow *T. versicolor*.
- The colonization and enzymatic production potential of *T. versicolor* was higher substrates (C1, C2 and C3; all with relative low C/N ratios respect the others.
- Soil colonization by *T. versicolor* was successful under both sterile and non-sterile conditions when amended with substrates C1, C2 and C3; as assessed by means of ergosterol.
- Higher laccase production was observed in non-sterile soil colonization experiments possibly as a response of *T. versicolor* to competition with native microflora.
- The fungus was able to degrade naproxene both under sterile and non-sterile soil cultures at different sampling times; mainly with the substrate C1.
- Substrate C1 permitted the highest ND24 values although it produced less biomass and laccase. This substrate was chosen for soil bioaugmentation using *T. versicolor* in further experiments.

CHAPTER 5

Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) by *Trametes versicolor*

In this chapter, attention will focus on polycyclic aromatic hydrocarbons (PAHs) degradation by Trametes versicolor. Such compounds are considered as priority pollutants due to their ubiquitous occurrence, bioaccumulation potential and carcinogenic activity. The following sections describe the capacity of T. versicolor to degrade such compounds in liquid medium, both in single and in mixtures, the subsequent identification of degradation mechanisms, enzymatic degradation, solid state and slurry degradation experiments.

A slightly modified part of this chapter has been presented and published as:

Tahseen Sayara, Eduard Borràs, Montserrat Sarrà, Glòria Caminal and Antoni Sánchez, 2010 "Bioremediation of PAHs-contaminated soil through composting: influence of bioaugmentation and biostimulation on the contaminants biodegradation" at *Chemosphere* (under revision).

Borràs, *et al.*, 2008. *Degradation of benzo[a]anthracene and benzo[k]fluoranthene by Trametes versicolor*. The third Intertanional Meeting on Environmental Biotechnology and Engineering (3IMEBE), Universitat de les Illes Balears, september 2008.

5.1 Occurrence and biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs)

5.1.1 Sources of PAHs

Polycyclic aromatic hydrocarbons (PAHs) are wide group of compounds composed of two or more fused benzenic rings in linear, angular or cluster arrangements. The aromatic ring is one of the structures more widely spread in nature, present in several molecules. PAHs are ubiquitous in the environment; particularly a 90% of the total are located in soil (Wild and Jones, 1995; Evans and Furlong, 2003). Due to their frequent occurrence and their toxicological properties, sixteen non-substituted target PAHs (see figure 5.1) have been included in the list of priority pollutants by the Environmental Protection Agency of the United States (EPA) to be monitored in industrial sites (Keith and Teillard, 1979). They are rather recalcitrant in the environment and are considered as major hazard to human and animal health. Also, its potential carcinogenicity makes them a case study.

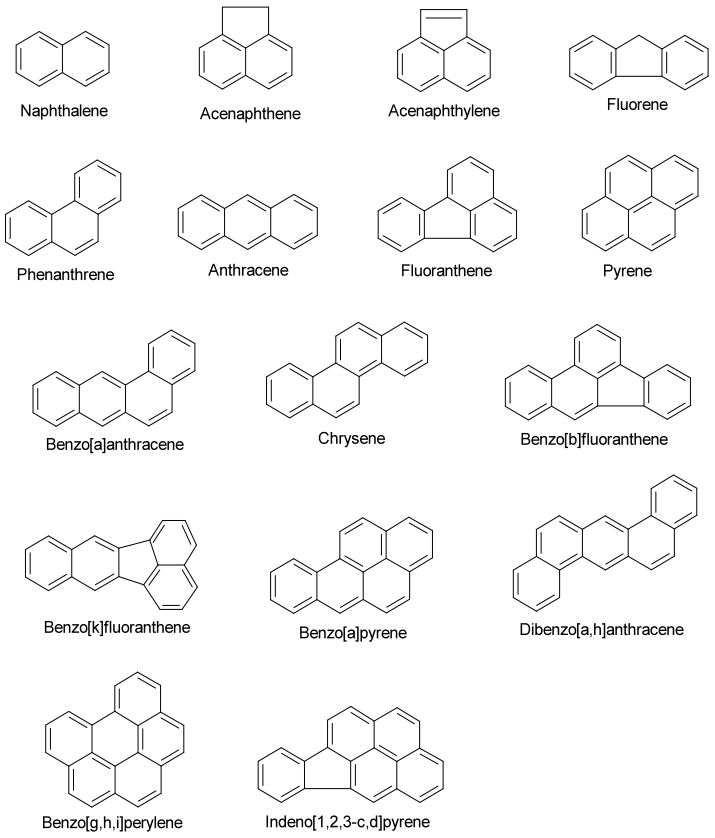


Figure 5.1 Molecular structure of the 16 PAHs selected as priority pollutants by the EPA.

Both natural and anthropogenic sources of PAHs can occur. In nature, their origin is related to pyrolisis of organic matter from wood or biomass. Also, heating of organic materials at low temperatures (100-150ºC) over an extended period of time can also produce these compounds. Petroleum and coal derivatives have a high content in PAHs.

The anthropogenic source of PAHs are related to the industrial processes, specially petroleum and carbon manufacturers, wood treatment plants, power plants, municipal incinerators and any other activity that implies the combustion of organic materials. Regularly the contaminated sites are associated to obsolete or active industrial plants where accidental spills or leaks in tanks came off. Domestic sources can occur in barbecues, domestic heating using biomass and exhaust vapors. These sources should be considered irrelevant when considering soil pollution sources. The highest PAHs levels have been detected so far in coal gasification sites ranging from 1 $\mu \cdot kg^{-1}$ to 300 $g \cdot kg^{-1}$ (Tornberg *et al.*, 2003; Bamfort and Singelton, 2005; Byss *et al.*, 2008; Loick *et al.*, 2009).

5.1.2 Physico-chemical and toxicity properties of PAHs

The main physico-chemical properties of the sixteen priority PAHs are listed in table 5.1. They are extremely hydrophobic compounds with an elevated melting point. They are very persistent in the environment due to its low solubility in water. In general terms, the more fused rings in the structure the lower the solubility. When introduced in soil, their equilibrium among solid, aqueous and vapor phases is low. PAHs are expected to adsorb on the hydrophobic surfaces of soil such as clays and organic matter (Onken and Traina, 1997). In soil, they are either trapped in the pores, fixed with covalent or hydrogen bond; also can bound during humification processes. The particular structure of each PAH conditions the feasibility of biodegradation by soil microorganisms.

Numerous PAHs exhibit acute carcinogenic, mutagenic and tetarogenic properties and may induce tumors even to single-dose exposures (Plat *et al.*, 2008). Other undesirable effects consist of negative effects toward reproduction and immunity (Eisler, 1987). Their effects have been established in many organisms including humans, birds, invertebrates, plants, amphibians and fishes. In the case of humans, exposure can come by inhalation, dermal contact and/or ingestion. They have been reported to show anti-estrogenic effects and so are listed as endocrine disrupting chemicals (Arcaro *et al.* 1999).

Special attention must be paid to benzo[a]pyrene, which has been classified as one of the most powerful carcinogens (Juasz and Naidu, 2000). In table 5.1, the potential of the target PAHs is represented in relation to that of benzo[a]pyrene.

Table 5.1 Physico-chemical properties and carcinogenic effects of the 16 priority PAHs by the EPA (adapted from: Nisbet and LaGoy, 1992; Bogan and Lamar, 1996; Zheng and Obbard, 2002a).

Compound	Effects*	Molecular Weight	Log K_{ow}	Solubility ($mg \cdot l^{-1}$)	Melting point (°C)	Ionization Potential (eV)	Carcino. factor[a]
Naphthalene	-	128	3.37	30	80	8.13	0.001
Acenaphtylene	-	152	4.07	16.1	92	8.22	0.001
Acenaphtene	-	154	4.33	3.47	96	7.86	0.001
Fluorene	1	166	4.18	1.98	116.5	7.88	0.001
Phenanthrene	1	178.24	4.57	1.29	101	8.03	0.001
Anthracene	1	178.24	4.54	0.07	216	7.43	0.01
Pyrene	1	202.26	5.18	0.135	156	7.53	0.001
Fluoranthene	1	202.26	5.22	0.26	111	7.90	0.001
Benzo[a]anthracene	1	228.28	5.61	0.011	162	7.56	0.1
Chrysene	1,2	228.8	5.86	0.003	254	7.59	0.01
Benzo[a]pyrene	1,2	252.32	5.91	$3.8 \cdot 10^{-3}$	179	7.12	1
Benzo[b]fluoranthene	2	252.32	5.80	$1.5 \cdot 10^{-3}$	168	7.65	0.1
Benzo[k]fluoranthene	2	252.32	6.06	$8 \cdot 10^{-4}$	217	7.48	0.1
Indeno(1,2,3-dc)pyrene	2	276	6.50	$1.9 \cdot 10^{-4}$	164	8.02	0.1
Dibenzo[a,h]anthracene	-	276	6.75	$5 \cdot 10^{-4}$	267	-	5
Benzo[g,h,i]perylene	-	276	7.23	$2.6 \cdot 10^{-4}$	278	-	0.01

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

Log K_{ow}: octanol/water partition coefficient.

[a]carcino. factor: carcinogenetic factor related to benzo[a]pyrene

5.1.3 Strategies to enhance PAHs bioavailability

The low bioavailability of PAHs is considered the major rate-limiting factor in the biodegradation of such compounds by both bacteria and fungi. Consequently, the enhancement of their solubility is a key factor when trying to improve their availability. Two major strategies have been carried out for such purpose: the use of co-solvents and the use of surfactants.

Surfactants are molecules that comprise a hydrophilic head and a hydrophobic tail conferring them amphiphilic structure. This structure allows them to aggregate

and form micelles (10 to 200 molecules) above a certain concentration known as critical micelle concentration (CMC). These compounds can be grouped as cationic or non-ionic according to the ionic and hydrophilic characteristics. PAHs are desorbed with surfactants though solid-liquid equilibrium (Alcántara *et al.*, 2009). The increase of bioavailability by surfactants comes from two factors. On one hand, the compound is solubilized in the organic fraction inside the micelles, known as micellar solubilisation. On the other hand, the decrease in surface tension of pore water can favor the transport of the compound from the solid to the aqueous fraction.

Several works have shown the positive effect of the addition of non-ionic surfactants such as Tween 20, Tween 40, Tween 80, Brij35 and Triton X-100 in PAHs degradation experiments with white-rot fungi, both in liquid culture and solid state (Thiem, 1994; Zheng and Obbard 2001; Ding *et al.*, 2008; Leonardi *et al.*, 2008; Giubilei *et al.*, 2009). Nevertheless, some other authors have pointed out some inhibitory effects on biodegradation given that surfactants were toxic to the organism or the compound could be retained inside the micelle being unavailable to fungi. On the contrary, some studies pointed out that non-ionic surfactants stimulate the secretion of ligninolytic enzymes. Therefore, before any experiment with surfactants for biodegradation purposes a study to select the optimal surfactant has to be performed to avoid these setbacks.

The use of a water/solvent mixture is an alternative for enhancing PAHs bioavailability. The solubility of these in organic solvents is significantly superior to that in water. In spite of this, their use presents disadvantages due to their elevated costs and toxicity towards living organisms. However, some experiences using bacteria and white-rot fungi have been performed (Field *et al.*, 1995). In experiments of enzymatic degradation with purified enzymes such as laccase, MnP and LiP, the use of acetone or ethanol as co-solvent has been successfully utilized (Collins *et al.*, 1996; Majcherczyk *et al.*, 1998; Eibes *et al.*, 2006).

Finally, another approach to PAHs degradation technique in liquid medium are the two-liquid-phase (TLP) bioreactors containing a non-aqueous-phase-liquid (NAPL) (Déziel, 1999). These types of liquids are used to enhance the biodegradation rate of poorly soluble compounds. The substrate to degrade diffuses from the water-immiscible phase to the aqueous phase, and the microorganisms located at the interface can effectively degrade the compounds. The mass transfer rate is favored by the increase of the surface area for partitioning (Marcoux *et al.*, 2000).

5.1.4 Mycoremediation of PAHs by white-rot fungi

Research on PAHs biodegradation using white-rot fungi has an interesting potential. Their non-specific extracellular enzymes enable them to degrade both lignin and a wide sort of pollutants. Another advantage is that this type of organisms does not require preconditioning to individual pollutants and avoid the uptake of toxic substances. Moreover, these fungi can degrade at particularly low concentrations. Their hyphae system makes them suitable for solid-state applications, such as soil remediation. Several white-rot fungi have been studied, namely: *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Bjerkandera adusta*, *Trametes versicolor*, *Irpex lacteus, Nematoloma frowardi, Pleurotus eyringy* and *Panus tigrinus*.

The majority of studies have been carried out using pure cultures in liquid medium in which PAHs were added using an organic solvent. Cultures are grown in dark under both stationary or agitated conditions and providing punctual aeration. This kind of experiments provides information on the fungus capacity to degrade the pollutant, toxic effects and the identification of degradation intermediates. However, it is difficult to extrapolate these to real soil contamination.

The use of techniques such as slurries (Zheng and Obbard, 2001, 2002; Valentin *et al.*, 2006, 2007), microcosms (Canet *et al.*, 2001; Mollea *et al.*, 2005) and composting (McFarland and Qiu, 1995) allows a more realistic approach. In small bioreactors fungi grow on a mixture of soil and water (slurries) or soil amended with organic substrates (microcosms). Soil composting by fungal bioaugmentation is a promising procedure that can reduce PAHs either stimulating the biodegradation or binding intermediates to soil organic matter (Singh, 2006). For bench scale experiments, soil can be artificially spiked or from taken from polluted sites. Even though these more realistic approaches, real field trials in contaminated sites is the most accurate manner to determine the applicability of white-rot fungi (Leštan and Lamar, 1996). Their interactions with native soil microflora during bioremediation treatments is still not well understood and remains as one of the most currently challenging areas of knowledge (Boonchan *et al.*, 2000; Baldrian, 2004; Gao *et al.*, 2010).

Another approach to degrade PAHs is the use of ligninolitic enzymes. These can be purified or supernatants extracted from fungal liquid cultures. In these techniques, PAHs are directly added to the reaction medium (Collins *et al.*, 1996;

Majcherczyk *et al.*, 1998; Johannes and Majcherczyk, 2000a; Covino *et al.*, 2010c) or desorbed from polluted soil using surfactants. Usually, the reaction medium contains co-solvents and/or surfactants. The yield of these techniques is usually increased by means of mediators that can be natural (Johannes and Majcherczyk, 2000; Cañas *et al.*, 2007; Camarero *et al.*, 2008) or synthetic (Majcherczyk, 1998). The main drawback of this approach is the accumulation of intermediate products in the medium, some of them even more toxic than parental compounds. When employing fungal cultures, these by-products may be degraded by fungal enzymatic machinery up to mineralization to CO_2 and consequently do not accumulate.

The reactive oxygen species (ROS) are low-molecular-weight agents derived from ligninolytic enzymes, and are employed in nature to diffuse trough wood cell walls and initiate polymeric decay. These agents can also attack and cleave PAHs. The most studied ROS include hydroxyl radicals (·OH), peroxil radicals (ROO·) and hydroperoxyl radicals (·OOH). Hydroxyl radicals are generated due to biofenton-like reactions by fungi (Guillén *et al.*, 2000; Hammel *et al.*, 2002; Gómez-Toribio, *et al.* 2009; Marco-Urrea *et al.*, 2010). Peroxil and hydroperoxil radicals occur as secondary radicals due to peroxidation of unsaturated fatty acids by ligninolytic enzymes (Moen and Hammel, 1994; Camarero *et al.*, 2008).

5.1.5 Metabolites derived from PAHs

Several reports on PAHs fungal degradation have been written in the last years (Sutherland et. al. 1995; Cerniglia, 1997; Canet *et al.*, 1999; Kanaly and Harayama, 2000; Mougin *et al.*, 2002; Antizar-Ladislao *et al.*, 2004; Sutherland, 2004). Fungi and bacteria can metabolize a wide variety of PAHs (Juhasz and Naidu, 2000) although the metabolic pathway used has certain differences. Some of the metabolites produced as intermediates during biodegradation by both fungi and bacteria are quite similar to those observed in mammals (Cerniglia and Gibson, 1980). In this section, a brief description of the different degradation pathways of PAHs by both bacteria and fungi is presented.

5.1.5.1 Bacterial metabolism

Nowadays, numerous genera of bacteria have been shown to oxidize PAHs. The bacterial degradation of PAHs has been extensively studied over the two past decades and a great diversity of bacteria are known to metabolize low-molecular-

weight PAHs and few genera high-molecular-weight. Most research has focused on the role of various species of *Rhodococcus, Mycobacterium, Alcaligenes, Pseudomonas, Beijerinckia, Staphylococcus, Arthrobacter, Nocardia* and *Gordona*. Most bacteria use PAHs as sole carbon source.

The majority of bacteria degrade PAHs using an aerobic pathway. This involves the initial oxidation of the benzene rings by dioxygenases that produce *cis*-dihydrodiols. These are converted to diphenols (catechol) by other dioxigenases (see figure 5.2). The subsequent ring cleavage may occur via *meta* or *orto* fision of the ring. Further catabolism results in the formation of tricarboxylic acids intermediates. Few bacteria catalyze the degradation of PAHs to trans-hydrodiols. The sulfate-reducing and methane-oxidizing metabolism has been attributed to the genus *Pseudomonas* but the knowledge is currently scarce (Haritash and Kaushik, 2009).

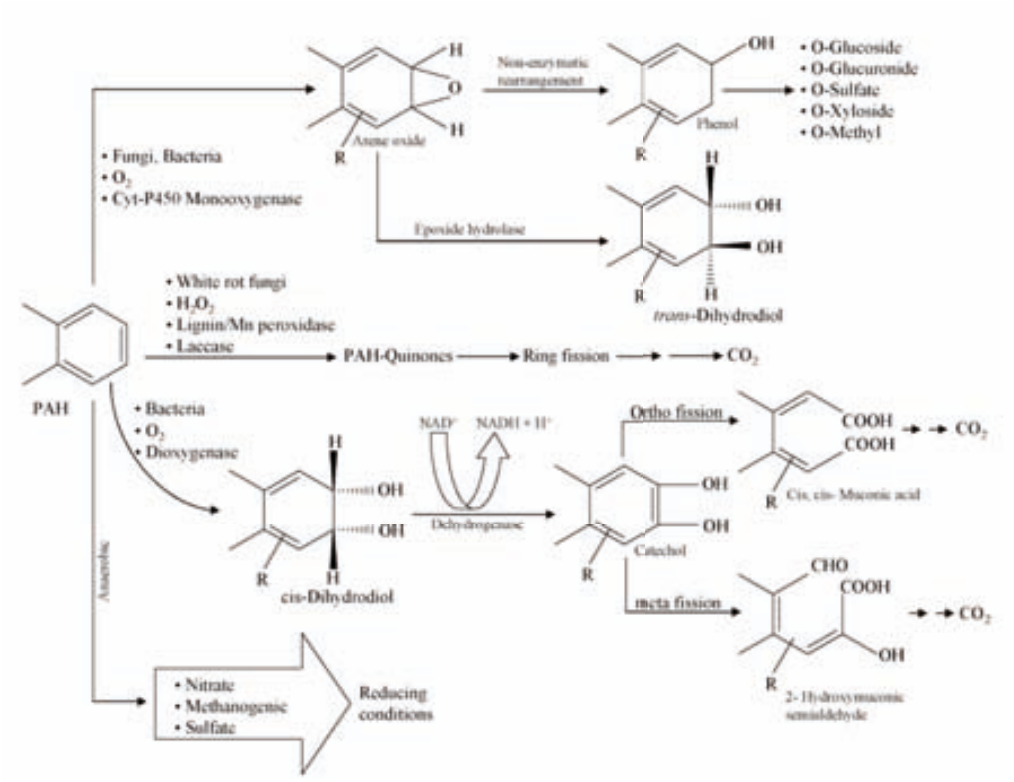


Figure 5.2 Initial degradation routes of PAHs by bacteria and fungi (Sutherland and Cerniglia 2001).

5.1.5.2 Fungi and yeast metabolism

Even though research on fungal metabolism has been extensive, the knowledge of fungal metabolism of PAHs is limited compared to that of bacteria (Singh, 2006). Metabolism of PAHs by fungi has a similar importance in the bioremediation to that of bacteria. Fungi cannot commonly use PAHs as sole carbon and energy

source; rather degrading them via co-metabolic processes (Wunder *et al.*, 1994; Casillas *et al.*, 1996). A few yeasts and filamentous fungi have been reported to be use certain PAHs such as anthracene, phenanthrene, pyrene and benzo[a]pyrene as sole carbon and energy sources (Romero *et al.*, 1998; Rafin *et al.*, 2000; Pan *et al.*, 2004). PAHs can also be stored in intracellular lipid vesicles (Verdin *et al.*, 2005).

In general terms, fungi are slower than bacteria at PAHs degradation. Fungi play a significant ecological role, as their polar and reactive metabolites can be mineralized or detoxified by soil bacteria. Fungal species that have demonstrated significant potential to metabolize such compounds are the Zygomicete *Cunninghamela elegans*, the Ascomycetes *Aspergilus niger* and *Penicillium* species.

Filamentous fungi and yeasts initially degrade the benzene rings of PAHs via the cytochrome P450 monooxygenase enzyme leading to the formation of an arene oxide (Figure 5.2) (Colombo *et al.*, 1996). These molecules are unstable intermediates and have never been isolated from fungal cultures. This may be further transformed into *trans*-dihydrodiol molecule via an epoxide hydrolase or via a non-enzymatic rearrangement to a phenol. A second phase can lead to the formation of conjugative products such as glucuronides, glucosides, xylosides and sulfates. These products are more water-soluble than other typical metabolites. They present non-mutagenic effects, while the initial oxidative products are more toxic and can form DNA adducts.

In addition, some non-ligninolytic fungi produce both ketones and quinones from some PAHs (Pothuluri *et al.*, 1992, 1993; Sutherland, 1992; Garon *et al.*, 2000; Mougin *et al.*, 2002), but the mechanisms by which they are formed are not currently well understood.

The group of lignin-degrading fungi also named white-rot have received considerable attention for their ability to degrade aromatic pollutants (Field *et al.*, 1993, Aust, 1995; Hammel, 1995; Pointing, 2001). The other major pathway for PAHs transformation involves several lignin-modifying enzymes (LMEs) such as extracellular lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Since these enzymes are extracellular and have low substrate specificity, research has focused on the use of these fungi for PAHs bioremediation. LMEs metabolize PAHs via reactions involving reactive oxygen species to produce quinones. All these compounds may be further degraded by ring fission enzymes (Hammel *et*

al., 1991). However, fungi can seldom mineralize PAHs to CO_2. Moreover, these fungi can also produce the intracellular enzymes cytochrome P450 and epoxide hydrolase (Bezael *et al*., 1996), thus degrading PAHs via non-ligninolitic pathway.

5.1.6 Aims of this chapter

The results of biodegradation experiments with several PAHs are presented in next sections. First of all, in section 5.2, are presented the degradation experiments of PAHs in submerged cultures both by fungal cultures and enzymatic assays. In a second step, in section 5.3, are presented the results related to the degradation pathways of fluorene, anthracene, phenanthrene, pyrene and benzo[a]anthracene by *Trametes versicolor*. Finally, in section 5.4, are presented different treatments of PAHs-spiked soils including slurry, biopiles and composting in Dewars vessels systems.

5.2. Degradation of model PAHs by *T. versicolor* in liquid medium

5.2.1 Screening of optimal surfactant for PAHs *in vivo* degradation

The surfactant addition effect on the biodegradation of PAHs is apparently highly variable and depends on many parameters (Fu and Alexander, 1995). Thus, it is important to select the proper conditions for each microorganism in order to optimize PAHs degradation. For the selection of the most advantageous surfactant, two high-molecular weight (HMW) PAHs were chosen: benzo[a]anthacene (BaA) and benzo[k]fluoranthene (BkF). The choice of these is due to its low solubility and its scarce degradation by bacteria. It was expected that the conditions that permit the degradation of such compounds will permit the degradation of lower molecular weight PAHs.

Three different non-ionic surfactants were added separately to the DM medium to increase the solubility of the compounds; Triton X-100, Tween 20 and Tween 80. Non-ionic surfactants were chosen due to their higher solubilization capacity as well as their lower cost compared to cationic and anionic ones (Alcántara *et al.*, 2009). This makes them interesting for further use in decontamination processes. For large-scale applications a cheaper surfactant would be needed to reduce costs while maintaining the degradation performance.

All surfactants were tested separately by adding 1 $g \cdot l^{-1}$ to the medium. For Triton X-100 the effective CMC is 0.12 $mg \cdot l^{-1}$ (Marcoux *et al.*, 2000). The effective CMC of Tween 20 is 0.06 $g \cdot l^{-1}$ (Alcántara *et al.*, 2009). And finally, the effective CMC of Tween 80 is 0.016 $g \cdot l^{-1}$. The concentration was over the CMC in every case, thus guaranteeing the enhancement in compounds solubilization. For a real scale application, the concentration should be optimized to guarantee degradation capacity while reducing its costs.

According to the results, glucose was completely depleted after 4 days of culture in DM medium in presence or not of surfactants. These revealed no toxic effects of any surfactant on fungal growth. Boyle *et al.* (1998) showed that both Triton X-100 and Tween 80 increase the accessibility of PAHs to white-rot fungi without interfering their growth. Another work (Tekere *et al.*, 2005) also demonstrated no toxic effect of Tween 20 towards *T. versicolor*. Laccase was detected in the broth along cultures; but MnP was not detected. These results are in accordance with

Acebes (2008), who reported that in a similar medium laccase was the only enzyme secreted by the fungus.

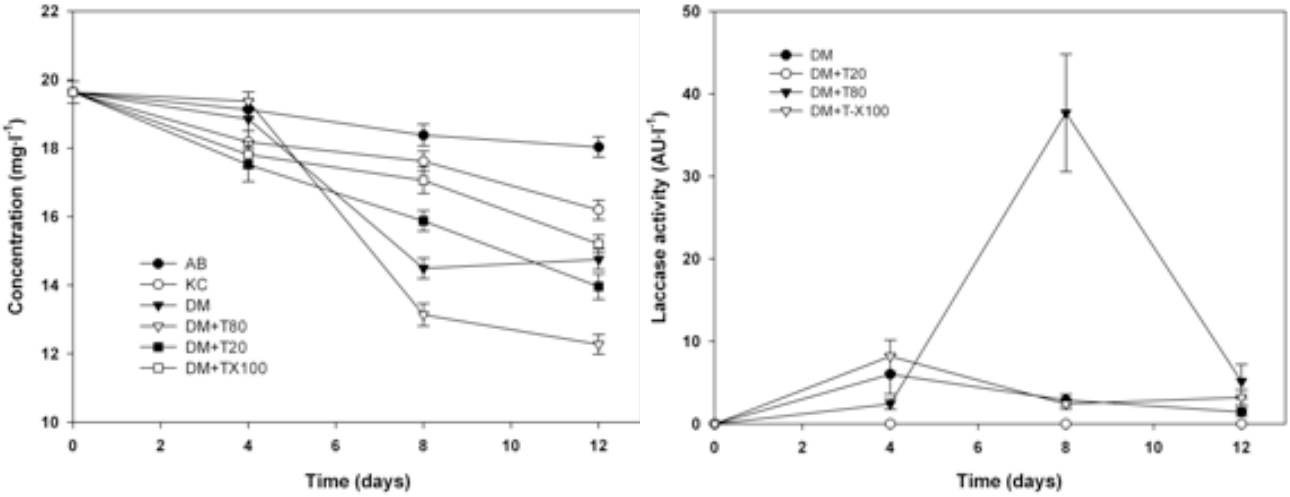


Figure 5.3a and 5.3b Time course concentration of BkF during 12 days of fungal culture (a). Laccase activity during the experiment (b). Errors are expressed as standard deviation of triplicates.

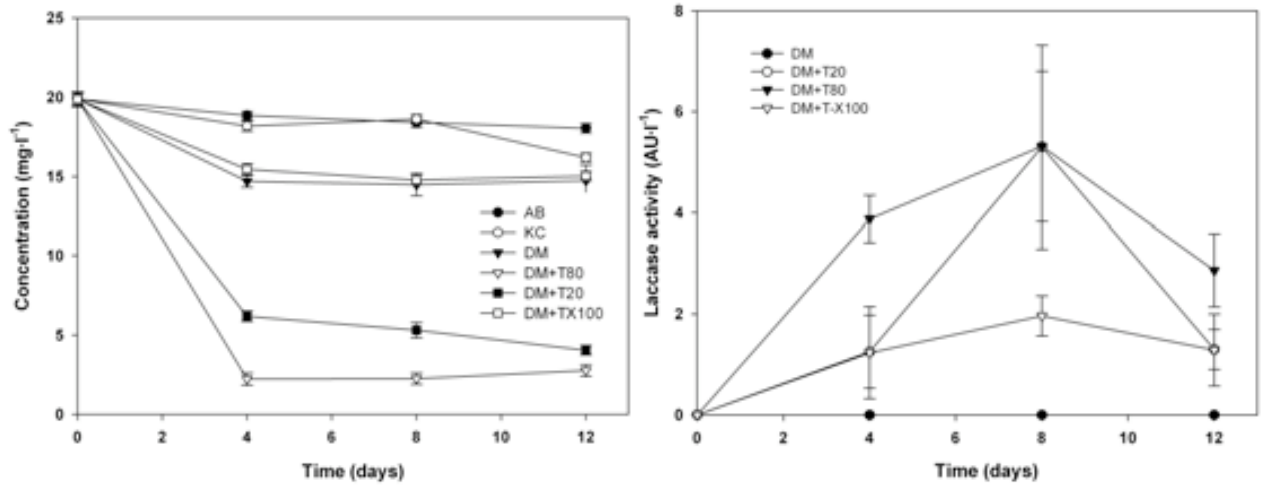


Figure 5.4a and 5.4b Time course concentration of BaA during 12 days of fungal culture (a).). Laccase activity during the experiment (b). Errors are expressed as standard deviation of triplicates.

Both compounds were degraded in DM medium (figures 5.3a and 5.4a) without any surfactant to 17.6% and 11.2% for BaA and BkF respectively. When Tween 20 was added to the degradation medium, the biodegradation increased to 73.4% and 18.5% for BaA and BkF respectively. This contrasted with the study of Tekere *et al.* (2005) that reported a 3% degradation of the initial BaA (25 $mg \cdot l^{-1}$) in stationary cultures of *T. versicolor*, in presence of Tween 20 ($0.5g \cdot l^{-1}$).

The addition of Tween 80 increased the biodegradation up to 81% and 24.9% for BaA and BkF respectively. Whilst Triton X-100 did not increase the biodegradation capacity, achieving biodegradation percentages similar to those

without presence of surfactants. Addition of Triton X-100 in TLP bioreactors did not enhance the degradation rate of PAHs (Marcoux *et al.*, 2000). It is also remarkable that the presence of surfactant such as Tween80, that contain unsaturated lipids, can increase the ligninolytic enzymes degradation through lipid peroxidation.

The degradation achieved without addition of surfactants could be attributed to the effect of surfactant-like compounds, named biosurfactants (Rosenberg and Ron, 1999). These are produced by the same degrading microorganisms and enhance the solubilisation of PAHs (Haritash and Kaushik, 2009). In culture conditions, with glucose as carbon source, the fungus could produce polysaccharopeptides (Cui and Chisti, 2003). These are released into the medium while pH declines along fermentation to 2.5, as given during biodegradation experiments favoring PAHs solubilization.

Inhibition experiments of the cytochrome P450 were performed as described in materials and methods. Biodegradation experiments with 1 mM ABT added in the medium were carried out. Table 5.2 summarizes the biodegradation percentages of both compounds after 12 days of fungal culture. Percentages are expressed respect to the initial concentration of 20 $mg \cdot l^{-1}$.

Table 5.2 Percentage of degradation of BaA and BkF after 12 days of fungal culture in presence or not of cytochrome P450 inhibitor (ABT).

	Biodegradation (%)	
Media	**Benzo[a]anthracene**	**Benzo[k]fluoranthene**
DM	17.6	11.2
DM + ABT	17.3	0.5
DM+T80	81.0	24.9
DM+T80+ABT	80.5	1.0

Results show that BaA degradation was not inhibited by presence ABT in the culture medium. Taking into account that this compound is easily degraded by laccase, as will be discussed later in section 5.2.3, it can be assumed that the initial step of oxidation is not controlled by Cyt-P450 but by laccase.

In the case of BkF a strong inhibition is observed when ABT was added in the culture medium. These results elucidate the possible implication of Cyt-P450 in the initial degradation mechanism of BkF. Further identification of diol-type metabolites could confirm this evidence.

The results obtained conclude the surfactant Tween 80 as the most suitable for further biodegradation experiments in liquid medium. For further applications, it would be necessary to optimize the surfactant concentration while maintaining the degradative capacity of the fungus.

5.2.2 Single PAHs *in vivo* biodegradation

This section presents the single PAHs biodegradation results in fungal cultures. Experiments were carried out separately for each PAH in defined medium containing 1 $g \cdot l^{-1}$ of Tween80. To asses the fungal enzymatic activity laccase was measured and glucose content was followed along the experiment. In figure 5.5 are shown the concentration along time of the PAHs studied in this section: fluorene, phenanthrene, anthracene, fluoranthene, pyrene and chrysene.

Fluorene was degraded by the fungus achieving 82% degradation after 12 days. Although the compound was initially slightly absorbed in the fungal biomass, the degradation was maintained along the experiment for 12 days. Collins *et al.* (1996) reported a 100% removal in whole cultures of *T. versicolor* after 11 days of culture in liquid medium. Whilst Tekere *et al.* (2005) reported a 78% removal in liquid media under stationary liquid cultures after 31 days.

Phenanthrene was degraded despite its elevated ionization potential (IP = 8.03 eV), which makes it not a substrate for laccase degradation. As for fluorene, the initial drop in the concentration was achieved mainly due to sorption on biomass. After that period a relevant pollutant removal (70%) was achieved. Collins *et al.* (1996) reported a 75% phenanthrene removal after 11 days of culture. Song (1997) described a mineralization of 10% by *T. versicolor* after 3 weeks of liquid culture supplemented with O_2. Han *et al.* (2004) demonstrated that an isolated of *T. versicolor* was capable to remove 70% of the initial content (25 $mg \cdot l^{-1}$) after 3 days. Moreover, Morgan *et al.* (1991b) described also phenanthrene degradation by the fungus in liquid cultures adding veratryl alcohol to the medium, showing a mineralization rate of 0,7 $\mu g \cdot g culture^{-1} \cdot day^{-1}$. Tekere *et al.* (2005) achieved a 49% removal in liquid media under stationary liquid cultures after 31 days. Sack and co-workers (1997) reported that 14% of the initial PHE was mineralized by *T. versicolor* in liquid cultures.

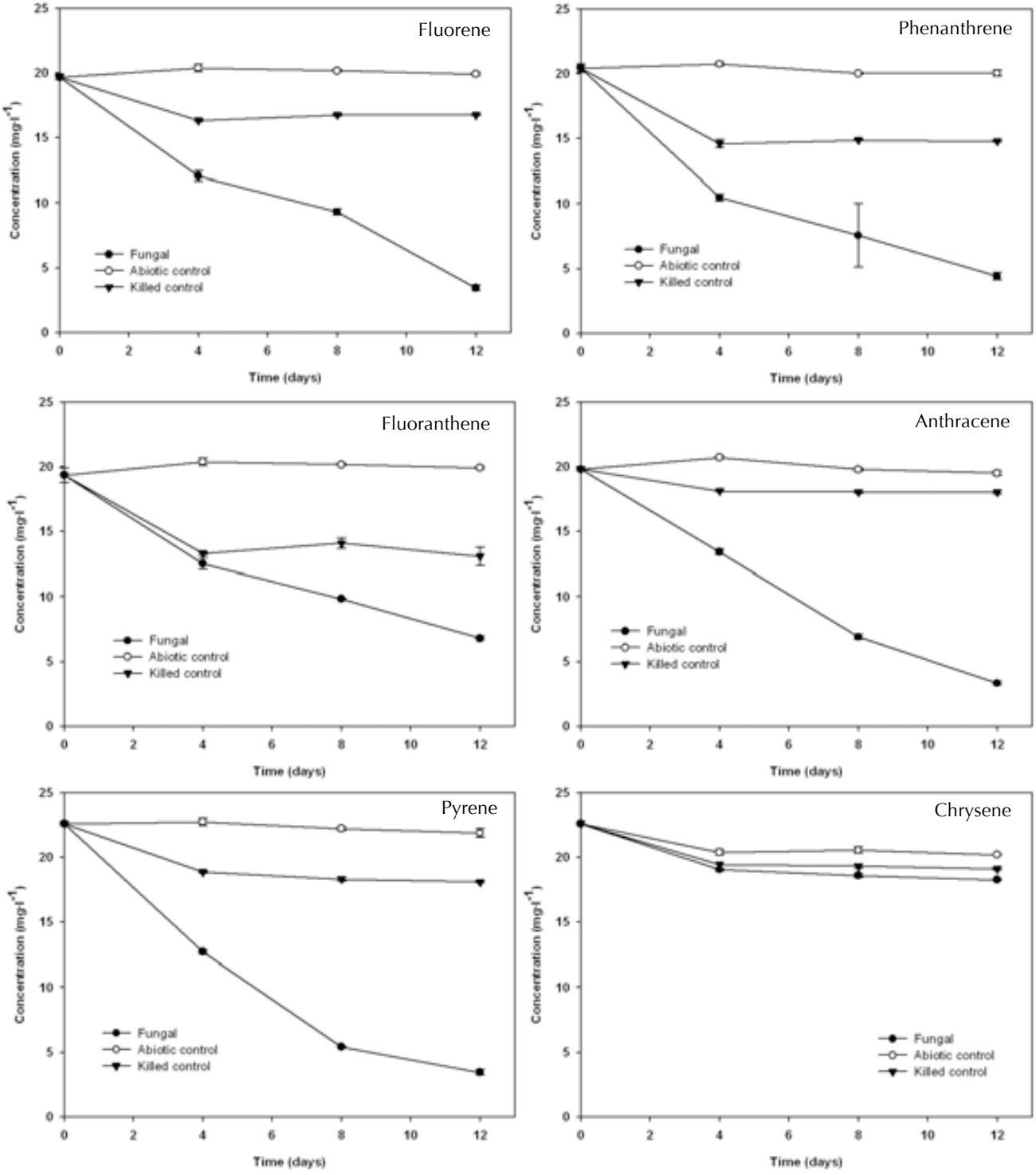


Figure 5.5 PAHs concentration during 12 days of fungal submerged culture in the experiment. PAHs testes were; fluorene, phenanthrene, anthracene, fluoranthene, pyrene and chrysene. Errors are expressed as standard error of triplicates.

Fluoranthene concentration decrease can be attributed to the biomass sorption process since the killed control and the fungal treatment present a similar profile at day 4. After that initial step, degradation occurs because the compound concentration in the fungal treatment decreases. At the end of the experiment a 58% of degradation was achieved.

After the sorption a biodegradation of 75% of remaining anthracene was achieved after 12 days of culture. This is similar to the previous reports for *T. versicolor*. Vyas and co-workers (1994) described a 60% degradation after 21 days culture in liquid medium, demonstrating that *T. versicolor* was faster at degradation respect other WRF. Tekere *et al.* (2005) reported a 54% removal in liquid media under stationary liquid cultures after 31 days.

The fungus degraded pyrene extensively, 82% respect to abiotic concentration at the end of the experiment. There was little sorption to biomass as the profile of killed control shows. This result contrasts with previous studies. Tekere *et al.* (2005) reported 0% degradation of initial pyrene (25 $mg \cdot l^{-1}$) for *T. versicolor* in stationary cultures, in presence of Tween20 (0.5$g \cdot l^{-1}$). Sack (1997) described a 2.4% mineralization of radiolabeled pyrene in liquid culture with *T. versicolor*.

No degradation was observed for chrysene, probably due to the low solubility of the compound that makes it non-bioavailable for the fungus and its enzymatic system. The concentration profile of the compound of fungal treatment is close to the concentration of killed control treatment.

Figure 5.6 shows the laccase activity and glucose concentration in the single PAHs degradation experiments. In general terms, no toxic effect towards the fungus was observed. An identical pattern was observed in all PAHs for glucose, which was completely depleted after 4 culture days in all cases. For the case of laccase, similar trends were also observed for all compounds, showing a characteristic peak of activity. After 4 days of culture in the case of fluoranthene, pyrene and chrysene or 8 days for the rest. Enzyme highest activities ranged from 16 to 80 $AU \cdot l^{-1}$. Enzyme profile could not elucidate whether enzyme was secreted as a response towards the pollutant or constitutively.

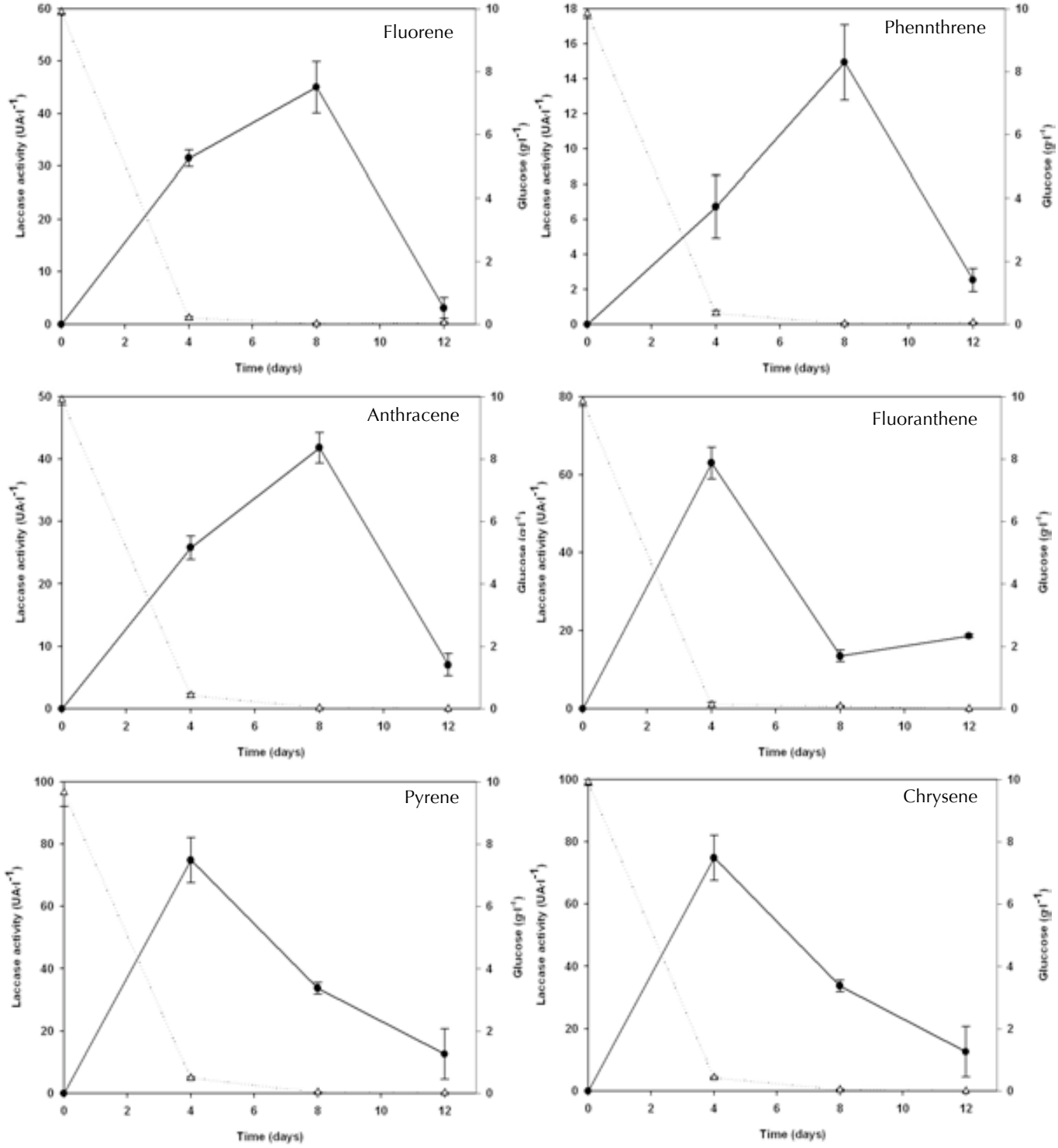


Figure 5.6 Glucose concentration and laccase activity during 12 days of fungal submerged culture in the degradation experiment of different PAHs; fluorene, phenanthrene, anthracene, fluoranthene, pyrene and chrysene. Errors are expressed as standard error of triplicates.

5.2.3 PAH-mixtures *in vivo* biodegradation

Earlier section focused on specific biodegradation of single PAHs. Since such compounds are found as mixtures in polluted sites or effluents, the effect of interaction during biodegradation in PAHs mixtures is important for a more realistic bioremediation approach. Some studies evidenced that PAHs degradation rates were different in single and mixed cultures (Haritash and Kaushik, 2009; Cajthaml *et al.*, 2008). In order to perceive possible interaction in the biodegradation capacity of the different PAHs an experiment with a mixture of the previously studies PAHs was performed. In addition to those, additional PAHs were added to the mixture: acenaphtylene, acenaphthene, benzo[b]fluoranthene and benzo[a]pyrene. The different PAHs were grouped into two different groups in order to make easier the analysis during the experiment: LMW (2-3 rings) and HMW (4-5 rings).

The results showed that glucose was consumed in a different pattern to that in single-PAH cultures. After 4 days of culture, close to $3g \cdot l^{-1}$ remained in the medium demonstrating some toxic effect towards the fungus. After 8 days was completely depleted (see figure 5.7). The enzymatic activity pattern was also different, because it was expected to reach a peak of activity between days 4 or 8, as observed in previous experiments. Nevertheless, laccase maximum activity was achieved at the end of the experiment. This general pattern denoted some toxic effects of PAHs towards the fungus, slowing down its metabolism or degrading capacity.

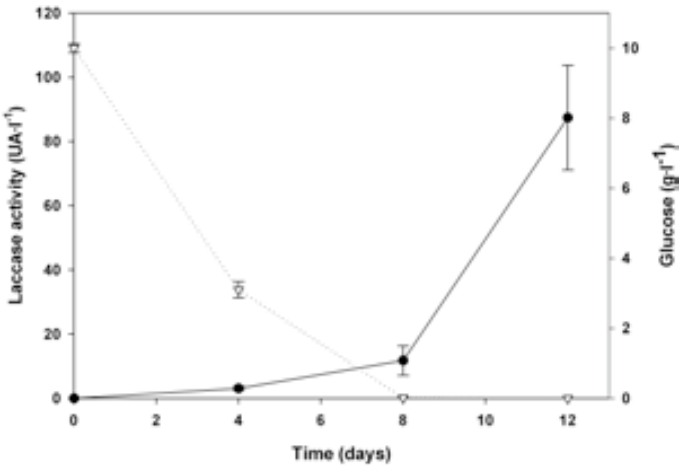


Figure 5.7 Glucose concentration and laccase activity during PAHs mixture biodegradation. Errors are expressed as standard deviation of triplicates.

Table 5.3 PAHs concentration along the experiment in fungal treatments, killed control and abiotic controls. Errors are expressed as standard error of triplicates.

	Concentration ($mg \cdot l^{-1}$) at 4 days of culture			**Concentration ($mg \cdot l^{-1}$) at 8 days of culture**			**Concentration ($mg \cdot l^{-1}$) at 12 days of culture**		
Compound	**Fungal**	**Killed control**	**Abiotic control**	**Fungal**	**Killed control**	**Abiotic control**	**Fungal**	**Killed control**	**Abiotic control**
Acenaphtylene	7.8 ± 0.3	9.5 ± 1.2	20.7 ± 1.8	5.4 ± 1.8	9.0 ± 0.8	19.3 ± 0.5	0.9 ± 0.7	7.3 ± 1.0	14.4 ± 4.0
Acenaphthene	8.5 ± 0.1	11.7 ± 0.9	26.6 ± 1.7	6.8 ± 1.7	13.1 ± 0.7	27.1 ± 0.5	2.3 ± 0.8	11.6 ± 1.2	21.8 ± 4.3
Fluorene	15.5 ± 0.3	17.9 ± 0.7	33.6 ± 0.6	11.9 ± 1.6	19.3 ± 0.6	33.5 ± 0.4	7.0 ± 1.3	17.0 ± 1.1	29.3 ± 2.6
Phenanthrene	11.5 ± 0.3	12.8 ± 0.5	25.1 ± 0.1	8.3 ± 1.0	14.2 ± 0.5	25.0 ± 0.3	5.5 ± 0.7	12.5 ± 0.9	22.1 ± 1.6
Anthracene	20.6 ± 0.3	21.2 ± 1.1	24.1 ± 0.1	19.1 ± 2.1	20.9 ± 0.4	24.8 ± 0.5	16.5 ± 1.3	20.6 ± 0.5	23.7 ± 0.7
Fluoranthene	12.0 ± 0.3	12.8 ± 0.6	28.7 ± 0.1	7.9 ± 1.0	14.4 ± 0.5	28.3 ± 0.3	5.5 ± 0.6	12.6 ± 1.0	24.7 ± 1.9
Pyrene	12.4 ± 0.3	12.2 ± 0.6	27.9 ± 0.2	7.5 ± 1.2	13.9 ± 0.6	27.2 ± 0.3	4.7 ± 0.6	12.3 ± 0.8	23.7 ± 1.9
Benzo[a]anthracene	20.4 ± 0.6	21.0 ± 1.1	28.0 ± 0.1	18.5 ± 1.8	21.9 ± 0.5	29.3 ± 0.3	17.7 ± 0.5	20.7 ± 0.6	26.7 ± 1.2
Chrysene	26.5 ± 0.4	27.3 ± 1.2	29.2 ± 0.1	26.3 ± 2.6	29.4 ± 0.3	31.7 ± 0.3	26.6 ± 0.4	28.7 ± 0.5	29.3 ± 0.9
Benzo[b]fluoranthene	17.6 ± 0.7	17.1 ± 0.3	25.5 ± 0.2	13.6 ± 1.2	17.1 ± 0.8	28.3 ± 2.3	13.9 ± 0.2	13.7 ± 2.0	27.5 ± 1.5
Benzo[k]fluoranthene	25.0 ± 0.5	23.7 ± 0.7	23.6 ± 0.1	24.1 ± 2.2	26.9 ± 0.3	28.4 ± 2.0	24.5 ± 0.3	24.3 ± 2.2	29.0 ± 1.0
Benzo[a]pyrene	24.4 ± 0.5	21.7 ± 1.5	34.4 ± 0.1	20.9 ± 1.9	23.8 ± 0.9	34.6 ± 0.4	19.7 ± 0.7	21.9 ± 0.7	31.1 ± 1.1
∑PAHs	202.0 ± 2.3	199.0 ± 3.7	327.4 ± 3.5	170.6 ± 2.4	223.9 ± 3.2	337. 8 ± 3.0	144.8 ± 2.2	203.1 ± 27	303.3 ± 5.6

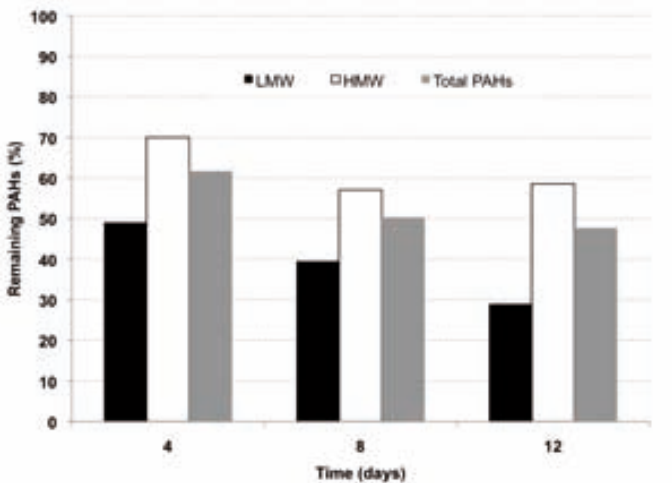


Figure 5.8 Remaining PAHs in the broth during time course of experiment.

Table 5.3 shows that the fungus was capable to degrade most PAHs from the mixture even though total concentration was 10-fold respect experiments of former section. The abiotic losses were scarce and sorption to fungal biomass was not negligible; both were taken into account for calculating biodegradation percentages. Phenanthrene, fluoranthene and fluorene were degraded to a lower extent to that obtained in single degradation experiment. Chrysene was degraded, whilst no degradation was observed in pure culture of the same compound. Benzo[k]fluoranthene and benzo[b]fluoranthene were not degraded in the experiment. In general terms, as showed in figure 5.8, PAHs removal was due mainly to LMW-PAHs

Table 5.4 Final percentage of degradation after fungal degradation in single-PAH and mixture-PAH after 12 days in submerged cultures.

	Degradation percentage (%)	
	Mixture-PAH	**Single-PAH**
Acenaphtylene	87.6	-
Acenaphthene	79.9	-
Fluorene	58.8	82
Phenanthrene	55.8	70
Anthracene	19.9	75
Fluoranthene	56.2	58
Pyrene	61.9	82
Benzo[a]anthracene	14.4	81
Chrysene	7.4	0
Benzo[b]fluoranthene	0.0	-
Benzo[k]fluoranthene	0.0	21
Benzo[a]pyrene	9.9	27*

* Data according to Hernandez (2008)

In table 5.4 the biodegradation percentages of the studied PAHs in previous sections are summarized. In general terms, a higher degradation yield was achieved in single-PAH cultures rather than in mixtures of PAHs. It has to be highlighted that chrysene was degraded in PAHs mixtures although it was not in single-PAH culture.

5.2.4 PAHs degradation by commercial *T. versicolor* laccase

The results presented in previous section demonstrate that *T. versicolor* is capable to degrade most of the 16 EPA's priority PAHs. In all experiments laccase was secreted in the broth by the fungus. Several authors have reported the ability of laccase to degrade PAHs in presence of co-solvents and mediators but it is not clear whether laccase can degrade PAHs without a co-solvent present in the medium. Moreover, the following experiments can provide information related to PAHs degradation metabolites related to lacasse activity.

An experiment with purified laccase in absence of co-solvents was carried out to asses whether laccase is capable to transform the PAHs in similar conditions to the fungal culture. The pH was adjusted to 4.5. Also the surfactant content was identical to that on the biodegradation experiments with the fungus (1 $g \cdot l^{-1}$). In addition, experiments without surfactant were also carried out.

In table 5.5 are listed the percentages of PAHs degradation and the concentration recovered after extraction with dichloromethane (DCM) in the abiotic controls. It can be observed that in medium that does not contain any surfactant, the degradation is scarce except for the more soluble compounds. These results contrast with those obtained by Majcherczyk *et al.* (1998). In that study, laccase was capable to degrade most PAHs. The presence of acetone as co-solvent in their experiment permitted more solubility of the compounds facilitating the attack by laccase and further degradation. Mougin (2002) also found that laccase was not capable to degrade Cry and BkF in presence of Twen 20 even in presence of acetonitrile as co-solvent.

The addition of both surfactants in the medium enhances the solubility of the compounds making the compounds more available to the enzyme. The maximum percentages of degradation were achieved in presence of both surfactants. It has been reported by Camarero *et al.* (2008) that laccase is capable to produce peroxyl radicals that can attack the PAHs in presence of the surfactant Tween 80.

These radicals are strong oxidizers that can attack the PAHs leading to their degradation. The role of detergents in the reactions of such compounds seems to be a key point for the biodegradation itself. Bacteria, yeasts and fungi under liquid culture can commonly produce these types of products.

Table 5.5 PAHs remaining concentration and degradation percentages with respect to the controls.

Compound	Concentration ($mg \cdot l^{-1}$)	Degradation percentage (%)			
		Lac + T80	Lac + T20	Lac	Boiled Lac
Acenaphtylene	23.2	20.3	19.5	6.9	0.0
Acenaphthene	24.2	13.9	7.3	5.4	0.0
Fluorene	31.6	15.5	9.6	5.8	0.0
Phenanthrene	22.8	7.9	12.1	0.0	0.0
Anthracene	24.4	13.4	10.2	1.8	0.0
Fluoranthene	25.9	13.0	18.2	0.0	0.0
Pyrene	25.3	15.0	19.8	2.2	0.0
Benzo[a]anthracene	26.1	9.0	11.8	0.0	0.0
Chrysene	28.0	0.0	0.0	0.0	0.0
Benzo[b]fluoranthene	26.6	19.0	23.3	4.2	0.0
Benzo[k]fluoranthene	26.7	5.4	9.8	0.0	0.0
Benzo[a]pyrene	31.4	20.0	22.1	3.5	0.0

Results prove that laccase is capable to degrade the different PAHs under similar conditions to those in the experimental with fungal cultures. The analysis of the organic fraction proved the appearance of new peaks in the chromatograms after the incubation with laccase. This suggested the production of oxidation products from the parental compounds. This evidence was supported by previous studies (Majcherscky *et al.*, 1998; Johannes *et al.*, 1998; Collins *et al.*, 1996) that suggested the formation of the corresponding quinones.

In order to improve the degradation achieved some mediators were added, each one per separate, to the reaction medium. The so-called laccase mediator system increases the laccase redox potential permitting the oxidation of compounds with a high ionization potential (IP) (Majcherczyk *et al.*, 1998; Mougin, 2002; Michizoe *et al.*, 2005). Thus, the addition of mediators increases the degradation percentages as well as the oxidation products level. This fact is important specially when trying to identify degradation products. This fact will be further discussed in the next section.

The mediators addition to the system increased the degradation after 24 hours. In presence of HOBT the degradation was higher respect the other mediators. The

HOBT addition to laccase system has been previously described as a strong mediator. Most PAHs were degraded extensively except chrysene, phenanthrene and fluoranthene. These three compounds were also scarcely degraded in the work presented by Majcherczyk *et al*. (1998) even thought they used a co-solvent.

Table 5.6 Laccase degradation percentages of PAHs in presence of different mediators.

Compound	Lac + HOBT +T80	Lac + VA +T80	Lac + ABTS +T80	Lac + DMHAP +T80
Acenaphtylene	98.5	96.6	20.6	36.2
Acenaphthene	98.8	45.1	32.4	103
Fluorene	96.8	23.8	13.0	8.8
Phenanthrene	9.7	1.2	5.5	0.0
Anthracene	70.2	59.8	33.4	19.4
Fluoranthene	8.2	0.0	6.0	0.0
Pyrene	50.9	15.8	9.7	3.6
Benzo[a]anthracene	73.8	15.6	9.3	3.2
Chrysene	4.8	0.0	5.1	0.0
Benzo[b]fluoranthene	31.1	22.6	17.5	14.8
Benzo[k]fluoranthene	17.7	4.1	7.1	2.3
Benzo[a]pyrene	85.7	40.1	42.4	34.2

Figure 5.9 shows a chromatogram after in vitro degradation in presence of HOBT. In red line are presented the peaks corresponding to the oxidation products and in blue the parental compounds. Significant amounts of oxidation compounds were accumulated in the medium. Some of these peaks coincided with those observed in the experiments of previous sections, arising from fungal degradation. This justified the use of this system in further experiments to identify by-products in the case laccase was implicated in the biodegradation mechanism.

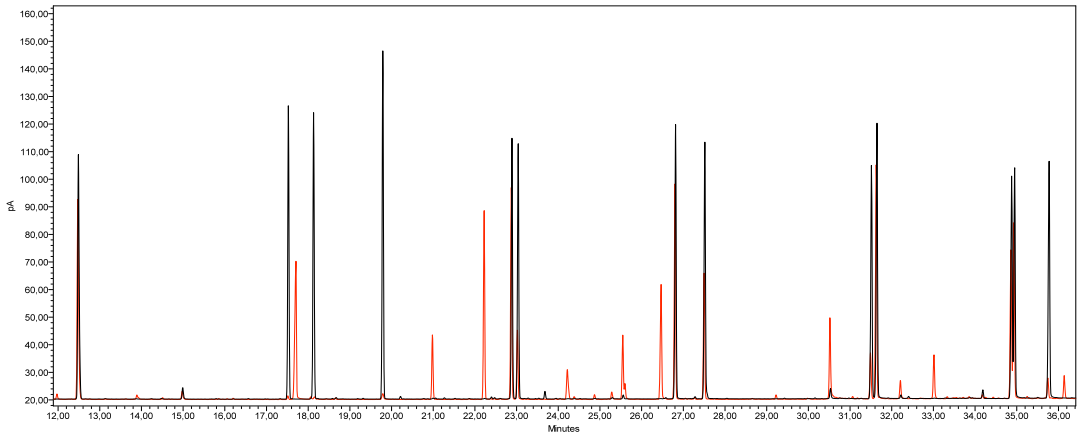


Figure 5.9 Chromatograms of the organic fraction (dichloromethane) before (black) and after (red) 24 hours of degradation with commercial laccase in presence of the mediator HOBT.

Some turbidity was observed at the end of the enzymatic degradation. This could be attributed to the formation of polymeric products (Majcherczyk *et al.*, 1998). They reported that in presence of the surfactant Tween 20 there was some copolymerization of PAHs. The analysis of the samples by size exclusion chromatography was necessary to confirm it but, unfortunately, it was not performed. Possible polymerization would have explained the decrease of BbF and BkF, that where degraded in our system but had never been described to be degraded by *Trametes* laccase.

The preceding results demonstrate the capacity of both fungal cultures and laccase-system to degrade PAHs separately or in mixtures. The comparison of the maximum degradation percentage can provide information whether laccase was the only enzyme responsible for such degradation. In general terms, the maximum degradation rates percentages were achieved with *in vivo* experiments. This difference in could be attributed to the fact that the fungus uses additional enzymatic systems to degrade the PAHs such as Cyt-P450 and glucose-oxidase. These can also contribute to the depletion of the compounds. Indeed, the by-products aroused from oxidation of PAHs can enter the metabolic system of the fungus favoring and extending biodegradation.

5.2.5 Conclusions

- It was established that the use of the nonionic surfactant Tween80 enhances the degrading capacity of *T. versicolor* towards PAHs as it increases the solubility of the pollutant in the liquid medium and can participate in generating ROS.
- Our strain of *T. versicolor* was capable to degrade in submerged culture acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene benzo[a]pyrene and benzo[k]fluoranthene. The individual removal efficiency depends on each PAH properties.
- It was established that Cyt-P450 is implicated in the initial cleavage of benzo[k]fluoranthene, but it was not implicated in initial the cleavage of benzo[a]anthracene. It was not studied for the other PAHs,.

- The fungus could also maintain the degradative abilities in PAHs mixtures at 10-fold total concentration. Even though, showing some toxic effects as a delay in glucose consumption and laccase secretion.
- It was demonstrated that laccase was capable to degrade PAHs in liquid culture without the presence of a co-solvent, in the same conditions to *in vivo* experiments.
- PAHs enzymatic degradation leads to the formation of several metabolic intermediate products that accumulate in the broth.
- It was demonstrated that PAHs laccase removal was lower than that obtained with *in vivo* experiments. Suggesting that other enzymatic systems can be implicated in PAHs removal.

5.3 Identification of PAHs degradation products

The aim of this section was to elucidate the degradation pathway of several PAHs by *T. versicolor*. The identification of intermediate metabolites was performed from aliquots taken both from fungal cultures and from enzymatic assays with purified laccase in presence of mediators. Different organic solvents were used to extract PAHs and the intermediates as described in section 3.6.11.

In literature, diverse analytical techniques have been used for study of the degradation products of PAHs. The results presented in this section employed the technique of GC/MS to identify products. Some of them were confirmed using the corresponding standards. The following results provide new information into the degradation mechanisms of fluorene, anthracene, acenapthylene, acenapthylene, phenanthrene, fluoranthene, pyrene and benzo[a]anthracene by *T. versicolor*. Some additional experiments of the individual intermediates were performed in order to elucidate the sequence of degradation accurately.

5.3.1 Fluorene (FLU)

5.3.1.1 Metabolites Identification

In biodegradation experiments with fungal cultures of fluorene (section 5.2.3), 9-fluorenone was detected as major metabolite after 4 days of culture when extracted using either DCM or ethyl acetate (see figure 5.10). Coupled with this product, another metabolite was detected in fungal cultures after 4-12 days and was identified as 9-H-fluoren-9-ol. A third product was identified from fungal cultures at day 12 and was identified as 9-H-fluoren-2-ol. Both 9-fluorenone and 9-H-fluoren-9-ol were confirmed using standards and were detected during the rest of the experiment.

In order to elucidate the role of laccase in the degradation of fluorene, experiments with purified enzyme and different mediators were also performed. The only dead-end metabolite identified was 9-fluorenone. Thus, it suggested that 9-fluorenone was formed from the transformation by laccase from the parental molecule.

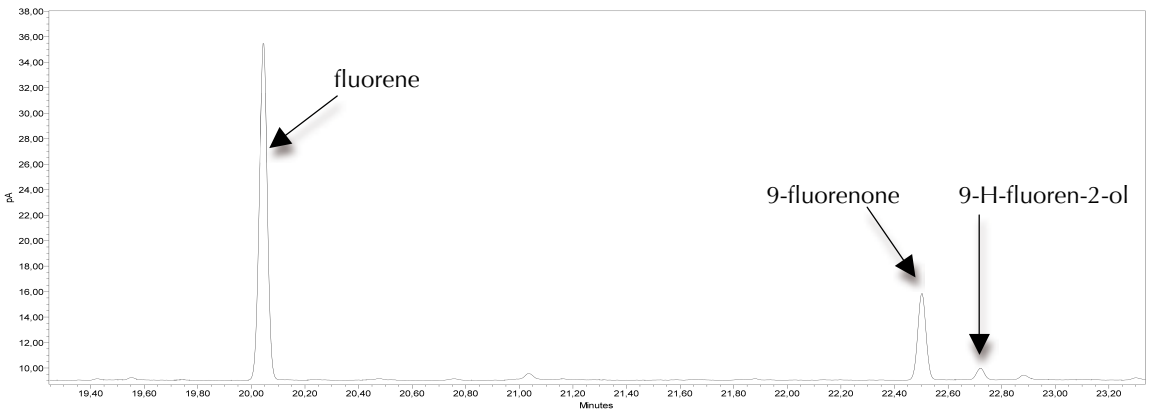


Figure 5.10 Chromatogram of the organic fraction (dichloromethane) after 4 days of fungal culture using GC/FID. Products were later identified using GC/MS.

Table 5.7 summarizes the characteristics of the metabolites identified from of fluorene degradation with purified laccase and fungal cultures.

Table 5.7 Retention data and electron impact mass spectral characteristics of fluorene metabolites. (* Lac: enzymatic assay; Fng: fungal culture)

Id.	t_R (min)	Degraded by*	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion
1	20.08	Lac/Fng	180	180 (100), 152 (42.1), 126 (7.5), 119 (1), 98 (3), 76 (12.6), 63 (7.4), 50 (2.6)	9-fluorenone [a]
2	20.13	Fng	182	181 (100), 165 (18.6), 152 (52.3), 139 (3.5), 126 (6.2), 91 (6.1), 76 (20.2), 63 (7.2), 51 (4.1)	9-H-fluoren-9-ol [a]
3	24.57	Fng	182	182 (100), 164 (20.7), 152 (40.4), 117 (6.3), 109 (3.6), 91 (9), 82 (13.3), 76 (17.3), 63 (7.7), 51 (4.9)	9-H-fluoren-2-ol

a: structures were identified with authentic standards

5.3.1.2 Metabolites degradation

After elucidating the major intermediates for fluorene degradation by *T. versicolor*, biodegradation experiments in DM medium containing Twen80 were performed to elucidate much clearly the degradation sequence. Both 9-fluorenone and 9-H-fluoren-9-ol were degraded in fungal culture as can be observed in figure 5.11 and 5.13. Aliquots analyzed by GC/MS demonstrated that in cultures of 9-Fluorenone the major metabolite formed was 9-H-fluoren-9-ol and vice versa. This evidence suggests an existing equilibrium between both compounds

regulated by the fungus. Enzymes such as cellobiose dehydrogenase and quinone-reductase, present in ligninolitic fungi (Roy *et al.*, 1996; Gómez-Toribio *et al.*, 2009) could be involved in this equilibrium.

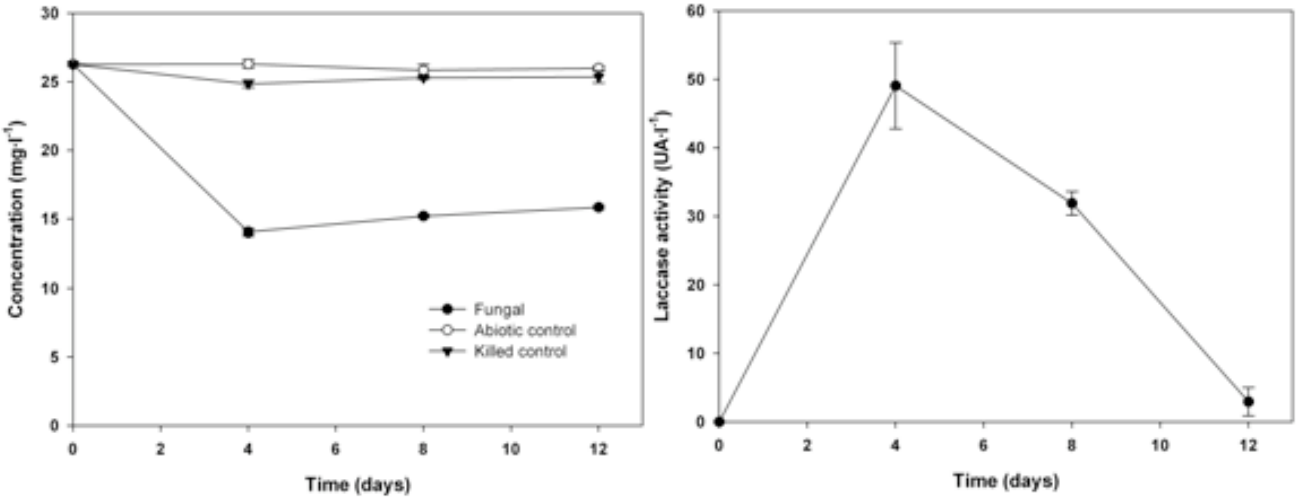


Figure 5.11a and 5.11b Time course degradation of 9-fluorenone by *T. versicolor* (a). Laccase activity during the experiment (b). Errors are expressed as standard error of triplicates.

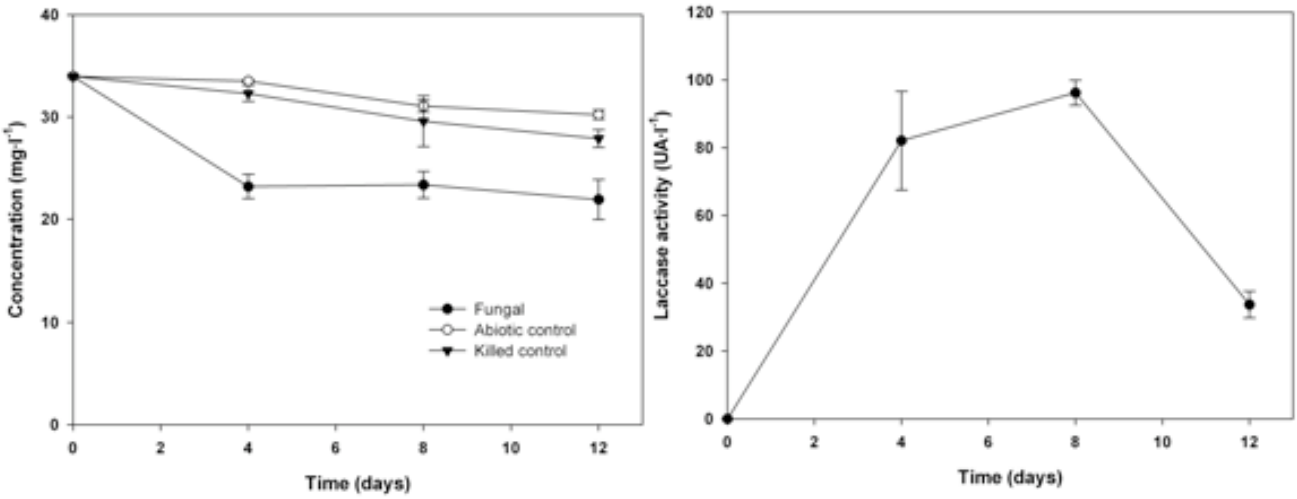


Figure 5.12a and 5.12b Time course degradation of 9-H-fluoren-9-ol by *T. versicolor* (a). Laccase activity during the experiment (b). Errors are expressed as standard error of triplicates.

It has been described by several autors (Majcherczyk *et al.,* 1998; Collins *et al.,*1996; Bressler *et al.,* 2000) that laccases are involved in the initial transformation of fluorene to 9-fluorenone. This metabolite was previously described in cultures of *P. chrysosporium* (Bogan *et al.*, 1996). In that work, authors suggested MnP as responsible for the transformation of fluorene to fluorenone via lipid peroxidation. During *in vitro* experiments, the initial fluorene was transformed to fluorenone stechiometrichaly in presence of oleic acid and Mn^{2+} in the reaction medium. Thus, laccase could transform the initial fluorene

via lipid peroxidation to 9-fluorenone. In our samples, oleic acid was detected also in the broth, both in fungal cultures and enzymatic assays. It is seems that mechanism showed in figure 5.13 is responsible for such biotransformation.

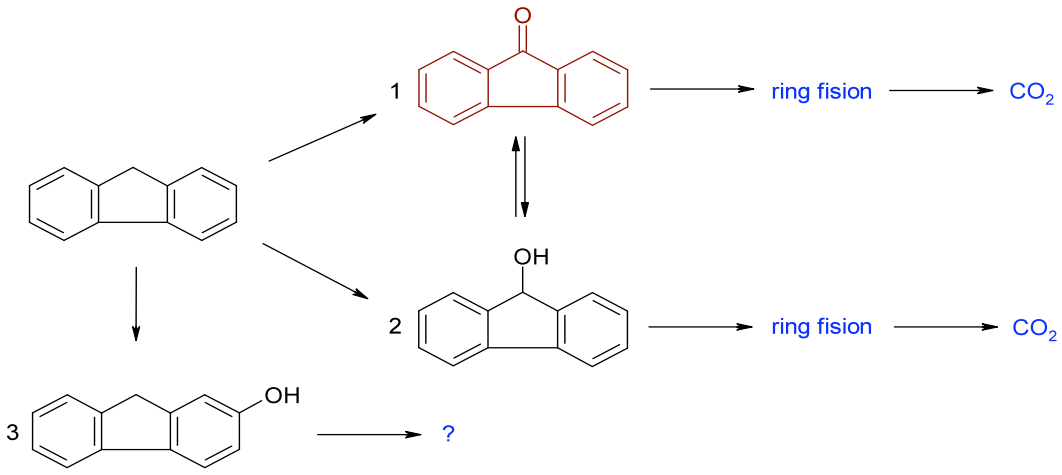


Figure 5.13 Proposed pathway of fluorene degradation by *T. versicolor*. Molecules are identified with the corresponding numbers

The other metabolite, 9-H-fluoren-9-ol, has previously been described as intermediate for *P. ostreatus* (Bezael *et al.* 1996a), *P. chrysosporium* (Bogan *et al.* 1996), *Cunninghamella* sp., *Berjkandera addusta* and *Dreschlera spicifera* (Garon *et al.* 2000). Bezalel and co-workers (1996) pointed out that cytochrome P450 is responsible for the initial attack of fluorene to form 9-H-fluoren-9-ol. The enzymatic system responsible for the formation of this metabolite is not clear. Since, in our case, no experiments with inhibitors of cytochrome P450 were performed for fluorene, it cannot be ensured the implication of this enzymatic system in this transformation.

Another possible origin of this product could be attributed to a later reduction of the quinone group from the fluorenone into an alcohol via a quinone reductase (Bogan *et al.,* 1996, Gómez-Toribio et al., 2009). Thus, further experiments should be performed to clarify the enzymatic system responsible for such transformation.

The metabolite identified as 9-H-fluoren-2-ol was detected only under fungal culture and few information of its possible origin is available. The formation of the product could be attributed to the hydroxylation by cytochrome P450 of the initial fluorene.

5.3.2 Anthracene (ANT)

5.3.2.1 Metabolites Identification

In biodegradation experiments in DM+T80 medium with *T. versicolor* cultures 9,10-anthraquinone was proved to be the major intermediate after 4-12 days of incubation while anthrone was also detected. Both products were later identified comparing the retention time with authentic standards. A third metabolite was detected from fungal cultures after 8 days, but not detected in any other sample. It was identified as ?-hydroxy-9,10-anthracenedione. The position of the hydroxyl group could not be precisely determined because of the lack of accuracy of the GC/MS and the lack of standards for this product. Table 5.8 summarizes the metabolites of anthracene degradation.

In degradation experiments using purified laccase of *T. versicolor* and mediators, 9,10-anthraquinone was identified as dead-end metabolite. None of the other previously mentioned metabolites were detected (see figure 5.14).

Table 5.8 Retention data and electron impact mass spectral characteristics of metabolites.detected after anthracene degradation (* Lac: enzymatic assay; Fng: fungal culture).

Id.	t_R (min)	Exp.*	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion
1	23.25	Fng	194	194 (100), 193 (12.4), 163 (13.3), 139 (8.5), 115 (4), 82 (11), 70 (4), 63 (4)	anthrone a
2	24.27	Fng	224	224 (100), 208 (5.2), 196 (72.9), 180 (6.7), 168 (92.5), 151 (10.1), 139 (94.1), 76 (50.7)	?-hydroxy-9-10-anthracenedione
3	25.54	Lac/Fng	208	208 (98.7), 180 (100), 152 (79.7), 126 (7), 99 (2.7), 76 (28.9), 63 (5.9), 50 (11.4)	9,10-anthraquinone a

a: structures were identified with authentic standards

Field *et al*. (1992) described the formation of anthraquinone by *T. versicolor* in submerged culture. In that work, they suggested that anthraquinone accumulated as a dead-end metabolite in *Bjerkandera* sp. and *Phanerochaete chrysosporium* but *T. versicolo*r was able to mineralize anthraquinone to CO_2. On the other hand, Vyas *et al*. (1994) showed that *T. versicolor* degraded anthracene by initial formation of anthraquinone and this was further degraded. Novotny *et al*. (2000) also detected anthraquinone as major metabolite in cultures of *P. chrysosporium* and *I. lacteus*. Although anthraquinone has been reported as possible toxic to some fungi (Anderson and Herysson 1996). No toxics effects were observed in our experiments.

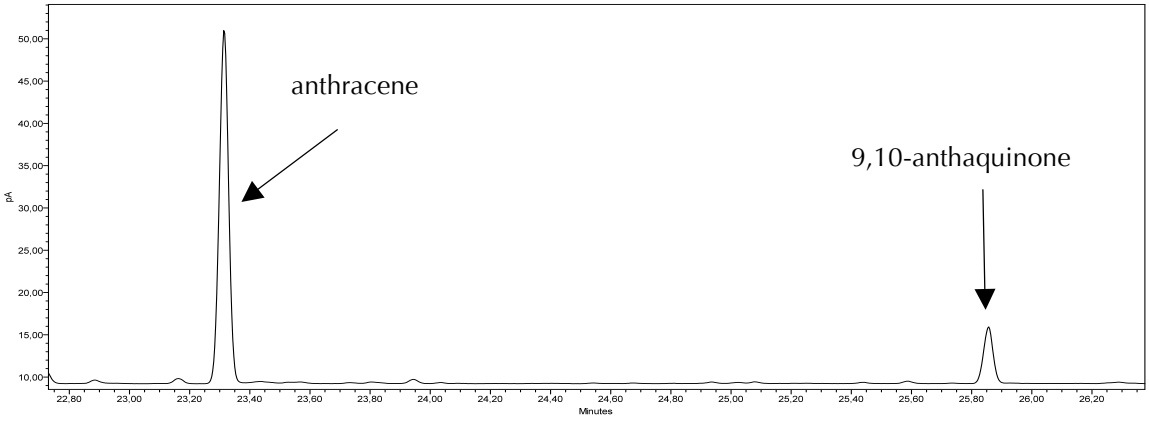


Figure 5.14 Chromatogram using GC/FID of the organic fraction (dichloromethane) extracted from experiment with purified laccase in presence of HOBT. Products were later identified using GC/MS.

Different authors have extensively described the formation of 9,10-anthraquinone in enzymatic degradation assays with purified laccase in presence of mediators (Johannes *et al.,* 1994; Collins *et al.,* 1996; Majcherczyk *et al.*, 1998; Cañas *et al.,* 2007).

Anthrone was firstly described as intermediate product in fungal cultures of *I. lacteus* by Cajthaml *et al*. (2002). Where a possible isomerization of an initial 9-hidroxy-anthracene that transformed to anthrone under thermal stress was pointed out. Eibes *et al*. (2006) detected anthrone as intermediate of anthracene in experiments with purified MnP from *Bjerkandera* sp. BOS55, suggesting its formation from a direct hydroxylation by •OH radicals during oxidative process. Thus, there is controversy on the current knowledge of which enzyme is actually responsible for its transformation.

The metabolite identified as ?-hydroxy-9,10-anthracenedione had never been described as intermediate product by *T. versicolor*. The same product was detected in *I. lacteus* cultures by Cajthaml *et al*. (2002). In that work, they attributed the hydroxylation of anthraquinone by Cyt-P450. Hence, this intracellular enzymatic system seems to be involved as well in the biodegradation process.

5.3.2.2 Metabolites degradation

Biodegradation experiments with pure anthraquinone and pure anthrone were performed in order to describe possible toxic effects towards the fungus and to identify possible intermediates obtaining them in higher amounts. All products detected are listed in table 5.9.

Table 5.9 Retention data and electron impact mass spectral characteristics of metabolites detected after degradation in fungal cultures of anthraquinone and anthrone.

Id.	t_R (min)	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion
5	14.65	166	148 (29.1), 104 (100), 76 (69.9), 50 (63.1), 43.9 (4.2)	1,2-benzenedicarboxilic acid
6	9.55	120	120 (41.5), 105 (100), 91 (0.8), 87 (5), 77 (56.1) 43 (29.5)	acetopthenone
3	25.54	208	208 (98.7), 180 (100), 152 (79.7), 126 (7), 99 (2.7), 76 (28.9), 63 (5.9), 50 (11.4)	9,10-anthraquinone[a]

a: structures were identified with authentic standards

After 12 days of fungal culture with anthraquinone more that 50% of the initial content was degraded (see figure 5.15) and 1,2-Benzenedicarboilic acid (an phtalic acid isomer) was detected. This metabolite was firstly described during the ligninolytic trasformation of anthracene by *I. lacteus* (Cajthaml *et al.,* 2002). Phtalic acid was formed from the degradation of anthraquinone by LiP of *I. lacteus* from 2-(2′-hydroxybenzoyl)-benzoic acid (HBBA), an intermediate formed from the degradation of anthaquinone by LiP. HBBA is a typical product in the metabolism of PAHs for white rot fungi (Cerniglia and Sutherland, 2006). This intermediate product was not detected in any of the samples analyzed in our experiment, but it seems to be the predecessor molecule to phtalic acid. Considering that *T. versicolor* produced laccase, it can be assumed that this enzyme may be responsible for such transformation. Another product detected from anthraquinone cultures was acetophenone, which could be formed from the degradation of 2-(2′-hydroxybenzoyl)-benzoic acid or from further transformation of phtalic acid.

Experiments with pure anthrone demonstrated that such compound showed no toxic effect towards the fungus. Degradation results of anthrone are not shown since the compound transformed spontaneously to anthraquinone that was then degraded by the fungus. Figure 5.16 shows the proposed anthracene degradation pathway of for *T. versicolor*.

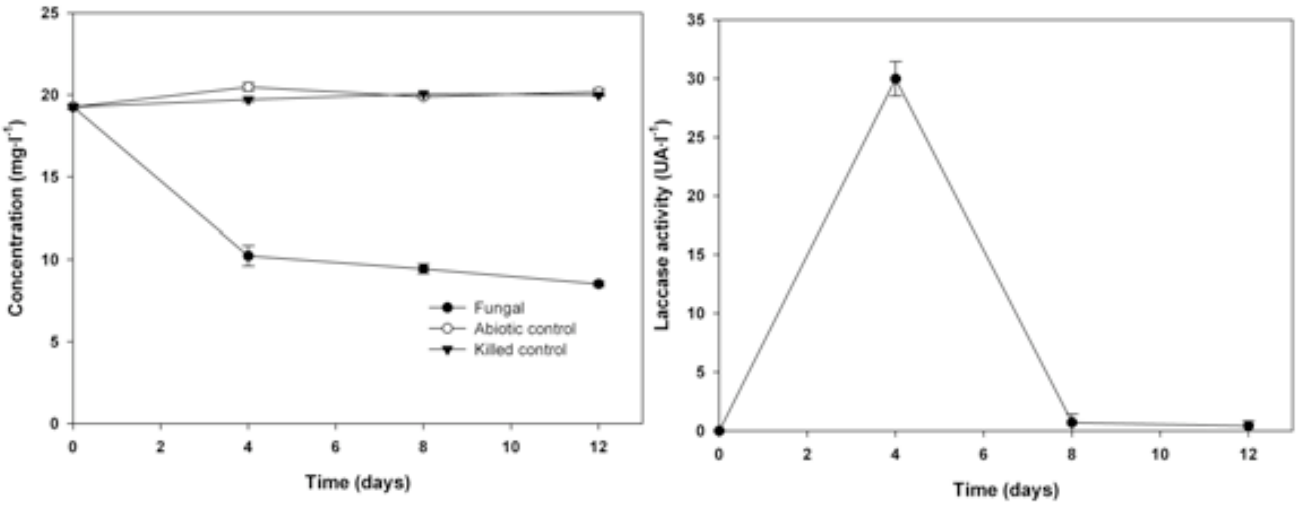


Figure 5.15a and 5.15b Time course degradation of anthraquinone by *T. versicolor* (a). Laccase activity during the experiment (b). Errors are expressed as standard error of triplicates.

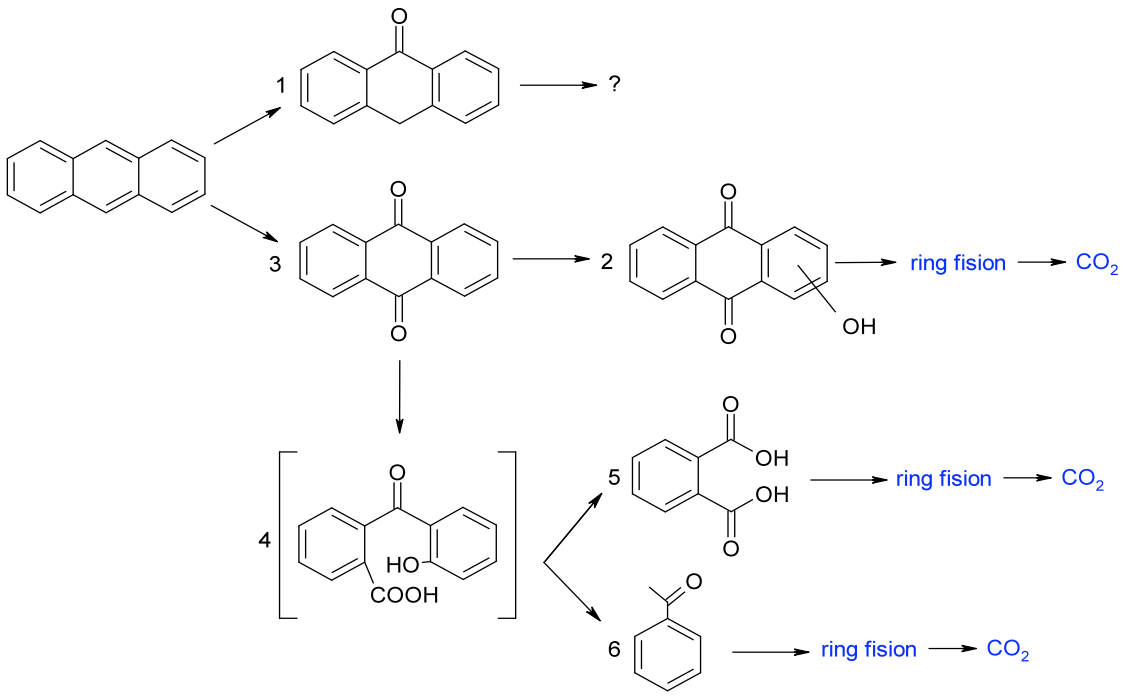


Figure 5.16 Proposed pathway of anthracene degradation by *T. versicolor*.

5.3.3 Acenaphthene (APE) and Acenaphthylene (APY)

The PAHs acenaphthene and acenaphthylene deserve special attention. Their degradation products have been studied together and the samples for analysis were taken from experiments from sections 5.2.3 and 5.2.4. In these, both compounds were present together. Moreover, their degradation pathways coincide in several metabolites (Johannes *et al.*, 1998).

Table 5.10 Retention data and electron impact mass spectral characteristics of acenaphthene and acenaphthylene metabolites. (* Lac: enzymatic assay; Fng: fungal culture)

Id.	t_R (min)	Exp.*	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion
1	18.43	Lac/Fng	168	168 (91.7), 140 (100), 114 (4.4), 113 (8.8), 98 (2.2), 74 (4.3), 70 (10.1), 62 (4.5)	acenaphthenone
2	20.94	Lac/Fng	170	170 (90), 169 (100), 152 (69.1), 141 (39.4), 127 (5.5), 115 (27), 98 (3.1), 90 (4.8), 84 (8.3), 76 (12), 63 (12.2), 50 (5)	acenaphthenol
3	24.14	Lac	182	182 (46.5), 154 (87.6), 126 (100), 98 (7.3), 87 (7.3), 74 (12.3), 63 (14.2), 50 (7.4), 43 (4.1)	1-2-acenaphthylenedione
4	24.85	Lac	184	184 (40.1), 155 (100), 139 (8.9), 127 (78.8), 87 (9.6), 51 (6.9), 44 (6.9)	1H,3H-naphtho[1,8-cd]pyran-1-one
5	26.44	Lac/Fng	198	198 (56.3), 154 (100), 126 (89.9), 122 (2.1), 99 (6.3), 74 (11), 63 (18.7), 50 (6.9)	1,8-naphthalic anhydride

The products identified as intermediates are listed in table 5.10. All of them were detected in enzymatic assays with purified lacase. Only three of them: 1,8-Naphthalic anhydride, acenaphthenol and acenaphthenone were also detected in samples from *in vivo* biodegradation experiments. Most identified intermediates coincide with the molecules previously determined by both Majcherczyk *et al.* (1998) and Johannes *et al.* (1998). In those works they proposed a common pathway for the degradation of both compounds via laccase in presence of the mediator HOBT. Accordingly, a pathway for the degradation of both products by *T. versicolor* is proposed in figure 5.17.

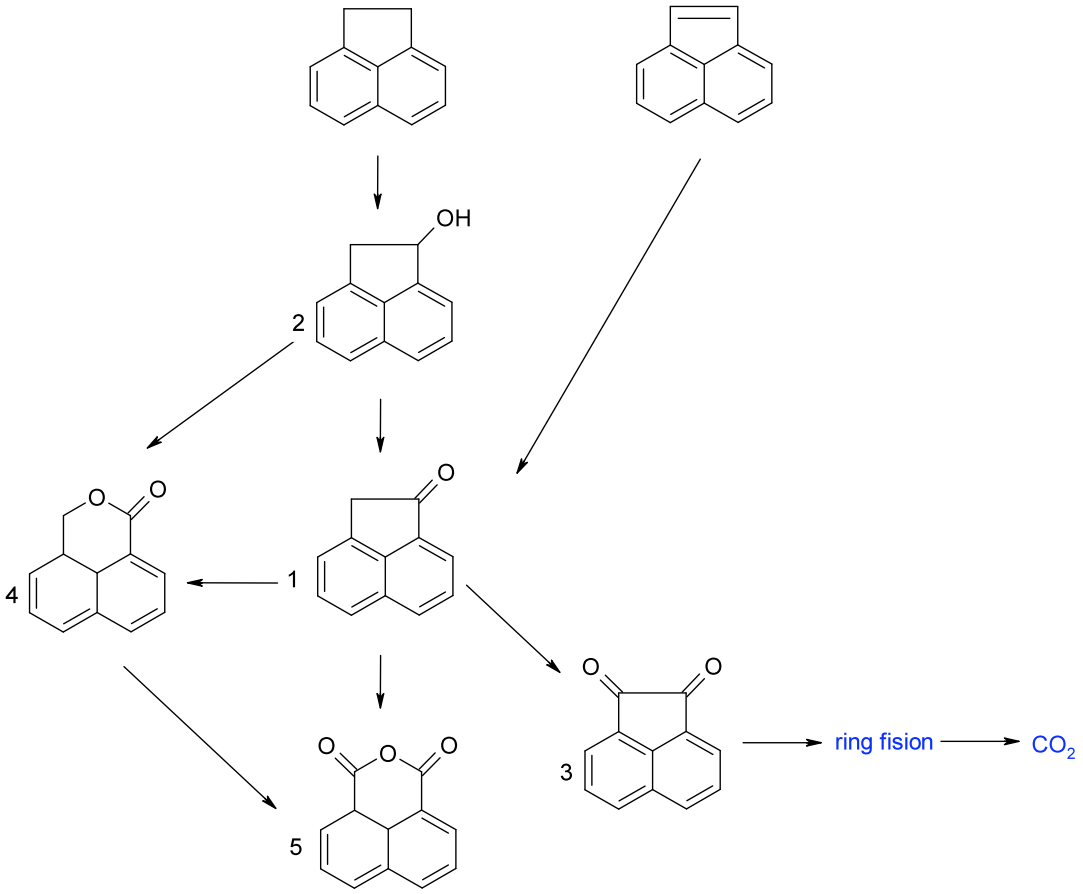


Figure 5.17 Proposed pathway of acenaphthene and acenaphthylene degradation by *T. versicolor*.

5.3.4 Phenanthrene (PHE)

A single metabolite was detected from phenanthrene degradation. In fungal cultures with PAHs mixtures (section 5.2.3) a metabolite identified as 9-phenanthrenol was detected after 8-12 (see table 5.11). The metabolite was not further detected, suggesting that was metabolized by the fungus itself.

In enzymatic degradation assays with laccase and mediators, phenanthrene was degraded but no intermediates were detected. Bömer et al. (1998) demonstrated the capacity of purified laccase of *Trametes hirsuta* to degrade phenanthrene to phenanthrene-9,10-quinone, 2,2`-diphenic acid and phenanthrene-9,10-dihydrodiol in presence of the mediator HOBT and unsaturated lipids via lipid peroxidation. The results of Moen and Hammel (1994) also supported this hypothesis. Mougin *et al.* (2002) also reported phenanthrene-9,10-quinone as metabolite after degradation assay employing purified laccase from *Pycnoporus cinnabarinus ss3*.

This identified intermediate is product of the attack in the phenanthrene K-region via Cyt-P450 under non-ligninolytic conditions as described for *Cunningamela elegans, Irpex lacteus, Saccharomyces racemosum, Aspergilus niger, P. chrysosporium, P. ostreatus* and *Cyclothyrium* sp. as reviewed Cerniglia and Sutherland (2006). Sack *et al.* (1997) reported phenanthrene *trans*-9,10-dihydrodiol as major metabolite after 63 day in nitrogen limited medium culture for *T. versicolor*. Cajthaml *el al.* (2002) suggested 9-phenanthrenol as precursor for methoxiphenanthrene for *I. lacteus*. Moreover, Casillas *et al.* (1996) suggested it as precursor of 9-phenanthryl-sulfate and 9-phenathryl-glucuronide. Sutherland *et al.* (1991) found that *P. chrysosporium* metabolizes phenanthrene to phenanthrene trans-3,4- and trans-9,10-dihydrodiols, 3-,4- and 9- phenanthrols and a glucoside conjugate of 9-phenanthrol. But none of the three compounds could be detected in our experiments.

Table 5.11 Retention data and electron impact mass spectral characteristics of phenanthrene metabolite.

t_R (min)	MW according to CI	m/z of fragment ions	Structural suggestion
27.66	194	194 (94.4), 166 (29.9), 165 (100), 139 (9.4), 115 (3.8), 97 (7.9), 69 (6.9)	9-phenathrenol

The results obtained suggest that *T. versicolor* could transform phenanthrene via the intracellular Cyt-P450. In this way, 9-phenanthrenol is a novel metabolite in the metabolism of phenanthrene by *T. versicolor*. The intermediate product phenanthrene-9,10-oxide is supposed to be the precursor of the 9-phenanthrenol. Figure 5.18 resumes the proposed pathway for the initial degradation of phenanthrene. The disappearance of such metabolite could be attributed to the formation of conjugative products, more soluble, probably easily degraded by the fungus and also a laccase substrate.

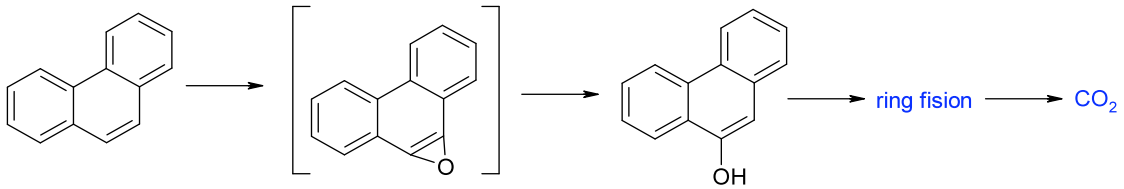


Figure 5.18 Proposed pathway of phenanthrene degradation by *T. versicolor*.

5.3.5 Fluoranthene (FLT)

Little information could be obtained from both fungal degradation experiments and enzymatic assays with fluoranthene. Toluene was the only product detected after 8 and 12 days of fungal culture when extracting with chloroform (see table 5.12). It could be assigned as an end pathway metabolite. This suggests a rapid transformation of this metabolites or its polymerization with other compounds present in the medium or the formation of conjugative products.

Table 5.12 Retention data and electron impact mass spectral characteristics of fluoranthene metabolite.

t_R (min)	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion
4.39	92	92 (100), 89 (4.1), 51 (5.8), 65 (10.5)	toluene

5.3.6 Pyrene (PYR)

When pyrene was degraded in *in vivo* experiments with fungal cultures in section 5.2.3 one metabolite was detected after 12 days of culture in a mixture of PAHs. This was identified as 1-hydroxypyrene, also known as 1-pyrenol.
In experiments with purified laccase, pyrene was degraded and an unidentified metabolite was detected. The latter could not be elucidated in the available structures library of the GC/MS employed. Probably, quinones formed as metabolites transform to polymeric compounds in presence of Tween80 as described by Majcherczyk *et al.* (1998).

The pyrene metabolism described in literature for *T. versicolor* indicates an initial hydroxylation on pyrene by Cyt-P450 and epoxide hydrolase (Sack *et al.*, 1997). Two major initial pathways are proposed for the transformation leading to final quinones or carboxylic acids. On one hand, the formation of pyrene *trans*-4,5-hydrodiol by *P. chrysosporium, P. ostreatus, Cyclothyrium* sp. and *T. versicolor*. On the other hand, the formation of 1-pyrenol by *C. elegans, A. niger* and *P. chrysosporium and A. aegerita*.

Table 5.13 Retention data and electron impact mass spectral characteristics of pyrene metabolite.

t_R (min)	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion
32.03	218	218 (100), 189 (80.8), 163 (6.1), 109 (10.8), 94 (37.4), 81 (5.7)	1-hydroxypyrene (1-pyrenol)

The identification of 1-pyrenol differs from the results of Sack *et al.* (1997), that identified pyrene *trans*-4,5-hydrodiol as metabolite for *T. versicolor*. Thus, our strain seems to degrade initially pyrene via a non-described pathway for the fungus. Lange *et al.* (1996) suggested that *Crinispellis stipitaria* formed different metabolites from pyrene degradation and this could depend on both the culture medium and the strain. Our results agree with this hypothesis. The enzymatic system responsible of such transformation seems to be Cyt-P450 and epoxide-hydrolase. Additionally, Brezna and co-workers (2006) demonstrated that Cyt-P450 of *Mycobacterium vanbaalenii* PYR-1 transformed pyrene into 1-pyrenol. This reinforces our hypotesis. The intermediate product pyrene-1,2-oxide is supposed to be the precursor of the 1-pyrenol, but was not detected in any sample. Further degradation could involve the formation of conjugative products. Figure 5.19 resumes the proposed pathway for the initial degradation of pyrene.

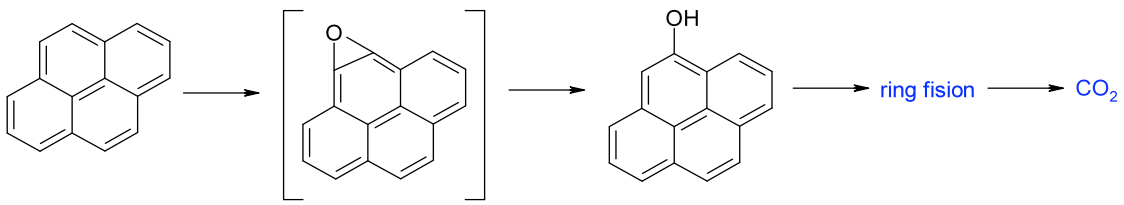


Figure 5.19 Proposed pathway of pyrene degradation by *T. versicolor*.

5.3.7 Benzoa[a]anthracene (BaA)

The knowledge on benzo[a]anthracene metabolism by white rot fungi is quite scarce despite having been reported to be degraded by cultures of *P. chrysosporium* (Ding *et al.*, 2008), *P. ostreatus* (Anderson and Henrysson, 1996), *I. lacteus* (Novotny *et al.*, 2000), *T versicolor* (Tekere *et al.*, 2005) and purified enzymes laccase in presence of mediators (Majcherczyk *et al.*, 1998; Mougin *et al.*, 2002). The major metabolite produced is 7,12-benzo[a]anthraquinone, that has been detected both for *P. ostreatus* and for *I. lacteus*. Cajthaml and co-workers (2006) proposed an accurate degradation pathway for *I. lacteus* where the initial step is the formation of the quinone. The purpose of these section is to contribute in expanding the knowledge on the metabolism of benzo[a]anthracene by *T. versicolor*.

5.3.7.1 Metabolites identification

The analysis of samples after the extraction with dichloromethane from 4 days fungal cultures in DM with Tween 80 proved the presence of a peak at 33 min (see figure 5.20) that was lately identified as 7,12-benzo[a]anthraquinone (BaQ). The same peak was detected after 8 and 12 days of culture. This evidence suggested that *T. versicolor* could metabolize the compound in a similar manner to what was prior described for ligninolytic enzymes.

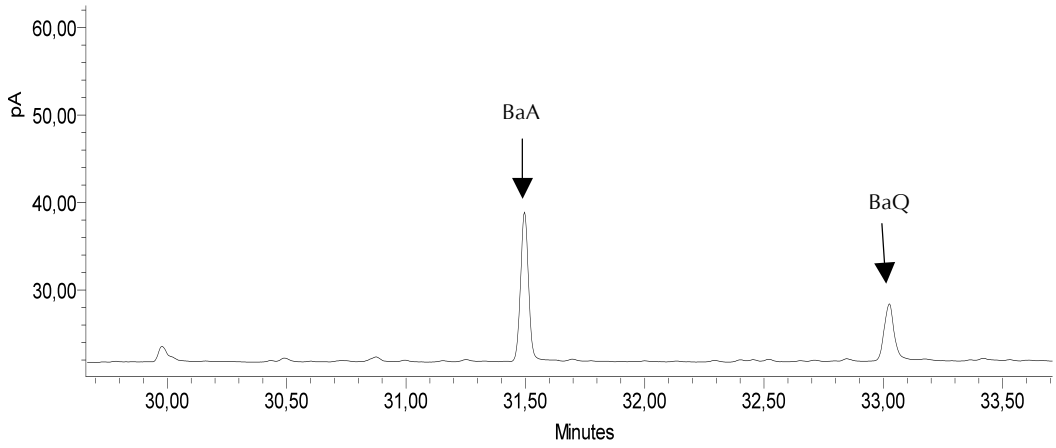


Figure 5.20 Chromatogram of the organic fraction after 4 days of fungal culture using a GC/FID. Products were later identified using GC/MS.

A complementary experiment of 6 days with fungi culture in DM medium containing Tween 80 was performed for a better understanding of the rapid degradation that had been observed in the previous experiments. Half of the initial BaA was degraded within the first 48 hours of experiment, as shown in figure 5.21. The final concentration matches with the concentration at 4 days found in the experiment presented in section 5.2.1. Samples for identification of metabolites were taken along the experiment, concentrated and then analyzed by GC/MS.

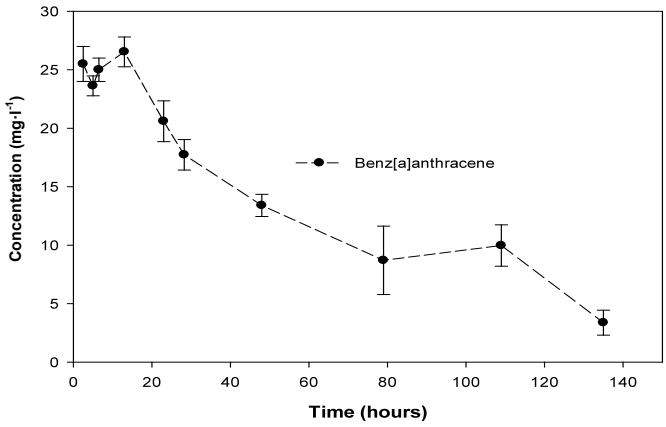


Figure 5.21 Time course degradation of Benzo[a]anthracene by *T. versicolor*.

Surprisingly, a huge peak appeared near 27 minutes, which did not correspond to any metabolite related to BaA structure (see figure 5.22). This peak was identified by GC/MS as oleic acid. This compound was accumulated in the broth and later its concentration decreased with the degradation of BaA, supporting the theory of lipid peroxidation catalysed by laccase produced by the fungus. An explanation for the appearance of oleic acid in the medium comes from the cleavage of the molecule of Tween 80. Thus, Tween 80 leads to the formation of oleic acid and this enhances the generation of oxygen reactive species that leads to the initial formation of BaQ that is further degraded into other intermediates.

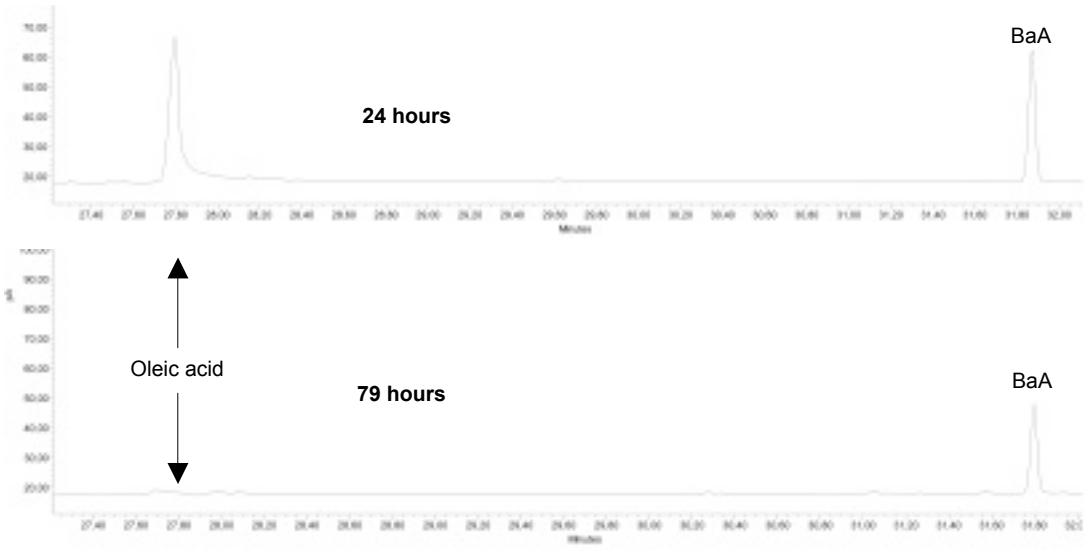


Figure 5.22 Appearance and disappearance of oleic acid during fungal degradation of BaA.

5.3.7.2 Enzymatic degradation of Benzo[a]anthracene

According to the information available at the moment, three different experiments were performed: inhibition of Cyt-P450 test (section 5.2.1) and enzymatic assays with commercial laccase.

The experiments with inhibitor showed no effect on degradation of benzo[a]anthracene, achieving a final degradation of 80.5% after 12 days of experiment, a similar value to experiments with no inhibitor. Hence, it was confirmed that Cyt-P450 does not play any role in the initial transformation of BaA by *T. versicolor* (see section 5.2.3).

Experiments with purified laccase demonstrated the capacity of the enzyme to degrade BaA in presence of HOBT, DMHAP and VA in medium containing Tween 80. In presence of HOBT and DMHAP, BaQ was identified as major metabolite (see table 5.14). Also, 4-hydroxy-1-tetralone and 1-tetralone were

detected as minor metabolites of enzymatic transformation of the parental molecule. Besides, oleic acid was detected in the medium, probably as a cleavage product of Tween80. This suggests that BaA is degraded by laccase via lipid peroxidation. A scheme of the proposed pathway for the enzymatic degradation of BaA by laccase is represented in figure 5.23.

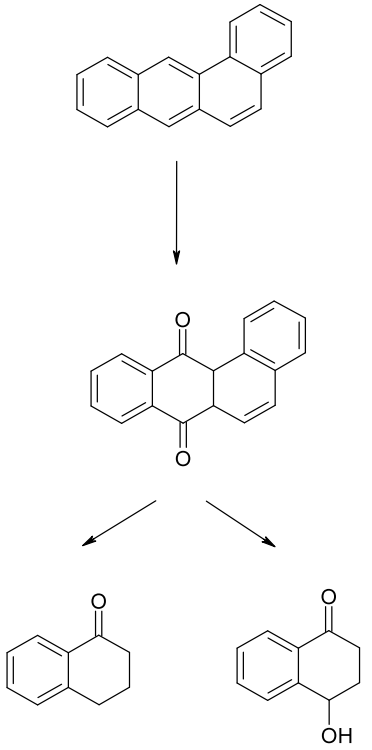


Figure 5.23 Proposed pathway for the degradation of BaA by laccase-mediator complex.

Table 5.14 Retention data and electron impact mass spectral characteristics of benzo[a]anthracene metabolites after degradation with purified laccase.

Id.	t_R (min)	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion
1	33.17	258	258 (100), 230 (29.6), 202 (56.2), 174 (0.3), 163 (2.2), 150 (5.5), 137 (0.8), 129 (10), 100.9 (0.3)	benzo[a]anthracene-7,12-dione a
2	15.86	146	146 (72.4), 130 (13.3), 118 (100), 115 (17.5), 91 (14.9), 90 (66.4), 77 (66), 63 (13.1), 51 (11.3)	1(2H)-naphthalenone (alpha-tetralone) a
3	9.27	162	162 (100), 147 (30), 134 (78.3), 120 (11.5), 106 (65), 77 (25), 51 (15.2)	4-hydroxy-1-tetralone a

a: structures were identified with authentic standards

Since laccase has been reported to be involved in lipid peroxidation (Bömher *et at.*, 1998; Srebotnik and Boisson, 2005) and the evidences in preceding results, more experiments were performed to prove whether lipid peroxidation occurred. Assays with purified laccase in presence of different surfactants were carried out using: Tween 80 (1 $g \cdot l^{-1}$), Tween 20 (1 $g \cdot l^{-1}$) and Tween 20 (1 $g \cdot l^{-1}$) + oleic acid (0,3 mM). Results are summarized in table 5.15.

Table 5.15 BaA enzymatic degradation (%) of in presence in different surfactants and oleic acid addition.

	Degradation (%)		
Mediator	**Tween 80**	**Tween 20**	**Tween 20 + oleic ac.**
None	36.9	11.4	25.5
ABTS	22.4	5.3	17.5
AV	77.5	14.8	27.8
HOBT	94.5	53.2	91.3
DMHAP	91.8	8.0	16.3

As indicated in table 5.15, the highest degradation of BaA was achieved in presence of Tween 80. In the reaction medium that contained Tween 20, the percentage of degradation decreased in respect to the values obtained in presence of Tween80. The addition of oleic acid increased the degradation about 2-fold and in the case of laccase + HOBT the degradation reached values similar to those obtained with Tween 80. This suggests that oleic acid is necessary for the degradation of BaA because it enhances the generation of reactive oxygen species that are responsible for the attack of BaA.

The intermediates listed in table 5.16 were detected after degradation of BaA. Several metabolites were detected after 79 hours of culture in samples extracted with dichloromethane and ethyl acetate.

Table 5.16 Retention data and electron impact mass spectral characteristics of benzo[a]anthracene metabolites detected after fungal degradation.

Id.	t (min)	MW according to CI	m/z of fragment ions (relative intensity)	Structural suggestion
1	33.17	258	258 (100), 230 (29.6), 202 (56.2), 174 (0.3), 163 (2.2), 150 (5.5), 137 (0.8), 129 (10), 100.9 (0.3)	benzo[a]anthracene-7,12-dione a
2	15.44	144	144 (100), 115 (86.9), 99 (3.5), 89 (9.7), 72 (9.8), 63 (11.2), 45 (11.9)	naphthalenol (1-naphthenol)
3	15.86	146	146 (72.4), 130 (13.3), 118 (100), 115 (17.5), 91 (14.9), 90 (66.4), 77 (66), 63 (13.1), 51 (11.3)	1(2H)-naphthalenone (alfa-tetralone) a
4	9.27	162	162 (100), 147 (30), 134 (78.3), 120 (11.5), 106 (65), 77 (25), 51 (15.2)	4-hydroxy-1-tetralone
5	19.37	146	134 (12.7), 105 (100), 77 (43), 51 (8.2)	phtalide
6	17.85	194	194 (4.2), 163 (100), 133 (20.2), 77 (10.5)	dimethyl phtalate
7	14.71	148	148 (26.9), 104 (100), 76 (76.3), 50 (45.4), 44 (7)	phthalic anhydride
8	7.74	108	108 (100), 90 (20.3), 63 (7.6), 56 (24.5), 51 (10.1), 41 (20.6)	o-cresol
9	5.93	106	106 (100), 105 (99.6), 80 (2.5), 77 (89.5), 51 (45)	benzenealdehide
10	3.33	92	92 (100), 89 (4.1), 51 (5.8), 65 (10.5)	toluene
11	15.58	138	138 (65.6), 121 (100), 109 (1.3), 93 (26.1), 65 (21.7), 53 (6.7)	4-hydroxy-benzoic acid
12	16.64	158	158 (100), 130 (47), 102 (74.3), 76 (57.7), 50 (31.6)	1,4-naphthalenedione
13	17.90	160	160 (81.8), 131 (16.6), 104 (100), 76 (45.1), 66 (6), 55 (4.2), 50 (23.8)	1,4-naphthalenediol
14	7.23	108	108 (100), 90 (20.3), 63 (7.6), 56 (24.5), 51 (16.1), 41 (20.6)	benzenemethanol

a: structures were identified with authentic standards

5.3.7.2 Metabolites degradation

In parallel, a biodegradation experiment of BaQ was performed in order to ensure that this was not an end-metabolite product and to detect intermediates. In figure 5.26, it can be observed that *T. versicolor* is capable to degrade 50% of the initial content of BaQ after 12 days. The intermediates detected matched those for BaA. Results so far indicated that BaA was transformed in a similar manner to that described for *I. lacteus* by Cajthaml and co-workers (2002). In order achieve a better understanding of the pathway, biodegradation experiments with alpha-tetralone and naphthalene-1,4-dione were carried out.

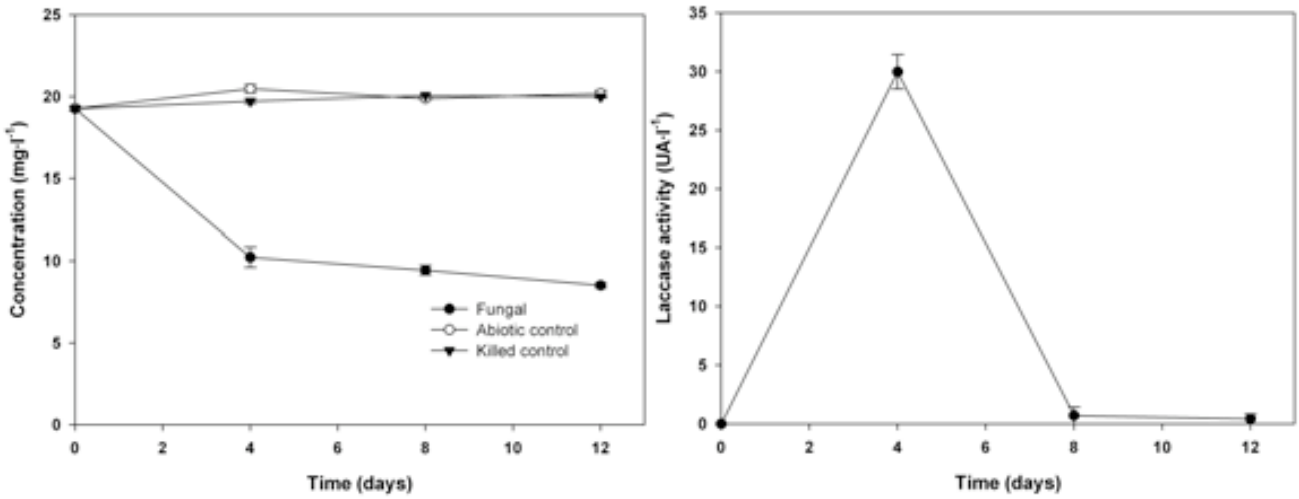


Figure 5.24a and 5.24b Time course degradation of 7,12-benzo[a]anthraquinone by *T. versicolor*. (a). Laccase activity during the experiment (b). Errors are expressed as standard error of triplicates.

The metabolite identified as alpha-tetralone was detected only when BaA was degraded by purified laccase. After *in vivo* biodegradation experiments 1-naphthenol was identified from both BaA and BaQ. Given that Cajhtaml *et al.* (2002) demonstrated that both compounds were related, a biodegradation experiment of alfa-tetralone was performed (see figure 5.25). About 91% of the initial content of alpha-tetralone was degraded after 4 days of culture. A new peak was detected in the chromatogram (see figure 5.26). Subsequent identification of the molecule by GC/MS confirmed that 1-naphthalenol was a metabolite produced by hydrogenation from alpha-tetralone. Since this transformation only occurred in presence on *T. versicolor,* it was demonstrated that the fungus is involved in such process. Two other metabolites appeared from the degradation of alpha-tetralone: phtalic anhydride and 1,4-naphthalenedione.

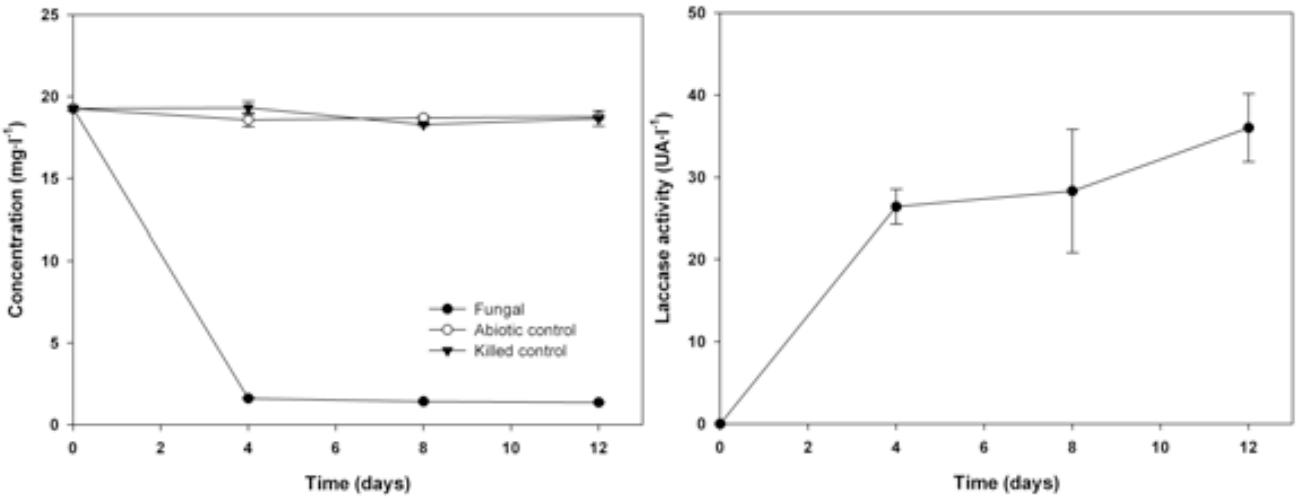


Figure 5.25a and 5.25b Time course degradation of alpha-tetralone by *T. versicolor* (a). Laccase activity during the experiment (b). Errors are expressed as standard error of triplicates.

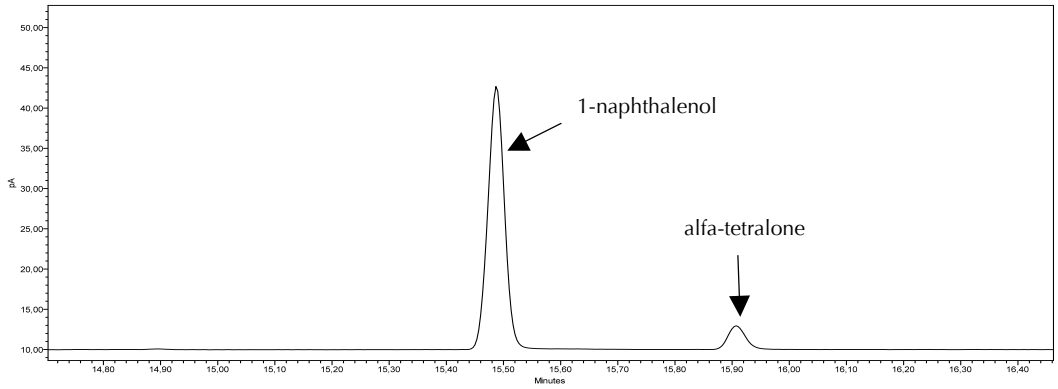


Figure 5.26 GC/FID chromatogram of the organic fraction from biodegradation of alpha-tetralone after 4 days of fungal culture. Products were later identified using GC/MS.

Biodegradation of 1,4-naphthalenedione was also carried out. This product was previously described as precursor intermediate of 4-hydroxy-1-tetralone in biodegradation of BaA by *I. lacteus* (Cajthaml *et al.*, 2002). It was reported to be a particularly toxic compound (Yen *et al.*, 2002). Results of biodegradation are not shown because the compound disappeared spontaneously both in presence of fungi and in the abotic control. No toxicity respect the fungus was observed. The product transformed spontaneously to 1,4-naphthalenediol via a hydroxylation process.

To summarize the results obtained from biodegradation of BaA and its metabolites by *T. versicolor* a scheme is proposed in figure 5.27.

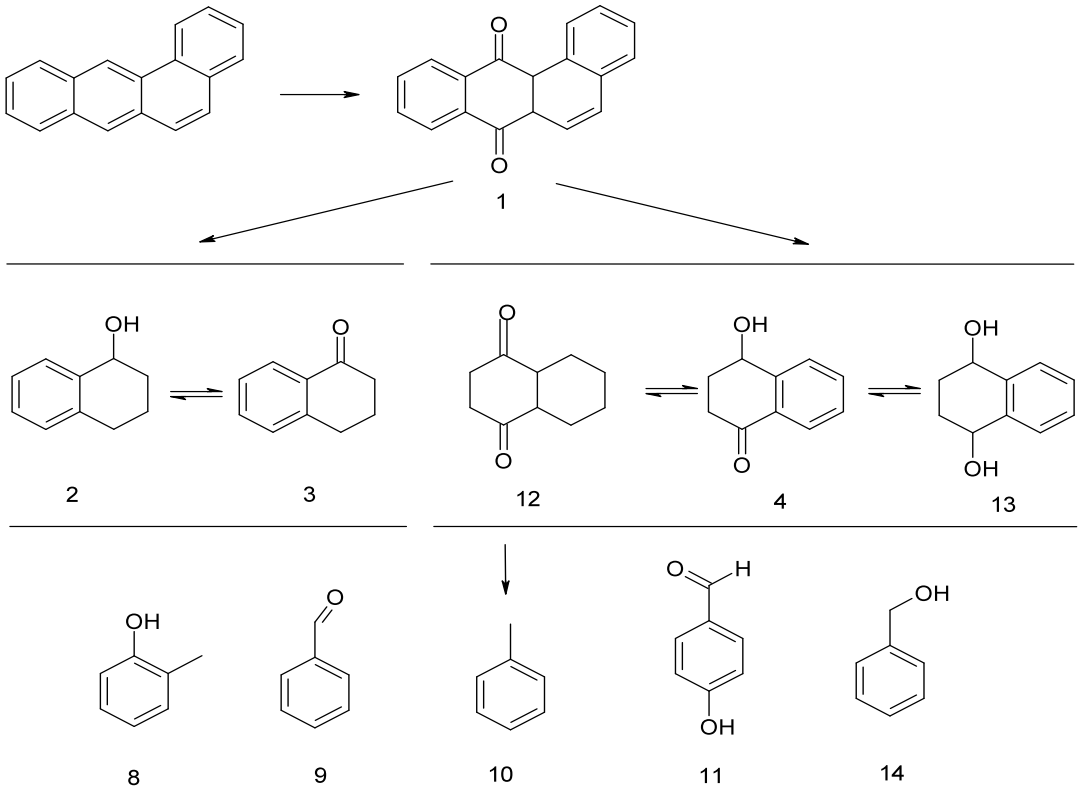


Figure 5.27 Proposed pathway for the degradation of BaA by *T. versicolor*. The metabolites with not clarified origin are bound with brackets.

5.3.8 Benzo[k]fluoranthene (BkF)

The analysis of the organic fraction after biodegradation of benzo[k]fluoranthene did not show any metabolite regardless of the organic solvent used as extractant. However, experiments with inhibitor of Cyt-P450 were performed showing a strong inhibition, reducing the biodegradation to near 0%. Thus, it seems that the formation of hydrodiols would be the most reasonable metabolite formation. These types of molecules were not detected in any sampling time. This could be explained by the rapid formation of a conjugative molecule, more soluble in water and possibly metabolized by the fungus. Further research is needed to elucidate the degradation mechanism of this compound.

5.3.9 Conclusions

- Novel PAH-degradation intermediates were Identified from fluorene, anthracene, phenanthrene, pyrene and benzo[a]anthracene degradation experiments in submerged cultures of *T. versicolor*.
- It was determined that the fungus was capable to remove or metabolize the intermediate products arising from parental PAHs in the case of fluorene, anthracene and benzo[a]anthracene. Thus not accumulating intermediate products.
- Degradation mechanism of benzo[a]anthracene was elucidated.
- The metabolites formed could be related to an enzymatic system responsible of such transformation.

5.4 Degradation of PAHs in soil system

In previous sections it has been well established the capacity of *T. versicolor* to degrade several PAHs and some of their metabolites. A step forward is the remediation of an artificially polluted soil because the pollutant bioavailability is affected by soil. In this environment, several properties such as organic matter and clay content, the presence of dissolved organic matter and soil aggregation can all together influence the bioavailability of the pollutant. Its entrapment can limit the availability to the enzymes responsible for the transformation (Gianfreda and Rao, 2004). In addition, enzymes can restrict their activity due to sorption or immobilization to soil particles (Burns, 1982).

Next results focus on the capacity of the fungus to degrade PAHs in soil employing different systems: biopile microcosms, slurries and aerated biopiles in Dewar vessels.

5.4.1 Biopile microcosms

Biodegradation experiments of the two target PAHs BaA and BkF were performed in a similar manner to that described in section 4.2.2.4 for soil colonization. The fungus was grown on lignocellulosic substrate and then mixed with soil to colonize it under sterile conditions. The difference relies on the soil, which was spiked either with BaA or BkF. Experiments could not be performed by triplicate due to contamination by other microorganisms.

The soil was rapidly colonized during the first week as assessed by visual observations in presence of both compounds. In terms of ergosterol per unit of dry mass, the fungus colonized the soil to the same extent as reported in section 4.2 when colonizing under sterile conditions. The concentration did not decrease during the 4 weeks of experiment, and laccase was detected. It is assumed that it was constitutive laccase since the range was quite close to that observed when colonizing non-polluted soil. Thus, the enzyme secretion was not stimulated by the presence of either pollutant.

With regard to the concentration of BaA and BkF, two different trends were observed. For the case of BkF, no decrease in concentration was achieved. Whilst in the case of BaA, near 50% of the spiked compound was removed from soil. The different trends respect to the liquid experiments can be attributed to the soil-pollutant interactions that made both compounds less bioavailable to the fungus.

The high K_{ow} and K_{oc} of these pollutants suggest that the compounds were trapped onto the organic matter and clay from soil reducing even more its bioavailability.

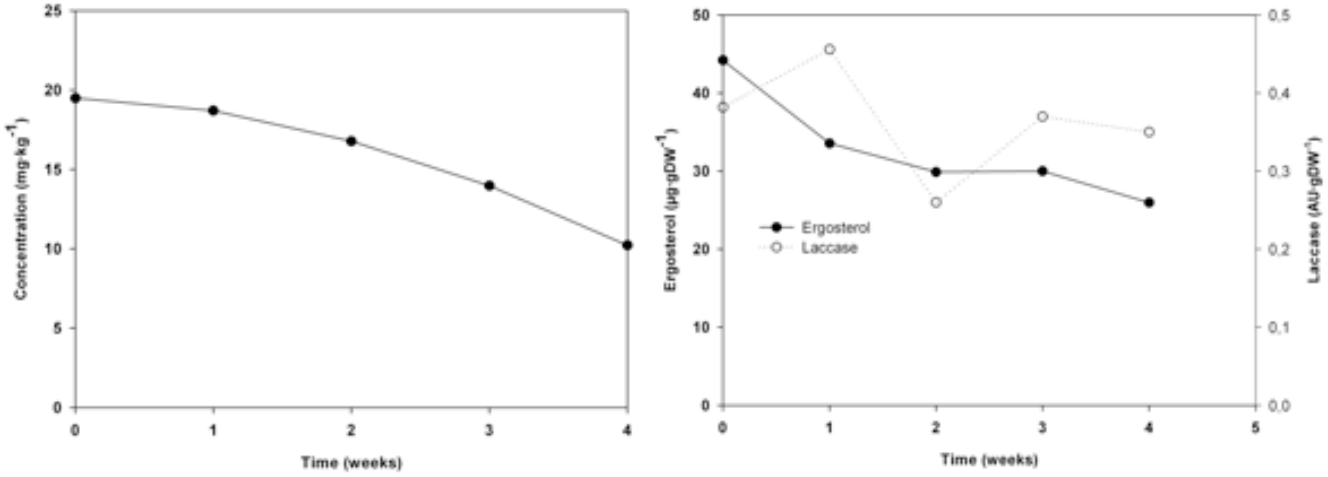


Figure 5.28a and 5.28b Concentration of BaA in soil (a); ergosterol and laccase activity during the experiment (b)

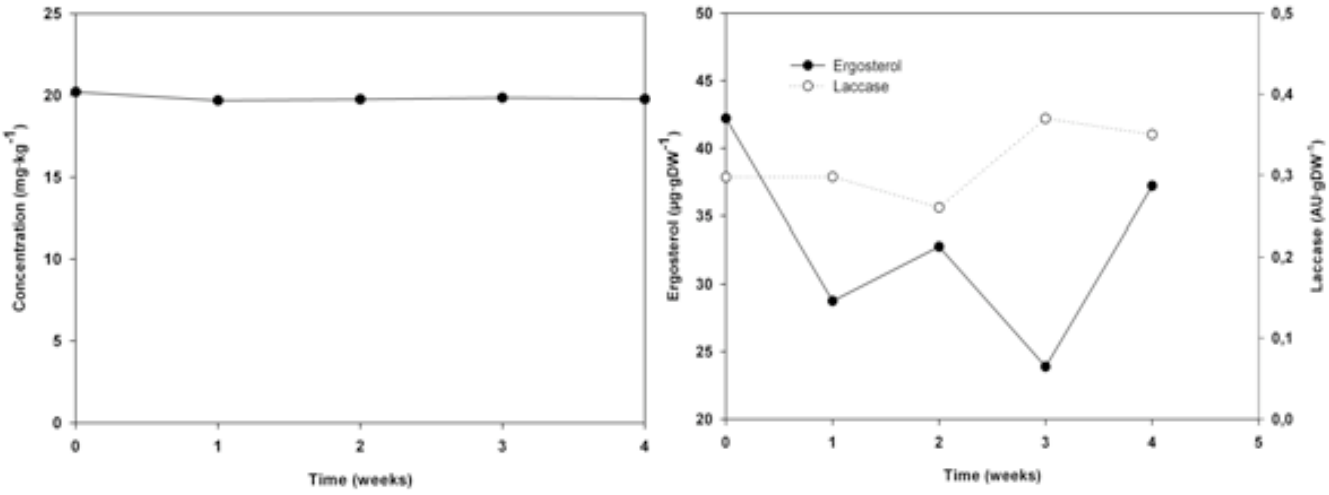


Figure 5.29a and 5.29b Concentration of BkF in soil (a); ergosterol and laccase activity during the experiment (b)

5.4.2 Slurries

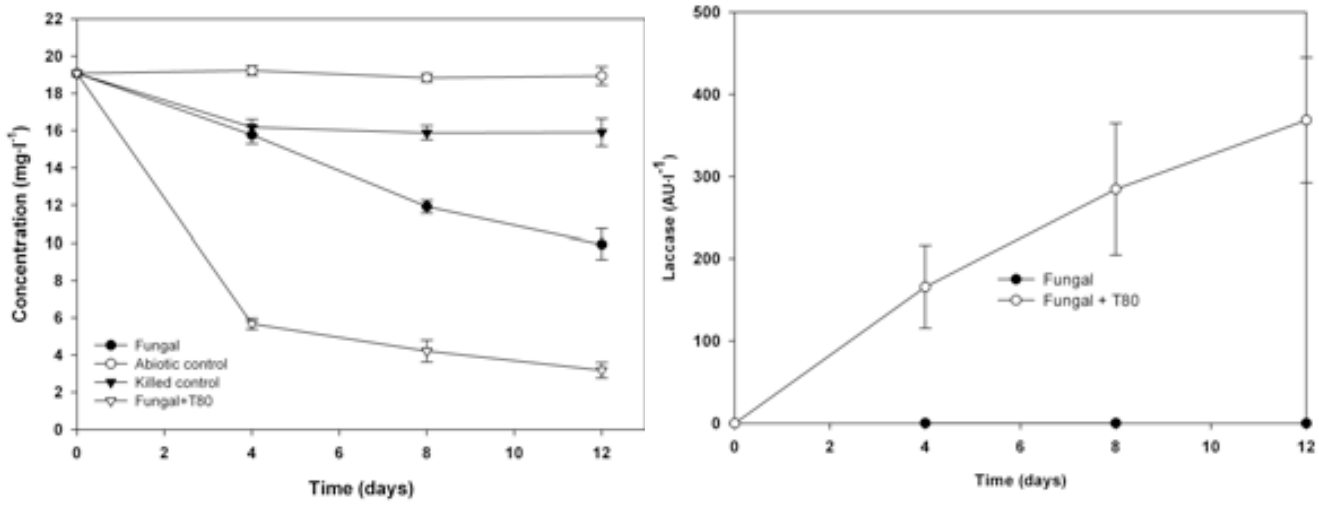
Biodegradation experiment of target PAHs, BaA and BkF, were performed employing the slurry technique as described in section 3.4.2.2. The experiments compared the fungus capacity to degrade both compounds in absence or not of Tween 80. The use of a non-ionic surfactant permits the mobilization of soil-bound PAHs (Thiem *et al.*, 1997). In the soil-aqueous system, PAHs solubilization takes place at surfactant concentrations greater than in aqueous systems (Liu *et al.*,

1992). Surfactant sorption onto soil particles results in a fraction being unavailable for micellar solubilization. This is known as effective CMC (CMC_{eff}) (Zheng and Obbard, 2002b). This later study reported a CMC_{eff} for Tween 80 of $6.19 \cdot 10^{-4}$ mol·l^{-1} in the same soil/water proportion employed in these experiments (1g/10mL). Thus the concentration of Tween 80 in the media guaranteed the effective solubilization of PAHs.

The soil-slurry bioreactors to eliminate PAHs using white-rot fungi have been satisfactorily described by Zheng and Obbard (2001). In that study, authors reported the elimination of four to six-ring PAHs by *P. chrysosporium* in a medium containing 0.4% (w/v) Tween 80.

In figure 5.30a and 5.31a are presented the concentration profiles of both BaA and BkF along the experiment. In the case of BaA, degradation rates were similar to those obtained in liquid experiments. Surprising results were obtained for the case of BkF, which was degraded more efficiently respect to the liquid and solid experiments. This later finding could be explained by a strong sorption of BkF onto the soil matrix due to its elevated K_{oc} (Kastner, 2000); that cannot permit the extraction of the compound.

The fungus consumed the initial glucose during the first 4 days in all cases. The laccase activity increased along the experiment, showing a dissimilar trend to that observed in section 5.2.1 (see figures 5.30b and 5.31b). In addition, it was not detected practically in absence of Tween 80. This can be explained by a partial sorption of the enzyme onto the soil organic matter and clays, which could immobilize the enzyme.



Figures 5.30a and 5.30b BaA concentration (a) and laccase activity along the experiment (b). Errors are expressed as standard error of triplicates.

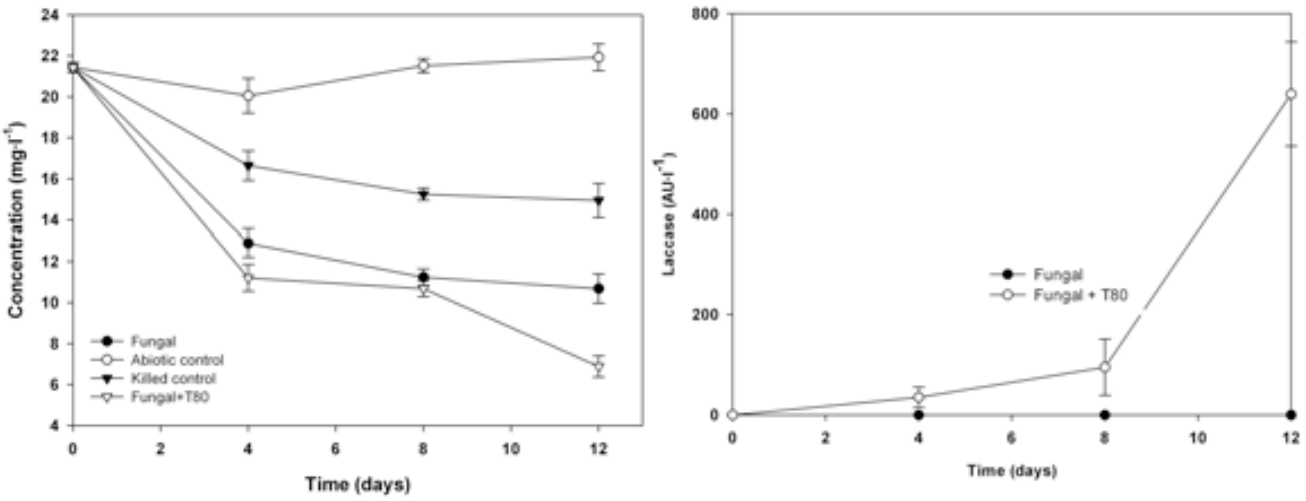


Figure 5.31a and 5.31b BkF concentration (a) and laccase activity along the experiment (b). Errors are expressed as standard error of triplicates.

5.4.3 Aerated biopiles (Dewar vessels)

An alternative approach to treat soils contaminated with PAHs is the composting process. In this section are compared the traditional composting process using native microorganisms and the composting process bioaugmented with *T. versicolor* for biodegradation purposes. All experiments were carried out under non-sterile conditions.

The contaminants were mixed together in a stock solution containing the PAHs. These include: flourene, phenanthrene, anthracene, flouranthene, pyrene, benzo[a]anthracene and chrysene. They were mixed together and used as contaminants during the experiment. The percentage of each compound as a part of the total PAHs (∑PAHs) was 30%, 28%, 9%, 20%, 3.5%, 3% and 6.5% respectively. They were then spiked into soil samples, where the applied concentration was adjusted to 1g∑PAHs·kg^{-1} (dry matter).

Afterward, the spiked soil was manually mixed with the proposed organic co-substrates at a ratio 1: 0.25 (soil: co-substrate, dry weight). In treatments where bioaugmentation was to be evaluated, inoculum (*T. versicolor*) was introduced (1ml·3g^{-1} of co-substrate) within the mixture. It was different to biopile microcosm system, in which the fungus was introduced grown on a lignocellulosic substrate. The porosity of the produced mixture was modified to ensure aerobic condition introducing bulking agent consisting of wood chips at a ratio of (1:1, v/v). In all treatments, tap water was added during the preparation of the composting mixture

to modify the water content according to the recommended values for composting process (40-60%). All the composting experiments were carried out in duplicates during 30 days of incubation. The experiment program was as follow:

- Treatment 1: contaminated soil + *T. versicolor* + compost + bulking agent
- Treatment 2: contaminated soil + *T. versicolor* + straw pellets + bulking agent
- Treatment 3: contaminated soil + compost + bulking agent
- Treatment 4: contaminated soil + *T. versicolor* + sterile compost + bulking agent

Also, duplicated Control (C) treatments which only contaminated soil (1g$\sum$PAHs·kg^{-1}) were employed in order to follow the PAHs degradation by the indigenous microorganism without any additives.

Figure 5.32 shows the progress of the temperature profile along the composting process. As a standard behavior in such process, the temperature began to increase in all treatments, indicating the oxidation process caused by the microbial activity within the composted materials (Ruggieri *et al.*, 2008) and co-substrates. Temperature profile varied among the different treatments typologies during the first 10 days, but they had almost the same behavior after that period.

The temperature trend went in distinct pathways especially in treatment 2, which was in the thermophilic ranges during the first week. The other treatments were always in the mesophilic ranges. This behavior was due to the easily degradable materials in the pelleted straw that permitted a rapid growth of the fungus (visual observations) in the vessel with the consequent increase of temperature. This increase in temperature could inhibit further degradation capacity of both the bioaugmented fungus and the native microbial population. Fungi present low temperature-stress tolerance and high temperature affects their survival ability.

Treatment typologies with compost as co-substrate varied among them especially during the first 10 days as mentioned before. In this context, temperature in treatments 1 and 3 were closed to each other, whereas in treatment 4, with sterilized compost, temperature was increased to less extent compared with non-sterilized (1 and 3), which is logically attributed to the absence of microorganism within the compost itself because of sterilization. In general terms, no inhibitory effects were observed as a result of PAHs presence. This indicated the high capacity and tolerance of both soil microflora and *T. versicolor*.

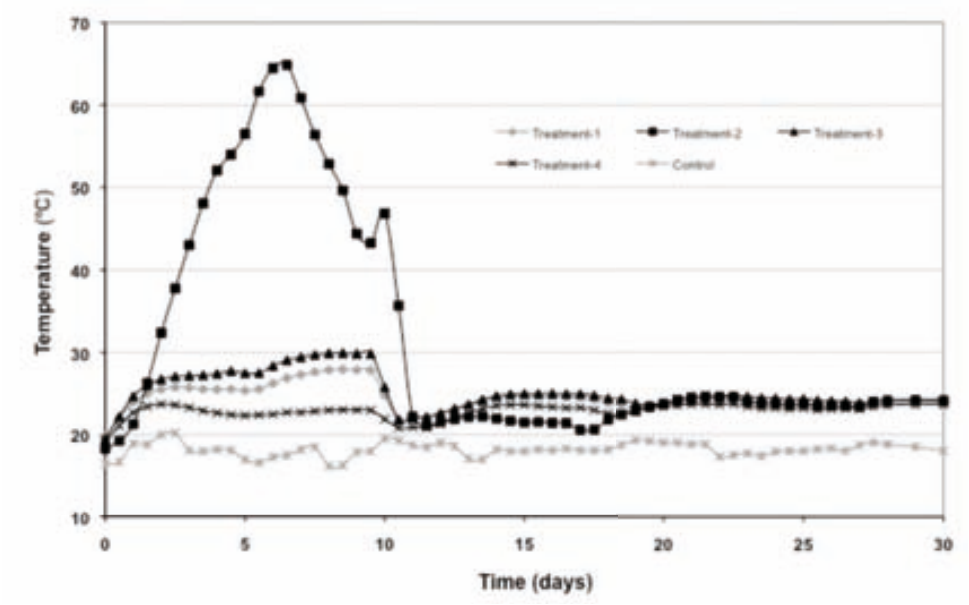


Figure 5.32 Temperature profile during the process.

The different configurations employed in this experiment demonstrated high potential of soil native microorganisms to eliminate PAHs. Figure 5.32 shows the remaining PAHs (as total PAHs) after 5, 10, 20 and 30 days of composting. Elimination rate of 89 % was achieved after 30 days in treatments with compost as co-substrates. In table 5.17 are listed the final concentration of the individual compounds. As it can be observed, the low-molecular-weight-PAHs were extensively degraded in all treatments (except 2); being BaA and Cry the less degraded in all cases. Nevertheless, degradation rate of 71% was achieved in treatments with pelleted straw as co-substrate. Only 29.5% of the PAHs were removed in the control (C).

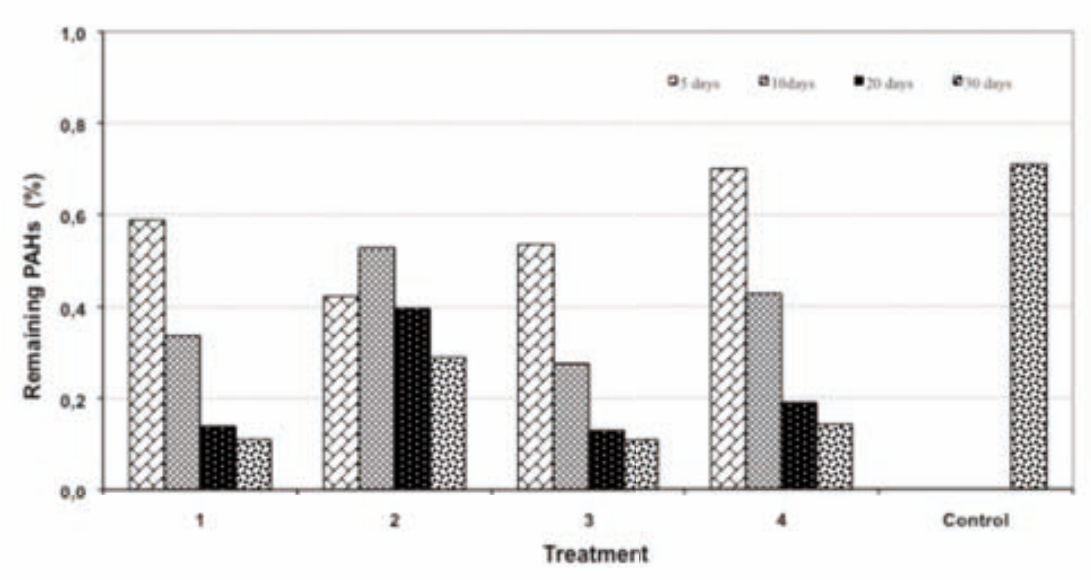


Figure 5.33. Remaining PAHs (%) after 5, 10, 20 and 30 days of composting in Dewar vessels.

It can be observed that treatments with compost as co-substrates followed the same trend either they were augmented with *T. versicolor* or not. This indicates that bioaugmentation with the fungus did not enhance the remediation process when compost was added. Moreover, in treatment 4, the degradation rate during the first 10 days was less than those obtained in the other treatments containing compost (1 and 3). This can be attributed to the initial absence of microorganisms in the compost as it had been sterilized. However, it is thought that it was able to re-colonize and support the indigenous microflora to eliminate PAHs; consequently 86% of the PAHs was degraded at the end of the experiment. These results are in accordance with previous results that demonstrated the capacity of stable OFMSW-compost in enhancing the degradation of such contaminants (Sayara *et al.*, 2009b; Gandolfi *et al.*, 2010).

Table 5.17 Remaining PAHs at the end of the process. Errors are expressed as standard error (n=2).

	Remaining concentration ($gPAHs \cdot kg_{soil}^{-1}$) in each treatment				
Compound	**1**	**2**	**3**	**4**	**Control**
Fluorene	8.6 ± 2.3	32.5 ± 6.1	11.5 ± 1.5	9.6 ± 4.7	150.7 ± 3.7
Phenanthrene	11.5 ± 1.3	36.5 ± 0.1	11.0 ± 2.0	10.8 ± 2.3	206.7 ± 3.9
Anthracene	6.2 ± 1.3	19.2 ± 6.4	6.9 ± 0.7	6.5 ± 3.4	56.5 ± 1.2
Fluoranthene	23.7 ± 6.3	111.2 ± 14.7	20.2 ± 2.5	21.5 ± 2.5	176.6 ± 1.1
Pyrene	3.9 ± 0.7	18.6 ± 2.5	3.4 ± 0.3	5.5 ± 1.2	26.8 ± 0.2
Benzo[a]anthracene	13.9 ± 1.3	21.7 ± 5.0	13.3 ± 0.8	19.0 ± 1.0	28.1 ± 0.7
Chrysene	42.4 ± 1.8	50.3 ± 4.1	41.5 ± 0.7	55.0 ± 0.8	60.7 ± 1.6
∑PAHs	110.1 ± 4.0	289.8 ± 6.3	107.7 ± 2.9	127.8 ± 5.6	705.9 ± 3.5

The most interesting results are those of treatment 2, as degradation was almost identical to other treatment during the first 10 days, but later it was less efficient with respect to the other treatments. The similarity during the first 10 days was essentially caused by the elimination of low molecular weight PAHs. Nevertheless, the difference was noted in eliminating high molecular weight-PAHs, due to fungi. Additionally, the temperature profile in this treatment was different from others (see figure 5.33). It was in the thermophilic rage during the first period which might inhibit further degradation by the fungus and/or native soil microflora.

In this regard, the applied co-substrate effect aroused as an important factor. It can be concluded that the bioremediation efficiency in this experimental setup relies on the components selectivity and co-substrate properties rather than its nutrients. Although the indigenous soil microorganisms were able to degrade some of the

PAHs as noticed in the control (29.5%), they were unable to degrade the more recalcitrant compounds like chrysene and benzo[a]anthracene. It is interesting to highlight that PAHs degradation rate was found to be fast during the first 10 days, mainly due to low weight compounds. But during the last stage it followed slow removal rate, which was most likely attributed to nutrients depletion needed for the microbial activity. Also, the remaining PAHs could be attributed to the non-available fraction due to physical interaction with the soil matrix. The same behavior has been documented in previous works (Margesin *et al.*, 2000; Hamdi *et al.*, 2007; Hafidi *et al.*, 2008; Silva *et al.*, 2009; Sayara *et al.*, 2010a).

Moreover, comparing the different treatments, it can be observed that the addition of *T. versicolor* did not enhance the remediation process as they followed the same trend especially for those were compost was applied. The obtained result are in accordance with some previous studies in which different kinds of white-rot fungi were used in an attempt to enhance the PAHs degradation, but no positive results were obtained (Baheri and Mysami, 2002; In der Wiesche *et al.*, 2003; Silva *et al.*, 2009; Karamalidis *et al.*, 2010). The regulation of temperature in treatment 2 could enhance the PAHs degradation efficiency so that it would avoid the fungal inhibition due to the termophilic range.

Indeed, native microorganisms present in soil and organic amendments were more effective than the inoculum as they were more adapted to that particular environment. In addition, the application of stable organic co-substrates which are believed to have a great portion of humic matter as part of their organic matter are more efficient to stimulate and enhance the degradation rate as the facilitate the desorption of PAHs (Gandolfi *et al.*, 2010; Karamalidis *et al.*, 2010; Sayara *et al.*, 2010a, 2010b; Tejada *et al.*, 2008).

Enzymatic activity analysis showed no laccase activity in any bioaugmented treatment at any of the sampling times. Even though visual observations, after 5 days, clearly indicated the presence of fungi within the vessels in treatments 1, 2 and 4. Consequently, most degradation probably occurred due to the indigenous soil microorganisms and those that were introduced within the compost itself. Probably laccase was attached to soil particles or organic matter and it could not be extracted.

In fact, introducing exogenous microorganism is not always effective and is known to have some risks specially the case of white-rot fungi that are not soil microorganisms. For instance, introducing an adequate co-substrate is usually

more efficient as observed by the obtained results and in chapter 4. The obtained results probably demonstrate that the compost was simultaneously able to act for both bioaugmentation and biostimulation.

Fungal biomass was measured in terms of µg ergosterol per g of soil (see figure 5.35). It was detected in all soil treatments but varied largely during the first 10 days depending on the amendment employed. In treatment 2, the biomass content increased quickly because of the high content in easily degradable organic matter available and adequate aeration which favored fungal growth. The intensive microbial activity was clearly noticed in the temperature profile that reached easily the thermophilic range in that period, as described before.

Treatments 1 and 3 had similar fungal biomass; although treatment 1 found to have a little more respect the other. This may be caused by the competitive influence that induced the indigenous microorganisms growth, or this was due to the bioaugmentation despite the absence of their enzymatic activity as discussed before. After 10 days of composting, all treatments had almost the same fungal biomass except treatment 3 as it was amended with sterilized compost.

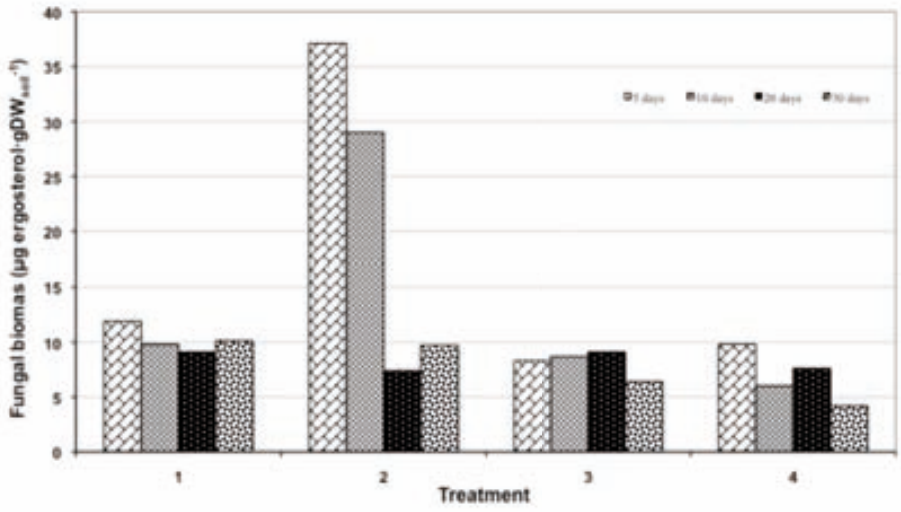


Figure 5.36. Fungal biomass evolution during the process.

PAHs biodegradation was also assessed by means of metabolic intermediates present in soil analyses. The presence of these intermediate products is considered as true indicator of such remediation process. Polar metabolites (see table 5.18) were identified from degradation of anthracene and fluorene in all treatments. The main product of fluorene was 9H-fluorenone that appeared at days 5 and 10. At day 20, 9H-fluorenone was not detected and 9H-fluorenol was detected. These two metabolites have been described as metabolites from degradation of fluorene by white rot fungi (see section 5.3.1). Two major

metabolites were detected from anthracene degradation: anthrone and anthraquinone. Both products have been extensively described as by-products during metabolism on anthracene for white-rot fungi (see section 5.3.2). Moreover, dibuthyl phtalate was detected, which has been described as a by-product of several PAHs degradation by fungi. Other PAHs metabolites could not be detected although its concentration decreased in the soil. These were possibly bonded to soil organic matter or degraded by native microflora.

Table 5.18 Metabolites identified by GC/MS during the process.

t_R (min)	MW according to CI	m/z of fragment ions	Structural suggestion
20.09	180	180 (100), 152 (42.1), 126 (7.5), 119 (1), 98 (3), 76 (12.6), 63 (7.4), 50 (2.6)	9-fluorenone
20.14	182	181 (100), 165 (18.6), 152 (52.3), 139 (3.5), 126 (6.2), 91 (6.1), 76 (20.2), 63 (7.2), 51 (4.1)	9-H-fluoren-9-ol
25.54	208	208 (98.7), 180 (100), 152 (79.7), 126 (7), 99 (2.7), 76 (28.9), 63 (5.9), 50 (11.4)	9,10-anthraquinone
25.28	278	149 (100), 121 (2.6), 104 (4.9), 93 (3.3), 76 (4.2), 57 (10.7), 41 (12.4)	dibutyl phtalate

5.4.4 Conclusions

- Biopile microcosms were not effective technique because of limitations in bioavailability of PAHs due to sorption to soil organic matter and clays.
- Slurry treatment permitted good biodegradation rates of target PAHs.
- The composting treatment technique in Dewar vessels did not permit better PAHs removal when bioaugmentating *T. versicolor* compared to addition of compost. Problems with temperature rising (termophilic range) occurred.
- The effect of biostimulation and bioaugmentation on PAHs-contaminated soil through composting was evaluated. The obtained results did no show any improvements due to bioaugmentation with *T. versicolor* with respect to classical biostimulation with compost. On the contrary, biostimulation using different organic co-substrates was able to improve the degradation rate.

CHAPTER 6

Degradation of creosote Polycyclic Aromatic Hydrocarbons by *Trametes versicolor*

Creosote is one of the most important soil pollutants worldwide. It is formed during high-temperature treatment of several woods and/or coal tar. PAHs represent 85% in weight of their composition. In the first section of the chapter, the ability of the fungus to remove the PAHs fraction from creosote in liquid, slurry and biopiles microcosms approaches is presented. The second section presents the soil bacteria effect on PAHs-removal ability of the fungus during soil creosote-remediation. This work is the result of collaboration with the Laboratory of Environmental Biotechnology in the Institute of Microbiology in Prague, Academy of Sciences of the Czech Republic.

A slightly modified part of this chapter has been published as:

Borràs, E., Caminal, G., Sarrà, M., Novotný, Č. 2010. Effect of soil bacteria on the ability of polycyclic aromatic hydrocarbons (PAHs) removal by *Trametes versicolor* and *Irpex lacteus* from contaminated soil. Soil Biology & Biochemistry 42, 2087-2093.

6.1 Degradation of cresote-PAHs by *Trametes versicolor*

The capacity of *Trametes versicolor* to colonize soil and to biodegrade efficiently PAHs has been well established in previous chapters. Taking into account this knowledge, this section shows the results related to the capacity of *T. versicolor* to degrade PAHs from creosote in liquid media, solid state and slurry systems.

6.1.1 Introduction to soil creosote pollution

6.1.1.1 What is creosote?

Creosote is the name for a group of compounds formed during high-temperature treatment of several woods and/or coal tar. The different creosotes can be classified in groups: wood creosote, coal tar creosote, coal tar, coal tar pitch and coal tar pitch volatiles. All of them vary in color, texture and application.

Wood creosote has been traditionally employed as medicine, demonstrating laxative and cough suppressing properties, as well as a disinfectant; but is rarely used this way today. Coal tar products are used in pesticides, insecticides, animal and bird repellants and fungicides. Coal tar creosote was mostly used in wood preservation industry.

6.1.1.2 Creosote pollution problematic

The wood-preserving industry has been using extensively many pesticides. The most important chemicals employed for that purpose are creosote, penta-chlorophenol (PCP) and CCA (copper, chrome and arsenate). These all can be grouped as the more broadly pesticides used in the course of the recent industrial era in terms of volume (Mueller et al., 1989). Only in the United States, $4.5 \cdot 10^7$ kg per year of creosote were employed and it is assumed that 40 percent of contaminated sites are due to PAHs-creosote (ATSDR, 1995).

The proper disposition of creosote does not emerge as neither an environmental problem nor threaten to human health. Wood preserving facilities are usually creosote-polluted sites (Potin *et al.*, 2004). Commonly, this contamination arises from leaking tanks, drippings, spills and leachates. In spite of this, accidental spills or inappropriate disposals can generate risks. Creosote enters soil and makes its way into the ground water, where it takes years to breakdown. Such contamination is commonly associated to surface soils, waters from treatment

lagoons as well as groundwater contaminated by leachates (Mueller *et al.*, 1989). Creosote was classified as probable human carcinogen agent (ATSDR, 2002)

6.1.1.2 Chemical composition

Creosote, a black colored and viscous fluid, is a complex mixture of compounds with diverse chemical structures. Coal tar creosote is formed (by weight) of around 85% PAHs, 10% phenolic compounds and 5% O-N-S-heterocyclic (see table 6.1). More than 300 chemicals have been identified in coal tar creosote, and it may contain another 10,000, many of which are harmful to living organisms (Hale and Aneiro, 1997). Thus, it is impossible to represent a chemical formula and structure for creosote. In consequence it results difficult to characterize, monitor and evaluate the removal and biotransformation of its constituents (Molnár *et al.*, 2007).

Table 6.1 Predominant PAHs in coal tar creosote (adapted from Mueller *et al.*, 1989).

Compound	Relative percentage (%, w)
Naphthalene	13
2-methylnaphthalene	13
Phenanthrene	13
Anthracene	13
1-methylnaphthalene	8
Biphenyl	8
Fluorene	8
2,3-dimethylnaphthalene	4
2,6-dimethylnaphthalene	4
Acenaphthene	4
Fluoranthene	4
Chrysene	2
Pyrene	2
Anthraquinone	1
2-methylanthracene	1
2,3-benzo[b]fluorene	1
Benzo[a]pyrene	1

6.1.1.3 Regulation of creosote use

The Scientific Committee of Toxicology, Ecotoxicology and Environment of the European Union dictated a series of studies that confirm the threats associated to creosote when this contains a concentration above 0.005% in mass of

benzo[a]pyrene. This committee expressed the risk of suffering cancer by consumers exposed to creosote as well as to products of wood treated with creosote.

The Directive 94/60/EC of the European Parliament regulates the use and commercialization of creosote and coal tar distillates used for wood treatment, as well as for other preparations that contain them. It limits the contents of benzo[a]pyrene, maximum of 50 $mg \cdot kg^{-1}$ (0.005% mass), and of extractable phenols with water, maximum of 30 $g \cdot kg^{-1}$ (3% mass). According to the modified arrangements of Directives 2001/90/EC and 2001/91/EC, creosote cannot be used in the treatment of wood. However, the use of creosote for wood treatment in industrial facilities or carried out by professionals for retreatment *in situ* is allowed if accomplishes the later requirements. In Spain, the Ministerial Order PRE/2666/02 adapts to the Spanish legislation the Directives 2001/90/EC and 2001/91/EC.

Today, creosote-treated wood can only be destined to industrial and professional uses if it fulfills certain requirements. It is allowed in railroad sleepers, on electrical and energy transmission, on telecommunication networks, for enclosures, for specific agricultural purposes, in bridges and in docks. Its use is forbidden in interiors of buildings, leisure parks, gardens and in other places of open-air leisure or in those that there is a risk of skin contact or in contact with products destined for human or animal consumption.

6.1.1.2 Remediation of creosote-polluted soils

Remediation of creosote-contaminated soils involved a wide spectrum of chemical and thermal technologies. These treatments include solvent extraction, thermal desorption, incineration, landfarming and others; all them accepted by the EPA (FRTR, 2002). These techniques require high amounts of energy, thus at high operational cost. Additionally, toxic by-products can arise from thermal remediation as suggested by Richter *et al.* (2000). Hence, attention in bioremediation as a promising technology has increased, as a more environmental friendly and cost effective alternative.

Creosote was considered during a long time as environmentally recalcitrant to microbial attack. But along the last three decades it has been well established that soil microorganisms are capable to degrade both PAHs and PCP, the major creosote components in polluted sites (Mueller *et al.*, 1989; Sabaté *et al.*, 2006;

Haritash and Kaushik, 2009). Bioremediation technologies have commonly relied on bacterial ability to remove PAHs from soil, via stimulation or bioaugmentation strategies. Bacteria bioremediation of PAHs requires uptake of these compounds from an aqueous phase prior to their intracellular metabolism. Bacteria are effective at degrading the PAHs containing 2-3 rings, using them as carbon sources (Cerniglia, 1992); while high molecular weight (HMW) PAHs, 4-6 rings, are normally recalcitrant to bacterial attack (Cerniglia, 1984). This is hampered by their little bioavailavility due to the low solubility, restricted transport through cell membrane as well as their binding to soil clay and/or organic matter (Alexander, 2000). However, in the last decade, research in biodegradation of HMW-PAHs has reported new bacterial isolates, namely *Mycobacterium* sp. and *Sphingomonas* sp., capable of degrading such compounds (Kanaly and Harayama, 2010).

Analogously, bioremediation using white-rot fungi has been extensively studied since the mid 1980's (Šašek *et al.*, 2003; Atagana *et al.*, 2006; Baldrian, 2008). Most of the limitations mentioned formerly for bacteria can be overcome by using fungi, as they penetrate the soil via hyphal elongation through the polluted matrix and secrete oxidative enzymes nearby the xenobiotics (Potin *et al.*, 2004). These qualities make them tolerant to highly toxic environments. When white-rot fungi are applied into soil, they are typically accompanied by the increase of ligninolytic enzymes (Šnajdr and Baldrian, 2006) and secretion of coupling enzymes (D'Annibale *et al.* 2005). Moreover, the monooxygenase system of Cyt-P450 may also be involved in degradation. Most common white-rot fungi studied for creosote-soil remediation include *Phanerochaete sp., Pleurotus ostreatus, Irpex lacteus, Bjerkandera adusta, Trametes sp.* and *Lentinus tigrinus*.

Several studies at bench scale have established the ability of white-rot fungi to degrade PAHs both in polluted soil (Field *et al.*, 1994; Andersson and Henrysson, 1996; Martens *et al.*, 1999; Lamar *et al.*, 2002; Novotny *et al.*, 2004; Leonardi *et al.*, 2007; Byss *et al.*, 2008; Covino *et al.*, 2010b) and in creosote-treated-wood (Galli *et al.*, 2008; Kim *et al.*, 2010; Covino *et al.*, 2010b). Field trials led to dissimilar results, their effectiveness depending on factors as selection of fungal strain, soil aging and inoculums type (Davis *et al.*, 1993; Lamar *et al.*, 1994; Lestan *et al.*, 1996; Eggen, 1999; Rama *et al.*, 2001). Arguably, the major limitation for *on site* bioremediation using white-rot fungi is the constraint for large preliminary research, which is both costly and time consuming (Baldrian, 2008)

6.1.2 Aims of this section

The results presented in this section focus on the ability of *T. versicolor* to degrade the PAHs from aromatic-creosote fraction. The mixtures of PAHs used for the experiments are obtained according to the procedure described in section 3.6.10.1. *T. versicolor* is tested as degrader in liquid, slurry and biopile-like microcosms.

6.1.3 Creosote-PAHs degradation in liquid medium

A liquid biodegradation experiment was performed as an initial approach to determine the capacity of *T. versicolor* to degrade PAHs from creosote. Experiments were carried out using a defined medium in presence and in absence of Tween 80. This non-ionic surfactant had already been proven to enhance PAHs degradation by the fungus (see chapter 5). The effect of creosote constituents towards fungus activity and degradation capacity was unknown.

As figure 6.1 shows, *T. versicolor* was able to consume the initial glucose. After 4 days, 2 $g \cdot l^{-1}$ of glucose remained in the medium, also when Tween 80 was added. Creosote had some toxic effects towards the fungus since the consumption rate was slower if compared to experiments of chapter 5. This effect can be attributed to other compounds present creosote mixture that.

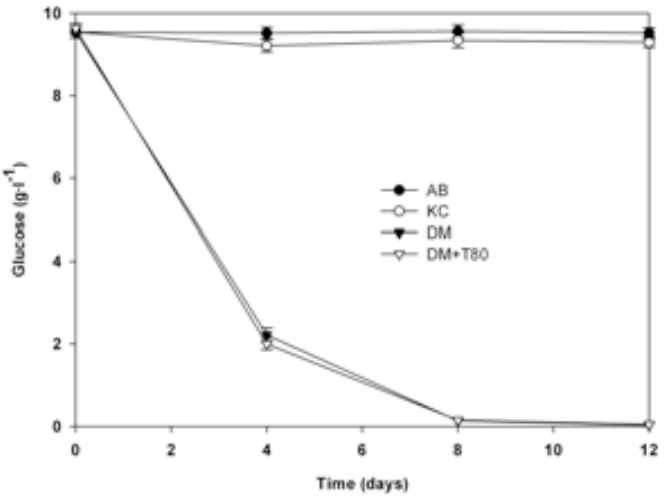


Figure 6.1 Glucose consumption along creosote degradation. Errors are expressed as the standard error of triplicates.

In terms of enzymatic machinery, laccase was secreted, thus demonstrating that the fungus was active (see figure 6.2). Two different patterns were observed. In the defined medium laccase increased along the experiment, reaching its maximum at the end of it (26 $AU \cdot l^{-1}$). Whereas the same medium containing Tween 80 it

reached a 7.5-fold maximum at day 8 of culture (195 AU·l^{-1}), with a posterior decrease; a similar pattern observed in section 5.2.2.

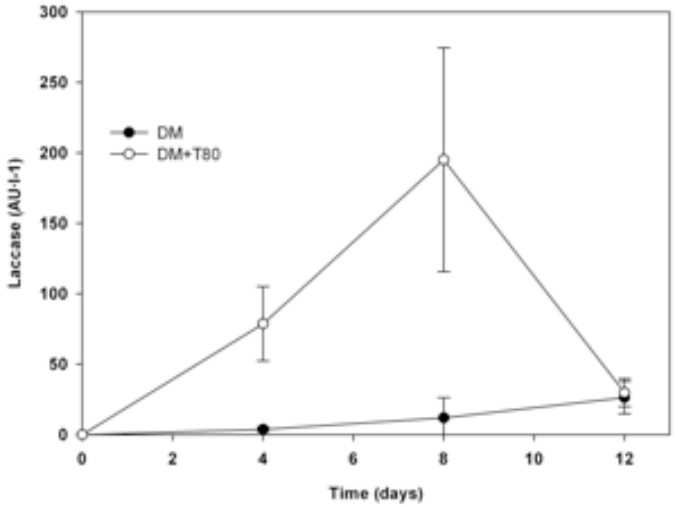


Figure 6.2 Laccase activity along the experiment. Errors are expressed as the standard error of triplicates.

Regarding to PAHs removal, figure 6.3 and table 6.2 shows the residual concentration of PAHs grouped by number of rings. Tables 6.3 and 6.4 present the PAHs concentration along the experiment. The abiotic and sorption losses were quantified by means of controls (table 6.4) and considered when calculating the biodegradation percentages.

In defined medium, 2-3 ring-PAHs, the most soluble and bioavailable compounds, were the most extensively degraded by the fungus, to a final 17.8% respect the abiotic control at day 12. Being all degraded except PHE and FLT, the two compounds with high IPs. The 4 ring-PAHs were degraded less extensively, to a final biodegradation of 4.8%, mainly due to the depletion of BaA, BkF and BbF. Finally, the 5-6 ring-PAHs were not degraded. To conclude, the fungus was only capable to degrade the low molecular weight PAHs, which are commonly more bioavailable to the enzymatic machinery of the fungus.

In general terms, the addition of Tween 80 (1g·l^{-1}) increased the biodegradation of PAHs by *T. versicolor*. The group of 2-3 ring-PAHs were degraded up to 26,7% respect the abiotic after 12 days of culture. All compounds in this group were degraded above 40% except for PHE and FLT, both biodegraded near 11%. The group of 4 ring-PAHs was degraded above 15% in all 3 sampling days. Biodegradation was mainly due to a general decrease in the concentration of all compounds except BbF. The 5-6 ring-PAHs were also degraded with the exception of the last sampling day, where the depletion of such compounds in the

killed control was higher than in the treatment sample. The depletion of BaP was the most important contribution to the decrease in concentration of this group.

Table 6.2 Degradation percentage of PAHs grouped by number of rings.

Number of rings	Degradation percentage (%)					
	Defined Medium			Defined Medium + Tween 80		
	4 days	8 days	12 days	4 days	8 days	12 days
2-3 rings	6.6	0.2	17.8	29.9	16.4	26.7
4 rings	9	0	4.8	18.1	15.1	15.8
5-6 rings	6.1	0	0	11.6	5.2	0

It is important to highlight that he PAHs removal occurred mainly before the first sampling period and was practically stopped during the rest of the experiment. Overall, PAHs were removed better in presence of the surfactant Tween 80. But the biodegradation efficiency was lower compared to that attained in the previous chapter in section 5.2.3. This fact could be attributed to the presence of other compounds in creosote that reduced the general degradation capacity of the fungal enzymatic machinery. Part of this would be also involved in the degradation of these additional compounds. Nevertheless, the fungus accomplished the purpose and the results opened the door to carry out experiments in slurry and solid-state systems.

Figure 6.3 Residual PAHs concentration along the creosote degradation experiment. Errors are expressed as the sum of the standard deviation (n=3) of each compound.

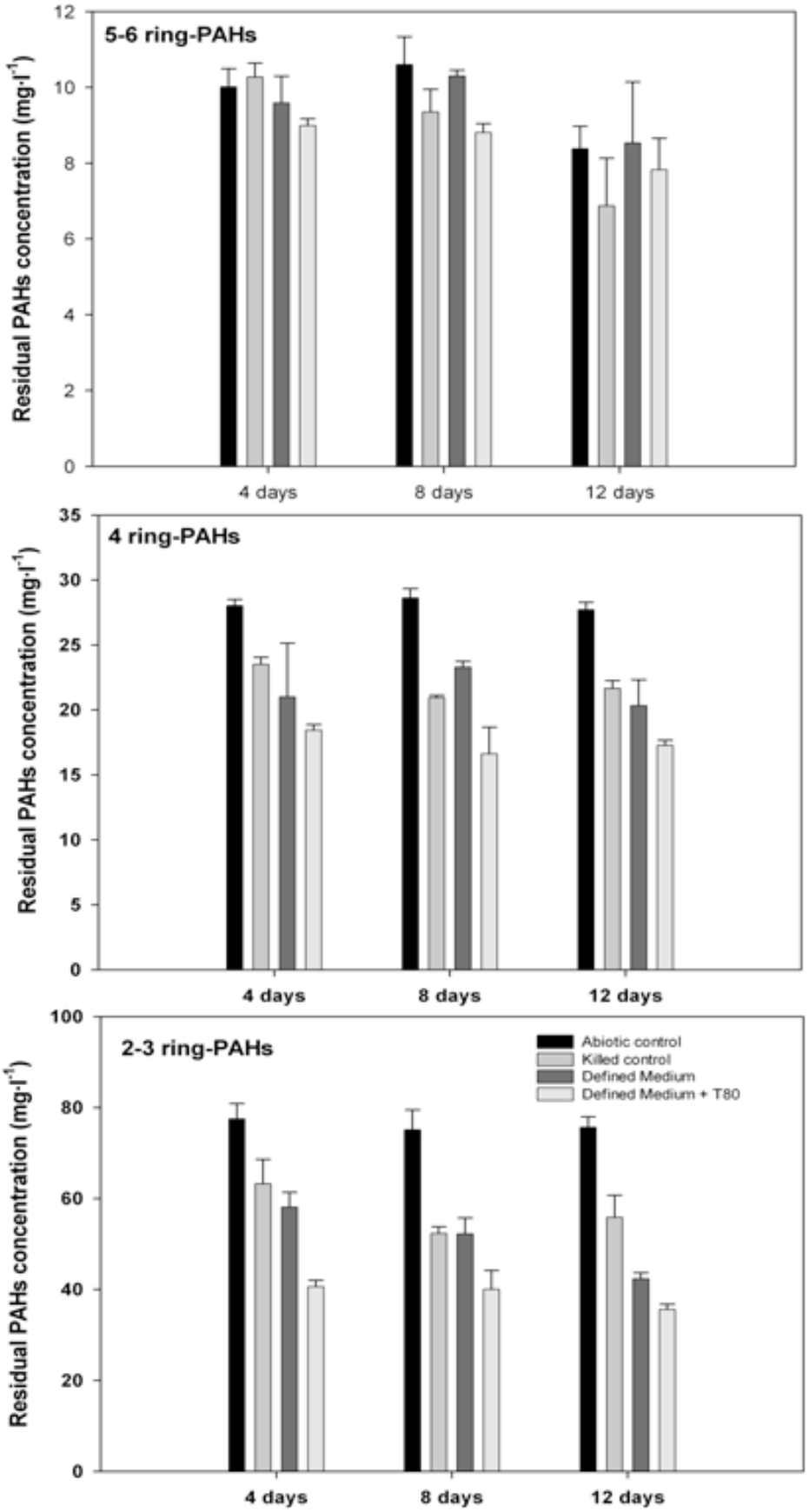


Table 6.3 Residual PAHs concentration in defined medium and defined medium containing Tween 80 along the creosote degradation experiment. Errors are expressed as standard deviation of triplicates. Errors of grouped PAHs are the sum of individual compounds.

Compound	Initial PAHs ($mg \cdot l^{-1}$)	PAHs concentration in Defined Medium ($mg \cdot l^{-1}$)			PAHs concentration in Defined Medium + T80 ($mg \cdot l^{-1}$)		
	0 days	4 days	8 days	12 days	4 days	8 days	12 days
Naphtalene	20.4 ± 1.9	12.5 ± 2.8	10.0 ± 3.2	5.6 ± 1.1	8.2 ± 1.4	7.7 ± 4.1	6.5 ± 1.1
Acenaphthylene	5.7 ± 0.3	3.1 ± 0.2	2.4 ± 0.3	1.7 ± 0.0	1.6 ± 0.1	1.5 ± 0.0	1.4 ± 0.0
Acenaphthene	1.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
Fluorene	15.7 ± 0.7	7.7 ± 0.6	6.5 ± 0.8	5.0 ± 0.2	3.4 ± 0.1	3.3 ± 0.5	2.9 ± 0.0
Phenanthrene	33.1 ± 0.8	19.1 ± 1.3	18.3 ± 0.8	16.6 ± 0.5	15.5 ± 0.3	15.7 ± 0.8	14.1 ± 0.2
Anthracene	5.1 ± 0.5	3.2 ± 0.2	2.8 ± 0.3	2.1 ± 0.1	1.9 ± 0.0	1.6 ± 0.1	1.5 ± 0.1
Fluoranthene	20.1 ± 0.2	12.0 ± 0.9	11.9 ± 0.4	11.0 ± 0.5	9.8 ± 0.2	10.2 ± 0.2	9.1 ± 0.2
Sum of 2-3 ring-PAHs	81.4 ± 1.2	58.1 ± 3.2	52.2 ± 3.5	42.4 ± 1.3	40.6 ± 1.4	40.0 ± 4.2	35.6 ± 1.2
Pyrene	13.8 ± 0.4	5.8 ± 4.1	7.9 ± 0.3	7.3 ± 0.4	6.2 ± 0.2	6.5 ± 0.3	5.8 ± 0.1
Benzo[a]anthracene	7.7 ± 0.5	5.1 ± 0.4	5.1 ± 0.2	4.6 ± 0.2	3.7 ± 0.2	3.7 ± 0.2	3.4 ± 0.2
Chrysene	6.5 ± 0.1	4.5 ± 0.3	4.5 ± 0.2	4.4 ± 0.5	3.5 ± 0.3	1.2 ± 2.0	3.2 ± 0.3
Benzo[b]fluoranthene	3.5 ± 0.1	2.5 ± 0.2	2.7 ± 0.1	2.1 ± 0.8	2.4 ± 0.1	2.4 ± 0.0	2.3 ± 0.0
Benzo[k]fluoranthene	5.3 ± 0.1	3.2 ± 0.3	3.2 ± 0.1	2.0 ± 1.7	2.7 ± 0.1	2.8 ± 0.1	2.6 ± 0.1
Sum of 4 ring-PAHs	48.1 ± 0.7	21.0 ± 4.1	23.3 ± 0.4	20.3 ± 2.0	18.4 ± 0.4	16.6 ± 2.1	17.3 ± 0.4
Benzo[a]pyrene	6.2 ± 0.2	6.1 ± 0.2	6.2 ± 0.1	5.8 ± 0.3	5.3 ± 0.1	5.1 ± 0.2	5.1 ± 0.1
Dibenzo[a.h]anthracene	3.1 ± 0.1	1.7 ± 0.6	2.3 ± 0.1	1.5 ± 1.3	2.1 ± 0.1	2.1 ± 0.0	1.8 ± 0.3
Indeno[1,2,3]pyrene	2.1 ± 0.1	0.4 ± 0.2	0.3 ± 0.0	0.2 ± 0.2	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.2
Benzo[g,h,i]perylene	1.9 ± 0.3	1.4 ± 0.1	1.6 ± 0.0	1.0 ± 0.9	1.4 ± 0.1	1.4 ± 0.0	0.9 ± 0.8
Sum of 5-6 ring-PAHs	22.1 ± 0.4	9.6 ± 0.7	10.3 ± 0.2	8.5 ± 1.6	9.0 ± 0.2	8.8 ± 0.2	7.8 ± 0.8
Total PAHs	151.6 ± 1.5	88.8 ± 5.3	85.9 ± 3.5	71.2 ± 2.9	68.0 ± 1.5	65.5 ± 4.7	60.7 ± 1.5

Table 6.4 Residual PAHs concentration in abiotic and killed control along the creosote degradation experiment. Errors are expressed as standard deviation of triplicates. Errors of grouped PAHs are the sum of individual compounds.

	Initial PAHs ($mg \cdot l^{-1}$)	PAHs concentration in Abiotic control ($mg \cdot l^{-1}$)			PAHs concentration in Killed control ($mg \cdot l^{-1}$)		
Compound	**0 days**	**4 days**	**8 days**	**12 days**	**4 days**	**8 days**	**12 days**
Naphtalene	20.4 ± 1.9	16.3 ± 1.7	13.6 ± 4.3	12.5 ± 1.3	15.9 ± 5.3	9.5 ± 1.5	11.8 ± 4.7
Acenaphthylene	5.7 ± 0.3	4.6 ± 0.7	4.4 ± 0.2	4.2 ± 0.3	3.8 ± 0.1	3.3 ± 0.1	3.4 ± 0.2
Acenaphthene	1.4 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	0.4 ± 0.1	0.5 ± 0.1
Fluorene	15.7 ± 0.7	12.1 ± 1.8	12.1 ± 0.7	12.3 ± 1.1	9.5 ± 0.3	8.8 ± 0.1	9.0 ± 0.4
Phenanthrene	33.1 ± 0.8	24.4 ± 1.8	24.7 ± 0.6	25.6 ± 1.1	18.2 ± 0.8	16.6 ± 0.1	17.1 ± 1.1
Anthracene	5.1 ± 0.5	4.3 ± 0.5	4.4 ± 0.3	4.5 ± 0.2	3.6 ± 0.1	3.2 ± 0.0	3.2 ± 0.2
Fluoranthene	20.1 ± 0.2	14.9 ± 1.2	15.1 ± 0.5	15.7 ± 1.1	11.8 ± 0.5	10.6 ± 0.1	10.9 ± 0.7
Sum of 2-3 ring-PAHs	81.4 ± 1.2	77.5 ± 3.4	75.1 ± 4.4	75.7 ± 2.3	63.2 ± 5.4	52.4 ± 1.5	55.8 ± 4.9
Pyrene	13.8 ± 0.4	9.9 ± 1.1	10.1 ± 0.3	10.5 ± 0.8	7.7 ± 0.4	6.9 ± 0.1	7.1 ± 0.4
Benzo[a]anthracene	7.7 ± 0.5	6.0 ± 0.8	6.2 ± 0.4	6.3 ± 0.4	5.2 ± 0.3	4.6 ± 0.1	4.7 ± 0.3
Chrysene	6.5 ± 0.1	5.5 ± 0.7	5.7 ± 2.2	5.7 ± 0.3	4.6 ± 0.3	4.1 ± 0.1	4.3 ± 0.2
Benzo[b]fluoranthene	3.5 ± 0.1	3.0 ± 0.2	3.1 ± 0.1	2.6 ± 0.2	2.7 ± 0.1	2.5 ± 0.1	2.5 ± 0.1
Benzo[k]fluoranthene	5.3 ± 0.1	3.6 ± 0.8	3.6 ± 0.2	2.6 ± 0.3	3.3 ± 0.1	2.9 ± 0.1	3.1 ± 0.2
Sum of 4 ring-PAHs	48.1 ± 0.7	28.0 ± 1.7	28.6 ± 2.3	27.7 ± 1.0	23.5 ± 0.5	20.9 ± 0.2	21.7 ± 0.6
Benzo[a]pyrene	6.2 ± 0.2	6.6 ± 0.5	6.6 ± 0.5	4.6 ± 0.4	6.4 ± 0.1	6.0 ± 0.1	6.1 ± 0.1
Dibenzo[a.h]anthracene	3.1 ± 0.1	2.4 ± 0.1	1.9 ± 0.6	1.3 ± 0.4	2.1 ± 0.3	1.4 ± 0.6	0.7 ± 1.3
Indeno[1,2,3]pyrene	2.1 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.7 ± 0.2	0.2 ± 0.2	0.5 ± 0.2	0.1 ± 0.2
Benzo[g,h,i]perylene	1.9 ± 0.3	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.2	1.6 ± 0.1	1.4 ± 0.0	0.00 ± 0.0
Sum of 5-6 ring-PAHs	22.1 ± 0.4	11.0 ± 0.5	10.6 ± 0.7	8.4 ± 0.6	10.3 ± 0.4	9.4 ± 0.6	6.9 ± 1.3
Total PAHs	151.6 ± 1.5	116.5 ± 3.9	114.3 ± 5.0	111.7 ± 2.6	97.0 ± 5.4	82.6 ± 1.6	84.4 ± 5.1

6.1.4 Creosote-PAHs degradation in slurry

Slurry bioreactors resulted effective for the degradation in soil environment for both BaA and BkF (see section 5.3.2). Thus, an experiment applying the same methodology was performed to assess the capacity of the fungus to degrade PAHs from creosote. Creosote was spiked into the soil and 500 mL bottles were employed as reactors. The ratio of soil/water was 1/10 (w/v).

According to glucose profile, the fungus was slightly inhibited at the beginning as near 2 $g \cdot l^{-1}$ remained in the broth at day 4. It was consumed completely after 8 days (see figure 6.4). This pattern is similar to that observed in the previous experiment and also from section 5.2.3. As regards to enzymatic activity, laccase was measured. A peak was achieved after 8 days of culture, showing a similar pattern described formerly as depicted in figure 6.5.

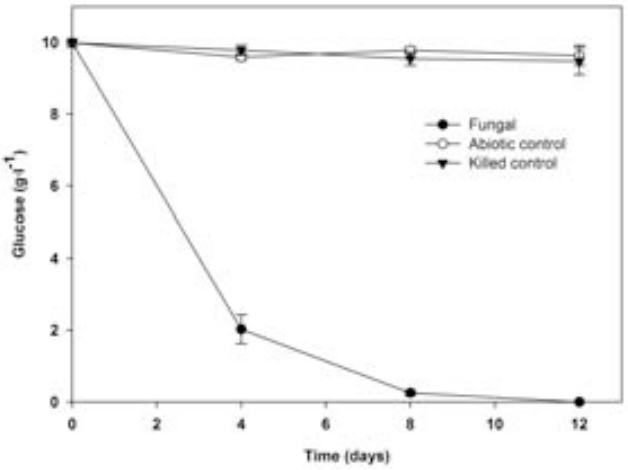


Figure 6.4 Glucose profile during creosote-PAHs degradation. Errors are the standard error of triplicates.

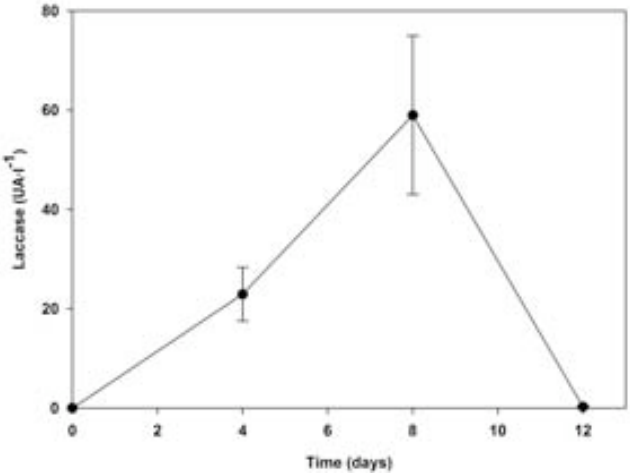


Figure 6.5 Laccase activity profile during experiment. Errors are the standard deviation of triplicates.

PAHs removal was mostly due to sorption onto soil particles, as can be observed in tables 6.5 and 6.6. Even though, the fungus was capable to degrade 34.4% of the initially spiked compounds. Low molecular weight PAHs, the 2-3 ring group, were degraded during the first 8 days of experiment. Degradations percentages surpassed those observed in previous section but were lower than those obtained in section 5.2.3. From that moment on, concentration slightly decreased until the end on the experiment; suggesting that biodegradation practically stopped.

This fact could occur due to two different phenomena. On one hand, the total amount of PAHs added was higher and also other compounds present in creosote could inhibit the fungal degrading ability. On the other hand, the low biodegradation could be attributed to bioavailability limitations. It is not clear whether the extracted PAHs were available for the fungus or were attached to soil particles and thus not available for the degrading machinery.

The sorption onto soil particles of 4 ring-PAHs was even more important as evidence in both controls. Only pyrene, benzo[a]anthracene and benzo[k]fluoranthene were slightly degraded by the fungus at the end of the experiment (32.3%, 13.6% and 12.6%, respectively).

The high molecular PAHs depletion was achieved only due to sorption or binding to soil particles. No compound belonging to 5-6 ring-PAHs group was degraded except for benzo[a]pyrene. Though, that low degradation percentage could not be differentiated from experimental procedure error.

Results suggest that the slurry system is appropriate for the low-molecular creosote-PAHs removal but not for the high molecular ones. Therefore, it seems that this approach is not suitable for a soil treatment process implementation since bacteria can accomplish satisfactory removal. This result contradicts the observations of section 5.4.2. Even though, it is important to highlight that in that section, slurry system was applied to lower concentration of single PAHs.

Table 6.5 PAHs concentration in abiotic and killed control along creosote-PAHs degradation. Errors are expressed as standard error of triplicates. Errors of grouped PAHs are the sum of individual errors.

	Initial PAHs ($mg \cdot l^{-1}$)	PAHs concentration in Abiotic control ($mg \cdot l^{-1}$)			PAHs concentration in Killed control ($mg \cdot l^{-1}$)		
Compound	**0 days**	**4 days**	**8 days**	**12 days**	**4 days**	**8 days**	**12 days**
Naphtalene	1.9 ± 0.1	1.6 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
Acenaphthylene	7.24 ± 0.1	6.3 ± 0.1	6.2 ± 0.3	6.5 ± 0.4	5.2 ± 0.1	5.3 ± 0.1	5.4 ± 0.1
Acenaphthene	2.1 ± 0.2	1.7 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	1.6 ± 0.1
Fluorene	12.7 ± 0.7	10.5 ± 0.2	10.4 ± 0.2	10.1 ± 0.1	9.6 ± 0.1	9.5 ± 0.2	9.2 ± 0.1
Phenanthrene	34.0 ± 0.2	30.8 ± 0.3	30.8 ± 0.4	30.5 ± 0.2	27.9 ± 0.3	27.2 ± 0.1	31.4 ± 4.0
Anthracene	10.4 ± 0.3	8.6 ± 0.1	8.3 ± 0.2	8.1 ± 0.2	7.1 ± 0.1	7.0 ± 0.3	7.1 ± 0.2
Fluoranthene	19.4 ± 0.4	16.3 ± 0.2	15.5 ± 0.2	15.5 ± 0.3	14.6 ± 0.3	15.1 ± 0.2	14.8 ± 0.2
Sum of 2-3 ring-PAHs	87.7 ± 0.8	75.8 ± 0.5	74.4 ± 1.0	74.1 ± 0.6	67.3 ± 0.5	67.0 ± 0.5	70.9 ± 0.5
Pyrene	16.5 ± 0.4	13.7 ± 0.1	13.6 ± 0.2	13.5 ± 0.2	11.6 ± 0.3	11.3 ± 0.1	10.9 ± 0.1
Benzo[a]anthracene	10.8 ± 0.4	5.9 ± 0.1	5.8 ± 0.4	5.6 ± 0.2	5.8 ± 0.1	5.6 ± 0.4	5.0 ± 0.1
Chrysene	11.1 ± 0.4	6.4 ± 0.4	5.0 ± 0.1	5.0 ± 0.3	6.0 ± 0.2	4.9 ± 0.1	4.9 ± 0.2
Benzo[b]fluoranthene	5.9 ± 0.2	3.2 ± 0.1	3.0 ± 0.2	3.1 ± 0.2	3.0 ± 0.1	2.8 ± 0.2	2.9 ± 0.2
Benzo[k]fluoranthene	8.1 ± 0.4	4.9 ± 0.1	4.0 ± 0.1	3.4 ± 0.5	4.6 ± 0.1	3.8 ± 0.1	3.6 ± 0.3
Sum of 4 ring-PAHs	52.4 ± 0.9	34.0 ± 0.4	31.4 ± 0.9	30.7 ± 0.7	31.1 ± 0.4	28.3 ± 0.5	27.3 ± 0.4
Benzo[a]pyrene	9.8 ± 0.4	5.7± 0.2	4.1 ± 0.2	4.2 ± 0.1	5.7 ± 0.2	4.0 ± 0.2	4.0 ± 0.1
Indeno[1,2,3]pyrene	1.9 ± 0.3	4.0 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	3.8 ± 0.1	2.4 ± 0.2	2.4 ± 0.1
Dibenzo[a.h]anthracene	6.5 ± 0.3	1.4 ± 0.1	1.3 ± 0.0	1.3 ± 0.1	1.2 ± 0.0	1.3 ± 0.1	1.1 ± 0.0
Benzo[g,h,i]perylene	4.7 ± 0.21	2.8 ± 0.1	2.3 ± 0.0	2.3 ± 0.1	2.7 ± 0.1	2.1 ± 0.1	2.0 ± 0.1
Sum of 5-6 ring-PAHs	22.9 ± 0.6	13.9 ± 0.2	10.2 ± 0.5	10.3 ± 0.2	13.5 ± 0.2	9.8 ± 0.3	9.5 ± 0.2
Total PAHs	163.1 ± 1.4	123.7 ± 0.7	116.1 ± 1.4	115.0 ± 0.9	111.9 ± 0.7	105.1 ± 0.7	107.7 ± 0.7

Table 6.6 PAHs concentration in defined medium containing Tween 80 along creosote-PAHs degradation and biodegradation percentage at each sampling time. Errors are expressed as standard error of triplicates. Errors of grouped PAHs are the sum of individual errors.

	PAHs concentration in Defined Medium + T80 ($mg \cdot l^{-1}$)			Biodegradation percentage (%)		
Compound	**4 days**	**8 days**	**12 days**	**4 days**	**8 days**	**12 days**
Naphtalene	1.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	3.3	5.2	12.7
Acenaphthylene	4.6 ± 0.2	3.5 ± 0.0	3.2 ± 0.6	10.0	28.6	34.7
Acenaphthene	1.2 ± 0.1	1.3 ± 0.3	1.0 ± 0.1	9.4	22.0	29.5
Fluorene	7.5 ± 0.4	5.7 ± 0.0	5.5 ± 0.3	20.3	36.8	36.7
Phenanthrene	19.2 ± 1.2	14.2 ± 0.1	14.1 ± 0.3	28.4	42.2	56.5
Anthracene	5.9 ± 0.4	4.7 ± 0.2	4.4 ± 0.2	13.7	28.2	33.9
Fluoranthene	10.6 ± 0.7	7.8 ± 0.1	7.7 ± 0.3	25.0	47.4	46.2
Sum of 2-3 ring-PAHs	50.4 ± 1.5	38.2 ± 0.3	37.1 ± 0.5	22.4	38.7	45.7
Pyrene	9.2 ± 0.5	6.8 ± 0.6	6.5 ± 0.3	17.9	33.4	32.3
Benzo[a]anthracene	6.0 ± 0.4	4.5 ± 0.1	4.3± 0.2	0.0	18.5	13.6
Chrysene	6.4 ± 0.4	4.8 ± 0.1	4.6 ± 0.2	0.0	1.5	5.0
Benzo[b]fluoranthene	3.3 ± 0.2	2.4 ± 0.1	2.5 ± 0.2	0.0	10.9	12.6
Benzo[k]fluoranthene	4.7 ± 0.3	3.6 ± 0.4	3.4 ± 0.1	0.0	4.2	6.7
Sum of 4 ring-PAHs	29.6 ± 0.9	22.1 ± 0.4	21.3 ± 0.3	4.2	19.7	19.6
Benzo[a]pyrene	5.6 ± 0.4	3.9 ± 0.2	3.8 ± 0.14	1.7	4.1	3.9
Indeno[1,2,3]pyrene	1.5 ± 0.2	1.1 ± 0.0	1.2 ± 0.3	0.0	0	0
Dibenzo[a.h]anthracene	3.9 ± 0.3	2.4 ± 0.7	2.8 ± 0.1	0.0	14.6	0
Benzo[g,h,i]perylene	2.7 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	0.0	1.2	0
Sum of 5-6 ring-PAHs	13.6 ± 0.5	9.4 ± 0.4	9.9 ± 0.2	0	3.7	0
Total PAHs	93.6 ± 1.8	69.7 ± 0.7	68.2 ± 0.6	14.8	30.5	34.3

6.1.5 Creosote-PAHs degradation in solid matrix employing mobilizing agents (MAs)

White-rot fungi growth in soil matrix allows them to access the pollutants, even those being only slightly bioavailable (Giubilei *et al.*, 2008). The addition of mobilizing agents (MAs), such as surfactants or oils, is supposed to enhance the PAHs bioavailability and has been previously used in soil mycoremediation (Bogan and Lamar, 1999; Marquez-Rocha *et al.*, 2000; Zheng and Obbard, 2001; Leonardi *et al.*, 2008). Most studies on this topic point out the need to find the proper MA for each fungus (Leonardi *et al.*, 2007). Likewise, MA applied to soil need to be in a concentration well above their critical micelle concentration in water (Haigh, 1994).

The partial objective of this subsection was to assess the capacity of *T. versicolor* to degrade the 16-EPA PAHs from creosote in spiked soil. The soil and the inoculum carrier are those employed in previous chapters. The fungus was pre-grown on a lignocellulosic substrate, and then spiked soil was added and subsequently mixed. Besides, mobilizing agents (MA) were added to soil to a final concentration of 1% (w/w) dissolved in water. MAs employed for the experiment were: Tween 20, Tween 80, Triton X-100, BS-400. The later is a low-cost commercial surfactant employed in soil remediation applications for hydrocarbons elimination and dispersion. Also, incubation controls, without MAs addition were performed. The initial humidity content was adjusted to 60% of the holding capacity. All treatments were carried out as biopile-like microcosm under sterile conditions so that all removal could be attributed to fungal activity.

Visual observation demonstrated that the fungus was capable of colonizing the spiked soil after the first week. From there on, the mycelia density increased from a qualitatively point of view. After 5 weeks ergosterol was measured. Results are shown in table 6.7. The fungus colonized the spiked soil achieving ergosterol values similar to those obtained in colonization of non-contaminated soil (see section 4.2.2.4). Consequently, no inhibitory effects were observed due to creosote spiking into soil.

In general terms, MAs addition did not suppose a decrease in viable biomass respect the addition of water. In spite of this, Tween 20 diminished significantly ($p \leq 0.001057$) the colonization ability of the fungus. However, the biomass levels were higher compared to those obtained in similar conditions for *I. lacteus, P. ostreatus, Phlebia* sp., *Allescheriella* sp. and *L. tigrinus* (Giubilei *et al.*, 2009;

Leonardi *et al.*, 2008; Covino *et al.*, 2010b). Similar results were reported for *Trametes hirsuta* (Boyle, 1996), which growth was not affected by the addition of cyclodextrins.

Table 6.7 Fungal growth after 5 weeks expressed as μg ergosterol·g^{-1}dry soil. The errors are expressed as standard error of the triplicates. Results of one-way ANOVA on the variation of ergosterol. (a: statistically significant; $p \leq 0.05$)

Mobilizing Agent	Ergosterol (μg·g^{-1}dry soil)	Probability (*p*)
no-MA	192.2 ± 27.5	0.134244
Tween 20	58.6 ± 10.9	0.001057a
Tween 80	153.1 ± 10.9	0.552819
Triton X-100	172.0 ± 22.3	0.124733
BS-400	133.1 ± 20.8	0.668616

The MA applied to the soil affected enzyme secretion. Laccase activity measured was similar to those ranges attained when colonizing soil using the same substrate (namely C1), see chapter 4. The addition of Tween20 diminished significantly laccase activity ($p \leq 0.000975$) whilst Tween 80 increased it 2-fold ($p \leq 0.000003$). No differences were observed for other MA respect the addition of water. Leonardi *et al.* (2008) found that Tween 20 and Tween 80 increased the laccase activity of *P. ostreatus* growing in soil. Similar findings were reported for *Phlebia* sp. by Giubilei *et al.* (2009). In that work, it was reported that the secretion of lignin modifying enzymes as response to MAs appears to be both enzyme and specie dependent. In addition, Svobodová *et al.* (2008) reported that laccase of *I. lacteus* was not affected by MAs since it was tighly bound to hyphae surface.

Table 6.8 Enzymatic activity after 5 weeks. The errors are expressed as standard error of triplicates. Results of one-way ANOVA on the variation of laccase (a: statistically significant; $p \leq 0.05$).

Mobilizing Agent	Laccase (AU·g^{-1} dry soil)	Probability (*p*)
no-MA	0.28 ± 0.03	0.61653
Tween 20	0.23 ± 0.05	0.000975a
Tween 80	0.67 ± 0.04	0.000003a
Triton X-100	0.35 ± 0.02	0.617578
BS-400	0.31 ± 0.01	0.113271

The PAHs degradation capacity of *T. versicolor* in solid matrix can be seen in tables 6.10 and 6.11. The group of 2-3 ring-PAHs was degraded extensively, specially ACE, APY, FLU and PHE. In all treatments above 65% respect the abiotic control. No differences were observed among different MAs and incubation

control (H_2O, none-MAs), except for BS-400. In which biodegradation reached the maximum. The group of 4 rings-PAHs was degraded, ranging from 33.8 to 52.8% respect the abiotic control. PYR, BaA and CRY exhibited the highest susceptibility to fungal degradation, above 20% for all compounds in all cases. The group of 5-6 ring-PAHs was less degraded, ranging from 17.4 to 34.8%. BaP and I1,2,3PYR the most extensively degraded compounds.

BS-400 addition of affected significantly the removal efficiency of PAHs ($p \leq 0.000078$), permitting the highest degradation. None of the other MAs enhanced significantly PAHs removal respect the no-addition of MA. Even though, all MAs allowed reliable biodegradation, especially for the low molecular weight PAHs. Triton X-100 and Tween 20 were the MAs that less favored biodegradation (33 and 38%). In the case of Tween 80 and no addition of MA, similar degradations were reached.

Table 6.9 shows the significance in removal efficiency of each PAHs depending on the MA added. Overall, BS-400 seems to be the best MA to enhance PAHs biodegradation from creosote polluted-soil. Tween 80 also enhanced the removal of several PAHs. No differences were observed among addition of surfactant and incubation control, which were reliable in liquid medium.

Table 6.9 Results of one-way ANOVA on the variation of each PAH removal respect the abiotic control with MAs addition. (a: statistically significant; $p \leq 0.05$)

	Probability (*p*)			
Compound	**T80**	**T20**	**TX-100**	**BS-400**
Acenaphthylene	0,193788	0,025969a	0,093207	0,000132a
Acenaphthene	0,203183	0,035664a	0,327118	0,038014a
Fluorene	0,000143a	0,136982	0,070169	0,000359a
Phenanthrene	0,000538a	0,006267a	0,000232a	0,000123a
Anthracene	0,523437	0,008426a	0,001162a	0,000064a
Fluoranthene	0,092084	0,495217	0,006994a	0,000738a
Pyrene	0,023239a	0,684503	0,144952	0,000259a
Benzo[a]anthracene	0,498526	0,408326	0,666051	0,000252a
Chrysene	0,004233a	0,378887	0,403698	0,000037a
Benzo[b]fluoranthene	0,001056a	0,001267	0,039935a	0,007566a
Benzo[k]fluoranthene	0,082178	0,020682a	0,500291	0,002693a
Benzo[a]pyrene	0,00011a	0,00982a	0,971773	0,001939a
Dibenzo[a.h]anthracene	0,021831	0,003339a	0,779152	0,025109a
Indeno[1,2,3]pyrene	0,133263	0,341012	0,266351	0,070062
Benzo[g,h,i]perylene	0,445058	0,447811	0,473793	0,001777a

Table 6.10 Residual concentration of PAHs in soil microcosms alter 5 weeks of treatment. Errors are expressed as standard error of triplicates. Errors of grouped PAHs are the sum of individual errors.

	Residual concentrations after 5 weeks (μg PAH·g^{-1}dry soil)					
Compound	**Abiotic Control**	**no-MA**	**T80**	**X-100**	**BS400**	**T20**
Acenaphthylene	4.3 ± 0.1	2.2 ± 0.2	2.5 ± 0.1	2.4 ± 0.2	2.0 ± 0.1	2.3 ± 0.0
Acenaphthene	0.6 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
Fluorene	13.2 ± 0.2	4.0 ± 0.8	2.1 ± 0.5	6.7 ± 0.7	2.4 ± 1.2	4.5 ± 0.1
Phenanthrene	32.3 ± 0.4	6.6 ± 0.6	5.4 ± 0.4	4.9 ± 0.2	4.5 ± 0.8	6.8 ± 0.8
Anthracene	6.3± 0.2	2.5 ± 0.2	3.0 ± 0.3	2.4 ± 0.1	2.1 ± 0.2	2.6 ± 0.2
Fluoranthene	23.3 ± 0.1	8.7 ± 0.0	9.5 ± 1.1	8.1 ± 0.9	6.8 ± 0.5	12.1 ± 0.1
Sum of 2-3 ringhs PAHs	79.9 ± 0.6	24.1 ± 1.0	22.8 ± 1.3	24.6 ± 1.1	17.8 ± 1.6	28.3 ± 0.9
Pyrene	17.4 ± 0.3	6.2 ± 1.2	5.9 ± 0.6	6.78± 1.5	3.9 ± 0.5	7.7 ± 0.3
Benzo[a]anthracene	9.2 ± 0.2	4.2 ± 0.3	4.4 ± 0.3	5.1 ± 0.5	3.6 ± 0.2	5.6 ± 0.2
Chrysene	8.8 ± 0.2	5.6 ± 0.2	6.2 ± 0.2	6.6 ± 0.4	4.7 ± 0.3	6.7 ± 0.3
Benzo[b]fluoranthene	5.23 ± 0.1	4.6 ± 0.2	4.3 ± 0.1	4.5 ± 0.0	4.4 ± 0.1	5.1 ± 0.1
Benzo[k]fluoranthene	5.7 ± 0.1	5.2 ± 0.0	5.6 ± 0.1	5.4 ± 0.1	5.2 ± 0.0	5.7 ± 0.1
Sum of 4 ringhs PAHs	46.4 ± 0.4	25.9 ± 1.4	26.5 ± 0.7	28.4 ± 1.6	21.9 ± 0.6	30.7 ± 0.4
Benzo[a]pyrene	5.7 ± 0.1	4.1 ± 0.3	2.2 ± 0.2	3.9 ± 0.7	2.7 ± 0.2	4.8 ± 0.1
Dibenzo[a.h]anthracene	6.4 ± 0.4	5.3 ± 0.1	6.2 ± 0.1	6.1 ± 0.5	5.2 ± 0.2	5.5 ± 0.2
Indeno[1,2,3]pyrene	5.4 ± 0.2	2.9 ± 0.1	4.3 ± 0.2	3.7 ± 0.4	3.2 ± 0.3	2.9 ± 0.1
Benzo[g,h,i]perylene	4.6 ± 0.1	4.5 ± 0.1	4.5 ± 0.1	4.5 ± 0.2	3.2 ± 0.7	4.5 ± 0.2
Sum of 5-6 ringhs PAHs	22.0 ± 0.4	16.7 ± 0.4	17.2 ± 0.3	18.2 ± 1.0	14.3 ± 0.8	17.8 ± 0.3
Total PAHs	148.3 ± 0.8	66.8 ± 1.8	66.5 ± 1.5	71.2 ± 2.2	54.1 ± 1.9	76.8 ± 1.0

Table 6.11 Degradation of PAHs after 5 weeks of treatment in biopile microcosms expressed as percentage respect the abiotic control.

	Biodegradation percentage (%)				
Compound	**no-MA**	**T80**	**X-100**	**BS400**	**T20**
Acenaphthylene	47.8	42.6	43.8	53.7	45.7
Acenaphthene	70.5	45.2	73.3	88.9	89.3
Fluorene	69.9	84.5	49.2	81.8	66.1
Phenanthrene	79.5	83.3	84.9	86.1	79.1
Anthracene	60.9	52.0	62.1	67.0	59.0
Fluoranthene	62.7	59,0	65.2	70.7	47.8
Sum of 2-3 ringhs PAHs	69.8	71.5	69.2	77.7	64.6
Pyrene	64.3	65.9	61.2	77.3	56.0
Benzo[a]anthracene	54.3	52.5	44.5	61.0	39.2
Chrysene	36.3	29.5	25.1	46.5	23.5
Benzo[b]fluoranthene	12.0	17.6	14.4	15.8	3.7
Benzo[k]fluoranthene	8.9	2.1	5.2	8.8	1.2
Sum of 4 ringhs PAHs	44.2	43.0	38.8	52.8	33.8
Benzo[a]pyrene	27.7	61.1	31.4	52.1	15.0
Dibenzo[a.h]anthracene	16.9	1.9	3.8	17.4	13.1
Indeno[1,2,3]pyrene	47.0	20.6	32.4	41.5	45.9
Benzo[g,h,i]perylene	1.7	1.1	1.4	29.6	1.2
Sum of 5-6 ringhs PAHs	23.9	21.6	17.4	34.8	19.2
Total PAHs	55.0	55.2	52.0	63.5	48.2

The results of this experiment suggested that the fungus was more efficient at degrading PAHs in solid-state approach rather than in liquid and slurry. However, PAHs removal could not be correlated neither to biomass nor laccase. Fungal hyphae extend through the soil; hence, it is easier to overcome biodisponibility restrictions of low soluble compounds. Similar findings have been recently observed in our laboratory for other low soluble compounds such as decabromodiphenyl ether (deca-BDE) (data not published).

Finally, more experiments should be carried out to elucidate the optimal duration of the treatment. It was not known whether degradation occurred in the initial stages or elongating the experiment would improve the final biodegradation yield.

5.1.6 Conclusions

- *T. versicolor* was validated as a creosote-PAHs biodegrader in liquid medium. Even though, maximum removals were achieved for the low molecular weight compounds.
- Slurry treatment permitted biodegradation of the low molecular PAHs in slurry treatment. Whereas the high molecular ones were scarcely degraded.
- The addition of MAs in soil affected in a dissimilarly the fungus ability to colonize soils. Tween 20 inhibited it while Tween 80 enhanced it.
- Tween 20 inhibited laccase secretion in biopile microcosms.
- Better PAHs removals were achieved in biopile microcosms with respect to liquid and slurry approaches.
- BS-400 allowed a significant enhancement in PAHs removal respect the other MAs. These did not enhance the removal respect the only addition of water.

6.2 Interactions of fungi and bacteria during PAHs-creosote soil remediation: the case of *Trametes versicolor* and *Irpex lacteus*

The data presented in this section is a collaboration issue with the Laboratory of Environmental Biotechnology from the Department of Ecology in the Institute of Microbiology in Prague, which belongs to the Academy of Sciences of the Czech Republic. The aim of this stage was to study the effects of soil bacteria on the ability of both *T.versicolor* and *Irpex lacteus* to remove PAHs-creosote during soil mycoremediation. The later fungus had been extensively studied in the Institute of Microbiology for PAHs removal (Cajthaml *et al.*, 2006, 2008; Novotný *et al.*, 2009).

6.2.1 White-rot fungi and bacteria interactions during PAHs soil remediation

PAHs are a group of omnipresent environmental organopollutants with toxic, mutagenic and carcinogenic properties whose accumulation in the environment results from both natural and industrial processes. Sixteen PAH molecules have been considered to be priority pollutants of the aquatic and terrestrial ecosystems by the U.S. Environmental Protection Agency (EPA). The potential of white-rot fungi to degrade recalcitrant organopollutants in both artificially spiked and industrially contaminated soils has been reported (Novotný *et al.*, 1999; Mougin, 2002; D'Annibale *et al.*, 2006; Covino *et al.*, 2010b). Dense mycelial growth and significant enzyme production are necessary to remove PAHs from contaminated soils. Typically, white-rot fungi are applied as inoculum growing on a convenient solid substrate from which the fungus colonizes the contaminated soil with explorative mycelium producing extracellular enzymes involved in PAH degradation (Novotný *et al.*, 2000; Šašek *et al.*, 2003; Byss *et al.*, 2008; Leonardi *et al.*, 2008). The degradation mechanism similar to that of litter-decomposting fungi (Steffen *et al.*, 2002), includes oxidative transformation of PAHs by peroxidases, laccase and Cyt-P450 monooxygenase producing trans-dihydrodiols, epoxides, quinones and phenols, and conjugation resulting in the formation of glucuronides, glucosides, sulfates, etc. (Singh, 2006).

I. lacteus has been demonstrated to extensively degrade PAHs in industrially-polluted soils (Šašek *et al.*, 2003; Byss *et al.*, 2008; Leonardi *et al.*, 2008) whereas

the results with *T. versicolor* show both high (Rama *et al.*, 2001) and low (Novotný *et al.*, 1999) biodegradation rates.

The degradation of or 3-4 ring PAHs can be easily catalised by bacteria, but scarcely degrade the high molecular weight PAHs (Gramss *et al.*, 1999). Only white-rot fungi are able to degrade the laters. For efficient degradation, the cooperation of indigenous microflora and white-rot fungi is required, being the later responsible for the initial oxidation of higher PAHs, with 5-6 rings, and the indigenous microflora responsible for the degradation of PAHs metabolites (Baldrian, 2008).

Competition with autochthonous soil microflora is an important factor in soil bioremediation by white-rot fungi but the knowledge of their interactions with native microbiota is poor (Gramss *et al.*, 1999; Mougin, 2002; Singh, 2006; Arun *et al.,* 2008; Baldrian, 2008). *T. versicolor* was shown to attack soil bacteria including pseudomonads (Thorn and Tsuneda, 1992) but soil pseudomonads can also inhibit growth of white-rot fungi (Radtke *et al.*, 1994). *Pleurotus ostreatus* was able to enhance the degradation of PAHs in non-sterile, artificially contaminated soils but it also inhibited growth of indigenous bacteria and changed the composition of the bacterial community (Andersson *et al.*, 2000), even eliminating most bacteria (Andersson *et al.*, 2003). Growth of *I. lacteus*, in contrast to *P. ostreatus* and *Phanerochaete chrysosporium*, was not inhibited by *Pseudomonas putida* and *Sphingomonas* sp. on malt extract/glucose- and phenazine-induction agar media (Novotný *et al.*, 2001). Successful colonization of non-sterile soils by white-rot fungi demonstrates the ability to overcome the adverse effect of soil microflora (Rama *et al.*, 2001; Singh, 2006; Leonardi *et al.*, 2008). Besides the presence of bacteria, fungi and their metabolites can also induce the production of fungal laccase implicated in PAHs degradation (Crowe and Olsson, 2001; Baldrian, 2004; Baldrian, 2006).

Polar metabolites produced by oxidation of high-molecular-weight PAHs by white-rot fungi can be mineralized by indigenous microflora isolated from sludges and forest soils (Meulenberg *et al.*, 1997; Kotterman *et al.*, 1998b), which can increase the biodegradation rate. However, this behavior is not omnipresent as documented by studies with *P. chrysosporium*, *P. ostreatus* or *T. versicolor* (Bhatt *et al.*, 2000; Canet *et al.*, 2001).

6.2.2 Aims of this section

As the interactions of fungi applied in soil with the soil bacteria and the effect of the latter on fungal biodegradation are poorly understood (Singh, 2006), the aim of this section was to investigate the effect of bacterial stress in the soil environment on two fungi with a potential for PAH biodegradation and soil colonization, *I. lacteus* and *T. versicolor*. The bacterial stress was represented either by the innate soil microflora or a defined mixture of the soil bacteria *P. aeruginosa* and *R. erythropolis* inoculated into the sterile soil. The effect was measured by the efficiency of removal of 16 EPA-recommended PAHs spiked into the non-contaminated soil, by the ability of the fungi to colonize the soil and by production and secretion of their extracellular MnP and laccase.

Competition with soil bacterial microflora is one of important factors in bioremediation of PAHs in contaminated soils by white-rot fungi that is not well understood (Field *et al.*, 1995a; Mougin, 2002; Baldrian, 2008). This effect was studied using the indigenous soil microflora and a defined mixture of two soil bacteria, *P. aeruginosa* and *R. erythropolis*.

6.2.3 Results

6.2.3.1 Soil colonization by fungi

The capacity of fungi to colonize PAH-polluted soil in the presence and absence of live bacteria was tested (table 6.12). Fungal growth was assessed by visual observation and measurement of the ergosterol content. Both fungal species were capable to colonize the soil but the colonization rate and intensity were different. *I. lacteus* showed higher capacity than *T. versicolor*. After two weeks the soil was almost fully colonized by *I. lacteus* whereas with *T. versicolor* it took almost four weeks and the mycelium was less dense. Once *I. lacteus* colonized the soil volume, it formed a massive mycelium layer over the soil and continued expanding all over the glass tube wall. In the case of *T. versicolor*, the mycelium formed a very thin layer over the soil and stopped its growth. The contents of ergosterol in the biomass of *I. lacteus* and *T. versicolor* were found to be 2.4 and 6.6 mg ergosterol·g^{-1} DW fungal biomass (Rodríguez-Rodríguez *et al.,* 2010a). In terms of ergosterol, despite various growth rates observable within five weeks with both fungi, each fungus attained similar growth yields in different soil treatments after 10 weeks growth. These values were 2-fold in *I. lacteus* (table 6.12) but this difference is much higher when expressed in the terms of biomass

due to the above-mentioned difference in the ergosterol content between the two fungi.

Table 6.12 Fungal growth after 5 and 10 weeks expressed as µg ergosterol·g^{-1}dry soil.

	Biomass content (µg ergosterol·g^{-1}dry soil)			
	Trametes versicolor		***Irpex lacteus***	
Treatment	**5 weeks**	**10 weeks**	**5 weeks**	**10 weeks**
NS	38.6 ± 1.8	51.0 ± 4.9	72.0 ± 10.3	96.6 ± 11.6
S	47.7 ± 3.0	47.0 ± 4.8	47.0 ± 9,4	104.7 ± 7.2
S+Bac	49.1 ± 8.6	52.0 ± 8.7	51.1 ± 9.5	91.8 ± 11.2

The errors are expressed as standard deviation of the triplicates. Different treatments: NS: nonsterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum (10^8 CFU·g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*).

In general terms, the ergosterol contents are lower than those obtained in previous sections for soil colonization. An explanation can come from the biopile system employed in this experiment: the soil was added as a layer above the lignocellulosic substrate colonized by the fungi, which grew as exploratory mycelia through the soil. In other soil experiments, the lignocellulosic substrate and the soil were mixed favoring the fungal growth as confirmed by higher ergosterol content.

A three-way ANOVA was performed to assess statistical significance of the data to show that the growth of neither fungus was inhibited by the presence of bacteria, either the autochthonous microflora or the introduced *P. aeruginosa* and *R. erythropolis* (table 6.10). These results partially confirmed growth interactions between *I. lacteus* and either *P. aeruginosa* or *R. erythropolis* observed in preliminary experiments on agar media using the method of Radtke *et al.* (1994) (not shown), where no inhibitory effects were observed on MEG and low-nitrogen mineral Kirk medium (Tien and Kirk, 1988) with either bacterium but clear growth inhibition was observed on complex LB medium with the two bacteria applied individually.

Both fungi demonstrated their capability to colonize soils in the presence of PAHs and live bacteria and thus did not verify the idea of *T. versicolor* as a fungus having problems in colonizing soil (Boyle, 1995; Martens and Zadrazil, 1998). Neither the indigenous soil microflora nor the two soil bacteria affected the growth of *T. versicolor* or *I. lacteus* in soil and thus our observations did not confirm fungal-bacterial antagonist relationships reported by Thorn and Tsuneda (1992) and Radtke *et al.* (1994). The growth of *I. lacteus* was faster in non-sterile

soil and its final growth yields expressed in mgDW·g^{-1} soil calculated from the ergosterol contents measured in the two fungal strains used were 5 to 6-fold compared to *T. versicolor* in both the presence and absence of bacteria in soil.

The growth yield values obtained for *T. versicolor* were comparable to those obtained by Rama *et al.* (2001) for *T. versicolor* growing in an industrially-polluted soil but 25-fold higher than those of *T. versicolor* colonizing a PAH-spiked chernozem soil (Novotný *et al.*, 1999). The growth yield of *I. lacteus* was 10-fold compared to that of *P. ostreatus* colonizing a PAH-spiked chernozem soil (Novotný *et al.*, 1999). The superiority of *I. lacteus* compared to *T. versicolor* observed during colonization of a sterile soil (Novotný *et al.*, 2001) was confirmed in this study but did not translate into the ability to degrade PAHs.

Table 6.13 Results of three-way ANOVA on the variation in ergosterol levels according to factors Fungus (*T. versicolor* vs. *I. lacteus*), Treatment (NS: nonsterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum (10^8 CFU·g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*) and Time (5 vs. 10 weeks). (a: statistically significant, $p \leq 0.05$).

Variable	F	d.f.	Probability
Fungus	135	1,24	0.00a
Time	96	1,24	0.00a
Treatment	1	2,24	0.34
*Fungus*Time*	54	1,24	0.00a
*Fungus*Treatment*	6	2,24	0.01a
*Time*Treatment*	1	2,24	0.38
*Fungus*Time*Treatment*	6	2,24	0.01a

6.2.3.2 Enzymatic activity

Laccase and MnP are extracellular enzymes produced by white rot fungi related to the capacity of PAHs degradation (Novotný *et al.*, 2004). Their activities in soil were measured from the soil fractions at 5 and 10 weeks (table 6.11). MnP was not detected for *T. versicolor*. Laccase was present in all *Trametes* cultures and its level was 3-9-fold higher in sterile soil in comparison with the soil where live bacteria were present. This is not in line with the observations of Baldrian (2004) showing a significant increase of laccase production in his strain of *T. versicolor* when interacting with soil bacteria. Crowe and Olsson (2001) also described an induction of laccase activity in *Rhizoctonia solani* in the presence of an antagonistic *P. fluorescens* on potato dextrose agar. On the other hand, the presence of innate bacteria in soil, in comparison with the sterilized soil, did not

affect or even completely suppressed the laccase activity of *P. ostreatus* and *Dichomitus squalens* in the soil, respectively (Lang *et al.*, 1998). No MnP activity was detected in the *T. versicolor* soil cultures (table 6.14). Novotný *et al.* (1999) reported low MnP activities with another strain of *T. versicolor* growing in rich chernozem soil whereas no activity was detected in a soil with low contents of organic carbon and nitrogen. This could explain the absence of MnP in our cultures since the C and N levels in our soil were comparably low.

Table 6.14 MnP and laccase activity in *T. vesicolor* and *I. lacteus* soil cultures after 5 and 10 weeks of experiment expressed as AU·g^{-1}dry soil.

		Enzyme activity (AU·g^{-1}dry soil)			
		MnP		**Laccase**	
Fungus	**Treatment**	**5 weeks**	**10 weeks**	**5 weeks**	**10 weeks**
Trametes versicolor	NS	nd	nd	2.3 ± 0.9	1.6 ± 0.2
	S	nd	nd	14.6 ± 2.9	13.2 ± 3.6
	S+Bac	nd	nd	1.5 ± 0.8	4.5 ± 1.3
Irpex lacteus	NS	1.5 ± 0.4	2.4 ± 0.3	0.4 ± 0.1	0.3 ± 0.0
	S	1.3 ± 0.2	4.5 ± 2.3	0.3 ± 0.1	0.4 ± 0.1
	S+Bac	3.6 ± 1.2	2.1 ± 0.8	0.4 ± 0.0	0.5 ± 0.0

The errors are expressed as standard deviation of the triplicates. Different treatments: NS: non-sterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum (10$^{}$ CFU·g$^{}$ *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*). nd: not detected

Both fungi are characterized by their production of MnP and laccase and, consequently, the mechanisms used in the biodegradation of PAHs can be considered similar in the two organisms (Collins and Dobson, 1996; Majcherczyk *et al.*, 1998; Cerniglia and Sutherland, 2001, Novotný *et al.*, 2004). *I. lacteus* expressed both enzymes (table 6.11). The specific MnP activities in *I. lacteus* ranged from 21 to 70 mAU·g^{-1} ergosterol, which was quite similar to 59 and 81 mAU·g^{-1} ergosterol measured in *P. ostreatus* and *T. versicolor* growing in chernozem soil, respectively (Novotný *et al.*, 1999). The enzyme levels slightly increased during cultivation, except for the culture where sterile soil was inoculated with two soil bacteria. Laccase levels in soil in the *I. lacteus* cultures were low, probably due to the firm binding of the enzyme to the fungal mycelium (Svobodová *et al.*, 2008). A three-way ANOVA was performed to assess statistical significance of the data (table 6.15).

Table 6.15 Results of three-way ANOVA on the variation in laccase and MnP levels according to factors Fungus (*T. versicolor* vs. *I. lacteus*), Treatment (NS: nonsterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum (10^8 CFU·g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*) and Time (5 vs. 10 weeks) (a: statistically significant, $p \leq 0.05$).

	Laccase			MnP		
Variable	**F**	**d.f.**	**Probability**	**F**	**d.f.**	**Probability**
Fungus	171	1,24	0.00a	476	1,24	0.00a
Time	5	1,24	0.03a	3	1,24	0.09a
Treatment	198	2,24	0.00a	3	2,24	0.06a
*Fungus*Time*	2	1,24	0.17	3	1,24	0.09a
*Fungus*Treatment*	163	2,24	0.00a	3	2,24	0.06a
*Time*Treatment*	0	2,24	0.71	2	2,24	0.14
*Fungus*Time*Treatment*	25	2,24	0.00a	2	2,24	0.14

6.2.3.3 PAHs removal

The recovery values measured immediately after spiking the soil for the individual PAHs were high (table 6.16) but the recovery was reduced in later phases of the experiment for most of the mixture components (tables 6.17 and 6.18). In order to quantify the PAHs removal by the fungi, abiotic controls were measured at each sampling time. PAHs abiotic losses represented 15 and 21% of the total PAHs after 5 and 10 weeks, respectively. NAP and APE were probably removed due to their high volatility but the rest of PAH molecules that were not recovered by the extraction method was probably sorbed to soil humate and clay fraction (Bogan *et al.*, 1999, Zang *et al.*, 2007, Cofield *et al.*, 2008).

Both fungi were capable to reduce the initial PAH content. *T. versicolor* showed more efficiency in PAH removal for all PAHs tested, compared to *I. lacteus*, although its capacity to colonize soil was significantly lower (tables 6.14 and 6.15). In *I. lacteus*, generally, the removal values were higher in the sterile soil without bacteria. This behavior was observed also in *T. versicolor*, namely in the case of PHE, FLT, PYR and BaP. The ability of both fungi to remove PAHs from the sterile soil was inhibited by the addition of the two soil bacteria, as was clearly shown for PHE, FLU and BaA in *T. versicolor* and PHE and FLU in *I. lacteus* (tables 6.14 and 6.15). In the case of ANT, whose amount was reduced by both fungi to about one half, this effect was very low or negligible.

Table 6.16 PAHs spiked in soil and recovered after Soxhlet extraction (μg PAH·g^{-1}dry soil)

	Concentration (μg PAH·g^{-1}dry soil)		
Compounds	**Spiked**	**Recovered**	**% Recovery**
Naphtalene	1.5 ± 0.0	1.8 ± 0.1	81.0
Acenaphthylene	17.4 ± 0.5	9.3 ± 0.5	53.6
Acenaphthene	4.7 ± 0.1	2.7 ± 0.2	56.3
Fluorene	26.4 ± 0.3	20.1 ± 1.6	76.2
Phenanthrene	71.2 ± 2.0	68.3 ± 3.0	96.0
Anthracene	21.4 ± 0.6	19.8 ± 0.6	92.4
Fluoranthene	42.9 ± 1.2	43.5 ± 0.3	101.4
Sum of 2-3 rings PAHs	185.6 ± 2.4	165.6 ± 3.5	82.2
Pyrene	31.2 ± 0.2	30.8 ± 0.8	99.0
Benzo[a]anthracene	20.9 ± 0.6	20.9 ± 1.0	99.9
Chrysene	18.3 ± 0.5	18.0 ± 0.9	98.4
Benzo[b]fluoranthene	10.7 ± 0.0	10.4 ± 0.2	96.6
Benzo[k]fluoranthene	12.7 ± 0.6	12.8 ± 0.3	100.5
Sum of 4 rings PAHs	93.8 ± 1.0	92.8 ± 1.6	99.0
Benzo[a]pyrene	13.9 ± 0.4	13.6 ± 0.8	98.1
Dibenzo[a.h]anthracene	8.0 ± 0.8	7.9 ± 0.2	97.9
Indeno[1,2,3]pyrene	nm	nm	nm
Benzo[g,h,i]perylene	7.5 ± 0.4	6.8 ± 0.2	90.0
Sum of 5-6 rings PAHs	29.45± 1.0	28.3 ± 0.8	96.0
Total PAHs	308.8 ± 2.7	286.7 ± 4.0	92.8

The errors are expressed as standard deviation of the triplicates. nm: not measured

The main effect of the presence of live bacteria in the soil, both the indigenous microflora and the two soil bacteria, was a significant decrease of degradation of PAHs by the two fungi. It was demonstrated for all three groups of PAHs in the case of *T. versicolor* and for 2-3-ring PAHs in the case of *I. lacteus*. Consequently, the residual total PAH concentrations exceeded those without bacteria by 15%. These results are in agreement with the observations with *P. ostreatus* where *P. putida* reduced the removal of ANT, PHE, FLT, PYR and BaP from industrially contaminated (Bhatt *et al.*, 2002). The competition for nutrients in the soil between the two types of microorganisms probably does not explain the adverse effect of bacteria (Rama *et al.*, 2001).

Table 6.17 Residual concentrations of PAHs expressed as μg PAH·g^{-1}dry soil in soil after treatment with *I. lacteus*.

	Residual concentrations after 5 weeks (μg PAH·g^{-1}dry soil)				**Residual concentrations after 10 weeks** (μg PAH·g^{-1}dry soil)			
Compound	**Abiotic control**	**NS**	**S+bac**	**S**	**Abiotic control**	**NS**	**S+bac**	**S**
Naphtalene	nm	nm	nm	nm	nm	nm	nm	nm
Acenaphthylene	7.7 ± 1.4	7.1 ± 0.7	6.2 ± 0.2	5.8 ± 0.3	6.0 ± 0.5	6.5 ± 1.3	4.7 ± 0.2	5.4 ± 0.4
Acenaphthene	nq	nq	nq	nq	nq	nq	nq	nq
Fluorene	10.9 ± 1.6	3.2 ± 0.9	1.2 ± 0.5	nq	8.5 ± 1.2	nq	nq	nq
Phenanthrene	51.5 ± 2.6	63.7 ± 2.0	48.1 ± 1.1	43.3 ± 1.3	48.2 ± 1.8	45.4 ± 1.1	38.4 ± 1.5	24.1 ± 0.8
Anthracene	18.8 ± 1.3	9.9 ± 0.6	8.8 ± 0.3	7.4 ± 0.4	14.2 ± 1.0	7.4 ± 0.7	7.2 ± 1.7	8.1 ± 0.7
Fluoranthene	41.3 ± 2.4	43.2 ± 1.5	40.6 ± 0.2	36.6 ± 1.6	39.6 ± 1.8	42.2 ± 1.1	39.8 ± 1.9	34.3 ± 1.3
Sum of 2-3 rings PAHs	130.2 ± 4.4	127.0 ± 2.8	104.9 ± 1.3	93.1 ± 2.0	116.4 ± 3.0	101.5 ± 2.1	90.2 ± 2.9	71.9 ± 1.8
Pyrene	33.2 ± 1.9	29.2 ± 1.3	30,3 ± 0.8	28.0 ± 0.9	31.6 ± 1.2	31.0 ± 1.7	28.4 ± 01.0	29.6 ± 0.5
Benz[a]anthracene	18.7 ± 0.4	18.5 ± 1.2	17.6 ± 1.3	16.2 ± 1.4	18.9 ± 0.6	16.3 ± 1.7	13.5 ± 1.0	15.7 ± 1.6
Chrysene	15.3 ± 0.7	17.3 ± 0.9	17.6 ± 1.5	16.8 ± 0.9	13.9 ± 0.7	15.2 ± 1.5	12.8 ± 0.6	13.8 ± 1.2
Benzo[b]fluoranthene	9.0 ± 0.2	10.5 ± 0.1	12.2 ± 0.0	11.5 ± 0.1	8.6 ± 0.3	10.2 ± 0.4	8.3 ± 0.1	9.3 ± 0.3
Benzo[k]fluoranthene	11.3 ± 0.3	12.9 ± 0.5	13.0 ± 1.1	12.5 ±0.2	11.3 ± 0.7	11.2 ± 0.1	9.1 ± 0.4	10.5 ± 0.6
Sum of 4 rings PAHs	87.5 ± 2.1	88.3 ± 2.1	90.6 ± 2.4	84.9 ± 1.9	84.3 ± 1.7	83.9 ± 2.9	72.0 ± 1.6	78.9 ± 2.1
Benzo[a]pyrene	11.1 ± 0.3	6.2 ± 1.3	6.6 ± 1.0	6.2 ± 1.3	10.7 ± 0.6	7.5 ± 1.3	5.6 ± 1.2	7.2 ± 0.8
Dibenzo[a.h]anthracene	7.3 ± 0.37	7.6 ± 0.9	6.7 ± 0.6	6.5 ± 0.8	7.4 ± 0.1	4.7 ± 0.2	4.2 ± 0.2	4.6 ± 0.1
Benzo[g,h,i]perylene	7.4 ± 0.04	5.8 ± 0.3	5.4 ± 0.1	5.3 ± 0.2	7.4 ± 0.3	1.8 ± 0.4	2.5 ± 0.4	2.5 ± 0.3
Sum of 5-6-rings PAH	25.9 ± 0.5	19.6 ± 1.5	18.7 ± 1.1	17.9 ± 1.5	25.5 ± 0.7	13.9 ± 1.4	12.3 ± 1.2	14.2 ± 0.8
Total PAHs	243.6 ± 4.9	234.9 ± 3.8	214.2 ± 2.9	195.9 ± 3.2	226.3 ± 3.5	199.3 ± 3.8	174.4 ± 3.6	165.1 ± 2.9

The errors are expressed as standard deviation of the triplicates. Different treatment: NS: non-sterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum (10^8 CFU·g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*). nm: not measured. nq: not quantifiable.

Table 6.18 Residual concentrations of PAHs expressed as μg PAH·g^{-1}dry soil in soil after treatment with *T. versicolor*.

	Residual concentration after 5 weeks (μg PAH·g^{-1}dry soil)				**Residual concentration after 10 weeks** (μg PAH·g^{-1}dry soil)			
Compound	**Abiotic control**	**NS**	**S+bac**	**S**	**Abiotic control**	**NS**	**S+bac**	**S**
Naphtalene	nm	nm	nm	nm	nm	nm	nm	nm
Acenaphthylene	7.7 ± 1.4	9.5 ± 0.3	8.5 ± 1.1	7.7 ± 0.9	6.0 ± 0.5	nq	2.3 ± 0.4	4.6 ± 0.5
Acenaphthene	nq	nq	nq	nq	nq	nq	nq	nq
Fluorene	10.9 ± 1.6	10.4 ± 1.5	5.9 ± 1.3	3.1 ± 0.9	8.5 ± 1.2	nq	nq	nq
Phenanthrene	51.5 ± 2.6	47.6 ± 1.7	38.4 ± 1.9	25.0 ±1.2	48.2 ± 1.8	37.0 ± 0.8	31.6 ± 1.4	21.3 ± 1.4
Anthracene	18.8 ± 1.3	18.3 ± 0.1	15.4 ± 0.3	10.7 ± 1.3	14.2 ± 1.0	7.9 ± 0.8	8.4 ± 0.9	6.1 ± 1.3
Fluoranthene	41.3 ± 2.4	29.8 ± 2.0	29.5 ± 1.0	24.8 ± 1.6	39.6 ± 1.8	29.1 ± 1.9	28.3 ± 0.8	17.1 ± 1.6
Sum of 2-3 rings PAHs	130.2 ± 4.4	106.1 ± 3.0	89.3 ± 2.7	63.6 ± 2.7	116.4 ± 3.0	74.0 ± 2.2	70.7 ± 1.9	49.1 ± 2.6
Pyrene	33.2 ± 1.9	26.1 ± 1.1	22.0 ± 1.0	20.8 ± 2.0	31.6 ± 1.2	20.8 ± 1.4	20.1 ± 1.3	18.1 ± 2.0
Benz[a]anthracene	18.7 ± 0.4	18.0 ± 1.0	16.0 ± 1.0	16.1 ± 0.9	18.9 ± 0.6	13.7 ± 0.1	13.9 ± 1.5	9.9 ± 1.5
Chrysene	15.3 ± 0.7	14.4 ± 0.6	13.7 ± 1.0	13.7 ± 0.9	13.9 ± 0.7	11.6 ± 0.3	12.6 ± 0.9	11.5 ± 1.8
Benzo[b]fluoranthene	9.0 ± 0.2	9.6 ± 0.2	9.1 ± 0.3	8.8 ± 0.5	8.6 ± 0.3	7.2 ± 0.1	8.1 ± 0.4	7.5 ± 0.6
Benzo[k]fluoranthene	11.3 ± 0.3	10.5 ± 0.8	9.7 ± 0.8	10.0 ± 0.8	11.3 ± 0.7	7.9 ± 0.3	8.3 ± 0.5	8.1 ± 0.4
Sum of 4 rings PAHs	87.5 ± 2.1	78.5 ± 1.8	70.4 ± 1.6	69.4 ± 2.5	84.3 ± 1.7	61.2 ± 1.5	62.9 ± 2.2	55.3 ± 3.1
Benzo[a]pyrene	11.1 ± 0.3	13.7 ± 0.1	12.8 ± 1.1	10.1 ± 2.0	10.7 ± 0.6	7.0 ± 0.6	6.8 ± 0.6	4.7 ± 1.7
Dibenzo[a.h]anthracene	7.3 ± 0.37	7.3 ± 0.2	7.2 ± 0.4	5.5 ± 0.5	7.4 ± 0.1	4.6 ± 1.9	5.6 ± 2.0	3.7 ± 0.1
Benzo[g,h,i]perylene	7.4 ± 0.04	7.33 ± 0.1	7.1 ± 0.5	7.4 ±0.3	7.4 ± 0.3	3.1 ± 1.7	5.0 ± 1.0	2.1 ± 0.1
Sum of 5-6-rings PAH	25.9 ± 0.5	28.3 ± 0.3	27.1 ± 1.3	22.9 ± 2.1	25.5 ± 0.7	14.7 ± 2.6	17.3 ± 2.3	10.5 ± 1.7
Total PAHs	243.6 ± 4.9	213.0 ± 3.5	186.8 ± 3.4	155.9 ± 4.2	226.3 ± 3.5	149.8 ± 3.7	150.8 ± 3.7	114.8 ± 4.2

The errors are expressed as standard deviation of the triplicates. Different treatment: NS: non-sterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum (10^8 CFU·g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*). nm: not measured. nq: not quantifiable.

PCA was performed to analyze the changes in PAHs concentrations by both fungi in the different treatments and time. The first two principal components (Fungus, Treatment) explained 53% and 29% of the data variability, respectively. A three-way ANOVA was performed to assess statistical significance of the data. The results indicated that *T. versicolor* was better in PAHs removal under the different treatments with respect to *I. lacteus* and increased the removal when increasing the treatment period (table 6.19).

Table 6.19 Results of three-way ANOVA on the variation in PC1 and PC2 according to factors Fungus (*T. versicolor* vs. *I. lacteus*), Treatment (NS: non-sterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum (10^8 CFU·g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*) and Time (5 vs. 10 weeks). (a: statistically significant, $p \leq 0.05$)

	PC1			PC2		
Variable	**F**	**d.f.**	**Probabitily**	**F**	**d.f.**	**Probabitily**
Fungus	1831	1,24	0.00a	695	1,24	0.00a
Time	482	1,24	0.00a	4341	1,24	0.00a
Treatment	148	2,24	0.00a	5	2,24	0.12
*Fungus*Time*	55	1,24	0.00a	729	1,24	0.00a
*Fungus*Treatment*	11	2,24	0.00a	84	2,24	0.00a
*Time*Treatment*	35	2,24	0.00a	38	2,24	0.00a
*Fungus*Time*Treatment*	49	2,24	0.00a	4	2,24	0.02a

Figures 6.5 and 6.6 show the removal of different groups of PAHs by both fungi. In the sterile soil, *T. versicolor* was capable to degrade 34% of the total PAHs within 5 weeks and this amount increased to 50% until 10 weeks. The degradation of 2-3 ring PAHs was significantly higher compared to the other PAHs after 5 weeks but this difference decreased after 10 weeks. The degradation capacity of *T. versicolor* was negatively affected in the presence of bacteria; the removal of the total PAHs after 10 weeks was reduced almost two-fold. The negative effect of living bacteria did not appear in the case of 4-ring PAHs (figure 6.6). *I. lacteus* removed only 20 and 27 % of the total PAHs within 5 and 10 weeks, respectively (figure 6.5). The inhibitory effect of bacteria was less pronounced than in *T. versicolor* and, in the presence of living bacteria, 5-6-ring PAHs were selectively degraded.

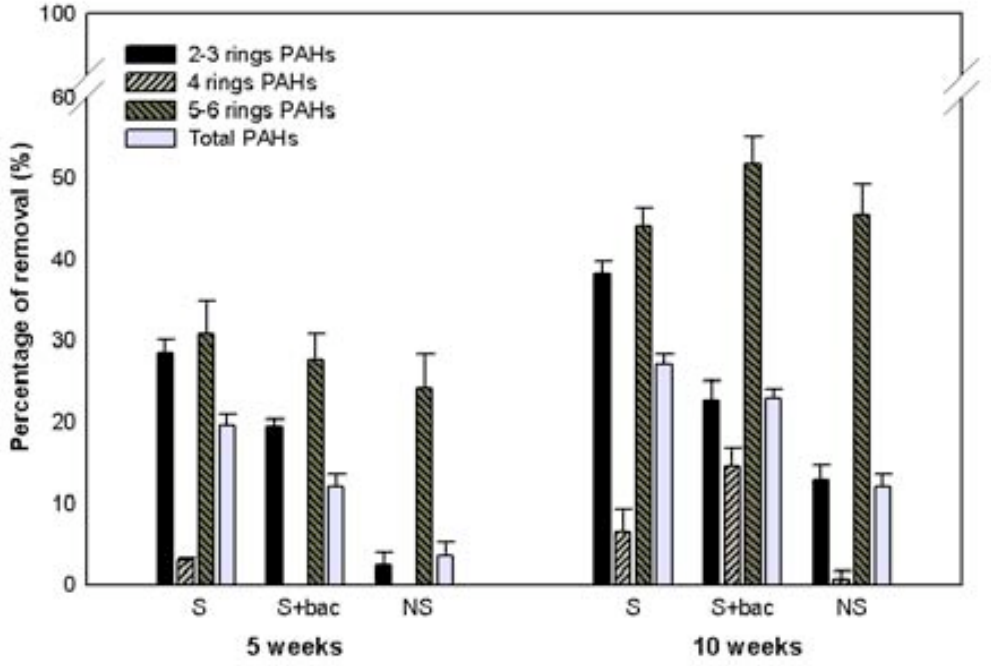


Figure 6.5 PAHs removal respect the abiotic control by *Irpex lacteus* expressed as percentage (% of PAHs inoculated).

Different treatments: NS: non-sterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum. The errors are expressed as standard deviation of the triplicates. (n=3). Different treatments: NS: non-sterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum (10^8 CFU·g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*).

In the absence of live bacteria, *T. versicolor* was clearly superior to *I. lacteus*, being capable of a faster and more extensive removal of PAHs despite its weaker growth in the soil. *I. lacteus* showed a very low efficiency of removal of PYR, BaA and BkF, compared to *T. versicolor*, whereas CHRY and BbF were degraded by neither fungus. The efficiency of *I. lacteus* was similar to previous studies using the artificially-spiked or industrially contaminated soils except for PYR, CHRY and BaP (Novotný *et al.*, 2000; Bhatt *et al.*, 2002). The removal of total PAHs by *T. versicolor* (49% within 10 weeks) was similar to the results obtained by Rama *et al.* (2001) in an industrially-contaminated soil (38% within 20 weeks), where also agriculture waste-pellets were used for inoculation. The behavior of both fungi in the absence of live bacteria thus seems to have been ´standard´ in our experiments.

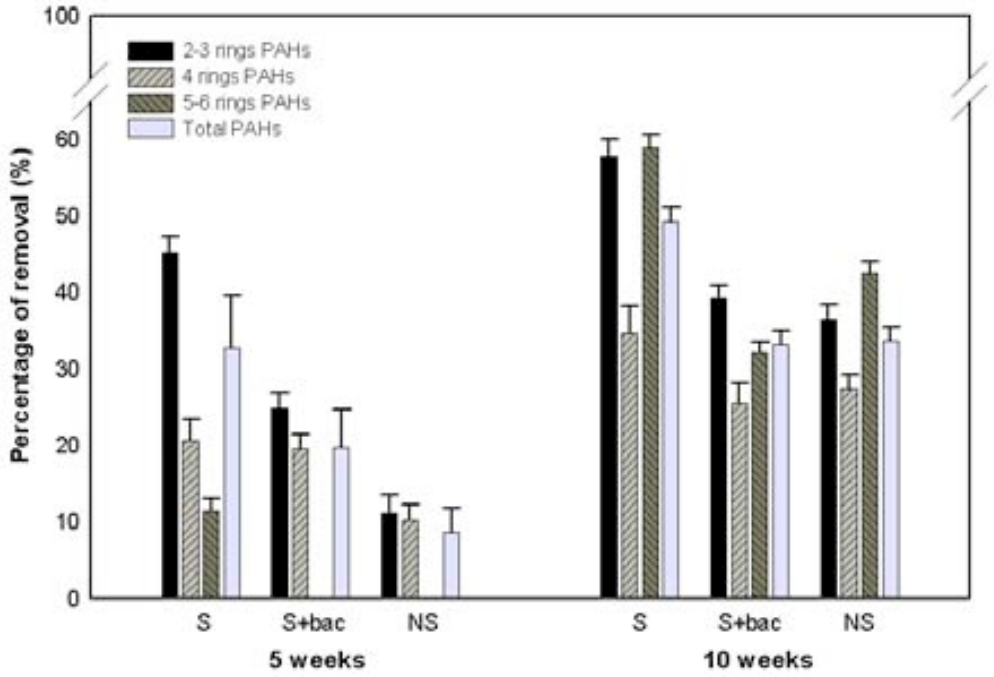


Figure 6.6 PAHs removal respect the abiotic control by *Trametes versicolor* expressed as percentage (% of PAHs inoculated).

Different treatments: NS: non-sterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum. The errors are expressed as standard deviation of the triplicates. (n=3). Different treatments: NS: non-sterile soil; S: sterile soil: S+bac: sterile soil + bacterial inoculum (10^8 CFU·g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*).

From the perspective of removal of the individual PAHs, however, the situation was more complex. Though *I. lacteus* and *T. versicolor* are expected to use similar biodegradation mechanisms the observed inhibition effects on the removal of individual PAHs were different. In *I. lacteus*, a strong bacterial inhibition was observed in the case of PHE and FLU. This inhibition was weaker in *T. versicolor*. No bacterial inhibition of ANT removal was observed in *I. lacteus* compared to a low inhibition in *T. versicolor*. In the case of 5-6-ring PAHs, significant bacterial inhibitions were observed in *T. versicolor* but not in *I. lacteus*. A weak enhancement of the removal rate of BaP and DaA, and of BghiP by *I. lacteus* was observed in the presence of *P. aeruginosa* and *R. erythropolis* and of the indigenous microflora, respectively. In *T. versicolor*, such an effect was demonstrated only for APY in the presence of both the indigenous microflora and *P. aeruginosa* and *R. erythropolis*. Similar fungal/bacterial synergistic effect was observed when the litter-decomposing fungus *Agrocybe aegerita* or the white-rot fungus *Kuehneromyces mutabilis* were inoculated into a non-sterile soil (Sack and

Fritsche, 1997). Also, during co-cultivation in a liquid medium of *Bjerkandera* sp. BOS55 with indigenous microflora from soils, activated sludge and PAH-adapted sediment, an increased mineralization of BaP occurred (Kotterman *et al.*, 1998b). Similarly, Boonchan *et al.* (2000) working with co-cultures of the soil-borne fungus *Penicillium janthinellum* with bacterial soil consortia in soil described an enhanced degradation of PYR, BaP and DaA, compared to the individual cultures. The extent of synergism in our study was rather small and did not manifest at the level of total PAHs.

Additionally, PAHs-degradation was assessed by identification of the metabolites produced along the experiment. In the case of *T. versicolor*, after 5 weeks of treatment the following metabolites were found in sterile soil: anthraquinone, acenaphtenone and dibutyl phtalate. After 10 weeks of treatment were detected also in sterile soil: dibutyl phtalate, 9H-fluoren-9-ol, 9H-fluoren-9-one and benzo[a]anthraquinone. For *I. lacteus*, in sterile soil, dibutyl phtalate was the only metabolite identified after 5 weeks; it was also found after 10 weeks together with 9H-fluoren-9-one. These findings suggest that in the other treatments, soil microflora degraded the polar metabolites.

6.2.4 Conclusions

The comparative study investigating the effect of bacteria on growth, biochemistry and biodegradation efficiency of two model species of white-rot fungi capable of PAH degradation using bacterial stress by indigenous soil microflora or selected soil bacteria demonstrated a significant decrease of degradation of total PAHs in the presence of live bacteria. Only weak fungal/bacterial synergistic effects were observed in the case of removal of APY and some 5-6-ring PAHs but the effects were not similar in the two fungi. The tested bacteria did not affect the capability of the two fungal organisms to colonize soil and did not influence the fungal growth yields. No specific effect of bacteria on the extracellular enzyme levels was detected. The study demonstrated the bacterial effect in soil under well-defined conditions and showed that more research is needed to investigate into the interactions between various bacterial and fungal species and their adverse and synergistic effects with the ultimate aim to control them in bioremediation technologies.

Chapter 7

Concluding remarks and future perspectives

Concluding remarks and future perspectives

The main concluding remarks of the present PhD project are listed below:

- High level of *T. versicolor* biomass (3gDW·l^{-1}) in pellet morphology (3 mm) can be obtained using a defined medium. Representing an important reduction in the cost (98%) respect to the previous used complex medium (malt extract). Air pulsed fluidized bioreactor with pH control at 4.5 is suitable for this proposal. Pellet production has been successfully scaled up from 1.5 L to 10 L bioreactor.

- All lignocellulosic substrates tested allow fungal growth. Nevertheless, a direct correlation between fungal biomass and biodegradation yield is not obvious. Consequently, the substrate for solid matrices bioremediation should be selected depending on the cost and required operational conditions such as size and mixture porosity.

- *T. versicolor* is able to biodegrade all PAHs studied and also their degradation metabolites. This is an important issue because no dead-end products are accumulated when using this microorganism as bioremediator agent.

- Although *in vitro* enzymatic experiments evidence the possible implication of *T. versicolor* laccase in PAHs degradation, it requires the presence of mediators. Additionally, other enzymatic systems such as cytochrome P450 and reductive enzymes are involved in fungal biodegradation of PAHs and their intermediates.

- PAHs degradation yield depends on the approach, being both the matrix and concentration key parameters. Thus, at low PAHs concentration (near 20 mg·l^{-1}) submerged cultures (liquid and slurry) allow achieving good removal yields when employing an appropriate surfactant like Tween 80. Whereas, at higher concentrations (above 150 mg·l^{-1}) best removal is obtained in solid-state culture, such as biopile microcosm. In this approach, surfactant is not required but the addition of the suitable surfactant (BS-400) improves significantly the bioremediation results.

- Even though most studies have been carried out under sterile conditions to evaluate *Trametes* degradation and colonization ability, results obtained under non-sterile conditions are promising. Consequently, *Trametes* is placed as a suitable agent to be applied in polluted soils bioremediation applications.

Although broad laboratory research has been carried out, further laboratory research should focus on the following topics:

- Apply *T. versicolor* for the treatment of aged PAHs-contaminated soil.
- To develop a suitable technology for real site restorage, by means of optimizing operational process conditions.
- To develop new tools to monitor fungal activity in solid matrices, bioavailability of pollutants, assess ecotoxicity and microbial diversity.

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Trametes versicolor pellets production: Low-cost medium and scale-up

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ABSTRACT

A cost analysis of a decolourization process using the ligninolytic fungus *Trametes versicolor* in pellet form was carried out. It established that the key to making the process cost-efficient was to reduce the cost of the culture medium for pellet production, which accounts for over 95% of the total cost of the process, due to the high price of malt extract. A cheaper defined medium was formulated in order to obtain spherical pellets approximately 3 mm in diameter. The pH of the medium played an important role in pellet production. Experiments were therefore conducted to allow the control of the pH. A stirred-tank bioreactor (1 L) was ruled out, since problems related to oxygen transfer and types of agitation have been shown to exert a great influence on pellet formation. Use of air-pulsed bioreactors solved these problems, allowing both pellet production and scale-up of the process in a 10 L air-pulsed bioreactor. The new pellet production process reduced the total cost by up to 94.4% per unit volume of wastewater treated. In a decolourization test the pellet s obtained from the new process showed an outstanding performance.

1. Introduction

Use of filamentous microorganisms, such as fungi, has been extensively studied in biotechnological processes for producing primary and secondary metabolites, antibiotics, enzymes, polysaccharides and vitamins [1–6].

When fungi are grown in submerged cultures a large number of factors contribute to development of any particular morphological form [3,7–11], ranging from dispersed mycelial filaments to densely interwoven mycelial masses, known as pellets, of highly varying compactness. Good control of mycelial morphology in fermentation could be important for many industrial applications.

Dispersed mycelial cultures display an increase in viscosity when the agitation rate is increased. Consequently, problems appear, related to mass transport of oxygen, heat and nutrients, along with an increase in biomass growth on the bioreactor walls, agitators, probes and baffles [12–16]. By contrast, pellet cultures solve such operational problems because fungi form aggregates of hyphae as they grow, exhibit low viscosity, approach Newtonian flow behaviour and do not adhere to any part of the bioreactor [17].

Obtaining uniform pellets of a desired size is not easy, since many factors influence pellet formation: inoculum size, type and age, genetic factors, ability to produce bioflocculants, medium composition, shear forces, pressure, temperature, medium viscosity and oxygen concentration.

White-rot fungi are used extensively in biodegradation processes [18–20]. The oxidative ability of these fungi is related to their extracellular and intracellular enzymatic system. Their non-specific enzymatic system allows them to degrade and/or mineralize a wide range of pollutants resistant to other microorganisms, such as dyes, PAHs, PCBs, pesticides, pentachlorophenols and endocrine disruptors [21–28].

Two major strategies have been developed for use of white-rot fungi for degradation of pollutants in bioreactors [29–31]. One is treatment with enzymes, whether purified or using broths from fungi cultures, such as laccases, manganese peroxidases and lignin peroxidases [32–35]. The other is direct degradation of pollutants using active cultures of fungi [36–38]. The main advantage of using fungi lies in the broad range of enzymes produced and the further transformation of the intermediate biodegradation products. On the other hand, use of enzymes allows easier operation and, in some cases, faster biodegradation.

This research team has developed a continuous process to degrade Gris Lanaset G [39] and has experience with degradation of other kinds of pollutants, such as endocrine disruptors [40], trichloroethylene (TCE) and tetrachloroethylene (PCE) using *Trametes versicolor* in pellet form [28,41,42].

The main aims of this research were to analyse the costs of the process, to develop a low-cost process for producing *T. versicolor* pellets on bioreactor scale and to scale-up pellet production.

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2. Materials and methods

2.1. Microorganism

T. versicolor was obtained from ATCC#42530. The fungus was maintained on 2% malt agar slants at 25 °C until use. Subcultures were routinely made.

2.2. Chemicals

Grey Lanaset G – a commercial mixture of several metal complex dyes (Cr and Co) – was kindly supplied by Ciba (ref. 080173.5). All other chemicals were reagent grade.

2.3. Mycelial suspension

A mycelial suspension of *T. versicolor* was obtained by inoculation of four 1 cm diameter plugs, from the fungus growing zone on 2% malt agar, in 150 mL of 2% (w/v) malt extract medium in a 500 mL Erlenmeyer flask. This was incubated at 25 °C at constant agitation (135 rpm, r = 25 mm). After 4–5 days a dense mycelial mass was formed. It was separated from the culture medium, resuspended in an equal volume of a sterile saline solution (0.8% (w/v) NaCl) and then disrupted with an X10/20 (Ystral GmbH) homogenizer. The resulting mycelial suspension was stored at 4 °C until use.

2.4. Conventional pellet production

The mycelial suspension was used to produce pellets by inoculating 1 mL of the suspension in 250 mL on a 2% (w/v) malt extract (ME) medium adjusted to pH 4.5 with 0.5 M NaOH in a 1-L Erlenmeyer flask. The medium had been sterilized at 120 °C for 30 min. The flask was incubated at 25 °C at constant agitation (135 rpm, r = 25 mm) for 5 days. The pellets formed were stored at 4 °C in sterilized saline solution (0.8% NaCl) until use.

2.5. Continuous dye treatment

A previously defined [30,43] 1500 mL air-pulsed bioreactor was used. The bioreactor was filled with the start-up medium which contained (per litre): 8 g glucose, 1.9 g NH_4Cl, 10 mL macronutrients solution which contained per litre: 20 g KH_2PO_4, 5 g $MgSO_4{\cdot}7H_2O$ and 1 g $CaCl_2$, 1 mL micronutrients solution which contained per litre: 1.5 g nitriltriacetic acid, 3 g $MgSO_4{\cdot}7H_2O$, 0.5 g $MnSO_4{\cdot}H_2O$, 1 g NaCl, 0.1 g $FeSO_4{\cdot}7H_2O$, 0.1 g $CoSO_4$, 0.1 g $ZnSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.01 g $CuSO_4{\cdot}5H_2O$, 0.01 g $AlK(SO_4)_2{\cdot}12H_2O$, 0.01 g H_3BO_3 and 0.01 g $NaMoO_4$, and 150 mg Grey Lanaset G. The pH was adjusted to 4.5 with 0.5 M NaOH.

The bioreactor was inoculated with pellets equivalent to 3.2 g DCW/L. Once the glucose concentration in the bioreactor had fallen to approximately 2 g/L, the simulated wastewater treatment was switched on, operating at a hydraulic retention time (HRT) of 24 h and a cellular retention time (CRT) of 21 days. The sterilized simulated wastewater contained (per litre): 150 mg Grey Lanaset G, 10 mL macronutrients solution, 1 mL micronutrients solution and 0.31 g glucose/(g DCW d).

2.6. Pellet production on bioreactor scale

Three different bioreactors were used for pellet production in batch mode.

One was a Biolab bioreactor (Braun) with a working volume of 900 mL, where air was blown in through a metal sparger under the impeller. The air flow varied, depending on the experiment. Details are given case by case.

The others were two glass air-pulsed bioreactors, one with a working volume of 1500 mL, the other 10 L [30,39]. The pulsed air flow was generated as previously defined [43]. The pulse frequency was 0.16 s^{-1}, allowing fluidization and liquid phase homogenization of the biomass.

The bioreactors were equipped with a pO_2 probe and a pH controller in order to maintain a pH of 4.5 and a constant temperature of 25 °C.

The defined medium (DM) proposed for pellet production contained (per litre): glucose, depending on the experiment, 2.1 g NH_4Cl, 10 mg thiamine, 100 mL macronutrients solution and 10 mL micronutrients solution. The pH was adjusted to 4.5 as described above.

The bioreactors were inoculated with 4 mL of mycelial suspension of *T. versicolor* per litre of medium.

2.7. Decolourization test

The decolourization tests were carried out on Erlenmeyer flask scale with 250 mL of medium that contained (per litre): 6 g glucose, 150 mg Grey Lanaset G, 1.168 g 2,2-dimethylsuccinate (DMS) buffer, 10 mL macronutrients solution and 1 mL micronutrients solution. The pH was adjusted to 4.5 as described above. The flasks were inoculated with pellets equivalent to 3.2 g DCW/L and were maintained at 25 °C at constant agitation (135 rpm, r = 25 mm) for 4 days.

2.8. Analytical methods

The glucose concentration was measured with a YSI 2000 enzymatic analyser from Yellow Springs Instruments and Co.

The biomass was measured as dry cell weight (DCW) with drying at 105 °C to constant weight.

Dye concentrations were determined by spectrophotometric measurements carried out at the visible maximum absorbance, 590 nm using a Varian UV/vis Cary spectrophotometer at 20 °C.

Pellet size was determined using a Meiji Labax magnifying glass (model SKT) at 40× with a calibrated ocular micrometer. The diameter was measured over a randomly selected 40-particle sample set. The confidence range was calculated using the t-student distribution.

2.9. Enzymatic test

Laccase activity was measured using a modified version [44] of the method for determination of manganese peroxidase (MnP) [45]. One activity unit (AU) was defined as the number of micromoles of DMP oxidized per minute. The DMP extinction coefficient was 10,000 M^{-1} cm^{-1}.

3. Results and discussion

3.1. Economic study on decolourization of Grey Lanaset G

Long-term continuous dye treatment (150 mg/L) was carried out in an air-pulsed bioreactor with retained pellets of *T. versicolor*. Fig. 1 shows the decolourization obtained for an HRT of 24 h [43] and CRT of 21 days [46]. Following the same method as in previous research, from day 21 one-third of the biomass was removed every 7 days. The average colour reduction was about 90% and the biomass was renewed four times.

The continuous biotechnological process consists of the following steps: pellet formation, start-up and continuous treatment. Treatment costs for this process in the steady state were evaluated. In the economic study, investment costs were not taken into

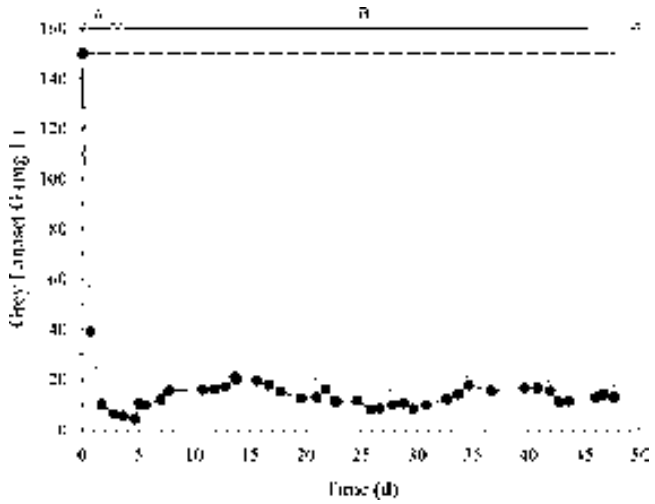

Fig. 1. Time course of influent dye concentration (- - -) and effluent concentration (●) in a bioreactor inoculated with *T. versicolor*. Operating conditions were: (A) start-up and (B) continuous treatment. The vertical arrows represent time when biomass renovations were carried out.

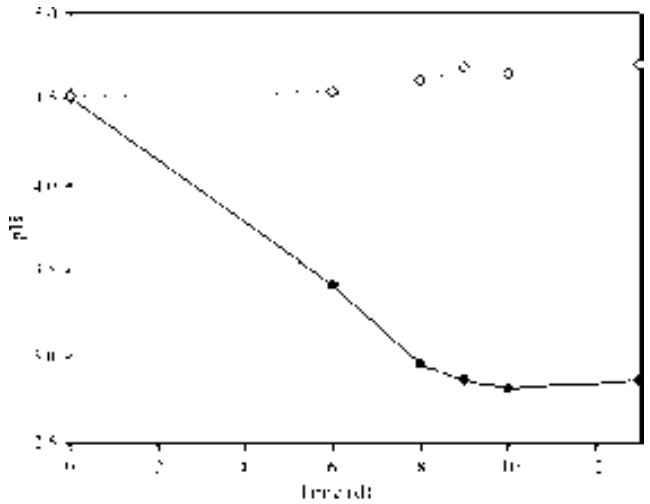

Fig. 2. Changes in pH of culture broth using defined medium (●) and malt extract medium (○).

account. Only the cost of continuous treatment, including the cost of the biomass production necessary for the continuous treatment, was considered.

Malt extract (ME) (2%) is the conventional growing medium for production of *T. versicolor* biomass in pellet form. The main disadvantage of this medium is that it is expensive (€42 kg^{-1} ME). After 5 days of growth 3 g DCW/L of pellets are obtained. The cost of ME for pellet formation is €0.28 g^{-1} DCW. Taking into account the data from the continuous experiment, analyzing the period in the stationary state from the first biomass renovation till 7 days after the last one, the raw materials cost for the pellet production required for treatment of 1 m^3 of simulated wastewater is €42.6 m^{-3} and the cost of raw materials for continuous operation is €0.66 m^{-3}. Table 1 summarizes the cost of raw materials used in this study.

Turning to the energy costs, the energy consumed by the devices used for pellet production and by the bioreactor during treatment is also taken into account, including equipment and sterilization of the culture medium in the pellet formation step, aeration, stirring and pumping. Energy costs are taken as €0.084854 kWh^{-1} (Fecsa-Endesa RD 1624/2006, Spain). The energy cost for pellet formation is €0.79 m^{-3} and the energy cost for the treatment €0.22 m^{-3}, giving a total energy cost for the process of €1.01 m^{-3}.

Consequently, the total cost required for the treatment is €43.61 m^{-3}, of which 97.7% is for the raw materials. To cut the cost of the process, it is therefore necessary to reduce the raw materials cost. For the continuous treatment the cost of raw materials (€0.66 m^{-3}) can be ignored compared with the cost of pellet formation (€42.6 m^{-3}). This, therefore, is the key to reducing the cost of the decolourization process.

Table 1
Cost of the different raw materials used in this study

Nutrients	Cost
Malt extract	€42.06 kg^{-1}
Glucose	€0.9 kg^{-1}
NH_4Cl	€0.5 kg^{-1}
Tiamine	€305.5 kg^{-1}
Macronutrients solution	€8.97 m^{-3}
Micronutrients solution	€2.01 m^{-3}

3.2. Study on reformulation of the pellet production medium and bioreactor-scale production

The size (approximately 3 mm) and spherical shape of the pellets obtained on laboratory scale are suitable for applications in treatment of dyes and textile wastewater in a fluidized bioreactor [30,43]. One of the objectives of this research is to obtain pellets of this shape and size on bioreactor scale. ME medium requires no buffer because no significant variation in the pH of the medium is observed during the growth stage. It is maintained within the optimum pH range for fungal growth (4.5). The initial phase of the study focused on formulation of an alternative medium (DM with 17 g/L of glucose and buffered with DMS) for fungal growth in pellet form. Results obtained on Erlenmeyer flask scale showed inhibition of growth due to the pH decrease in the medium. Fig. 2 shows the pH decrease in the cultures with DM, while the pH was maintained at around 4.5 in ME medium cultures. Buffers other than DMS were tested, but none of them was able to maintain a pH of around 4.5 (data not shown). Tavares and col. [47] describe the same behaviour for pH during the glucose consumption period of *T. versicolor* growing in a DM. This is related to the synthesis of organic acids, such as oxalic and glycoxalic acids, associated with the fungal primary metabolism when glucose is consumed [48]. Other authors have also reported that mediums containing ammonium salt and weak buffers tend to turn acidic during fungal growth [3]. In order to control pH during the pellet formation period, next experiments were conducted with a laboratory-scale bioreactor.

Experiments on bioreactor scale were conducted on a stirred-tank bioreactor (Biolab) with low mechanical agitation (135 rpm) to avoid break-up of the pellets. At the end of the growing period with the DM (glucose, 10 g/L) a huge amount of biomass was observed, but mostly as fluffy hyphae aggregates not in compact pellet form. There have been reports [9] that a low concentration of oxygen tends to lead to formation of fluffy pellets that are easily broken up by mechanical agitation. As this experiment was carried out without pO_2 measurements, the team was unaware of possible oxygen limiting conditions during the experiment. But the results and observations during the culture time seem to confirm that oxygen was limited. The oxygen saturation percentage was measured in further experiments.

In order to solve the problems of biomass morphology and excessive biomass growth, the next experiment halved the amount of carbon source (7 g/L) while maintaining the same C/N ratio as

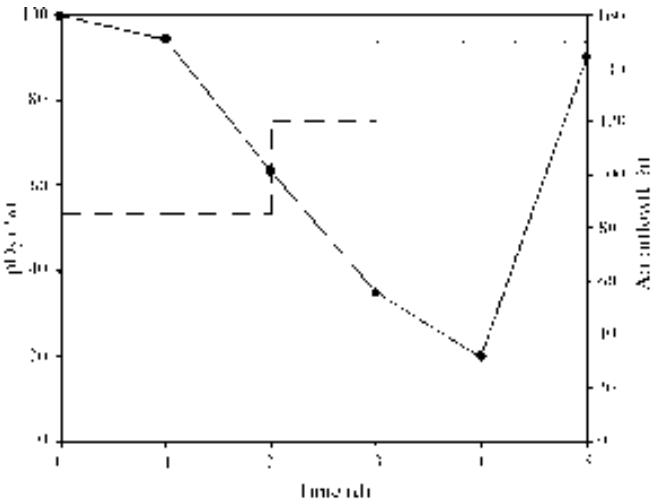

Fig. 3. Changes in air flow (–·–·–) and pO_2 (●) during pellet production using the Biolab bioreactor.

the two previous experiments above described. Fig. 3 shows the changes in inlet air flow and dissolved oxygen (pO_2). The high oxygen consumption is related to the fungal growth and the pO_2 increase on day 5 coincides with the glucose depletion (data not shown).

After day 3 the air inlet flow was increased to 150 L/h, equivalent to 1.6 vvm, but this was not enough to maintain a pO_2 level of over 30%. Increases in the agitation rate and the air flow supply were considered with a view to enhancing the oxygen concentration, but in the end that strategy was not chosen in order to avoid problems such as foam formation and possible pellet break-up. Formation of clumps was favoured instead of pellet morphology. The diameter of the few pellets obtained in this experiment was 1.66 ± 0.54 mm, much smaller than that obtained with ME medium on Erlenmeyer flask scale (approximately 3 mm).

To solve the operational problems related to mechanical agitation, further experiments were performed in an air-pulsed bioreactor. The main characteristic of this kind of bioreactor is the good oxygen transfer and homogenization, with less mechanical stress and without the fungi attaching to the walls or instrumentation, since both the mix and fluidization of pellets are obtained by means of air flow. Fig. 4 shows that pO_2 remained over 70%

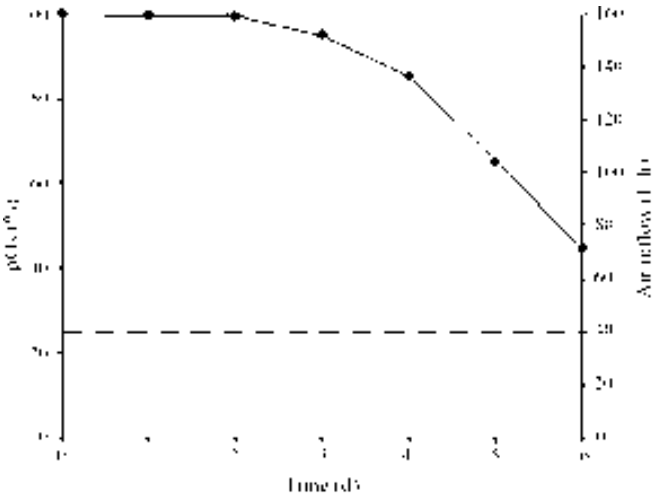

Fig. 4. Changes in air flow (–·–·–) and pO_2 (●) during pellet production using the 1500 mL air-pulsed bioreactor.

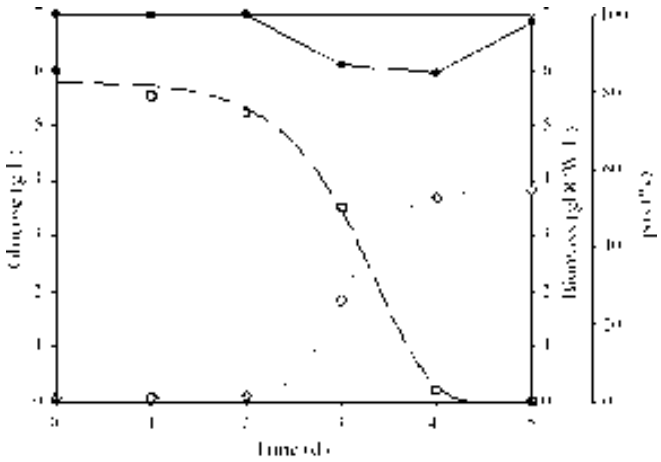

Fig. 5. Changes in glucose (□), biomass (◊) and pO_2 (●) during pellet production using the 10 L air-pulsed bioreactor.

with an air flow of 0.44 vvm. At the end of the experiment, the biomass was completely in pellet form with an average diameter of 3.09 ± 0.20 mm.

The good results obtained for pellet formation in a laboratory-scale bioreactor encouraged the team to scale-up the pellet formation process with a pilot-scale air-pulsed bioreactor (10 L). The reactor was designed for treatment of effluents containing dyes [30,39] but, in this study, was also used for pellet production. Fig. 5 shows that it was possible to guarantee a pO_2 level between 60% and 100% with only 0.25 vvm, indicating an improvement in oxygen transfer compared with the air-pulsed laboratory-scale bioreactor. The total biomass obtained in the pilot bioreactor was 3.89 ± 0.39 g/L, all in the form of pellets 3.05 ± 0.21 mm in size, similar to that obtained in the air-pulsed bioreactor on laboratory scale.

3.3. Validation of pellets in a decolourization test

Once the growth medium and the method of pellet formation had been defined on pilot scale, the suitability of the *T. versicolor* pellets obtained in this way for application in decolourization processes was studied. A decolourization test was conducted on Erlenmeyer flask scale. After 24 h of treatment a decolourization rate of 95% was achieved (see Fig. 6).

3.4. Comparison of process costs

Taking into account the amount of pellets per litre obtained with the DM, the cost of the DM for pellets production is €0.055 g^{-1} DCW. Therefore, for the continuous treatment the cost of biomass production is €0.838 m^{-3}.

Table 2 compares the costs of the process with ME medium and with DM. It shows that the total cost was reduced by 96%. The main contribution to this cost reduction came from the big reduction in the raw materials costs, but energy costs were also cut because pellets obtained with DM are produced in an air-pulsed bioreactor,

Table 2
Comparison of the total cost for the decolourization process with different pellet production media

	Malt extract	Defined medium
Raw materials (€/m^3)	43.26	0.838
Energy (€/m^3)	1.01	0.88
Total	44.27	1.72

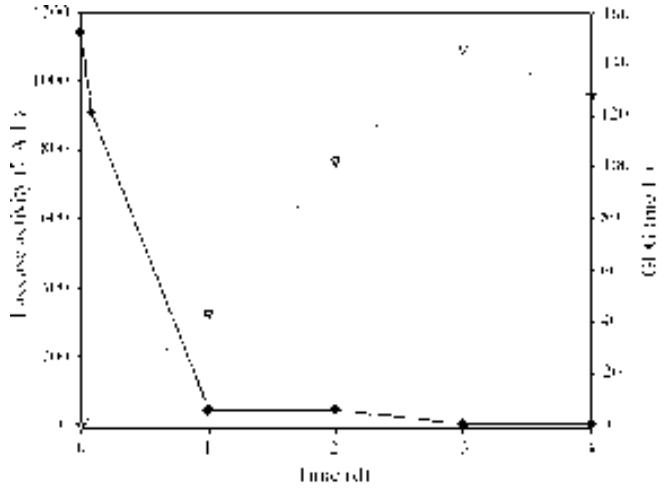

Fig. 6. Changes in laccase activity (▽) and colour (●) during the decolourization test at Erlenmeyer flask scale using pellets obtained with DM.

where aeration needs are lower than in the Biolab bioreactor and no mechanical agitation is required.

4. Conclusions

A new procedure has been developed to obtain pellets of *T. versicolor* for use in dye decolourization treatment or other bioremediation processes. The newly defined medium significantly reduced (by 96%) the cost of the whole process in terms of €/m^3 of wastewater treated. Problems related to the oxygen concentration and types of agitation have been shown to exert a great influence on pellet formation. Use of air-pulsed reactors solved these problems because it allows better oxygen transfer and smoother agitation. The process can be scaled up and the biomass obtained is usable for bioremediation processes such as decolourization of dyes.

Acknowledgements

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Effect of soil bacteria on the ability of polycyclic aromatic hydrocarbons (PAHs) removal by *Trametes versicolor* and *Irpex lacteus* from contaminated soil

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ABSTRACT

The effect of bacteria represented by indigenous soil microflora or a mixture of soil bacteria *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* on fungal growth, extracellular enzyme production and polycyclic aromatic hydrocarbons (PAHs) biodegradation efficiency in soil of white-rot fungi *Trametes versicolor* and *Irpex lacteus* was investigated. Both fungi were able to colonize soil. The growth yields measured by ergosterol were about two-fold in *I. lacteus* after 10 weeks. Laccase was produced in *T. versicolor* cultures in the presence or absence of bacteria but live bacteria reduced the laccase levels in soil about 5 times. Manganese-dependent peroxidase (MnP) was not detected in *T. versicolor* cultures. The amounts of MnP and laccase in *I. lacteus* cultures were not affected by the presence of bacteria. *T. versicolor* was more efficient in PAH removal for all PAHs tested although its capacity to colonize soil was lower. The removal rates of PAHs by *T. versicolor* in sterile soil were 1.5-fold, 5.8-fold and 1.8-fold for 2–3-ring, 4-ring and 5–6-ring PAHs, compared to *I. lacteus*, respectively. *I. lacteus* showed a low efficiency of removal of pyrene, benzo[a]anthracene and benzo[k]fluoranthene, compared to *T. versicolor*, whereas chrysene and benzo[b]fluoranthene were degraded by neither fungus. The main effect of the presence of the indigenous microflora or *R. erythropolis* and *P. aeruginosa* was a significant decrease of degradation of total PAHs by both *T. versicolor* and *I. lacteus*. Weak fungal/bacterial synergistic effects were observed in the case of removal of acenapthylene, benzo[a]pyrene, dibenzo[a,h]anthracene and benzo[g,h,i]perylene by *I. lacteus* and acenapthylene by *T. versicolor*. However, the bacterial effects were different in the two fungi. PAH abiotic losses represented 15 and 21% of the total PAHs after 5 and 10 weeks, respectively; naphthalene and acenaphthene were removed from the soil due to volatilization.

1. Introduction

PAHs are a group of omnipresent environmental organopollutants with toxic, mutagenic and carcinogenic properties whose accumulation in the environment results from both natural and industrial processes. 16 PAH molecules have been considered to be priority pollutants of the aquatic and terrestrial ecosystems by the U.S. Environmental Protection Agency. The potential of white-rot fungi to degrade recalcitrant organopollutants in both artificially-spiked and industrially-contaminated soils has been shown (Novotný et al., 1999; Mougin, 2002; D'Annibale et al., 2006; etc.). Dense mycelial growth and significant enzyme production are necessary to remove PAHs from contaminated soils. Typically, white-rot fungi are applied as inoculum growing on a convenient solid substrate from which the fungus colonizes the contaminated soil with explorative mycelium producing extracellular enzymes involved in PAH degradation (e.g. Novotný et al., 2000; Šašek et al., 2003; Leonardi et al., 2008; Byss et al., 2008). The degradation mechanism similar to that of litter-decomposting fungi (Steffen et al., 2002), includes oxidative transformation of PAHs by peroxidases, laccase and cytP450 monooxygenase producing trans-dihydrodiols, epoxides, quinones and phenols, and conjugation resulting in the formation of glucuronides, glucosides, sulfates, etc. (Singh, 2006).

Irpex lacteus has been demonstrated to extensively degrade PAHs in industrially-polluted soils (e.g. Šašek et al., 2003; Byss et al., 2008; Leonardi et al., 2008) whereas the results with *Trametes versicolor* show both high (Rama et al., 2001) and low (Novotný et al., 1999) biodegradation rates.

Competition with autochthonous soil microflora is an important factor in soil bioremediation by white-rot fungi but the knowledge of their interactions with soil microbiota is poor (Mougin, 2002; Singh, 2006; Arun et al., 2008). *T. versicolor* was shown to attack soil bacteria

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including pseudomonads (Thorn and Tsuneda, 1992) but soil pseudomonads can also inhibit growth of white-rot fungi (Radtke et al., 1994). *Pleurotus ostreatus* was able to enhance the degradation of PAHs in non-sterile, artificially contaminated soils but it also inhibited growth of indigenous bacteria and changed the composition of the bacterial community (Andersson et al., 2000). Growth of *I. lacteus*, in contrast to *P. ostreatus* and *Phanerochaete chrysosporium*, was not inhibited by *Pseudomonas putida* and *Sphingomonas* sp. on malt extract/glucose- and phenazine-induction agar media (Novotný et al., 2001). Successful colonization of non-sterile soils by white-rot fungi demonstrates the ability to overcome the adverse effect of soil microflora (Rama et al., 2001; Singh, 2006; Leonardi et al., 2008). Besides the presence of bacteria, fungi and their metabolites can also induce the production of fungal laccase implicated in PAHs degradation (Crowe and Olsson, 2001; Baldrian, 2004).

Polar metabolites produced by oxidation of high-molecular-weight PAHs by white-rot fungi can be mineralized by indigenous microflora isolated from sludges and forest soils (Meulenberg et al., 1997; Kotterman et al., 1998), which can increase the biodegradation rate. However, this behavior is not omnipresent as documented by studies with *P. chrysosporium*, *P. ostreatus* or *T. versicolor* (Bhatt et al., 2000; Canet et al., 2001).

As the interactions of fungi applied in soil with the soil bacteria and the effect of the latter on fungal biodegradation are poorly understood (Singh, 2006), the aim of our study was to investigate the effect of bacterial stress in the soil environment on two fungi with a potential for PAH biodegradation and soil colonization, *I. lacteus* and *T. versicolor*. The bacterial stress was represented either by the innate soil microflora or a defined mixture of the soil bacteria *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* inoculated into the sterile soil. The effect was measured by the efficiency of removal of 16 EPA-recommended PAHs spiked into the non-contaminated soil, by the ability of the fungi to colonize the soil and by production and secretion of their extracellular MnP and laccase.

2. Materials and methods

2.1. Fungal strains

T. versicolor ATCC 42530 and *I. lacteus* 617/93 were acquired from the American Type Culture Collection, Manassas, USA and the Culture Collection of Basidiomycetes, Academy of Sciences of the Czech Republic, Prague, respectively, and maintained by monthly subculturing on 20 g l^{-1} malt extract agar slants (pH 4.5) at 25 °C. Fungal mycelial suspensions were obtained by blending 7-d mycelium growing on the MEG medium (20 g l^{-1} malt extract, Scharlau, Spain, 10 g l^{-1} glucose, pH 4.5).

2.2. Bacterial strains

P. aeruginosa CCM 1960 and *R. erythropolis* CCM 2595 (both Czech Collection of Microorganisms, Masaryk University, Brno) were kindly provided by K. Malachová (University of Ostrava, Ostrava) and M. Pátek (Institute of Microbiology of the ASCR, v.v.i., Prague), respectively. Both strains were maintained by monthly subculturing on tryptone agar slants (0.8 g l^{-1} tryptone, 0.5 g l^{-1} yeast agar, 0.25 g l^{-1} NaCl, pH 7.0) at 25 °C. Overnight, liquid tryptone medium cultures grown at 25 °C were used as for inoculation.

2.3. Chemicals and solid-state substrate

Chemicals: creosote (Sugelabor-Chem Service, UK), dichloromethane (DCM) (SDS, France), alumina (Merck Chemical, Spain), ergosterol (Sigma–Aldrich, Spain), acetone (Panreac, Spain). PAHs standards were purchased from Dr. Ehrenstorfer (Germany). Any other chemicals used were of the analytical grade.

Wheat–straw pellets (ATEA Praha s.r.o., Czech Republic) were used as a lignocellulosic support for fungal growth in solid-state cultures.

2.4. Soil

The soil was collected from the A horizon of an agricultural site at Prades (Catalunya, Spain), sieved through a 5 mm steel mesh and stored in the dark at 4 °C. The soil had the following physical characteristics: pH_{wat} 5.1, pH_{KCl} 4.5, organic matter 2%, sand 77.35%, silt 12.10%, clay 10.55%, C_{org} 1.28%, N_{tot} 0.12% and water holding capacity 12.1% (all w/w). The soil was sterilized by X-ray irradiation when required.

2.5. PAH mixture preparation and soil contamination

The PAH mixture was obtained according to the method 3611B (EPA), by cleaning up 5 g of creosote with alumina to obtain the base-neutral aromatic compounds fraction. The samples were evaporated and dissolved in DCM to obtain a stock solution.

The soil was artificially contaminated by adding 300 μl of the stock solution to 9 g of dry soil and airdried for 24 h at the room temperature in a flow box. To obtain a homogeneous distribution of PAHs, the soil was mixed with a metal stick. The initial PAH concentration was determined 24 h after spiking the soil. The soil samples were stored in the freezer before analysis less than 24 h.

Final concentrations of PAHs in the soil and the percentage of recovery are shown in Table 1. A lower recovery of low MW PAHs was probably due to their volatility.

2.6. Experimental set-up

Amounts of 3 g of straw pellets in 100 ml tubes (2 mm ID) were moistened with 6 ml deionized water. The tubes were stoppered with cotton plugs and autoclaved twice (121 °C, 30 min) within two days. The substrate was then inoculated with 1 ml of the mycelial

Table 1
PAHs spiked in soil and subsequently recovered by Soxhlet extraction (μg PAH g^{-1} dry soil).

PAHs		Concentration (μg PAH g^{-1} dry soil)		
Compounds	Acronym	Spiked	Recovered	% Recovery
Naphtalene	NAP	1.5 ± 0.0	1.8 ± 0.1	81.0
Acenaphthylene	APY	17.4 ± 0.5	9.3 ± 0.5	53.6
Acenaphthene	APE	4.7 ± 0.1	2.7 ± 0.2	56.3
Fluorene	FLE	26.4 ± 0.3	20.1 ± 1.6	76.2
Phenanthrene	PHE	71.2 ± 2.0	68.3 ± 3.0	96.0
Anthracene	ANT	21.4 ± 0.6	19.8 ± 0.6	92.4
Fluoranthene	FLT	42.9 ± 1.2	43.5 ± 0.3	101.4
Sum of 2–3-rings PAHs		185.6 ± 2.4	165.6 ± 3.5	82.2
Pyrene	PYR	31.2 ± 0.2	30.8 ± 0.8	99.0
Benzo[a]anthracene	BaA	20.9 ± 0.6	20.9 ± 1.0	99.9
Chrysene	CRY	18.3 ± 0.5	18.0 ± 0.9	98.4
Benzo[b]fluoranthene	BbF	10.7 ± 0.0	10.4 ± 0.2	96.6
Benzo[k]fluoranthene	BkF	12.7 ± 0.6	12.8 ± 0.3	100.5
Sum of 4-rings PAHs		93.8 ± 1.0	92.8 ± 1.6	99.0
Benzo[a]pyrene	BaP	13.9 ± 0.4	13.6 ± 0.8	98.1
Dibenzo[a.h]anthracene	DaA	8.0 ± 0.8	7.9 ± 0.2	97.9
Indeno[1,2,3]pyrene	I123P	nm	nm	nm
Benzo[g,h,i]perylene	BghiP	7.5 ± 0.4	6.8 ± 0.2	90.0
Sum of 5–6-rings PAHs		29.45 ± 1.0	28.3 ± 0.8	96.0
Total PAHs		308.8 ± 2.7	286.7 ± 4.0	92.8

The errors are expressed as standard deviation of the triplicates. nm: not measured.

suspension and incubated at 25 °C until the mycelium colonized the substrate completely (2 weeks). Then a layer of the spiked soil (9 g) was added on the surface of each solid-state culture. At this point, in the treatments of sterile soil amended with bacteria, 10^8 bacterial CFU g^{-1} dry soil were added and humidity content was adjusted to 60% of the water holding capacity. Humidity was maintained by adding deionized water once per week.

Three different types of treatments were performed: with the sterile soil (S), with the non-sterile soil (NS), and with the sterile soil inoculated with *P. aeruginosa* and *R. erythropolis* (S + Bac). For each treatment and sampling time, three replicates were used for the quantification of PAHs, enzymatic assays and the biomass quantification, respectively. Triplicate abiotic controls with the sterile soil were included to quantify the abiotic losses of PAHs.

2.7. Ergosterol extraction and quantification

Ergosterol was analyzed in homogeneously mixed samples of the soil employing a modified method used by Novotný et al. (1999). An amount of 0.5–0.8 g soil removed from the soil culture was placed in a test tube and extracted with 1 ml cyclohexane and 3 ml KOH–methanol mixture (10% w/v) for 90 min at 70 °C. Ultrasonication was applied for the first 15 min (Selecta, Spain). Then 1 ml distilled water and 2 ml cyclohexane were added; the tube was vortexed for 30 s and centrifuged at 3500 rpm for 5 min. The organic phase was recovered and the aqueous phase was washed twice with 2 ml cyclohexane. The organic phases were pooled and evaporated to dryness under N_2. The dry sterol residue was dissolved in 1 ml methanol (15 min, 40 °C), vortexed for 30 s and centrifuged in Eppendorf vials (6000 rpm, 3 min). Finally the solution was transferred to amber vials and analyzed in a Dionex 3000 Ultimate HPLC equipped with a UV detector at 282 nm, (reverse phase Grace Smart RP18 column, 250 mm × 4 mm, particle size 5 μm). Methanol was isocratically supplied at 1 ml min^{-1}. The ergosterol content was expressed in micrograms per gram of solid dry weight (μg g DW^{-1}).

2.8. Laccase and MnP extraction and quantification

The extraction of enzymes was carried out using the modfied method of Lang et al. (1998) where 30 mL sodium acetate buffer (0.16 M, pH 5) were added to 3 g of a homogenized soil sample and shaken for 30 min at 4 °C. 1.5 mL aliquots of the extracts were transferred to Eppendorf vials and centrifuged (15,000g, 15 min) and the supernatant was then analyzed. Laccase activity was measured using the first step of the method for determination of MnP (Wariishi et al., 1992) where 2,6-dimethoxy phenol (DMP) was oxidized by laccase in the absence of Mn^{2+}. One activity unit (AU) was defined as the number of micromoles of DMP oxidized per minute. The DMP extinction coefficient was 24,800 M^{-1} cm^{-1}. MnP was measured with the same method in the presence of Mn^{2+} (Wariishi et al., 1992).

2.9. PAH extraction and quantification

The soil (9 g) was separated from the straw pellet (3 g) substrate and PAHs were extracted by Soxhlet (4 h, Buchi B-811, Switzerland) using DCM/acetone mixture (200 mL 1:1, v/v). The extracts were concentrated under N_2 and dissolved in DCM. PAH concentrations in the DCM extracts were measured using an Agilent 6890N gas chromatograph equipped with a flame ionization detector (ZB-5HT INFERNO capillary column, 30 m length, ID 0.25 mm, thickness 0.25 μm).

Identification and quantification of PAHs were based on matching their retention times with a mixture of PAH standards. The standard curves were linear in a concentration range of 1–50 mg l^{-1}.

2.10. Statistical analyses

Three-way ANOVA and principal component analysis (PCA) were performed using Statistica 7.0 software package (StatSoft, USA).

3. Results

3.1. Soil colonization by fungi

Capacity of the fungi to colonize the PAH-polluted soil in the presence and absence of live bacteria was tested (Table 2). Fungal growth was assessed by visual observation and measurement of the ergosterol content. Both fungal species were capable to colonize the soil but the colonization rate and intensity were different. *I. lacteus* showed more capacity to colonize the soil than *T. versicolor*. After two weeks the soil was almost fully colonized by *I. lacteus* whereas with *T. versicolor* it took almost four weeks and the mycelium was less dense. Once *I. lacteus* colonized the soil volume, it formed a massive mycelium layer over the soil and continued expanding all over the glass tube wall. In the case of *T. versicolor*, the mycelium formed a very thin layer over the soil and stopped its growth. The contents of ergosterol in the biomass of *I. lacteus* and *T. versicolor* were found to be 2.4 and 6.6 mg ergosterol g^{-1} DW fungal biomass (cf. Rodríguez-Rodríguez et al., 2010). In terms of ergosterol, despite various growth rates observable within five weeks with both fungi, each fungus attained similar growth yields in different soil treatments after 10 weeks growth. These values were two-fold in *I. lacteus* (Table 2) but this difference is much higher when expressed in the terms of biomass due to the above-mentioned difference in the ergosterol content between the two fungi.

A three-way ANOVA was performed to assess statistical significance of the data to show that the growth of neither fungus was inhibited by the presence of bacteria, either the autochthonous microflora or the introduced *P. aeruginosa* and *R. erythropolis* (Table 3). These results partially confirmed growth interactions between *I. lacteus* and either *P. aeruginosa* or *R. erythropolis* observed in preliminary experiments on agar media using the method of Radtke et al. (1994) (not shown) where no inhibitory effects were observed on MEG and low-nitrogen mineral Kirk medium (Tien and Kirk, 1988) with either bacterium but clear growth inhibition was observed on the complex LB medium containing tryptone and yeast extract with the two bacteria applied individually.

3.2. Enzymatic activity

Laccase and MnP are extracellular enzymes produced by white-rot fungi that are related to the capacity of degradation of PAHs (Novotný et al., 2004). Their activities in soil were measured from the soil fractions at 5 and 10 weeks (Table 4). MnP was not detected in *T. versicolor*. Laccase was present in all *Trametes* cultures and its

Table 2
Fungal growth after 5 and 10 weeks of experiment expressed as μg ergosterol g^{-1} dry soil.

Treatment	Biomass content (μg ergosterol g^{-1} dry soil)			
	Trametes versicolor		*Irpex lacteus*	
	5 weeks	10 weeks	5 weeks	10 weeks
NS	38.6 ± 1.8	51.0 ± 4.9	72.0 ± 10.3	96.6 ± 11.6
S	47.7 ± 3.0	47.0 ± 4.8	47.0 ± 9,4	104.7 ± 7.2
S + Bac	49.1 ± 8.6	52.0 ± 8.7	51.1 ± 9.5	91.8 ± 11.2

The errors are expressed as standard deviation of the triplicates. Different treatments: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum (10^8 CFU g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*).

Table 3
Results of three-way ANOVA on the variation in ergosterol levels according to factors Fungus (*T. versicolor* vs. *I. lacteus*), Treatment: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum (10^8 CFU g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*) and Time (5 vs. 10 weeks). (a: statistically significant).

Variable	F	d.f.	Probability
Fungus	135	1.24	0.00a
Time	96	1.24	0.00a
Treatment	1	2.24	0.34
Fungus × *Time*	54	1.24	0.00a
Fungus × *Treatment*	6	2.24	0.01a
Time × *Treatment*	1	2.24	0.38
Fungus × *Time* × *Treatment*	6	2.24	0.01a

Table 5
Results of three-way ANOVA on the variation in laccase and MnP levels according to factors Fungus (*T. versicolor* vs. *I. lacteus*), Treatment: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum (10^8 CFU g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*) and Time (5 vs. 10 weeks) (a: statistically significant).

	Laccase			MnP		
Variable	F	d.f.	Probability	F	d.f.	Probability
Fungus	171	1.24	0.00a	476	1.24	0.00a
Time	5	1.24	0.03a	3	1.24	0.09a
Treatment	198	2.24	0.00a	3	2.24	0.06a
Fungus × *Time*	2	1.24	0.17	3	1.24	0.09a
Fungus × *Treatment*	163	2.24	0.00a	3	2.24	0.06a
Time × *Treatment*	0	2.24	0.71	2	2.24	0.14
Fungus × *Time* × *Treatment*	25	2.24	0.00a	2	2.24	0.14

level was 3–9 times higher in sterile soil in comparison with the soil where live bacteria were present. It is in contradiction with the observations of Baldrian (2004) showing a significant increase of laccase production in his strain of *T. versicolor* when interacting with soil bacteria. Crowe and Olsson (2001) also described an induction of laccase activity in *Rhizoctonia solani* in the presence of an antagonistic *Pseudomonas fluorescens* on potato dextrose agar. On the other hand, the presence of innate bacteria in soil, in comparison with the sterilized soil, did not affect or even completely suppressed the laccase activity of *P. ostreatus* and *Dichomitus squalens* in the soil, respectively (Lang et al., 1998). No MnP activity was detected in the *T. versicolor* soil cultures (Table 4). Novotný et al. (1999) reported low MnP activities with another strain of *T. versicolor* growing in rich chernozem soil whereas no activity was detected in a soil with low contents of organic carbon and nitrogen. This could explain the absence of MnP in our cultures since the C and N levels in our soil were comparably low.

I. lacteus expressed both enzymes (Table 4). The specific MnP activities in *I. lacteus* ranged from 21 to 70 mU/μg ergosterol, which was quite similar to 59 and 81 mU μg^{-1} ergosterol measured in *P. ostreatus* and *T. versicolor* growing in chernozem soil, respectively (Novotný et al., 1999). The enzyme levels were slightly increasing during cultivation, except for the culture where the sterile soil was contaminated with the two soil bacteria. The laccase levels in soil in the *I. lacteus* cultures were low, probably due to the firm binding of the enzyme to the fungal mycelium (cf. Svobodová et al., 2008).

A three-way ANOVA was performed to assess statistical significance of the data (Table 5).

3.3. PAH removal

In order to quantify the PAH removal by the fungi, the abiotic controls were measured at each sampling time and used for correction. The PAH abiotic losses represented 15 and 21% of the total PAHs after 5 and 10 weeks, respectively. The recovery values measured immediately after spiking the soil for the individual PAHs were high (Table 1) but the recovery was reduced in later phases of the experiment for a majority of the mixture components (Tables 6 and 7). NAP and APE were probably removed due to their high volatility but the rest of PAH molecules that were not recovered by the extraction method was probably sorbed to soil humate (cf. Bogan et al., 1999; Zang et al., 2007; Cofield et al., 2008).

Both fungi were capable to reduce the initial PAH content. *T. versicolor* showed more efficiency in PAH removal for all PAHs tested, compared to *I. lacteus*, although its capacity to colonize soil was significantly lower (Tables 6 and 7; Table 2). In *I. lacteus*, generally, the removal values were higher in the sterile soil without bacteria. This behavior was observed also in *T. versicolor*, namely in the case of PHE, FLT, PYR and BaP. The ability of both fungi to remove PAHs from the sterile soil was inhibited by the addition of the two soil bacteria, as was clearly shown for PHE, FLU and BaA in *T. versicolor* and PHE and FLU in *I. lacteus* (Tables 6 and 7). In the case of ANT whose amount was reduced by both fungi to about one half, this effect was very low or nonexistent.

PCA was performed to analyze the changes in PAHs concentrations by both fungi in the different treatments and time. The first two principal components (Fungus, Treatment) explained 53% and 29% of the data variability, respectively. A three-way ANOVA was performed to assess statistical significance of the data. The results indicated that *T. versicolor* was better in PAHs removal under the different treatments with respect to *I. lacteus* and increased the removal when increasing the treatment period (Table 8).

Figs. 1 and 2 show the removal of different groups of PAHs by the two fungi. In the sterile soil, *T. versicolor* was capable to degrade 34% of the total PAHs within 5 weeks and this amount increased to 50% until 10 weeks. The degradation of 2–3-ring PAHs was significantly higher compared to the other PAHs after 5 weeks but this difference decreased after 10 weeks. The degradation capacity of *T. versicolor* was negatively affected in the presence of bacteria; the removal of the total PAHs after 10 weeks was reduced almost two-fold. The negative effect of living bacteria did not appear in the case of 4-ring PAHs (Fig. 1). *I. lacteus* removed only 20 and 27% of the total PAHs within 5 and 10 weeks, respectively (Fig. 2). The inhibitory effect of bacteria was less pronounced than in *T. versicolor* and, in the presence of living bacteria, 5–6-ring PAHs were selectively degraded.

Table 4
MnP and laccase activity in *T. vesicolor* and *I. lacteus* soil cultures after 5 and 10 weeks of experiment expressed as AU g^{-1} dry soil.

Fungus	Treatment	Enzyme activity (AU g^{-1} dry soil)			
		MnP		Laccase	
		5 weeks	10 weeks	5 weeks	10 weeks
Trametes versicolor	NS	nd	nd	2.3 ± 0.9	1.6 ± 0.2
	S	nd	nd	14.6 ± 2.9	13.2 ± 3.6
	S + Bac	nd	nd	1.5 ± 0.8	4.5 ± 1.3
Irpex lacteus	NS	1.5 ± 0.4	2.4 ± 0.3	0.4 ± 0.1	0.3 ± 0.0
	S	1.3 ± 0.2	4.5 ± 2.3	0.3 ± 0.1	0.4 ± 0.1
	S + Bac	3.6 ± 1.2	2.1 ± 0.8	0.4 ± 0.0	0.5 ± 0.0

The errors are expressed as standard deviation of the triplicates. Different treatments: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum (10^8 CFU g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*). nd: not detected.

4. Discussion

Competition with soil bacterial microflora is one of important factors in bioremediation of PAHs in contaminated soils by white-rot fungi that is not well understood (Field et al., 1995; Mougin, 2002). This effect was studied using the indigenous soil microflora and

Table 6
Residual concentrations of PAHs expressed as μg PAH·g^{-1}dry soil in soil after treatment with *I. lacteus*.

PAH	Residual concentrations after 5 weeks (μg PAH g^{-1} dry soil)				Residual concentrations after 10 weeks (μg PAH g^{-1} dry soil)			
	Abiotic control	NS	S + bac	S	Abiotic control	NS	S + bac	S
Naphtalene	nm	nm	nm	nm	nm	nm	nm	nm
Acenaphthylene	7.7 ± 1.4	7.1 ± 0.7	6.2 ± 0.2	5.8 ± 0.3	6.0 ± 0.5	6.5 ± 1.3	4.7 ± 0.2	5.4 ± 0.4
Acenaphthene	nq	nq	nq	nq	nq	nq	nq	nq
Fluorene	10.9 ± 1.6	3.2 ± 0.9	1.2 ± 0.5	nq	8.5 ± 1.2	nq	nq	nq
Phenanthrene	51.5 ± 2.6	63.7 ± 2.0	48.1 ± 1.1	43.3 ± 1.3	48.2 ± 1.8	45.4 ± 1.1	38.4 ± 1.5	24.1 ± 0.8
Anthracene	18.8 ± 1.3	9.9 ± 0.6	8.8 ± 0.3	7.4 ± 0.4	14.2 ± 1.0	7.4 ± 0.7	7.2 ± 1.7	8.1 ± 0.7
Fluoranthene	41.3 ± 2.4	43.2 ± 1.5	40.6 ± 0.2	36.6 ± 1.6	39.6 ± 1.8	42.2 ± 1.1	39.8 ± 1.9	34.3 ± 1.3
Sum of 2–3-rings PAHs	130.2 ± 4.4	127.0 ± 2.8	104.9 ± 1.3	93.1 ± 2.0	116.4 ± 3.0	101.5 ± 2.1	90.2 ± 2.9	71.9 ± 1.8
Pyrene	33.2 ± 1.9	29.2 ± 1.3	30,3 ± 0.8	28.0 ± 0.9	31.6 ± 1.2	31.0 ± 1.7	28.4 ± 01.0	29.6 ± 0.5
Benz[a]anthracene	18.7 ± 0.4	18.5 ± 1.2	17.6 ± 1.3	16.2 ± 1.4	18.9 ± 0.6	16.3 ± 1.7	13.5 ± 1.0	15.7 ± 1.6
Chrysene	15.3 ± 0.7	17.3 ± 0.9	17.6 ± 1.5	16.8 ± 0.9	13.9 ± 0.7	15.2 ± 1.5	12.8 ± 0.6	13.8 ± 1.2
Benzo[b]fluoranthene	9.0 ± 0.2	10.5 ± 0.1	12.2 ± 0.0	11.5 ± 0.1	8.6 ± 0.3	10.2 ± 0.4	8.3 ± 0.1	9.3 ± 0.3
Benzo[k]fluoranthene	11.3 ± 0.3	12.9 ± 0.5	13.0 ± 1.1	12.5 ± 0.2	11.3 ± 0.7	11.2 ± 0.1	9.1 ± 0.4	10.5 ± 0.6
Sum of 4-rings PAHs	87.5 ± 2.1	88.3 ± 2.1	90.6 ± 2.4	84.9 ± 1.9	84.3 ± 1.7	83.9 ± 2.9	72.0 ± 1.6	78.9 ± 2.1
Benzo[a]pyrene	11.1 ± 0.3	6.2 ± 1.3	6.6 ± 1.0	6.2 ± 1.3	10.7 ± 0.6	7.5 ± 1.3	5.6 ± 1.2	7.2 ± 0.8
Dibenzo[a.h]anthracene	7.3 ± 0.37	7.6 ± 0.9	6.7 ± 0.6	6.5 ± 0.8	7.4 ± 0.1	4.7 ± 0.2	4.2 ± 0.2	4.6 ± 0.1
Benzo[g,h,i]perylene	7.4 ± 0.04	5.8 ± 0.3	5.4 ± 0.1	5.3 ± 0.2	7.4 ± 0.3	1.8 ± 0.4	2.5 ± 0.4	2.5 ± 0.3
Sum of 5–6-rings PAH	25.9 ± 0.5	19.6 ± 1.5	18.7 ± 1.1	17.9 ± 1.5	25.5 ± 0.7	13.9 ± 1.4	12.3 ± 1.2	14.2 ± 0.8
Total PAHs	243.6 ± 4.9	234.9 ± 3.8	214.2 ± 2.9	195.9 ± 3.2	226.3 ± 3.5	199.3 ± 3.8	174.4 ± 3.6	165.1 ± 2.9

The errors are expressed as standard deviation of the triplicates. Different treatment: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum (10^8 CFU g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*). nm: not measured. nq: not quantifiable.

a defined mixture of two soil bacteria,*P. aeruginosa* and *R. erythropolis*. In the absence of live bacteria, *T. versicolor* was clearly superior to *I. lacteus*, being capable of a faster and more extensive removal of PAHs despite its weaker growth in the soil. *I. lacteus* showed a very low efficiency of removal of PYR, BaA and BkF, compared to*T. versicolor*, whereas CHRY and BbF were degraded by neither fungus. The efficiency of *I. lacteus* was similar to previous studies using the artificially-spiked or industrially-contaminated soils except for PYR, CHRY and BaP (Novotný et al., 2000; Bhatt et al., 2002). The removal of total PAHs by *T. versicolor* (49% within 10 weeks) was similar to the results obtained by Rama et al. (2001) in an industrially-contaminated soil (38% within 20 weeks), where also agriculture waste–pellets were used for inoculation. The behavior of both fungi in the absence of live bacteria thus seems to have been'standard' in our experiments.

Both fungi are characterized by their production of MnP and laccase and, consequently, the mechanisms used in the biodegradation of PAHs can be considered similar in the two organisms (cf. Collins and Dobson, 1996; Majcherczyk et al., 1998; Cerniglia and Sutherland, 2001). The main effect of the presence of live bacteria in the soil, both the indigenous microflora and the two soil bacteria, was a significant decrease of degradation of PAHs by the two fungi demonstrated for all three groups of PAHs in the case of *T. versicolor* and for 2–3-ring PAHs in the case of *I. lacteus*. Consequently, the residual total PAH concentrations exceeded those without bacteria by 15%. These results are in agreement with the observations with *P. ostreatus* where *P. putida* reduced the removal of ANT, PHE, FLT, PYR and BaP from industrially contaminated (Bhatt et al., 2002). The competition for nutrients in the soil

Table 7
Residual concentrations of PAHs expressed as μg PAH·g^{-1}dry soil in soil after treatment with *T. versicolor*.

PAH	**Residual concentration after 5 weeks** (μg PAH g^{-1}dry soil)				**Residual concentration after 10 weeks** (μg PAH g^{-1} dry soil)			
	Abiotic control	NS	S + bac	S	Abiotic control	NS	S + bac	S
Naphtalene	nm	nm	nm	nm	nm	nm	nm	nm
Acenaphthylene	7.7 ± 1.4	9.5 ± 0.3	8.5 ± 1.1	7.7 ± 0.9	6.0 ± 0.5	nq	2.3 ± 0.4	4.6 ± 0.5
Acenaphthene	nq	nq	nq	nq	nq	nq	nq	nq
Fluorene	10.9 ± 1.6	10.4 ± 1.5	5.9 ± 1.3	3.1 ± 0.9	8.5 ± 1.2	nq	nq	nq
Phenanthrene	51.5 ± 2.6	47.6 ± 1.7	38.4 ± 1.9	25.0 ± 1.2	48.2 ± 1.8	37.0 ± 0.8	31.6 ± 1.4	21.3 ± 1.4
Anthracene	18.8 ± 1.3	18.3 ± 0.1	15.4 ± 0.3	10.7 ± 1.3	14.2 ± 1.0	7.9 ± 0.8	8.4 ± 0.9	6.1 ± 1.3
Fluoranthene	41.3 ± 2.4	29.8 ± 2.0	29.5 ± 1.0	24.8 ± 1.6	39.6 ± 1.8	29.1 ± 1.9	28.3 ± 0.8	17.1 ± 1.6
Sum of 2–3-rings PAHs	130.2 ± 4.4	106.1 ± 3.0	89.3 ± 2.7	63.6 ± 2.7	116.4 ± 3.0	74.0 ± 2.2	70.7 ± 1.9	49.1 ± 2.6
Pyrene	33.2 ± 1.9	26.1 ± 1.1	22.0 ± 1.0	20.8 ± 2.0	31.6 ± 1.2	20.8 ± 1.4	20.1 ± 1.3	18.1 ± 2.0
Benz[a]anthracene	18.7 ± 0.4	18.0 ± 1.0	16.0 ± 1.0	16.1 ± 0.9	18.9 ± 0.6	13.7 ± 0.1	13.9 ± 1.5	9.9 ± 1.5
Chrysene	15.3 ± 0.7	14.4 ± 0.6	13.7 ± 1.0	13.7 ± 0.9	13.9 ± 0.7	11.6 ± 0.3	12.6 ± 0.9	11.5 ± 1.8
Benzo[b]fluoranthene	9.0 ± 0.2	9.6 ± 0.2	9.1 ± 0.3	8.8 ± 0.5	8.6 ± 0.3	7.2 ± 0.1	8.1 ± 0.4	7.5 ± 0.6
Benzo[k]fluoranthene	11.3 ± 0.3	10.5 ± 0.8	9.7 ± 0.8	10.0 ± 0.8	11.3 ± 0.7	7.9 ± 0.3	8.3 ± 0.5	8.1 ± 0.4
Sum of 4-rings PAHs	87.5 ± 2.1	78.5 ± 1.8	70.4 ± 1.6	69.4 ± 2.5	84.3 ± 1.7	61.2 ± 1.5	62.9 ± 2.2	55.3 ± 3.1
Benzo[a]pyrene	11.1 ± 0.3	13.7 ± 0.1	12.8 ± 1.1	10.1 ± 2.0	10.7 ± 0.6	7.0 ± 0.6	6.8 ± 0.6	4.7 ± 1.7
Dibenzo[a.h]anthracene	7.3 ± 0.37	7.3 ± 0.2	7.2 ± 0.4	5.5 ± 0.5	7.4 ± 0.1	4.6 ± 1.9	5.6 ± 2.0	3.7 ± 0.1
Benzo[g,h,i]perylene	7.4 ± 0.04	7.33 ± 0.1	7.1 ± 0.5	7.4 ± 0.3	7.4 ± 0.3	3.1 ± 1.7	5.0 ± 1.0	2.1 ± 0.1
Sum of 5-6-rings PAH	25.9 ± 0.5	28.3 ± 0.3	27.1 ± 1.3	22.9 ± 2.1	25.5 ± 0.7	14.7 ± 2.6	17.3 ± 2.3	10.5 ± 1.7
Total PAHs	243.6 ± 4.9	213.0 ± 3.5	186.8 ± 3.4	155.9 ± 4.2	226.3 ± 3.5	149.8 ± 3.7	150.8 ± 3.7	114.8 ± 4.2

The errors are expressed as standard deviation of the triplicates. Different treatment: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum (10^8 CFU g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*). nm: not measured. nq: not quantifiable.

Table 8
Results of three-way ANOVA on the variation in PC1 and PC2 according to factors Fungus (*T. versicolor* vs. *I. lacteus*), Treatment (NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum (10^8 CFU g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*)) and Time (5 vs. 10 weeks). (a: statistically significant).

	PC1			PC2		
Variable	F	d.f.	Probabitily	F	d.f.	Probabitily
Fungus	1831	1.24	0.00a	695	1.24	0.00a
Time	482	1.24	0.00a	4341	1.24	0.00a
Treatment	148	2.24	0.00a	5	2.24	0.12
Fungus × *Time*	55	1.24	0.00a	729	1.24	0.00a
Fungus × *Treatment*	11	2.24	0.00a	84	2.24	0.00a
Time × *Treatment*	35	2.24	0.00a	38	2.24	0.00a
Fungus × *Time* × *Treatment*	49	2.24	0.00a	4	2.24	0.02a

between the two types of microorganisms probably does not explain the adverse effect of bacteria (Rama et al., 2001).

From the perspective of removal of the individual PAHs, however, the situation was more complex. Though *I. lacteus* and *T. versicolor* are expected to use similar biodegradation mechanisms the observed inhibition effects on the removal of individual PAHs were different. In *I. lacteus*, a strong bacterial inhibition was observed in the case of PHE and FLU. This inhibition was weaker in *T. versicolor*. No bacterial inhibition of ANT removal was observed in *I. lacteus* compared to a low inhibition in *T. versicolor*. In the case of 5–6-ring PAHs, significant bacterial inhibitions were observed in *T. versicolor* but not in *I. lacteus*. A weak enhancement of the removal rate of BaP and DaA, and of BghiP by *I. lacteus* was observed in the presence of *P. aeruginosa* and *R. erythropolis* and of the indigenous microflora, respectively. In *T. versicolor*, such an effect was demonstrated only for APY in the presence of both the indigenous microflora and *P. aeruginosa* and *R. erythropolis*. Similar fungal/bacterial synergistic effect was observed when the litter-decomposing fungus *Agrocybe aegerita* or the white-rot fungus *Kuehneromyces mutabilis* were inoculated into a non-sterile soil (Sack and Fritsche, 1997). Also, during co-cultivation in a liquid medium of *Bjerkandera* sp. BOS55 with indigenous microflora from soils, activated sludge and PAH-adapted sediment, an increased mineralization of BaP occurred (Kotterman et al., 1998). Similarly, Boonchan et al. (2000) working with co-cultures of the soil-borne fungus *Penicillium janthinellum* with bacterial soil consortia in soil described an enhanced degradation of PYR, BaP and DaA, compared

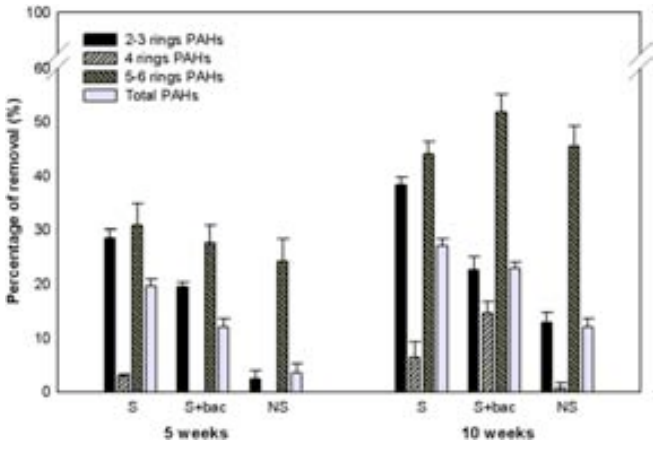

Fig. 1. PAHs removal with respect to the abiotic control by *Irpex lacteus* expressed as the percentage PAHs applied. Different treatments: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum. The errors are expressed as standard deviation of the triplicates. ($n = 3$). Different treatments: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum (10^8 CFU g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*).

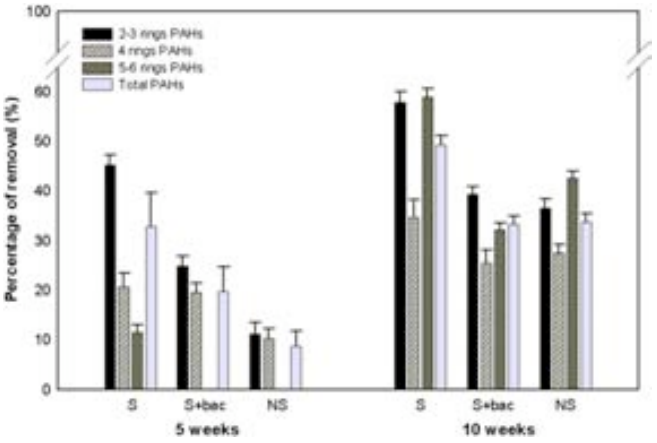

Fig. 2. PAHs removal with respect to the abiotic control by *Trametes versicolor* expressed as the percentage of PAHs applied. Different treatments: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum. The errors are expressed as standard deviation of the triplicates. ($n = 3$). Different treatments: NS: non-sterile soil; S: sterile soil: S + bac: sterile soil + bacterial inoculum (10^8 CFU g^{-1} *Pseudomonas aeruginosa* and *Rhodococcus erythropolis*).

to the individual cultures. The extent of synergism in our study was rather small and did not manifest at the level of total PAHs.

Both fungi demonstrated their capability to colonize soils in the presence of PAHs and live bacteria and thus did not confirm the image of *T. versicolor* as a fungus having problems in colonizing soil (cf. Boyle, 1995; Martens and Zadrazil, 1998). Neither the indigenous soil microflora nor the two soil bacteria did affect the growth of *T. versicolor* or *I. lacteus* in soil and thus our observations did not confirm fungal–bacterial antagonist relationships reported by Thorn and Tsuneda (1992) and Radtke et al. (1994). The growth of *I. lacteus* was more rapid in non-sterile soil and its final growth yields expressed in mg biomass g^{-1} soil calculated from the ergosterol contents measured in the two fungal strains used were 5–6-fold compared to *T. versicolor* in both the presence and absence of bacteria in soil. The growth yield values obtained for *T. versicolor* were comparable to those obtained by Rama et al. (2001) for *T. versicolor* growing in an industrially-polluted soil but 25 times higher than those of *T. versicolor* colonizing a PAH-spiked chernozem soil (Novotný et al., 1999). The growth yield of *I. lacteus* was 10-fold compared to that of *P. ostreatus* colonizing a PAH-spiked chernozem soil (Novotný et al., 1999). The superiority of *I. lacteus* compared to *T. versicolor* observed during colonization of a sterile soil (Novotný et al., 2001) was confirmed in this study but did not translate into the ability to degrade PAHs.

5. Conclusions

The comparative study investigating the effect of bacteria on growth, biochemistry and biodegradation efficiency of two model species of white-rot fungi capable of PAH degradation using bacterial stress by indigenous soil microflora or selected soil bacteria demonstrated a significant decrease of degradation of total PAHs in the presence of live bacteria. Only weak fungal/bacterial synergistic effects were observed in the case of removal of APY and some 5–6-ring PAHs but the effects were not similar in the two fungi. The bacteria tested did not affect the capability of the two fungal organisms to colonize soil and did not influence the fungal growth yields. No specific effect of bacteria on the extracellular enzyme levels was detected. The study demonstrated the bacterial effect in soil under well-defined conditions and showed that more research is needed to investigate into the interactions between

various bacterial and fungal species and their adverse and synergistic effects with the ultimate aim to control them in bioremediation technologies.

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Bioremediation of PAHs-contaminated soil through composting: Influence of bioaugmentation and biostimulation on contaminant biodegradation

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ABSTRACT

The degradation of several polycyclic aromatic hydrocarbons (PAHs) in soil through composting was investigated. The selected PAHs included: fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, and chrysene, with concentrations simulating a real creosote sample. The degradation of PAHs (initial concentration 1 g of total PAHs kg^{-1} dry soil) was assessed applying bioaugmentation with the white-rot fungi *Trametes versicolor* and biostimulation using compost of the source-selected organic fraction of municipal solid waste (OFMSW) and rabbit food as organic co-substrates. The process performance during 30 days of incubation was evaluated through different analyses including: dynamic respiration index (DRI), cumulative oxygen consumption during 5 days (AT_5), enzymatic activity, and fungal biomass. These analyses demonstrated that the introduced *T. versicolor* did not significantly enhance the degradation of PAHs. However, biostimulation was able to improve the PAHs degradation: 89% of the total PAHs were degraded by the end of the composting period (30 days) compared to the only 29.5% that was achieved by the soil indigenous microorganisms without any co-substrate (control, not amended). Indeed, the results showed that stable compost from the OFMSW has a greater potential to enhance the degradation of PAHs compared to non-stable co-substrates such as rabbit food.

1. Introduction

According to the U.S. Environmental Protection Agency, polycyclic aromatic hydrocarbons (PAHs) are recognized as priority pollutants (Mackay et al., 1992). These pollutants are introduced in the environment as a result of natural or anthropogenic activities (Johnson et al., 2005). The deleterious properties of PAHs, such as high toxicity and carcinogenicity, have made their remediation a critical need. Today, several technologies are available to deal with these environmental contaminants (Khan et al., 2004). However, each technology has its own optimal operation conditions, which are modulated by the contaminant properties (physicochemical) or by the prevailing environment conditions. The cost of a remediation technology is a crucial factor in its implementation. In this regard, bioremediation, which relies principally on microorganisms to degrade the target contaminants, is a promising technology because of its efficiency and cost-effectiveness. In this context, composting technology is one of the biological approaches applied for remediating PAHs-contaminated soil (Cajthaml et al., 2002; Sayara et al., 2009). Nevertheless, several factors, such as the presence of specific degraders, toxicity, concentration, bioavailability, and nutrient content, are believed to influence the biodegradation of PAHs (Antizar-Ladislao et al., 2004; Cajthaml and Šašek, 2005; Covino et al., 2010; Gandolfi et al., 2010; Sayara et al., 2010a,b).

For effective bioremediation of PAHs, the overall degradation and removal rate of the contaminants must be faster than the natural attenuation processes (Mohan et al., 2008). Accordingly, bioremediation of contaminated soil is usually carried out either by stimulating the indigenous organisms through providing a favorable environment or nutrients needed for increasing the microbial activity, or by bioaugmentation through introducing single strains or consortia of microorganisms with the desired catalytic capabilities to improve the biodegradation process (Khan et al., 2004; Covino et al., 2010). In some cases, both biostimulation and bioaugmentation are applied simultaneously (Lang et al., 1998; Hamdi et al., 2007; Mohan et al., 2008; Mrozik and Piotrowska-Seget, 2010).

Several bacteria and filamentous fungi have been reported to detoxify and degrade PAHs (Boonchan et al., 1998; Hamdi et al.,

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2007; Borràs et al., 2010). In this regard, although the use of white-rot fungi as PAHs degraders has been extensively studied in liquid cultures, their application for bioremediation of contaminated soil still needs further investigation, especially in the case of treatments with complex-solid matrices, such as composting. The ability of white-rot fungi as degraders to decompose several compounds including PAHs is attributed to their non-specific enzymatic system, including the ligninolytic enzymes and the cytochrome P450 (Hamdi et al., 2007; Borràs et al., 2010). For bioaugmentation purposes, a wide range of white-rot fungi have been used to remediate PAHs-contaminated soils (Mohan et al., 2008; Mrozik and Piotrowska-Seget, 2010). Nevertheless, not all white-rot fungi are able to colonize PAHs-polluted soil due to competition with the indigenous microflora (Lang et al., 1998). Also, their degradation is correlated with the bioavailability of pollutants (Covino et al., 2010), which influences the overall process behavior.

In spite of some drawbacks and the complexity of bioaugmentation, the use of fungi for bioremediation is receiving more attention as they are rapidly incorporated by the soil matrix. Also, they have the ability to grow in environments with low nutrient concentration, low humidity, and acidic conditions (Mollea et al., 2005). Several studies have described the successful application of bioaugmentation in soil remediation processes with different organic contaminants (Mohan et al., 2008; Covino et al., 2010; Teng et al., 2010). Synergistic degradation by white-rot fungi and bacteria can also occur during the bioremediation of PAHs-contaminated soil since fungi can initially cleave the aromatic ring and then bacteria are able to further degrade the resulting metabolites. However, in some studies the introduced microorganisms fail at degrading or enhancing the depletion of the target contaminants (Wiesche et al., 2003; Silva et al., 2009; Karamalidis et al., 2010).

During the composting process and as a result of the microbial activity, the temperature increases to reach the thermophilic range (>45 °C), especially when easily biodegradable organic matter is available in the composted matrix (Ruggieri et al., 2008). Consequently, the tolerance of the implanted exogenous microorganisms to such temperature will be a key factor. Also, the introduced microorganisms find themselves in a new environment where they have to compete with the indigenous microorganisms (Lang et al., 1998).

The present study investigates the impact of bioaugmentation and biostimulation on the bioremediation of PAHs-contaminated soil. Bioaugmentation was carried out using a white-rot fungus (*Trametes versicolor* ATCC 42530), whereas biostimulation was performed using two organic co-substrates that differ significantly on the basis of their degree of stability and their organic content. Thus we used a compost obtained from the organic fraction of municipal solid waste (OFMSW) and rabbit food, which was proposed to serve as an easily available lignocellulosic substrate. In this study we examined the effect on the performance of a PAH-remediation process of the degree of stability and of the organic matter content of the co-substrates, together with the impact of adding exogenous microorganisms.

2. Materials and methods

2.1. Soil

The soil used in this experiment was an agricultural soil collected from the surface horizon (0–30 cm) in Prades (Tarragona, Spain). Texture analysis demonstrated that it consisted of 73.4% sand, 18.6% silt, and 8% clay. It was classified as sandy loam soil according to the U.S. Department of Agriculture classification. It was air-dried and sieved to 2 mm to remove any debris, and kept at 4 °C until use. Preliminary analysis demonstrated that the soil was uncontaminated, as no PAHs were detected before the soil was used in the experiments. Other properties of the soil are presented in Table 1.

2.2. PAHs contaminants

Contaminated sites are commonly found to be polluted by several types of creosote components. Consequently, different PAHs listed among the 16 U.S. EPA priority pollutants that are typically found in contaminated soils were obtained from Sigma–Aldrich (Spain), to be used as target soil-contaminants. These PAHs include: fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, and chrysene. A stock mixture solution was prepared by mixing each PAH in the required ratio to obtain an individual weight percentage (w/w) of, respectively, 30, 28, 9, 20, 3.5, 3, and 6.5%. These percentages were chosen to mimic the concentration that prevailed in a real creosote sample (Creosote lot 42-13B, Chem Service, SUGELABOR S.A, Spain) and which was determined using Method 3611B of the U.S. EPA, where the volatile part was ignored. The stock solution contained a total of 7 g of the PAHs mixture dissolved in 1000 ml of dichloromethane. Afterward, the stock solution was spiked into the soil to obtain a resulting concentration of 1 g of PAHs kg^{-1} of dry soil in all the experiments. The soil was left until the dichloromethane had completely evaporated (1 day).

2.3. Organic co-substrates

Compost derived from the source-selected OFMSW and rabbit food (in weight percentage alfalfa 30%, wheat husk 30%, barley 9%, soy 8%, beet 4%, and impurities 11%) in the form of pellets was used as organic co-substrates and inoculum carrier (bioaugmented treatments) during the experiments. Compost was obtained from a composting plant located in Barcelona, whereas rabbit food was obtained from a commercial market (Suprem, Barcelona). These two co-substrates were selected to evaluate the effect of the organic matter stability on the bioremediation processes. On the basis of their dynamic respiration index (DRI), the degree of stability of the two co-substrates differed significantly. Moreover, the two co-substrates presented a notable difference in their organic matter content, which presumably supplies organic chemicals that are lacking in soil but essential to support microbial growth and catabolic activities toward PAHs. The major properties of the co-substrates are presented in Table 1.

2.4. Fungal strain preparation

The fungus *T. versicolor* ATCC 42530 was acquired from the American Type Culture Collection. The strain was maintained by sub-culturing every 30 days on 2% malt extract (w/v) agar slants

Table 1
Characteristics of the organic amendments used and soil.

Parameter/material	Soil	Compost	Rabbit food
Moisture content (%, wb)[a]	6.64 ± 0.01	32.82 ± 0.2	11.23 ± 0.12
Organic matter content (%, db)[b]	3.68 ± 0.35	43.54 ± 0.16	91.54 ± 0.07
Total organic carbon (%, db)[b]	1.26 ± 0.02	25.27 ± 0.33	48.78 ± 2.36
Total Kjeldahl nitrogen (%, db)[b]	0.65 ± 0.14	2.14 ± 0.51	3.13 ± 0.33
pH	6.7 ± 0.02	8.37 ± 0.01	6.01 ± 0.14
Elec. conductivity (mS/cm)	0.2 ± 0.01	4.92 ± 0.13	3.99 ± 0.23
Dynamic respiration index ($mg\ O_2\ g^{-1}\ OM\ h^{-1}$)	–	1.12 ± 0.08	6.52 ± 0.11

[a] wb: wet basis.
[b] db: dry basis.

(pH 4.5) at 25 °C. Fungal mycelial suspensions were obtained by blending the mycelium grown for 7 days on a malt extract medium that contained 20 g l^{-1} of malt extract, and the pH was adjusted to 4.5 with NaOH or HCl.

2.5. Laboratory-scale composting reactors

The composting process was set up using Dewar® vessels with an operation capacity of 4.5 l. The vessels were modified and conditioned to operate in batch-mode. According to previous works, these reactors proved their efficiency to simulate composting processes. More details about the composting system configuration, including the reactors and the monitoring tools, can be found elsewhere (Sayara et al., 2009).

2.6. Experimental composting system

The artificially contaminated soil prepared as described in Section 2.2 and containing 1 g of the PAH mixture kg^{-1} soil was manually mixed with the organic co-substrates at a ratio 1:0.25 (soil:co-substrate, dry weight). In treatments where the bioaugmentation was to be evaluated, the inoculum (*T. versicolor*) was introduced (1 ml of fungal suspension/3 g of co-substrate) in the mixture. The fungal inoculum was set to obtain a biomass corresponding to approximately 3.5 μg of ergosterol ml^{-1}. In order to ensure aerobic conditions, a bulking agent consisting of wood chips was introduced at a ratio of 1:1 (v/v). This bulking agent was considered as non-biodegradable under laboratory conditions. In all treatments, tap water was added during the preparation of the composting mixture to modify the water content according to the recommended values for the composting process (50–60%). All the composting experiments were carried out in duplicate during 30 days of incubation. Previous experiments using this composting system have shown that a 30-day period was sufficient to achieve a significant degradation of most PAHs (Sayara et al., 2010a,b). The experimental setup was as follows:

Treatment 1: contaminated soil + *T. versicolor* + compost + bulking agent
Treatment 2: contaminated soil + *T. versicolor* + rabbit food + bulking agent
Treatment 3: contaminated soil + compost + bulking agent
Treatment 4: contaminated soil + *T. versicolor* + sterile compost + bulking agent

No treatment using only rabbit food alone (without *T. versicolor*) was included since previous results based on factorial experimental design studies had clearly demonstrated that the addition of rapidly biodegradable organic matter alone to soil inhibited the PAHs biodegradation process (Sayara et al., 2010a,b). Duplicate control (C) treatments consisting of contaminated soil (1 g of PAHs kg^{-1} of dry soil) alone were used to monitor the PAHs biodegradation by indigenous microorganisms without any additive. For treatment 4, the compost was sterilized by autoclaving at 121 °C for 30 min. Previous work has demonstrated that these conditions are sufficient to obtain a practical disappearance of the compost respiration activity (Pagans et al., 2007).

2.7. Sampling

The degradation of PAHs was monitored after 5, 10, 20, and 30 days of composting. Before sampling, the reactors content was homogenized by manual mixing and representative samples of about 30–40 g were collected and used for carrying out the analyses. Remixing the composting matrix during sampling is also necessary to re-establish the porosity, which decreases as a result of compaction (Ruggieri et al., 2008) and organic matter degradation. Also, water content of the composting treatments was adjusted, if necessary, at the same time.

2.8. Analytical methods

Moisture content, organic matter content (OM), Kjeldahl nitrogen, total carbon content, pH, and electrical conductivity were determined according to standard methods (The US Department of Agriculture and The US Composting Council, 2001). All the results are presented as average of duplicates with standard deviation.

2.9. Respiration indices

These tests were used to evaluate and compare the microbial activity prevailing in the applied co-substrates and the composting mixtures of each treatment. Specifically, a dynamic respirometer was built as described by Adani et al. (2006). Briefly, about 150 g of sample were placed in a 500-ml Erlenmeyer flask and incubated in a water bath at 37 °C. Meanwhile, previously humidified air was continuously supplied to the sample to ensure aerobic conditions. Two respiration indices were calculated from the oxygen vs. time curve:

I) Dynamic respiration index (DRI): This value represents the average oxygen uptake rate during the 24 h of maximum activity measured as oxygen uptake rate (OUR).
II) AT_5: This represents the cumulative oxygen consumption during 5 days of maximum respiration activity without considering the lag phase.

The DRI and AT_5 values are expressed in mg O_2 g^{-1} OM h^{-1} and mg O_2 g^{-1} OM, respectively. More details about the respiration test and the system configuration can be obtained elsewhere (Ponsá et al., 2010a).

2.10. PAHs and PAHs metabolites analysis

PAHs in the composting treatments were quantified by gas chromatography (GC) according to a protocol described previously (Sayara et al., 2010b). PAHs metabolites were identified by GC–MS using an Agilent HP 6890 Series II GC coupled to a mass selective detector by electronic impact ionization (Agilent HP 5973) and a HP5-MS (Agilent) column (30 m × 0.25 mm × 0.25 μm). The operating conditions of the chromatograph were as follows: injector (splitless 1 min), 320 °C; injection volume, 1–3 μl (depending on the sample); oven temperature, 50 °C (1 min); ramp, 7 °C min^{-1}; final temperature, 320 °C; and carrier gas, He at 0.7 ml min^{-1}. The detector worked in a solvent delay mode (3.2 min) and the mass range measured was 40–400 (*m*/*z*). The detected products were identified by comparing the mass spectra with data from the Wiley 7® library.

2.11. Laccase extraction and quantification

The extraction of laccase was carried out according to a modified method described by Lang et al. (1998). One and one half milliliters of the extract were transferred to Eppendorf vials and centrifuged at 15,000 × *g* for 15 min. The supernatant was then analyzed. Laccase activity was measured using the first step of the method for determination of manganese peroxidase (MnP) (Wariishi et al., 1992), where 2,6-dimethoxy phenol (DMP) is oxidized by laccase. One unit of activity (AU) was defined as the number of micromoles of DMP oxidized per minute. The DMP extinction coefficient was 24,800 M^{-1} cm^{-1}.

2.12. Ergosterol extraction and quantification

Ergosterol was analyzed in homogeneously mixed samples of the soil-phase cultures according to Borràs et al. (2010). 0.5–0.8 g from each sample were removed and placed in a test tube to be extracted with a mixture of 1 ml cyclohexane and 3 ml of KOH–methanol solution (10% w/v) for 90 min at 70 °C. Ultrasonication was applied for the first 15 min (Selecta, Spain). Then 1 ml of distilled water and 2 ml of cyclohexane were added; the tube was vortexed for 30 s and centrifuged at 3500 rpm for 5 min. The organic phase was recovered and the aqueous phase was washed twice with 2 ml of cyclohexane. The organic phases were pooled and evaporated to dryness with nitrogen gas. The dry sterol residue was dissolved in 1 ml methanol for 15 min at 40 °C, vortexed for 30 s, and centrifuged in Eppendorf vials at 6000 rpm for 3 min. Finally the resulting solution was transferred to amber vials and analyzed in a Dionex 3000 Ultimate HPLC equipped with a UV detector at 282 nm, using a reverse-phase Grace Smart RP18 column (250 mm × 4 mm, particle size 5 μm). Methanol was isocratically supplied at 1 ml min^{-1}. The ergosterol content was expressed in micrograms per gram of solid dry weight (μg g^{-1}, dry weight).

3. Results and discussion

3.1. Characteristics of the organic co-substrates

As shown in Table 1, the organic co-substrates have a considerable source of organic matter that presumably can support the microbial activity needed for the bioremediation process. However, it is noteworthy that rabbit food is richer in organic matter (91.4%) than compost (43.5%), where the total organic carbon represents 48.78%, compared to 25.25% in compost. These contents clearly contributed to the different DRI values; the value was very high (6.5 mg O_2 g^{-1} OM h^{-1}) for rabbit food compared to the OFMSW compost (1.12 mg O_2 g^{-1} OM h^{-1}), considered stable. It is noteworthy that the stability degree of an organic substrate can reflect the availability of some chemical components like humic matter in the organic substrate. Such property is believed to play a major role in the soil bioremediation process as it facilitates PAHs desorption and consequently makes them more available for the microorganisms (Margesin and Schinner, 1997; Sayara et al., 2010b).

3.2. The composting process

Fig. 1 shows the evolution of the temperature profiles during the composting process. As expected for a composting process, an initial temperature rise was the result of the exothermal oxidation process caused by the microbial metabolism (Ruggieri et al., 2008).

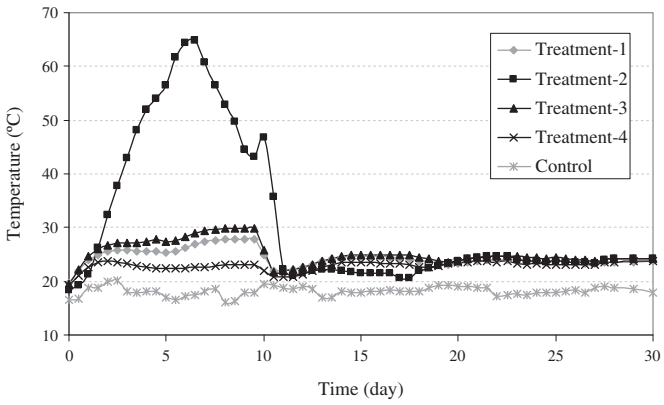

Fig. 1. Temperature profiles during the composting process.

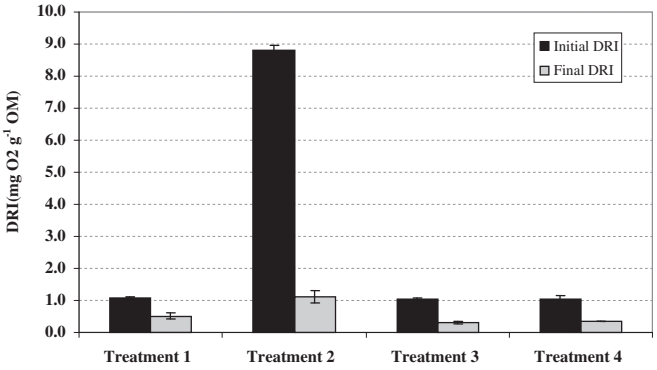

Fig. 2. Initial and final (after 30 days) values of the dynamic respiration index (DRI) of the composted materials.

The temperature profiles varied among the different treatments during the first 10 days, but afterward the patterns were similar for all treatments as the easily biodegradable fraction was depleted. In treatment 2, the temperature reached the thermophilic range after the first week, whereas all other treatments remained in the mesophilic range. This difference reflects the availability of easily biodegradable materials in treatment 2 (Table 1). However, as the available organic matter was depleted, the temperature decreased and the composted materials moved forward to the maturation phase. In this context, temperature profiles in treatments 1 and 3 were similar, whereas in treatment 4 with sterilized compost, the temperature increased to a lesser extent compared to non-sterilized compost (treatments 1 and 3). This is probably due to the absence of the compost microorganisms as a result of sterilization. The DRI values (Fig. 2) corroborated the temperature behavior of each treatment, indicating the suitability of the DRI test to monitor the progress of the organic matter stability during the composting process (Ponsá et al., 2010b).

3.3. Degradation of PAHs

Fig. 3 shows the remaining PAHs (as total PAHs) after 5, 10, 20, and 30 days of composting. Degradation yields reaching 89% after 30 days were obtained in treatments 1 and 3 where compost was added as co-substrate. Nevertheless, a degradation rate of 71% was achieved in treatments with rabbit food as co-substrate whereas only 29.5% of the PAHs were degraded in the control (C). Although four duplicate treatments were performed, a correlation coefficient of 0.95 was obtained. Obviously, treatments with compost as co-substrate followed

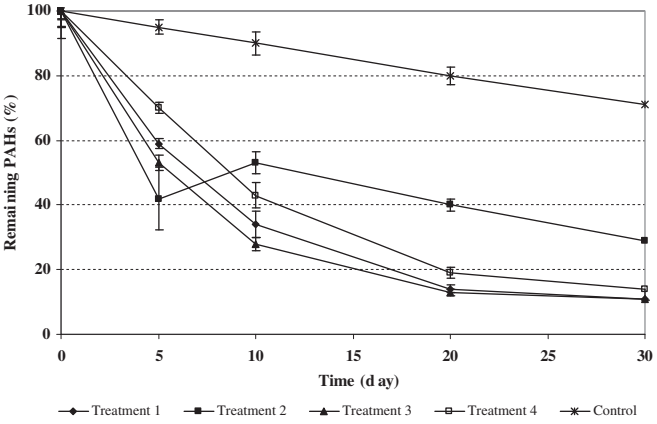

Fig. 3. Remaining PAHs (percentage) after 5, 10, 20, and 30 days of composting.

the same trend whether they were augmented with *T. versicolor* or not, indicating this organism did not contribute significantly to enhancing the bioremediation process. Moreover, in treatment 4, the degradation rate was slightly lower than for treatments 1 and 3, which might reflect a probable contribution of the compost microorganisms to the degradation of PAHs since the compost of treatment 4 was previously sterilized. These results are in accordance with previous reports where composted OFMSW demonstrated a high capacity to enhance the biodegradation of PAH-contaminated soils compared to the other amendments (Tejada et al., 2008; Sayara et al., 2009; Gandolfi et al., 2010). Results that draw attention are those obtained in treatment 2, which differed significantly from the other treatments. During the first 10 days the rate of PAH depletion was similar for all treatments as a result of the degradation of low-molecular-weight PAHs. Additionally, thermophilic temperature during the first period (Fig. 1) could have facilitated the volatilization of some of these low-molecular-weight PAHs (Margesin and Schinner, 1997). Then, the temperature increase to high levels (>55 °C) in treatment 2 might have affected/inhibited both the bacterial and fungal activities, slowing down further degradation of the PAHs (Sayara et al., 2009). On the other hand, the microbial community might have exhibited a preference for the more easily degradable substrate over the more recalcitrant ones. Consequently lower degradation rates were obtained at the end of the process. In this regard, the co-substrate appears to be an important factor affecting the efficiency of the bioremediation, which is most likely dependent on the selectivity of the components and degree of stability of the co-substrate rather than on its organic matter content (Sayara et al., 2009, 2010b).

Although the indigenous soil microorganisms were able to degrade some of the PAHs as noticed in the control experiment (29.5%), the provision of favorable conditions for these microorganisms is needed to enhance their activity. It is interesting to note that the degradation of PAHs was found to be fast during the first 10 days, but during the last stage it followed a slower removal rate, which was most likely due to the depletion of the nutrients needed for the microbial activity. The same behavior has been documented in previous works (Margesin et al., 2000; Cajthaml et al., 2002; Hamdi et al., 2007; Hafidi et al., 2008; Silva et al., 2009; Sayara et al., 2010b).

Bioaugmentation has been applied in an attempt to accelerate the degradation rate. Unfortunately, the addition/inoculation of *T. versicolor* did not enhance the remediation process, as the same trend was seen in the treatments where compost alone was applied. These results corroborate other studies in which fungi were used unsuccessfully to enhance the degradation of PAHs (Baheri and Meysami, 2002; Wiesche et al., 2003; Silva et al., 2009; Karamalidis et al., 2010). Indeed, native soil microorganisms and the microbial flora from the OMFSW compost were presumably better adapted to this particular environment. Regarding this point, it must be also considered that although fungi, and particularly *T. versicolor*, is the focus of the study, efficient degradation of PAHs can be achieved by prokaryotes, as has been reported in previous studies (Al-Mailem et al., 2010; HuiJie et al., 2011). Besides providing active microorganisms, the stable organic co-substrates improve the degradation rate by providing humic matter that facilitates the desorption of PAHs (Tejada et al., 2008; Gandolfi et al., 2010; Karamalidis et al., 2010; Sayara et al., 2010a,b).

The effect of incubating the compost at a fixed temperature of 37 °C on the degradation of PAHs was evaluated using the ideal conditions described in Section 2.9. Under those conditions, the remaining total PAH component after five days was 28%, 47%, 38%, and 48% for treatments 1, 2, 3, and 4, respectively. Accordingly, a fixed temperature of 37 °C was found to enhance the degradation process to a notable extent in treatment 1 compared to treatment 3,

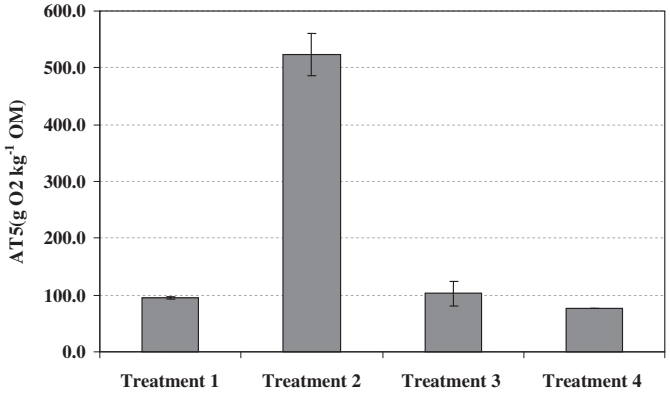

Fig. 4. Values of the cumulative oxygen consumption during 5 days (AT_5) in the treatments.

suggesting that in this situation, both the exogenous and indigenous microorganisms contributed synergistically to the degradation of PAHs although no enzymatic laccase activity was observed during the incubation period. On the contrary, in treatment 2 and under the same conditions, less degradation was obtained in spite of easily available organic matter. The amount of oxygen consumed during 5 days in treatment 2 was higher than for the other treatments (Fig. 4); however, the higher activity most likely reflected the degradation of the easily degradable organic matter rather than the PAHs. These observations confirm that stable organic co-substrate and mesophilic temperature are most suitable for this bioremediation process (Haderlein et al., 2006; Sayara et al., 2010a,b).

3.4. Respiration tests

Microbial activity in each treatment was monitored by measuring its DRI at the beginning and at the end of the incubation period (after 30 days) (Fig. 2). The AT_5 value was also determined (Fig. 4) for each treatment. This information is a useful indicator of the microbial activity found within the treatments, reflecting any change that could take place in the treated materials (Ponsá et al., 2010a). The initial microbial activity of the treatment with rabbit food (treatment 2) was high when compared to other treatments (Figs. 2 and 4). However, the composting period was able to stabilize the material. Thus, DRI values are similar for all treatments by the end of the process. It is important to point out that

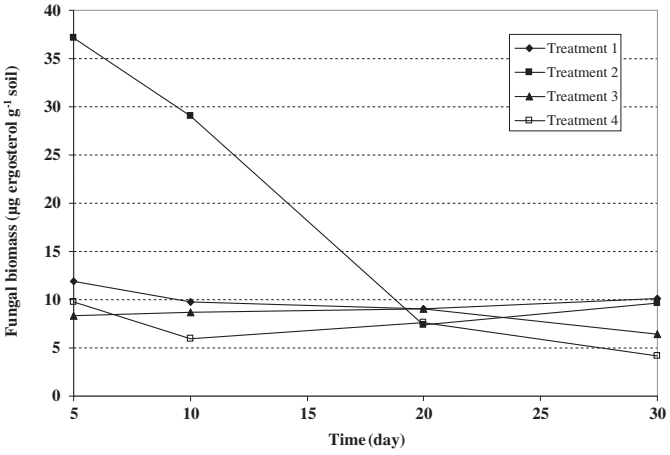

Fig. 5. Fungal biomass evolution in the different treatments during the composting process. Initial fungal biomass was not available for the different treatments.

Table 2
Identification of metabolites produced during the composting process.

Retention time (min)	Molecular mass	*m/z* of fragment ions (relative intensity)	Structural suggestion
20.09	180	180 (100), 152 (42.1), 126 (7.5), 98 (3), 76 (12.6), 63 (7.4), 50 (2.6)	9-fluorenone
20.14	182	181 (100), 165 (18.6), 152 (52.3), 139 (3.5), 126 (6.2), 91 (6.1), 76 (20.2), 63 (7.2), 51 (4.1)	9-H-fluoren-9-ol
25.54	208	208 (98.7), 180 (100), 152 (79.7), 126 (7), 99 (2.7), 76 (28.9), 63 (5.9), 50 (11.4)	9,10-anthraquinone

bioaugmentation did not affect the microbial activity since the initial DRI values were very similar for treatments 1, 3, and 4. This is corroborated by the AT_5 values. Excluding the AT_5 value of treatment 2, the rates of PAHs biodegradation after 5 days are in accordance with AT_5 values, where treatments 1 and 3 presented almost the same results. Nevertheless, it is noteworthy to mention that treatment 4 was less active (Fig. 4) due to the presence of sterilized compost, which was reflected by a lower AT_5 value and a lower level of PAHs degradation (30%) (Fig. 3) during that period. Usually, as the first period of bioremediation is characterized by a rapid decrease in the contaminants' concentration, especially those of low-molecular-weight (Hamdi et al., 2007; Silva et al., 2009; Sayara et al., 2010b), the values of AT_5 were a reliable measure of the biological activity within the composted materials.

3.5. Enzymatic activity and fungal growth

As some of the PAHs are characterized by their low bioavailability (chrysene, benzo(a)anthracene), we have introduced *T. versicolor* with an extracellular enzymatic system in an attempt to enhance their degradation. Normally, *T. versicolor* produces laccase, which is the enzyme presumed to be involved in the degradation of PAHs. However, laccase was not detected in our assays. Therefore, the resulting biodegradation was probably caused by the indigenous microorganisms of the composted materials. However, since in this study no samples were collected before 5 days, we are unable to conclude whether the implanted fungus had any effect on the degradation of PAHs before this time.

Success when introducing exogenous microorganisms is not always totally guaranteed, especially in the case of white-rot fungi, which are not soil microorganisms (Borràs et al., 2010). For instance, introducing an adequate co-substrate is usually more efficient, as the added compost is most likely simultaneously providing both the microflora (bioaugmentation) and nutrients (biostimulation) (Sayara et al., 2009). According to Lang et al. (1998) and Mrozik and Piotrowska-Seget (2010), several biotic factors can influence the bioaugmentation process, being usually the competition between the indigenous and exogenous microorganisms for the limited carbon sources as well as the antagonistic interactions and predation by protozoa and bacteriophages. Also, native species diversity may act as a resistance barrier to the invasion of non-native species (Kennedy et al., 2002). These factors play an essential role in the bioaugmentation process and its final results.

Regarding the fungal biomass that was measured in terms of ergosterol per grams of soil, which is an important indicator of the viable fungal biomass, it was found present in all soil treatments but varied greatly during the first 10 days, depending on the amendment used (Fig. 5). In treatment 2, it can be seen that the biomass content quickly increased because of the availability of high amounts of easily biodegradable organic matter (Table 1) and adequate aeration, which were favorable for fungal growth conditions. This was corroborated by the temperature rise (Fig. 1) and high oxygen consumption (Fig. 4). It is important to remark that high temperatures might influence the microbial growth, especially because temperatures above 55 °C normally disturb the microorganisms. In treatments 1 and 3 the fungal biomasses were similar. This is probably due to the fact that the implanted fungus had to compete with the indigenous microorganisms.

3.6. Identification of degradation products

Aerobic biodegradation of the studied PAHs was followed by monitoring the metabolites. The presence of these metabolites can be considered an indicator of the bioremediation process, although the PAH load can be reduced through humification processes involving organic matter from soil and compost (Ferrarese et al., 2008). Polar metabolites (Table 2) derived from anthracene and fluorene were identified in all treatments. The main product of fluorene was 9H-fluorenone, which was detected at days 5 and 10. At day 20, 9H-fluorenone was not detected but 9H-fluorenol was. These two metabolites have been described as metabolites from the degradation of fluorene by white-rot fungi (Bezalel et al., 1996). Two major metabolites were detected from anthracene: anthrone and anthraquinone. Both compounds have previously been reported as by-products during metabolism of anthracene by white-rot fungi (Hu et al., 2009). The products from biodegradation of the other PAHs could not be detected although its overall concentration decreased in the soil. One hypothesis could be that degradation products were bonded to organic matter of soil or were further degraded by fungi or native microflora.

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Soil colonization by *Trametes versicolor* grown on lignocellulosic materials: Substrate selection and naproxen degradation

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ABSTRACT

The ability of *Trametes versicolor* to degrade soil pollutants has been widely studied. However, the use of such fungus in real soil applications has lead to dissimilar results mainly due to soil colonization limitations. Therefore, it is important to investigate techniques to improve the survival of this white rot fungus in soils. In the present study, several processed and unprocessed low-cost lignocellulosic substrates were employed as inoculum carriers for fungal growth prior to application in soil for bioaugmentation. The fungal growth was determined by means of laccase activity and ergosterol determinations; additionally, the degrading capacity was measured by the naproxen degradation test (ND24). Although *T. versicolor* was able to colonize all materials, the colonization and enzymatic production was higher on processed agricultural wastes with relative low C/N ratios than in raw lignocellulosic substrates. Soil colonization was successful under both sterile and non-sterile conditions when amended with processed agricultural wastes, yielding even higher laccase production in non-sterile conditions. Moreover, *T. versicolor* was able to degrade significant amounts of spiked naproxen after 24 h in sterile and non-sterile soil cultures, showing the best results when using a material based on wheat straw as carrier.

1. Introduction

The ability of white rot fungi (WRF) to degrade soil pollutants has been broadly studied (Pointing, 2001; Gao et al., 2010), even though such contaminants are seldom present in wood, the natural habitat of this ecophysiological group of fungi. In addition, it is important to investigate ways to improve the survival of WRF in polluted soils, an unusual habitat to such organisms. This research is especially difficult due to problems associated to fungal biomass measurement in solid state and the detection of ligninolytic enzymes. Moreover, overcoming competition with soil native microflora maintaining the fungal degrading capacity remains challenging.

Soil is distinct from wood environment in many aspects. It contains a reduced amount of nutrients, in different forms than wood and presents a heterogeneous spatial distribution (Baldrian, 2008). The growth of WRF in soils is limited due to the reduced accessibility to nutrients, particularly carbon and nitrogen (Boyle, 1995). Most of the protocols to employ WRF for soil bioaugmentation have been adopted from mushroom growers (Bennet et al., 2001) and consist in the production of fungal inocula from lignocellulosic wastes in a process known as solid-state fermentation (SSF). Generally, WRF are introduced into soil pre-grown on these inexpensive lignocellulosic substrates used as inoculum carriers, such as corn–cobs, sawdust, wood chips and/or wheat straw, which are subsequently mixed with the polluted soil (Barr and Aust, 1994).

Usually, the larger the inoculum biomass, the faster and more successful is the establishment of the fungus in the soil (Leštan et al., 1996). Special care is required when balancing the carbon and nitrogen ratio in the substrates, which has a significant influence on the degrading performance of WRF. The selection of a suitable inoculum carrier can easily overcome the lack of nutrients and allow soil colonization (Lang et al., 1997; Mougin et al., 1997; Borràs et al., 2010).

In soil, colonization ability of WRF is affected due to factors such as porosity, pH, temperature and the presence of toxic compounds (Baldrian, 2008). However one of the major issues affecting this ability is the presence of indigenous soil microorganisms. Liang and McFarland (1994) demonstrated the striking difference at colonizing

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sterile and non-sterile soil. Under non-sterile conditions, interactions of fungi with soil microorganisms result in a higher demand of nutrients from the inoculum substrate for an optimal colonization (Gramss, 1979). Different species of WRF can be grouped as weak (*Ganoderma* sp., *Dichomitus* sp.) or strong competitors (*Pleurotus* sp., *Phanerochaete* sp., *Trametes* sp.) depending on their ability to colonize non-sterile soil (Walter et al., 2005; Šnajdr and Baldrian, 2006; Baldrian, 2008). However, previous studies reported contradictory results when trying to inoculate *Trametes versicolor* into soil (Novotný et al., 1999; McErlean et al., 2006; Ford et al., 2007; Borràs et al., 2010).

Soil colonization usually correlates with the increase of activity of ligninolytic enzymes and coupling enzymes (Lang et al., 1998; D'Annibale et al., 2005; Šnajdr and Baldrian, 2006). This fact is interesting for applications in bioremediation, since these enzymes are responsible for the transformation of some xenobiotics. Organic compounds and humic substances present in lignocellulosic substrates can act as natural enzyme-mediators, thus increasing the degradation capacity of fungi in soil (Zavarzina et al., 2004).

The monitoring of fungi in bioremediation and/or soil colonization processes is based on the estimation of fungal biomass, enzymatic activity and biodegradation capacity. Ergosterol quantification is an indicator of active fungal biomass in the solid matrix (Bååth, 2001; Barajas-Aceves et al., 2002). Laccase is the enzyme secreted in highest amounts by *T. versicolor* and is involved in biodegradation mechanisms as well as interactions with other microorganisms (Baldrian, 2004, 2006). Additionally, it acts as an indicator of the enzymatic machinery state of the fungus. Finally, ND24 is a novel biodegradation test developed to monitor the degradation capacity status of the fungus (Rodríguez-Rodríguez et al., 2010b); it employs naproxen as a model spiked xenobiotic of fast removal, whose degradation is evaluated after 24 h.

The present work aims to assess the potential of several agro-industrial wastes to support the growth of *T. versicolor*, with respect to biomass and enzymatic production for further application in soil bioaugmentation. Degradation ability of the fungus is also evaluated in spiked soil using biopile-like microcosms systems amended with the lignocellulosic substrates as inoculum carriers.

2. Materials and methods

2.1. Chemicals and fungal strain

Naproxen ((S)-(+)-methoxy-α-methyl-2-naphthalene acetic acid, 98%) and ergosterol (ergosta-5,7,22-trien-3β-ol, >95%) were obtained from Sigma–Aldrich Co (St. Louis, MO).

The strain *T. versicolor* ATCC 42530 was obtained from the American Type Culture Collection and maintained by subculturing every 30 days on 2% malt extract agar slants (pH 4.5) at 23 °C. Blended mycelial suspension was prepared according to Borràs et al. (2010).

2.2. Substrate materials and soil

2.2.1. Lignocellulosic substrates

Several lignocellulosic substrates were employed for the culture of *T. versicolor* in solid-state fermentation. They were grouped in two different types of substrate: i. - agricultural wastes: maize stalks (N1), wheat straw (N2), rice husks (N3), pine stardust (N4) - and ii. - agricultural wastes processed for animal feeding: wheat straw pellets (P1, ATEA Praha s.r.o., Czech Republic), and two kinds of rabbit feedstock (P2, Suprem®, Catalunya, Spain and P3, Figueres®, Catalunya, Spain).

2.2.2. Soil

Soil collected from an agricultural site at Prades (Catalunya, Spain) was sieved through a 5 mm steel mesh and stored in the dark at 4 °C. Soil physical characteristics were: pH_w 5.1, pH_{KCl} 4.5, organic matter 2%, sand 77.35%, silt 12.10%, clay 10.55%, C_{org} 1.28%, N_{tot} 0.12%, water holding capacity 12.1% (w/w) and total heterotrophic aerobic bacterial count 2.4×10^5 CFU g^{-1}.

2.3. Experimental procedures

2.3.1. Cultivation conditions on substrates

Cultures were performed in 24 × 150 mm tubes (Barloworld Scientific Ltd., Staffordshire, UK) containing 3 g DW of each lignocellulosic substrate and autoclaved at 120 °C during 30 min. Tubes were inoculated with 1 mL mycelial suspension and humidity was adjusted to 60% of the water holding capacity. Cultures were incubated in static conditions at 25 °C. Additional cultures were analogously performed in 161 mL serum bottles (Wheaton, Millville, NJ) closed with teflon lined butyl stoppers and aluminium rings in order to determine CO_2 accumulation. Humidity was adjusted to the necessary holding capacity. Triplicate cultures were sacrificed for analyses in every sampling time.

2.3.2. Soil colonization

Biopile experiments were performed in tubes with the fungus pre-grown (7 days) on each lignocellulosic substrate as described in Section 2.3.1. An amount of 9 g of soil was added to the mixture and then homogenized. Cultures were incubated at 25 °C in static conditions until sampling. Triplicate cultures were sacrificed for analyses in every time-point.

2.3.3. Naproxen degradation test (ND24)

A test based on naproxen degradation in 24 h (ND24) previously defined by Rodríguez-Rodríguez et al. (2010b) was employed as an indicator to compare the degrading ability of the fungus in soil cultures amended with different substrates. Tubes described in Section 2.3.1 were filled with 0.5 g substrate and inoculated with the fungus. After 7 days 1.5 g soil were added and the content was mixed to obtain a homogeneous matrix. Triplicate 12 d-old and 21 d-old cultures were spiked with a naproxen stock solution to give a final concentration of ~0.1 mg g^{-1} DW. Additional triplicate cultures previously autoclaved were employed as heat-killed controls at every time-point. After 24 h, the complete content of the cultures was lyophilized (Virtis Sentry freeze-drying equipment, Gardiner, NY) and then subjected to soxhlet extraction (Buchi 811, Switzerland) using methanol as solvent and with the following operation parameters: *phase 1*: 20 cycles at temperature level 14; *phase 2*: 10 min, at temperature level 14 and inert gas (N_2); *phase 3*: 30 min, at temperature level 5 and inert gas (N_2). The residue of the extraction was dissolved in methanol and adjusted to 10 mL. Aliquots (1.5 mL) were centrifuged at 10,000 rpm during 5 min and the supernatant was transferred to amber HPLC vials for later analysis. Results were expressed as the percentage of naproxen degradation in 24 h by comparing the remaining naproxen concentrations in active cultures with those in triplicate heat-killed controls. The naproxen extraction efficiency was over 95%.

2.4. Analytical methods

2.4.1. Substrates characterization

Elementary composition of the substrates was conducted with the elemental analyzer EA3011 (EuroVector, Milan, Italy) by burning the samples at 1200 °C in oxygen atmosphere and subsequent quantification by gas chromatography (Barone et al., 2010). Water holding capacity was determined as described by CEN (1999), and pH_w and pH_{KCl} were determined according to CEN (2000).

2.4.2. Ergosterol quantification

Ergosterol was extracted from homogeneously-mixed samples of substrate or substrate/soil cultures as previously described

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Table 1
Physicochemical properties and elemental composition of the lignocellulosic substrates.

Substrate[a]	Field capacity ($gH_2O \cdot g^{-1}$ DW)	C (% w)	H (% w)	N (% w)	O (% w)	S (% w)	C/N (w/w)	pH_{H_2O}	pH_{KCl}
P1	3.13	41.51	5.89	1.03	51.6	<1%	40.15	5.65	5.66
P2	3.33	40.65	6.10	3.46	49.8	<1%	11.75	5.35	5.15
P3	3.37	39.93	6.21	2.50	51.4	<1%	15.98	5.31	5.13
N1	1.03	45.15	6.48	0.57	47.8	<1%	79.67	3.99	3.44
N2	3.66	42.69	5.99	0.83	50.5	<1%	51.39	6.96	6.75
N3	7.2	46.60	6.11	0.04	47.2	<1%	1075.32	5.33	4.79
N4	2.3	40.80	5.38	0.33	53.5	<1%	121.32	3.96	3.47

[a] P1: wheat straw pellets, P2: Suprem®, P3: Figueres®, N1: maize stalks, N2: wheat straw, N3: rice husks, N4: pine stardust.

(Borràs et al., 2010). Briefly, 0.5–0.8 g samples were weighed and extracted with a mixture of 1 mL cyclohexane and 3 mL KOH-methanol solution (10% w/v) for 90 min at 70 °C (sonicating for the first 15 min). Then 1 mL distilled water and 2 mL cyclohexane were added; the tube was vortexed for 30 s and centrifuged at 3500 rpm for 5 min. The organic phase was recovered and the aqueous phase was washed twice with 2 mL cyclohexane. The organic phases were pooled and evaporated to dryness with nitrogen. The residue was dissolved in 1 mL methanol for 15 min at 40 °C, vortexed for 30 s and centrifuged at 6000 rpm for 3 min. Finally the resultant solution was transferred to amber vials and analyzed in a Dionex 3000 Ultimate HPLC (Sunnyvale, CA) equipped with an UV detector at 282 nm, using a reverse phase Grace Smart RP18 column (250 mm × 4 mm, particle size 5 μm, Deerfield, IL). Methanol was isocratically supplied at 1 mL min^{-1} as eluent and retention time was ~6.8 min. Ergosterol content was expressed as milligrams per gram of solid dry weight (mg g^{-1} DW).

2.4.3. Laccase activity

Laccase was first extracted according to a modified method by Lang et al. (1998): 30 mL sodium acetate buffer (0.16 M, pH 5) were added to 3 g of homogenized sample and shaken for 30 min at 4 °C; 1.5 mL from the extracts were centrifuged at 15,000 *g* for 15 min and the supernatant was then analyzed. Enzymatic activity was measured using a modified version of the method for manganese peroxidase determination (Wariishi et al., 1992). The reaction mixture consisted of 200 μL sodium malonate (250 mM, pH 4.5), 50 μL 2,6-dimethoxyphenol (DMP, 20 mM) and 600 μL sample. DMP is oxidized by laccase even in the absence of a cofactor. Changes in the absorbance at 468 nm were monitored for 2 min at 30 °C. Results were expressed as activity units (U) per gram of solid dry weight. One U was defined as the number of micromoles of DMP oxidized per min. The DMP extinction coefficient was 24,800 M^{-1} cm^{-1}.

2.4.4. CO_2 determination

CO_2 was analyzed with a HP5890 gas chromatograph equipped with a FID detector (Martín-González et al., 2011). The chromatographic separation was done by injection of 100 μL of gas sample in a Porapac-Q column (3 m 1/8″ column) at 70 °C (isothermal conditions). Helium (5 bar) was used as carrier gas. The retention time was approximately 1.8 min.

2.4.5. Naproxen quantification

Naproxen analyses were performed using a Dionex 3000 Ultimate HPLC equipped with an UV detector as previously described (Rodríguez-Rodríguez et al., 2010b).

3. Results and discussion

3.1. Substrates characterization

The selection of an adequate support to perform soil bioremediation with WRF is essential because the success of the process depends on guaranteeing both the colonization of the solid matrix and the degrading ability of the fungus throughout the treatment period. Therefore, intrinsic parameters of the substrates influence these abilities. Elemental composition and other physicochemical features of the studied substrates are shown in Table 1. In general, fungal growth is favoured at pH values in the acidic range and maximum laccase activity occurs at pH around 5 for *T. versicolor* (Tavares et al., 2006; Stoilova et al., 2010), while values over 7.4 have shown inactivation of WRF enzymes (Lu-Chau et al., 2004). Consequently, all substrates seemed suitable for both growth and laccase production, even though the pH values of N1 and N4 were slightly acid.

Although production of ligninolytic enzymes has been typically associated with a limitation of N, the relation C/N has been considered as a better indicator of ligninolytic activity than the absolute values of C and N. Reports of optimal C/N are quite dissimilar for production of ligninolytic enzymes in *T. versicolor*. Studies in liquid media suggest that optimal ratios range from 10 to 46 (Fakoussa and Frost, 1999; Lorenzo et al., 2002; Rodríguez Couto et al., 2002). In the present case, substrates N3 and N4 showed high ratios (>100), opposite to wheat straw pellets P1, P2 and P3 which presented low ratios (<50), meanwhile values for N1 and N2 were in the middle range. Nevertheless, it has to be pointed out that the accessibility of both C and N by the fungus is not clear. Therefore, these ratios are not directly comparable. These results are in accordance with those obtained by Yadav and Tripathi (1991) that used wheat bran and wheat straw to culture *T. versicolor* in solid-state fermentation. The optimal culture conditions were 55% moisture and pH 5.5.

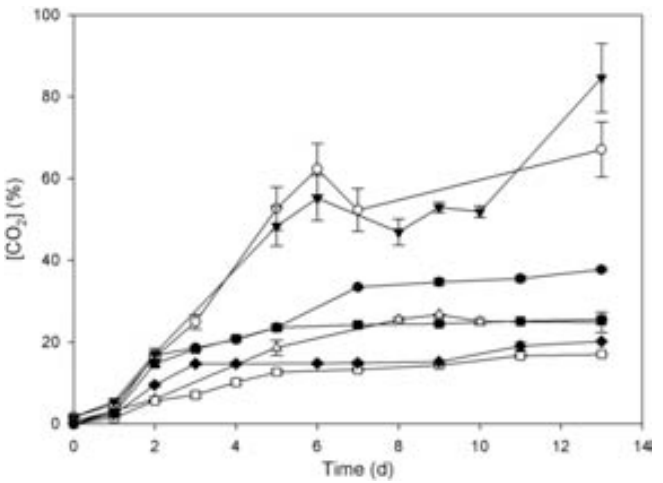

Fig. 1. CO_2 accumulation profiles during colonization of lignocellulosic substrates P1, P2, P3, N1, N2, N3, and N4 by *T. versicolor*. Water content of the cultures was equivalent to 60% the holding capacity for each substrate. Values plotted are means ± standard error for triplicate cultures.

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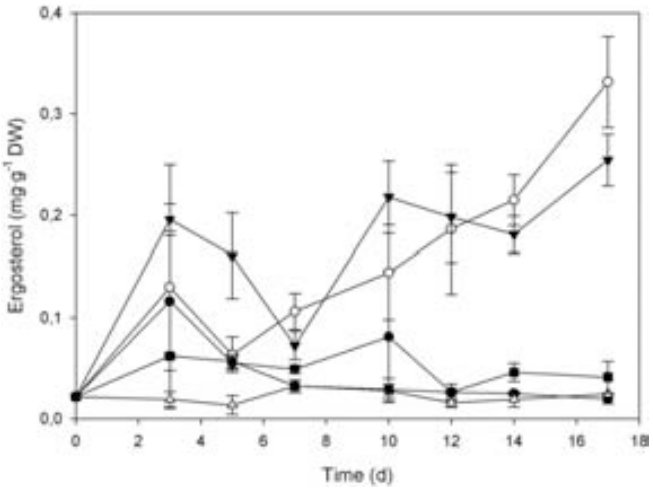

Fig. 2. Colonization of lignocellulosic substrates P1, P2, P3, N1 and N2 by *T. versicolor*. Biomass expressed as ergosterol content per unit of dry weight. Values plotted are means ± standard error for triplicate cultures.

3.2. Colonization of substrates by T. versicolor: screening

An initial colonization screening by *T. versicolor* under sterile conditions was performed in order to identify the most suitable substrates for fungal growth. Visual observations were accompanied by the determinations of CO_2 production, ergosterol content and laccase activity.

CO_2 accumulation profiles showed slight differences according to the water content (from 20% to 80% of the holding capacity) within the same substrate (data not shown), although production tended to increase as the humidity also increased. The optimal humidity content was 60% of the water holding capacity. However, different profiles were obtained for the different substrates. Fig. 1 shows the accumulation of CO_2 during substrate colonization with a water content equivalent to 60% of the maximum holding capacity. Growth on substrates P2 and P3 yielded the higher CO_2 concentrations after 13 d (>60%), followed by P1, N2 and N1 (between 25% and 40%), and finally N4 and N3 (<20%). These results, together with the visually determined poor colonization, led to rule out N4 and N3 for further experiments.

Measures of ergosterol content and laccase activity seemed to corroborate the previous results. Growth on P2 and P3 yielded high amounts of biomass with maximum values of 0.33 and 0.25 mg g^{-1} DW after 17 d, clearly surpassing the values from P1, N1 and N2 (Fig. 2). An important increase in ergosterol content was observed with P1 after 3 d, similar to that with P2, but unlike the latter, the ergosterol level gradually decreased up to the end of the culture period. Ergosterol values ranging from 0.084 to 0.464 mg g^{-1} DW have been reported for the WRF *Pleurotus ostreatus* in wheat straw and corncob cultures (Pant and Adholeya, 2007).

With respect to enzymatic activity, growth on substrate P2 yielded the highest laccase values, with a peak of 12.21 U g^{-1} DW on day 10 of culture (Fig. 3), which clearly surpassed the maximum activity detected with the remaining substrates. Laccase peaks were 1.11 and 1.37 U g^{-1} DW on days 7 and 12 for substrates P1 and P3 respectively. In spite of posterior decay after the peak values, important levels of activity were still detected up to the end of the culture period for P1, P2 and P3. Activity in substrates N1 and N2 was lower and detected only after 7 d with negligible values after day 10. These results correlate with the poor growth observed both visually and by ergosterol or CO_2 production.

Overall, growth and activity yielded better results in the substrates containing lower C/N ratios, in contrast to previous reports of enzymatic production by WRF (Pointing et al., 2000). Based on the results from this part of the screening, the substrates N1 and N2 were ruled out for subsequent experimentation.

3.3. Colonization of T. versicolor in sterile soil

T. versicolor pre-grown on either P1, P2 and P3 substrates was employed to inoculate sterile soil in order to describe the colonization profiles in terms of ergosterol and laccase activity. A lag phase in ergosterol content was observed due to the process of mixing the soil with the inoculum, which lasted until day 4 for P2 and P3, and day 6 for P1 (Fig. 4a). However, colonization of the matrix was accomplished afterwards, reaching maximum values of 0.09 (day 15), 0.28 (day 13) and 0.23 (day 13) mg g^{-1} DW for colonization with substrates P1, P2 and P3, respectively. Similar ergosterol profiles have been obtained in colonization of sewage sludge by analogous solid-phase systems with *T. versicolor* (Rodríguez-Rodríguez et al., 2010a). Meanwhile, very low levels (up to 0.002 mg g^{-1} DW) were accomplished in soil colonization with *T. versicolor* exploratory mycelium (Novotný et al., 1999).

Results of enzymatic activity are shown in Fig. 4b. As observed in the substrate colonization experiments, laccase activity was also higher when the substrate P2 was employed. However, the

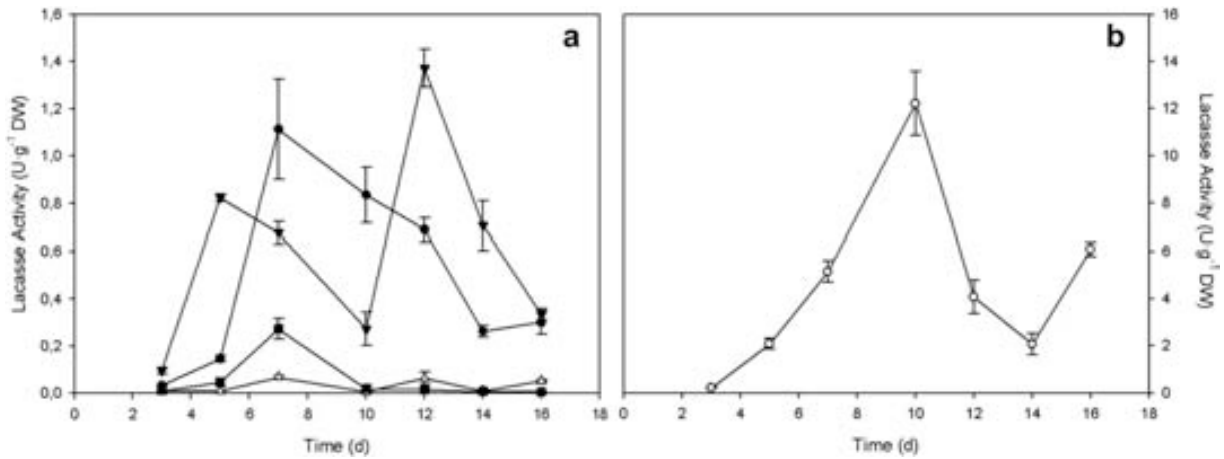

Fig. 3. Colonization of lignocellulosic substrates P1, P3, N1 and N2 (3a) and P2 (3b) by *T. versicolor*. Laccase activity expressed as U per unit of dry weight. Values plotted are means ± standard error for triplicate cultures.

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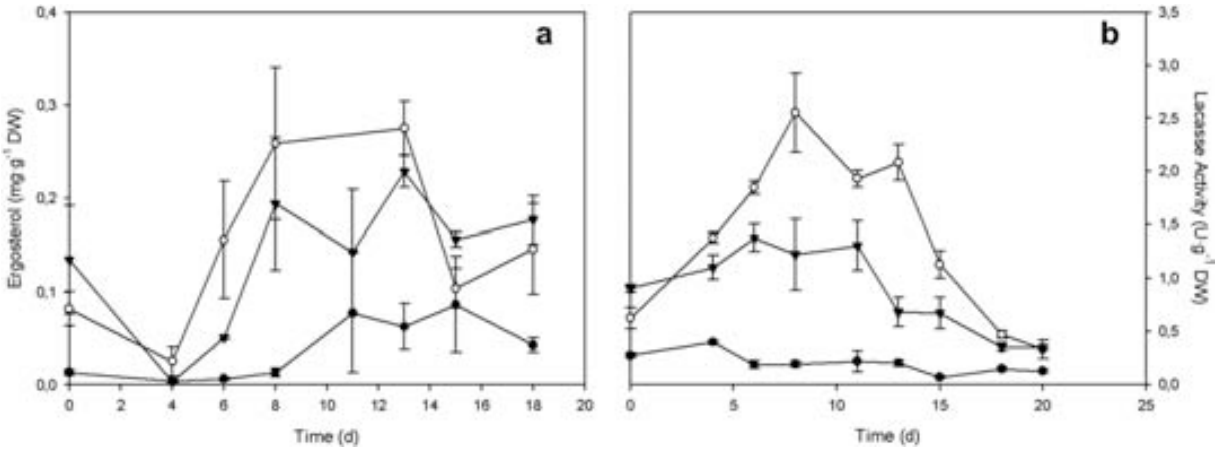

Fig. 4. Colonization of sterile soil by *T. versicolor* pre-grown on substrates P1, P2 and P3. Biomass (4a) expressed as ergosterol content per unit of dry weight and laccase activity (4b) expressed as U per unit of dry weight. Values plotted are means ± standard error for triplicate cultures.

maximum value of 2.55 U g^{-1} DW achieved by day 8 was almost 5-fold lower compared to values obtained in the experiments described in Section 3.2. A reduction of about 3-fold was also observed in the maximum value for P1, while P3 was the only substrate that yielded the same levels of activity (peak of 1.38 U g^{-1} DW) if compared to results in experiments without soil. Peak values ranging from 4 to 7 U g^{-1} DW were reported by Rodríguez-Rodríguez et al. (2010a) during sludge colonization by *T. versicolor*.

3.4. Colonization of *T. versicolor* in non-sterile soil

The next research step aimed to evaluate the performance of the substrates to support soil colonization by *T. versicolor* under non-sterile conditions, as a more real approach towards a bioremediation process. Experiments were analogous to those described in Section 3.3, but using non-sterile soil.

Values of ergosterol content were higher than those obtained with sterile soil when the substrates P2 and P3 were used (Fig. 5a), although this finding might be due to the presence of other fungi. Nonetheless, colonization was accomplished, as it was supported by visual examination. Growth was even clearer in the case of substrate P1, for which the final concentration of ergosterol (maximum of 0.11 mg g^{-1} DW) was 5 times higher than at initial time. Leštan and Lamar (1996) demonstrated the capacity of *T. versicolor* to colonize unsterilized soil when inoculated with sawdust pellets. Meanwhile, Tuomela et al. (2002) demonstrated that *T. versicolor* colonizes soil more efficiently than *P. ostreatus* and *Phanerochaete chrysosporium* when wheat straw is employed as lignocellulosic substrate. Interesting results were observed from laccase measures (Fig. 5b). Enzymatic activity yielded from the cultures amended with substrates P2 and P3 clearly surpassed that obtained in sterile conditions. Increases of 4-fold (9.38 U g^{-1} DW) and 9-fold (12.55 U g^{-1} DW) were obtained for the peak values with P2 and P3, respectively. This suggests an important fungal activity during colonization, in spite of the competition that usually takes place in the microbial ecosystems. It has been demonstrated that interaction between *T. versicolor* and microorganisms in non-sterile soil causes an increase in laccase activity (Baldrian, 2008). It is remarkable that levels over 1.5 U g^{-1} DW were still detectable by the end of the experiment with these substrates. In the case of P1, laccase activity was always below 1 U g^{-1} DW, but it was detectable all the time. Given that the extracellular ligninolytic enzymatic system of WRF has been established as an antagonistic agent against microbial soil populations, this increase in laccase activity during colonization can be interpreted as a response of *T. versicolor* to interactions with soil microorganisms (Thorn and Tsuneda, 1992; Baldrian, 2004, 2006). In this respect, White et al. (2000) suggested

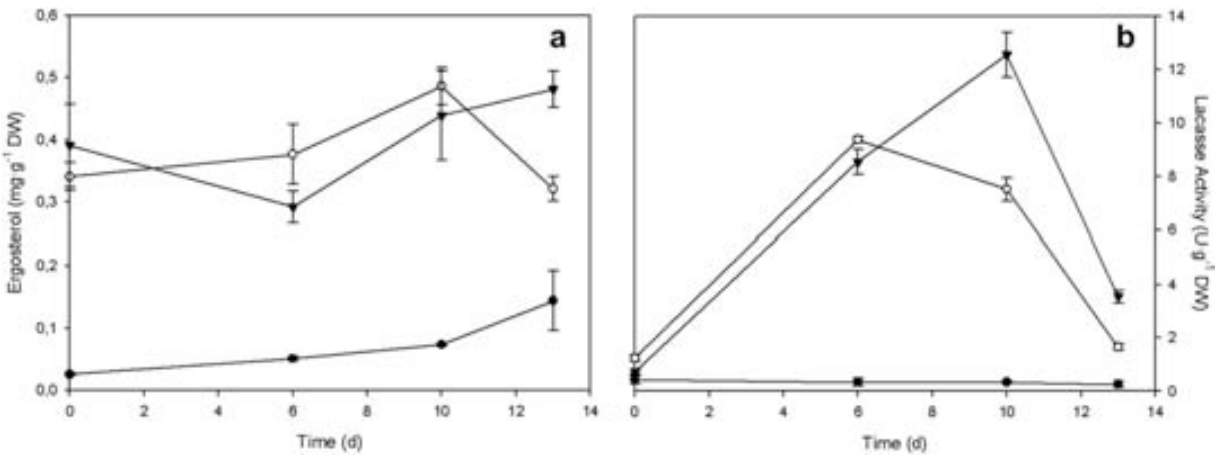

Fig. 5. Colonization of non-sterile soil by *T. versicolor* pre-grown on substrates P1, P2 and P3. Biomass (5a) expressed as ergosterol content per unit of dry weight and laccase activity (5b) expressed as U per unit of dry weight. Values plotted are means ± standard error for triplicate cultures.

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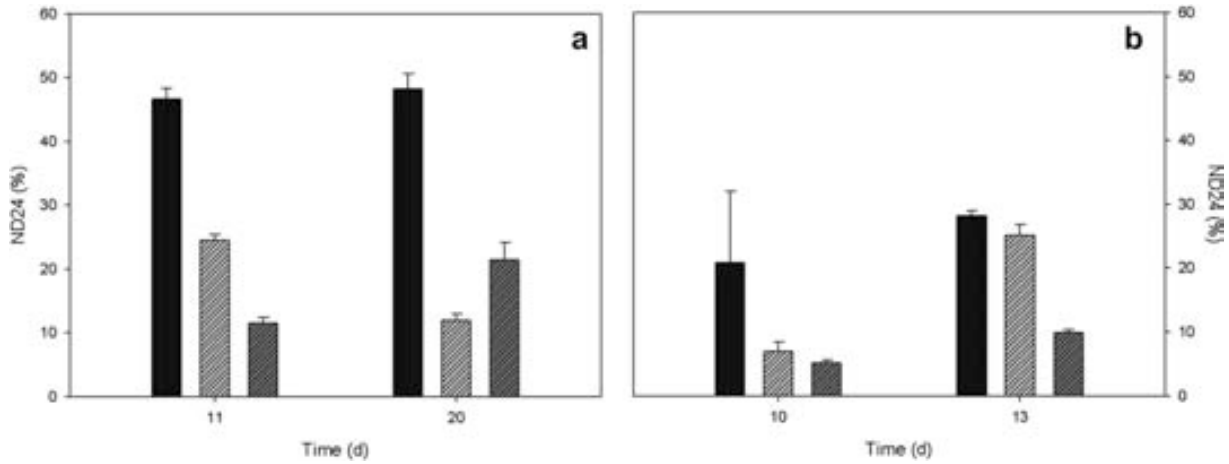

Fig. 6. Naproxen degradation (ND24) by *T. versicolor* during colonization of soil amended with substrates P1, P2 and P3. Sterile soil (6a) and non-sterile soil (6b). Values plotted are means ± standard deviation for triplicate cultures.

that both *T. versicolor* and *P. chrysosporium* produce hydrophobic compounds that enhance their survival when growing in soil by preventing bacterial growth.

Overall results indicate good colonization of soil by *T. versicolor*, although the performance was better with the substrates P2 and P3 over P1.

3.5. Degradation capacity of *T. versicolor* in soil amended with substrates

After demonstrating colonization of soil and enzymatic activity of the fungus, it was necessary to assess the ability of *T. versicolor* to degrade pollutants in that matrix. Therefore, the ND24 test was employed as a measure of the degrading ability of the fungus both in sterile and non-sterile conditions. The test is based on quantifying the degradation percentage of naproxen, an emerging-pollutant, 24 h after spiking the solid matrix.

Fig. 6a shows the results of ND24 obtained under sterile conditions after 11 d and 20 d of culture. Despite presenting lower ergosterol and laccase activity levels, naproxen degradation was higher when the substrate P1 was employed with ND24 values of around 50%. However, values with P2 and P3 were on the range of 10–25% at both time-points. Though laccase and the intracellular cytochrome P-450 complex have been involved in naproxen degradation (Marco-Urrea et al., 2010), in this case the extent of degradation was not proportional to the activity of laccase or the biomass. Similar findings were obtained during colonization of sewage sludge, with peak values of ND24 of ~56% for 17 d-cultures (Rodríguez-Rodríguez et al., 2010b).

Results from the experiments under non-sterile conditions are shown in Fig. 6b. Degradation was globally lower if compared to that achieved in sterile conditions, probably due to competition interactions with the soil microbiota. However, the trend was quite similar: higher ND24 values were obtained in soil amended with substrate P1 (in the range of 20–30%), while they were usually below 10% when P2 or P3 were used. Only 25.1% degradation was accomplished with substrate P2 at day 13. As far as the authors know, this is the first report of naproxen degradation in soil by WRF.

Though the substrates P2 and P3 seemed to be the most suitable for soil colonization in terms of biomass and laccase production, the ND24 test demonstrated that substrate P1 might be more suitable for possible soil bioremediation approaches under sterile and non-sterile conditions. Nevertheless, it is important to note that the degrading activity of *T. versicolor* was evaluated using a specific pollutant, and therefore this capacity should be tested against other compounds when applied for specific bioremediation purposes.

4. Conclusions

The colonization and enzymatic production potential of *T. versicolor* was higher on slightly processed wastes with relative low C/N ratios than in raw residues. Soil colonization was successful under both sterile and non-sterile conditions when amended with the substrates P1, P2 and P3, yielding even higher laccase production in non-sterile systems. Moreover, *T. versicolor* was able to degrade important amounts of spiked naproxen after 24 h in the sterile soil cultures, and particularly with the substrate P1 in non-sterile conditions. Interestingly, less biomass and laccase were produced with substrate P1, which reported the higher degradation values. Therefore, *T. versicolor* seems to be a potential microorganism for soil-bioaugmentation processes.

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