UNIVERSIDAD DE LA FRONTERA
Facultad de Ingeniería, Ciencias y Administración
Programa de Doctorado en Ciencias de Recursos Naturales

Degradation of polycyclic aromatic hydrocarbons by the white-rot fungus *Anthracophyllum discolor* Sp4 and nanoclay-immobilized manganese peroxidase

Doctoral Thesis
In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Sciences in Natural Resources
by

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TEMUCO – CHILE 2010

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are world widely distributed environmental pollutants that are recalcitrant, and have bioaccumulation and carcinogenic potential. The metabolic versatility of white-rot fungi and the broad substrate range of extracellular enzymes are useful for PAH oxidation. The main purpose of this work was to evaluate the role of manganese peroxidase (MnP) produced from *Anthracophyllum discolor* Sp4, a white-rot fungus from Southern Chile, in the degradation of PAHs and to evaluate the efficiency of nanoclay-immobilized enzyme in the degradation of these compounds in liquid medium and soil.

Different culture conditions for enhancing MnP production by the white-rot fungus *A. discolor* Sp4 were evaluated (Chapter 3). Effects of agitation, addition of lignocellulosic material (wheat grain), the inducer $MnSO_4$ and the surfactant Tween 80 on the MnP production in Kirk medium inoculated with *A. discolor* Sp4 were studied. The enzyme was purified and characterized under the selected culture conditions. At laboratory scale, a culture system for MnP production using tubes containing glass beads was evaluated. Two methods for measuring MnP activity were compared. The highest MnP production was obtained in Kirk medium supplemented with wheat grain and 0.25 mM $MnSO_4$ as inducer, incubated under static conditions at 30°C, reaching a maximum of 1,354 U l^{-1} after 13 days of incubation. This MnP production was 10 times higher than the production under basal conditions (stirring, without wheat grain and without inducer). The presence of Tween 80 (0.05% v/v) maintained a high and constant MnP activity during the incubation period. Two isoenzymes were purified (35 and 38 kDa, respectively). MnP enzyme produced by *A. discolor* Sp4 presented a maximal activity in the pH range between 4.5 and 5.5, a relatively high temperature tolerance (50ºC) and a high catalytic activity for 2,6-dimethoxyphenol and hydrogen peroxide. Using tubes containing glass beads, it was possible to obtain and even enhance MnP activity. In addition, this technique reduces samples volume,

simultaneously allowing a destructive sampling. The MBTH/DMAB assay has showed a higher sensitivity compared to the 2,6-DMP assay, and proved to be a suitable method for the determination of MnP in colored samples such as culture fluids supplemented with lignocellulosic support.

The potential of *A. discolor* Sp4 to degrade PAHs in liquid medium was studied evaluating the ligninolytic enzymes production (Chapter 4). In addition, 3, 4 and 5-ring PAH removal efficiency in a contaminated soil bioaugmented with *A. discolor* Sp4 in absence and presence of indigenous soil microorganisms was investigated. PAH mineralization in soil was also determined. *A. discolor* Sp4 was able to degrade phenanthrene, anthracene, fluoranthene and pyrene (simultaneously and individually) in Kirk medium associated to a high production of ligninolytic enzymes, particularly MnP. A high removal capability for phenanthrene (62%), anthracene (73%), fluoranthene (54%), pyrene (60%) and benzo*(a)*pyrene (75 %) was observed in autoclaved soil inoculated with *A. discolor* Sp4 in absence of indigenous microorganisms, associated with the production of manganese peroxidase (MnP). The metabolites detected of PAH degradation were anthraquinone, phtalic acid, 4-hidroxy-9-fluorenone, 9-fluorenone and 4,5-dihydropyrene. *A. discolor* Sp4 was able to mineralize 9% of phenanthrene. A less efficient removal of anthracene (83% vs. 62%), fluoranthene and pyrene (83% vs. 43%) was observed in soil bioaugmented by *A. discolor* in presence of indigenous microorganisms compared to the soil without the fungus (biostimulation). Under these conditions, the potential of *A. discolor* Sp4 to degrade benzo*(a)*pyrene was not observed. The main factor affecting the colonization of non-autoclaved soil by *A. discolor* Sp4 was the presence of indigenous soil microorganisms, which may compete with the fungus for the lignocellulosic substrate as a source of carbon and nitrogen, inhibiting the fungal growth.

In this study, immobilization of MnP on nanoclays was also described (Chapter 5). Approximately 75% of the enzyme was immobilized on the nanoclays through physical adsorption. Immobilized MnP from *A. discolor* Sp4 increased its stability to temperature and pH as compared to the free enzyme. PAH removal in soil by free or nanoclay-immobilized MnP (enzymatic bioremediation) in contaminated soil was evaluated.

Approximately 65% of removal efficiency for anthracene was obtained in soil by immobilized enzyme after 24 h, being about 3-fold higher compared with the control (free MnP). The action of indigenous microorganisms in the PAH degradation was not observed during this period.

The results of this study demonstrate that MnP from *A. discolor* Sp4 plays an important role in the PAH degradation and that this enzyme immobilized on nanoclays has a high potential as biocatalyst for *in-situ* bioremediation.

Degradación de hidrocarburos aromáticos policíclicos por el hongo de pudrición blanca *Anthracophyllum discolor* Sp4 y por manganeso peroxidasa inmovilizada en nanoarcillas

Resumen

Hidrocarburos aromáticos policíclicos (PAHs) son contaminantes ampliamente distribuídos en el medio ambiente, recalcitrantes, y poseen un potencial de bioacumulación y una actividad carcinogénica. La versatibilidad metabólica de los hongos de pudrición blanca y el amplio rango de sustrato de sus enzimas extracelulares permiten la oxidación de PAHs. El objetivo principal de este trabajo fue evaluar el rol de manganeso peroxidasa (MnP) producida por *Anthracophyllum discolor* Sp4, un hongo de pudrición blanca del Sur de Chile, en la degradación de PAHs y evaluar la eficiencia de esta enzima inmovilizada en nanoarcillas en la degradación de estos compuestos en medio líquido y en suelo.

Se evaluaron diferentes condiciones de cultivo para aumentar la producción de MnP por el hongo de pudrición blanca *A. discolor* Sp4 (Capítulo 3). Se estudiaron los efectos de la agitación, de la adición de un material lignocelulósico (grano de trigo), de un inductor como $MnSO_4$ y de un surfactante como Tween 80 en la producción de MnP en medio Kirk inoculado con *A. discolor* Sp4. Bajo las condiciones de cultivo seleccionadas, se purificó y caracterizó la enzima. A escala laboratorio, se evaluó la producción de MnP en un sistema de cultivo usando tubos conteniendo perlas de vidrio. Se compararon además dos métodos enzimáticos para medir la actividad de MnP. La mayor producción de MnP fue obtenida en medio Kirk suplementado con grano de trigo y $MnSO_4$ 0,25 mM como inductor, bajo condiciones estáticas a 30°C, alcanzando un máximo de 1.354 U l^{-1} luego de 13 días de incubación, actividad 10 veces más alta que la obtenida en condicion basal (agitación, sin grano de trigo ni inductor). La presencia de Tween 80 (0,05% v/v) mantuvo una actividad de MnP alta y constante durante el período de incubación. Dos isoenzimas (de 35 y 38 kDa respectivamente) fueron purificadas. MnP de *A. discolor* Sp4 presentó estabilidad para un rango de pH entre 4,5-5,5, una tolerancia a la temperatura relativamente alta (50ºC) y una alta actividad catalítica para 2,6-dimetoxifenol y peróxido de hidrógeno. En tubos

conteniendo perlas de vidrio, fue posible reproducir e incluso aumentar la actividad de MnP en comparación a cultivos en matraces Erlenmeyer. Además, esta técnica reduce el volumen de muestras permitiendo además un muestreo destructivo. Finalmente, el método MBTH/DMAB presenta una mayor sensibilidad en comparación con el método 2,6-DMP, siendo un método adecuado para la determinación de MnP en muestras coloreadas como los medios de cultivo suplementado con soportes lignocelulósicos.

Se estudió el potencial de *A. discolor* para degradar PAHs en medio líquido evaluando a la vez la producción de enzimas ligninolíticas (Capítulo 4). Además, se investigó la eficiencia de remoción de PAHs con 3, 4 y 5 anillos en un suelo contaminado bioaumentado con *A. discolor* en ausencia y presencia de microorganismos endógenos del suelo. Se determinó también la mineralización de PAHs en suelo. *A. discolor* Sp4 degradó eficientemente fenantreno, antraceno, fluoranteno y pireno (en forma simultanea e individual) en medio Kirk, produciendo gran cantidad de enzimas ligninolíticas, particularmente MnP. Se observó una alta capacidad de remoción de fenantreno (62%), antraceno (73%), fluoranteno (54%), pireno (60%) y benzo*(a)*pireno (75%) en suelo autoclavado inoculado con *A. discolor*. Se detectaron metabolitos de la degradación de PAHs como antraquinona, ácido ftálico, 4-hidroxi-9-fluorenone, 9-fluorenone y 4,5-dihidropireno. *A. discolor* Sp4 fue capaz de mineralizar un 9% de fenantreno. Se observó una menor remoción de antraceno (83% vs. 62%), fluoranteno y pireno (83% vs. 43%) en suelo bioaumentado con *A. discolor* Sp4 en presencia de microorganismos endógenos en comparación con el suelo sin el hongo (bioestimulación). Bajo estas condiciones, no se evidenció el potencial de *A. discolor* Sp4 de degradar benzo*(a)*pireno. El principal factor que afectó la colonización de *A. discolor* Sp4 en el suelo no autoclavado fue la presencia de microorganismos endógenos del suelo, los cuales pueden competir con el hongo por el sustrato lignocelulósico como fuente de carbono y nitrógeno, inhibiendo el crecimiento fúngico.

En este estudio, se evaluó además la inmovilización de MnP en nanoarcillas (Capítulo 5). Los resultados indican que la enzima fue inmovilizada en nanoarcillas mediante adsorción física en un 75%. En comparación con la enzima libre, MnP inmovilizada de *A. discolor* Sp4 alcanzó una mayor estabilidad frente a cambios de temperaturas y pH. Se evaluó la

remoción de PAHs por la adición de MnP libre o inmovilizada en nanoarcillas (bioremediación enzimática) en un suelo contaminado. Se observó un 65% de eficiencia de remoción de antraceno en suelo por la enzima inmovilizada luego de 24 h de tratamiento, siendo 3 veces más alta en comparación al control (MnP libre). No se observó la acción de microorganismos endógenos en la degradación de PAHs durante ese período.

Los resultados de este estudio demuestran que MnP de *A. discolor* Sp4 juega un rol importante en la degradación de PAHs y que esta enzima inmovilizada en nanoarcillas presenta un alto potencial como biocatalizador en la remediación *in-situ* de ambientes contaminados.

Acknowledgements

I am very grateful to CONICYT, to Professor María de La Luz Mora, Director of the PhD. Program in Sciences of Natural Resources and to many people who have contributed to the completion of this work.

Thank you to Professor María Cristina Diez, my supervisor, to invite me to participate in the project FONDECYT Nº 1050614 and for giving me the opportunity to work in the Environmental Biotechnology Lab.

Thank you to Olga Rubilar, Gonzalo Tortella, Mariela Bustamante, Grabriela Briceño, Saul Sanhueza, Raphael Cuevas, María Eugenia González and Soledad Pinto, for making the lab a nice place to work at.

Thanks to Dr. Erick Sheuermann for his support in chromatographic analysis.

Leticia, María del Pilar, John and Co. of the Department of Microbiology (SLU, Uppsala, Sweden) for giving me the opportunity to stay with my family in Sweden and to enjoy that beautiful time. To all, I have no words to thank you for the enormous support and for making me feel welcome in the lab.

Thanks to Lorena Leal, Nancy Ortiz, Janet Guiñez, Pedro Parada, for administrative assistance.

All my "classmates" for the constant support, valuable advice and friendship. Thanks to Marcelita, Aixa, Rubén, Marcela C., Oscar, Nicolás, Laura.

Dr. Leal, Dr. Alvear, Dr. Lienqueo and Dr. Hormazábal. Thanks for interesting discussions.

Professor Michael Seeger, thanks for your unconditional support during these last "10 years".

A ***Rodrigo***, por estar siempre cerca apoyándome, además de entregarme cada día una solución simple a todos mis múltiples problemas "científicos" o "logísticos".

A mis hijos ***Aitana*** y ***Mateo*** quienes han nacido y crecido viéndome estudiar, pero que siempre han logrado capturarme para compartir con ellos gran parte del día.

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Abbreviations

Å	Angstrom
ABTS	2,2´-azinobis(3-ethylbenzothiazoline-6-sulphonate)
A. discolor	*Anthracophyllum discolor*
Ant	anthracene
BaP	benzo*(a)*pyrene
^{14}C	carbon-14
CDH	cellulose deshidrogenase
cDNA	complementary deoxyribonucleic acid
DCP	dichlorophenol
2,4-DCP	2,4-dichlorophenol
2,4,6-TCP	2,4,6-trichlorophenol
2,5-DMP	2,5-dimetylphenol
DMAB	3-(dimethylamino)benzoic acid
2,6-DMP	2,6-dimethoxyphenol
Ea	activation energy
EPA	Environmental Protection Agency
Fluor	fluoranthene
GC-MS	gas chromatography-mass spectrometry
GC-FID	gas chromatography-flame ionization detector
G-MEA	glucose malt extract agar
GSH	glutathione
His_{12}-OPH	N-terminal dodehistidine tag
HPT	hydroxybenzotriazole
IEP	isoelectric point
IEF	preparative isoelectric focusing
IP	ionization potential
k	kinetic rate constant
L	laccase
LiP	lignin peroxidase

MBTH	3-methyl-2-benzothiazolinone
MnP	manganese peroxidase
OPH	organophosphorus hydrolase
PAHs	polycyclic aromatic hydrocarbons
Phe	phenanthrene
PCF	pentachlorophenol
POPs	persistent organic pollutants
Pyr	pyrene
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
ToMO	toluene o-xylene monooxigenase
TEM	transmission electronic microscopy

°C	degree Celsius
°K	degree Kelvin
eV	electronvolt
h	hours
min	minutes
kDa	kilodaltons
kg	kilogram
kJ	kilojoule
M	molar
mM	millimolar
µM	micromolar
l	litre
ml	millilitre
µl	microlitre
rpm	revolutions per minute
T	temperature

Outline of the thesis

The research reported in the thesis was conducted at the Center of Environmental Biotechnology, Scientific and Technological Bioresource Nucleus, Universidad de La Frontera, Temuco, Chile, at the Department of Chemical and Biotechnology Engineering, Faculty of Physical and Mathematical Sciences, University of Chile and at Uppsala BioCenter, Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden.

The thesis consists of six chapters. The first chapter contains a brief introduction including the hypothesis and objectives of the study (Chapter 1: Introduction).
The second chapter describes an overview of polycyclic aromatic hydrocarbons and degradation processes (Chapter 2: Theorical background).
The third chapter reports the enhanced production of manganese peroxidase of the Chilean white-rot fungus *Anthracophyllun discolor* Sp4 as well as the purification and biochemical characteristics of this enzyme (Chapter 3: Enhanced production of manganese peroxidase from *Anthracophyllum discolor* Sp4 and its characterization)
The fourth chapter describes the degradation of polycyclic aromatic hydrocarbons by the *Anthracophyllum discolor* Sp4 in liquid medium and in soil (Chaper 4: Degradation of polycyclic aromatic hydrocarbons by the Chilean white-rot fungus *Anthracophyllum discolor* Sp4).
The fifth chapter reports the immobilization of manganese peroxidase on nanoclays and the degradation of polycyclic aromatic hydrocarbons by this nanoclay-immobilized enzyme in comparison to free MnP (Chapter 5: Degradation of polycyclic aromatic hydrocarbons by free and nanoclay-immobilized manganese peroxidase from *Anthracophyllum discolor* Sp4).
The sixth chapter analyzes the results of this thesis and summarizes some concluding remarks (Chapter 6: General discussion and concluding remarks).

1

Introduction

1. Introduction

The contamination of soils with xenobiotic compounds due to industrial activities is widespread. Particularly, the contamination of soils with polycyclic aromatic hydrocarbons (PAHs) may cause environmental and human health problems since these compounds are toxic, mutagenic and has a carcinogenic potential. PAHs consist of fused benzene rings with two or more rings. Sixteen PAHs have been selected by the Environmental Protection Agency (EPA) of the United States of America as priority pollutants. These 16 EPA PAHs include phenanthrene, anthracene, fluoranthene, pyrene and benzo*(a)*pyrene, among others.

The use of white-rot fungi in pollutants biodegradation has received increasing attention in the past two decades. White-rot fungi have shown to be able to degrade complex aromatic molecules such as PAHs. These microorganisms possess a non-specific extracellular system, constituted of ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (L) and cofactors. The expression of this complex ligninolytic system is regulated by the onset of secondary metabolism by nitrogen, carbon, and/or sulphur limitation (Kotterman, 1998). In particular, *Anthracophyllum discolor* Sp4 is a Chilean white-rot fungus isolated from decayed wood in the rain forest of southern Chile with potential for degrading azodyes and clorophenols, and produces ligninolytic enzymes, especially MnP (Rubilar et al., 2007; Tortella et al., 2008).

Several reports indicate that MnP enzyme has a fundamental role in PAH degradation by means of the complex Mn^{3+}- chelate or free radicals formed by lipid peroxidation (Moen and Hammel, 1994; Bogan y Lamar, 1995). The detoxification of recalcitrant compounds using white-rot fungi requires large treatment periods, producing great amount of biomass (López, 2006). The fungal growth is usually susceptible to environmental conditions changes during the process and to the action of predators in soil (Gianfreda and Bollag, 1994). Due to these disadvantages, new alternatives of decontamination treatments have been developed. The application of immobilized enzymes is one of the most studied treatments. Proteins have a strong affinity to bind to various soil constituents involving changes in the protein structure and stability (Sarkar et al., 1989; Gianfreda and Bollag,

2002). Carriers of natural origin such as nanoparticles of allophane have proportioned a high stability to enzymes through its immobilization (Calabi et al., 2009).

Based on this background, the role of MnP in PAH degradation by the white-rot fungus *Anthracophyllum discolor* Sp4, the adequate conditions for the immobilization of MnP on nanoclays and the application of immobilized MnP in the bioremediation of contaminated soils with PAHs, have been studied in this thesis.

1.1 Hypothesis

Hypothesis 1
It is proposed that MnP enzyme of the white-rot fungus *Anthracophyllum discolor* Sp4 isolated from decayed wood in the rain forest of southern Chile, plays a pivotal role in the degradation of polycyclic aromatic hydrocarbons (PAHs) through oxidation.

Hypothesis 2
It is proposed that MnP enzyme immobilized on nanoclays is able to oxidize PAHs in soil.

1.2 Objectives

General objectives

The aim of this work was to evaluate the role of MnP produced from *Anthracophyllum discolor* Sp4 in the degradation of PAHs and to evaluate the efficiency of this nanoclay-immobilized enzyme in the degradation of these compounds in liquid medium and soil.

Specific objectives

1. To evaluate the optimal culture conditions to enhance MnP production by *A. discolor* Sp4 and to purify and characterize this enzyme.

2. To evaluate the MnP role in PAH degradation by *A. discolor* Sp4 and the biotic and abiotic factors involved in the bioremediation of soil contaminated with PAHs by *A. discolor* Sp4.

3. To determine the experimental conditions for maximizing the stability of nanoclay-immobilized MnP.

4. To evaluate the PAH degradation efficiency by nanoclay-immobilized MnP in liquid solution.

5. To evaluate the biotic and abiotic factors involved in the bioremediation of a soil contaminated with PAHs, by nanoclay-immobilized MnP.

2

Theoretical background

2. Theoretical background

2.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants (POPs) consisting of two or more fused benzene rings. PAHs originate from natural and/or anthropogenic sources. Natural sources of PAHs include forest fires and volcanic eruptions (Keith and Telliard, 1979). These compounds also can be produced by plants as biogenic precursors (Wilcke et al., 2000; Hafner et al., 2005) and during pyrolysis through the exposure of sediments to high temperatures during sediment diagenesis (Bamforth and Singleton, 2005). Anthropogenic sources include incomplete burning of organic matter as emissions during fossil fuel burning (Page et al., 1999), municipal solid waste incineration, and petroleum spills and discharge (Cerniglia and Heitkamp, 1989). These compounds are considered priority organic pollutants by the U.S. Environmental Protection Agency (EPA) due to their teratogenic, mutagenic and carcinogenic properties (Blumer, 1976; WHO, 1983; U.S. EPA, 1987; 2008). Some PAHs are resistant to degradation, accumulate in terrestrial and aquatic ecosystems, and maintain their toxic properties (Cerniglia, 1993). Most PAHs have low aqueous solubilities, vapor pressures and high octanol/water partitioning coefficients (Sims and Overcash, 1983; Mackay and Callcott, 1998; Oleszczuk and Baran, 2003). Because of these chemical properties, PAHs have a high affinity for association with organic carbon material (humus) in soil (Alexander, 1995) and lipid tissues (Cerniglia, 1984). High molecular weight PAHs have low water solubility, which reduces their bioavailability for microorganisms. Furthermore, PAHs adsorb to mineral surfaces such as clays and organic matter in soil. Irreversible sorption may increase in old PAHs contaminated soils, which results in lower chemical and biological extractability of these pollutants. Therefore, the bioavailability of PAHs correlates directly to their persistence in the soil (Bamforth and Singleton, 2005).

PAHs are widely distributed in soils and sediments, groundwater and the atmosphere and can be transported long distances as gases or aerosols, reaching rural and remote areas (Barra et al., 2006). They have been detected in sediments (Coates et al., 1997; Ohkouchi et

al., 1999), soils (Ellis et al., 1991; Lundstedt et al., 2003), aquifers and groundwater (Zein et al., 2006), and atmospheric deposits (Lim et al., 1999). PAH levels in soils vary between 1 μg kg^{-1} and 300 g kg^{-1} depending on the source of contamination. Atmospheric levels of PAH have been found to be between 60 μg m^{-3} and 3 mg m^{-3} air (Bamforth and Singleton, 2005).

There is currently no international standardized norm for the identification and assessment of contaminated sites (Weber et al., 2008). However, some studies have focused on detecting and identifying the origin of PAH environmental levels in specific parts of the world such as Chile (Table 2.1). The International Mussel Watch (IMW) Program, the United Nations Environment Program (UNEP), the National Oceanic, and Atmospheric Administration's National Status & Trends (NOAA´s NS&T) monitored several contaminants such as PAHs in bivalve mussels in Central and South America between 1991 and 1992 (Sericano et al., 1995). High total PAH concentrations (1.6 mg kg^{-1}) were detected in mussels in Punta Arenas (Sericano et al., 1995) (Table 2.1). Total PAH concentrations in mussels in Valdivia ranged from 0.03 mg kg^{-1} to 0.09 mg kg^{-1} in 2001 and from 0.03 mg kg^{-1} to 0.05 mg kg^{-1} in 2002 (Palma-Fleming et al., 2008). In Valparaíso, total PAH concentrations in mussels ranged from 0.03 mg kg^{-1} to 0.25 mg kg^{-1} in 2001 and from 0.01 mg kg^{-1} to 0.03 mg kg^{-1} in 2002, respectively. In addition, special attention has been paid to PAH levels in lake sediments (Table 2.1) in locations like Lake Icalma (3.182 mg kg^{-1}) (Daly and Wania, 2005).

The presence of PAHs in these remote ecosystems may be due to dry and wet deposition, direct and indirect discharges, and/or surface runoff from the drainage basin (Fernández et al., 1999; Quiroz et al., 2005; Barra et al., 2006). PAHs produced by plants as biogenic precursors may contribute significantly to total PAH concentrations in remote areas (Wilcke et al., 2000; Hafner et al., 2005). As described in the Northern Hemisphere, high mountain lakes could act as natural traps for atmospherically transported PAHs (Fernández et al., 1999). Some of these sites present concentrations as high as those observed in urban or industrial areas. Total PAH background levels of 1 – 5 mg kg^{-1} soil are considered common in urban areas. The levels detected in industrial areas can be 10 to 20 times higher

(Wilson and Jones, 1993). Barra et al. (2006) reported that local contaminating sources such as human influence caused the presence of PAHs levels in the Andean mountain soils. Quiroz et al. (2005) linked the presence of these contaminants to PAH diagenesis from organic matter coming from the water column from in-lake sources. Despite the presence of a small anthropogenic signal at the core, no correlation was detected with anthropogenic sources such as forest fires or biomass combustion (Quiroz et al., 2005).

Table 2.1 PAH levels detected in different environments.

Place	PAH level (mg kg^{-1})	Sample	References
CHILE			
Punta Arenas	1.60	mussels	Sericano et al., 1995
Valdivia	0.09	mussels	Palma-Fleming et al., 2008
Valparaíso	0.25	mussels	Palma-Fleming et al., 2008
Lake Galletue	0.38	sediments	Barra et al., 2006
Lake Icalma	3.18	sediments	Barra et al., 2006
EUROPE			
Central and Western Europe	0.18–1.1	sediments	Fernández et al., 1999
Eastern Europe	13–18	sediments	Fernández et al., 1999
Boreal forest (Norway)	< 0.01	soil	Jensen et al., 2007
Coastal meadow (Denmark)	0.25	soil	Johnsen and Karlson, 2005
Rural town (Estonia)	0.30	soil	Trapido, 1999
Forest (Norway)	1.04	soil	Jensen et al., 2007
City (Estonia)	4.53	soil	Trapido, 1999
Motorway (Denmark)	4.36	soil	Johnsen et al., 2006
Gasworks soil (Denmark)	493	soil	Lindhart et al., 1994
UNITED STATES			
Street dust < 2.0 μmg	23.7	soil	Rogge et al., 1993

2.2 Microbial degradation of PAHs

Significant interest has been generated in the development of technologies for detoxifying PAH-contaminated wastes and soils because PAHs pose a human health risk. PAH degradation by microorganisms is one of the most important technologies in this field. Microbiological processes are inexpensive, non-destructive and environmentally friendly

(less input of chemicals and energy and no generation of waste) (Providenti et al., 1993; Fedi et al., 2001).

PAHs are removed from contaminated sites mainly by microbial degradation. However, PAHs also can undergo volatilization, photooxidation, chemical oxidation, bioaccumulation, adsorption and adhesion to the soil matrix (Wild et al., 1991; Cerniglia, 1993).

The feasibility of degrading or mineralizing a pollutant depends on a wide variety of environmental and microbial factors. Some of the factors that may affect the availability of contaminants and the survival of the microorganisms that are introduced include concentration and physicochemical properties of PAHs, physicochemical properties of soil (moisture content redox conditions, temperature, pH, electron acceptors, organic matter concentration), seasonal factors, inorganic nutrient availability, depth, diffusion, predation and competition by indigenous microbiota (Cerniglia and Heitkamp, 1989; Sims and Overcash, 1983; Pagga, 1997; Martín et al., 2004).

2.2.1 Factors affecting PAH degradation

2.2.1.1 Abiotic factors

a) Composition, physicochemical properties and concentration of PAHs

PAHs exist in the environment as a complex mixture of substituted and unsubstituted fused benzene rings. Their physicochemical properties may determine their susceptibility to microbial degradation (MacGillivary and Shiaris, 1994). A PAH mixture with increasing alkyl substitution is more resistant to degradation than unsubsitituted-fused benzene rings. Enhanced degradation may happen if cooxidation in a PAH mixture occurs because it increases the microbial attack of more recalcitrant PAHs (MacGillivary and Shiaris, 1994). By contrast, the presence of a single compound or combination of compounds may inhibit the degradation of a PAH mixture due to the toxicity produced in the microbial population (Bauer and Capone, 1988). For instance, naphthalene is strongly toxic in a PAH mixture

and can inhibit the degradation of other PAHs that would normally be biodegraded (Riser-Robert, 1998).

The properties of PAHs vary with their molecular weight (Table 2.2). In general, volatility and solubility decrease as molecular weight and the tendency to adsorb to organic matter increase. One measure of volatility of PAHs is their vapor pressure. As the molecular weight of a PAH increases, its vapor pressure decreases. PAHs with low molecular weight are more volatile. The PAH degradation rate also depends on the ionization potential (IP) of these compounds. PAHs with low IP value are more susceptible to π-electron removal and the formation of PAH cation radical than compounds with high IP value (Cavelieri and Rogan, 1985).

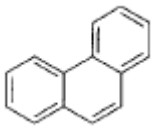
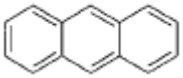
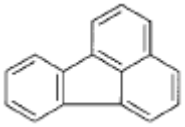
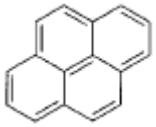
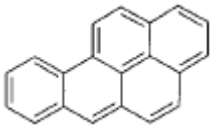
The concentration of a PAH also can affect its biodegradability and toxicity to microorganisms. High concentration of a PAH may inhibit degrading microorganisms, and the concentration at which inhibition occurs will vary with the compound (Alexander, 1985). Concentrations of PAHs in the range of 1 to 100 ppm in soil are not generally considered toxic to bacteria and fungi (Riser-Robert, 1998).

b) Physicochemical properties of soil

Temperature, salinity and pH are environmental variables that also control the degradation rate. Several studies found a significant correlation between temperature and PAH degradation rates (Bauer and Capone, 1985; Shiaris, 1989a). The degradation rate increases as the temperature increases. The salinity and pH of a contaminated site may influence the species composition of PAH-degrading consortia affecting the degradation rate (Shiaris, 1989b). PAHs degradation is affected by the presence of oxygen and nutrients. In fact, PAH-degrading microorganisms incorpore oxygen into the PAH ring during the initial enzymatic attack (Cerniglia, 1984) and low oxygen concentration may affect the PAH oxidation by aerobic microorganisms. Nutrients such as nitrogen and phosphorus may stimulate PAH degradation by microorganisms. However, the effect of nutrient limitation may vary depending on the ecosystem of contaminated site (MacGillivary and Shiaris, 1994).

The adsorption of PAHs into the soil matrix could be the most important factor in the behavior of PAHs in the soil environment. PAHs have high organic carbon adsorption coefficient (K_{oc}) value (Table 2.2), reflecting the high affinity of these compounds to adsorb out of solution onto soil organic material. PAHs may form complexes with the humic fraction of the soil or by attaching to reactive sites on the surfaces of organic colloids, producing more recalcitrant molecules (Alexander, 1999). Adsorption to soil constituents will affect the rate of volatilization, diffusion or leaching, as well as the availability of PAHs to microbial degradation.

Table 2.2 Physicochemical properties of some PAHs.

PAH compound	Number of rings	Chemical Structure	Solubility (mg l^{-1}) [a, b]	K_{oc} [c]	K_{ow} [c]	Ionization potential (IP) (ev) [d]
Phenanthrene	3		1.10	4.36	4.6	8.03
Anthracene	3		4.5×10^{-2}	4.42	4.5	7.43
Fluoranthene	4		2.6×10^{-1}	6.38	5.2	7.90
Pyrene	4		1.32×10^{-1}	4.80	5.2	7.53
Benzo*(a)*pyrene	5		3.8×10^{-3}	8.30	6.0	7.21

K_{ow}: octanol-water partition coefficient
K_{oc}: organic carbon adsorption coefficient
[a] Mackay and Callcott (1998)
[b] Sims and Overcash (1983)
[c] Oleszczuk and Baran (2003)
[d] Bogan and Lamar (1995)

2.2.1.2 Biotic factors

A biotic factor is the metabolic ability of microorganisms. The biotic factors that affect the microbial degradation of organic compounds include direct inhibition of enzymatic and the proliferation processes of degrading microorganisms (Rieser-Robert, 1998). PAH-degrading microorganisms could occur naturally in contaminated soil and require certain nutrients and aerobic or anaerobic soil conditions in order for biodegration to occur. Many soil microbes produce antibiotics that can affect the viability of other microorganisms (Radtke et al., 1994), while some microorganisms produce biosurfactants (Plante et al., 2008), which enhance the availability of PAHs.

2.2.2 PAH-degrading microorganisms

Many microorganisms are capable of transforming xenobiotic compounds and demonstrate the ability to metabolize both lower and higher molecular weight PAHs (Chen et al., 1999; Martín et al., 2004). For example, several microorganisms that are capable of degrading PAHs are mostly aerobic, gram negative and mobile. *Pseudomonas paucimobilis*, *Rhodococcus* sp., *Mycobacterium* sp., *Acinetobacter* sp., *Beijerinckia* sp., *Moraxella* sp., *Micrococcus* sp., *Arthrobacter* sp. and *Sphingomonas* sp. are microbial species that can degrade PAHs (Efroymson and Alexander, 1991; Kanaly and Harayama, 2000; Pizzul et al., 2006). Most bacteria have been found to oxygenate PAHs initially via a dioxygenase in order to form *cis*-dihydrodiol, which can be further oxidized to catechols (Pothuluri and Cerniglia, 1994) (Fig. 2.1). Over the past few decades, the isolation and characterization of degrading bacteria have opened a wide field in the study of their metabolic pathways and possible genetic modifications for more efficient degradation routes (Mrozik et al., 2003; Seeger et al., 2006; Saavedra et al., 2010).

Fungi are able to transform and even mineralize a large spectrum of organic compounds due to their enzymatic system and development of a mycelium capable of colonizing different types of substrates and accessing the pollutants in the soil (Pointing, 2001). Most fungi oxidize PAHs via a cytochrome P-450-catalyzed by a monooxygenase to form

reactive arene oxides, which produce trans-dihydrodiols by means of enzymatic hydration (Pothuluri and Cerniglia, 1994) (Fig. 2.1). White-rot fungi have metabolic versatility, and the non-specific function of their extracellular enzymes allows oxidizing PAHs to form quinones (Rabinovich et al., 2003) (Fig. 2.1). These features have led to research on the use of white-rot fungi as degradative agents in the PAHs bioremediation.

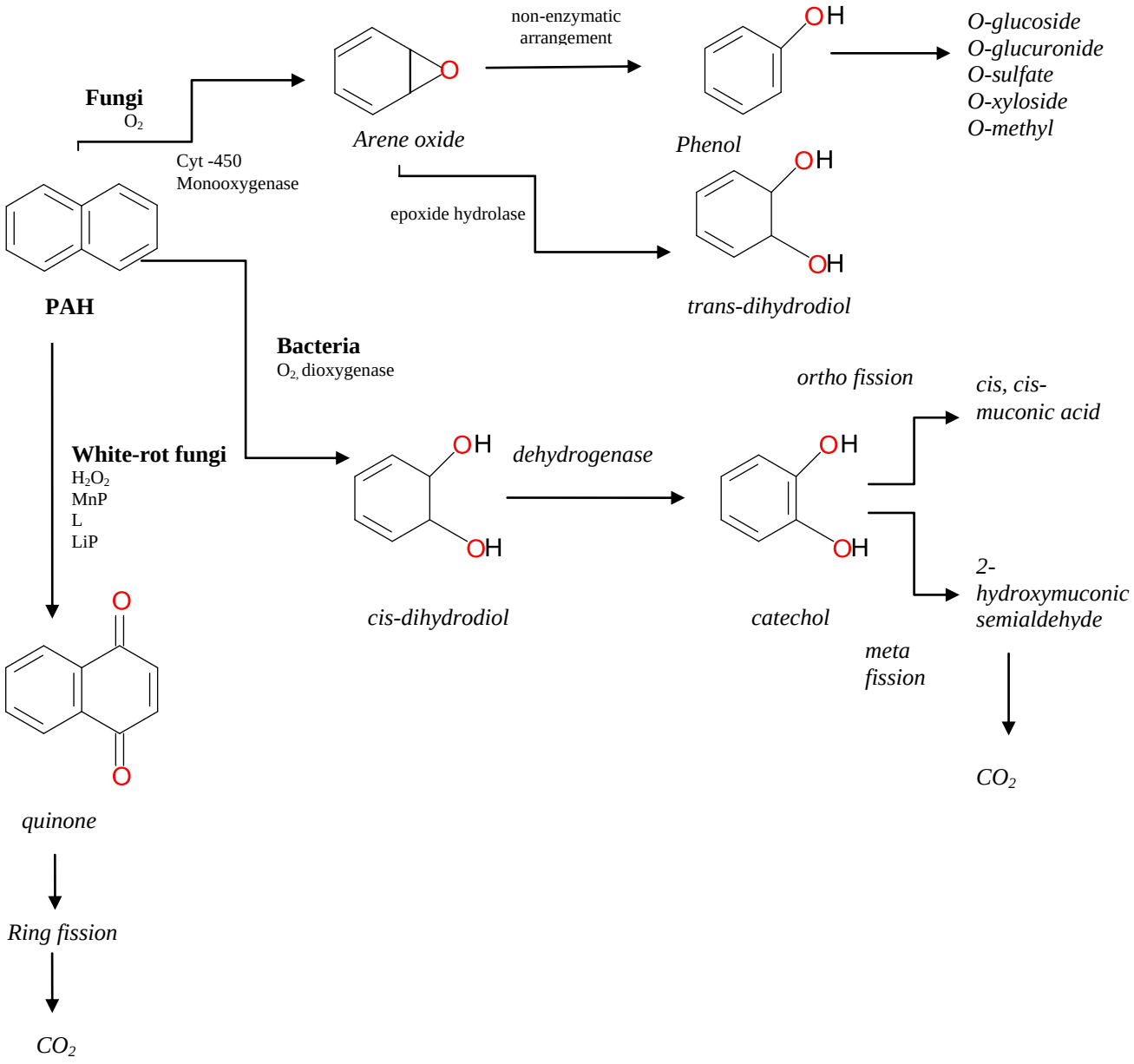


Figure 2.1 Microbial oxidation of PAHs via monooxygenase, dioxigenase and ligninolytic enzymes (adapted from Cerniglia and Heitkamp, 1989; Haritash and Kaushik, 2009).

2.2.3 Biodegradation of PAHs by white-rot fungi

White-rot fungi are known to degrade complex molecules such as PAHs through their extracellular enzymatic system (Hammel et al. 1986; Bumpus 1989, Sutherland et al., 1991; Pointing 2001). The extracellular ligninolytic enzyme system of white-rot fungi, which consists of peroxidases and laccases, initiates a free radical attack on PAHs via a single electron transfer to form more polar products (Collins et al., 1996; Steffen et al., 2002a, Xiao et al., 2003).

Hammel et al. (1991) showed that *Phanerochaete chrysosporium* was able to degrade anthracene to phtalic acid via 9,10-anthraquinone. Moen and Hammel (1994) reported formation of 2,2-diphenic acid from phenanthrene oxidation by *Phanerochaete chrysosporium*. Cajthaml et al. (2006) studied the capacity of *Irpex lacteus* to degrade benzo*(a)*anthracene and presented a detailed degradation route of this compound, including the breakage of specific rings. Using compounds labeled with ^{14}C, several studies found that white-rot fungi are able to mineralize PAHs to CO_2 (Bezalel et al., 1996; Wolter et al., 1997).

For a successful bioremediation of contaminated soils by white-rot fungi, it is necessary to determine the best conditions for enhancing the fungal growth and activation of the enzymatic system in soil (Pointing, 2001). Several studies have looked to elucidate the dynamic of white-rot fungi in a bioremediation process in order to find the best conditions for optimizing PAH biodegradation (Kotterman et al., 1998; Canet et al., 2001; Bishnoi et al., 2008; Byss et al., 2008; Anastasi et al., 2009).

2.2.3.1 Production of ligninolytic enzymes

One of important aspects to consider in PAHs degradation by white-rot fungi has been the correlation of degradation or mineralization rates with the production of ligninolytic enzymes. Bogan et al. (1996) observed that periods of high-level MnP transcript and enzymatic activity could be correlated to a maximal oxidation rate of fluorene and crysene

in soil. This supports the hypothesis that PAHs are oxidized in soil by mechanisms dependent on MnP. Anastasi et al. (2009) observed a correlation between the high pyrene degradation in sterile soil microcosms by a consortium of three basidiomycetes isolated from compost with the production of ligninolytic enzymes. The results indicated that ligninolytic enzymes such as laccase and manganese independent peroxidase play an important role in the degradation process. Novotny et al. (2004) reported that high levels of extracellular ligninolytic enzymes could be related to high biodegradation rates. However, other biochemical systems such as membrane-associated cytochrome P450 mono-oxygenase system, hydroxyl radicals and the level of H_2O_2 produced by white-rot fungi also should be considered in a bioremediation process (Novotny et al., 2004).

In order for successful bioremediation to take place, enzymatic enzymes levels must be available in large quantities. The expression of ligninolytic enzymes depends on the level of gene transcription regulated by several factors. For example, a low nitrogen level, elevated concentrations of dissolved oxygen (Leatham and Kirk, 1983; Heinzkill et al., 1998), slight agitation of the cultures and the addition of an inducer can stimulate white rot fungi growth and enzyme production (Wesenberg et al., 2003). Some fungi, such as *P. chrysosporium*, produce different redox-active enzymes depending on the nutrient conditions (Cameron and Aust, 1999). *P. chrysosporium* grown in a cellulose medium will tend to produce cellulose dehydrogenase (CDH). The same strain will induce a peroxidase secretion when grown under low nitrogen levels (Cameron and Aust, 1999). The addition of inducers such as manganese and copper can stimulate MnP and laccase production, respectively. Copper has been reported to be a strong laccase inducer in several species, such as *T. versicolor* (Collins and Dobson, 1997), *P. chrysosporium* (Dittmer et al., 1997), *Pleurotus eryngii* (Palmieri et al., 2000) and *Daedalea quercina* (Baldrian, 2004a). The expression of MnP in fungal cultures is dependent on Mn^{2+} that regulates *mnp* gene transcription (Ma et al., 2004). The effects of Cu and Mn are quite variable depending on the culture conditions and the form of copper or manganese added.

2.2.3.2 Role of indigenous microflora

The co-existence of white-rot fungal inoculum and indigenous microflora is desirable for reaching PAHs mineralization as consortia (Kotterman et al., 1998; Gramss et al., 1999). Fungal extracellular enzymes catalyze PAH oxidation, generating more polar and soluble metabolites, such as quinones, phtalate or diphenic acid (Kotterman, 1998). Increased polarity and water solubility are important factors for the enhancement of the bioavailability of these metabolites and their mineralization by native microorganisms present in contaminated sites (Kotterman et al., 1998) (Fig. 2.2).

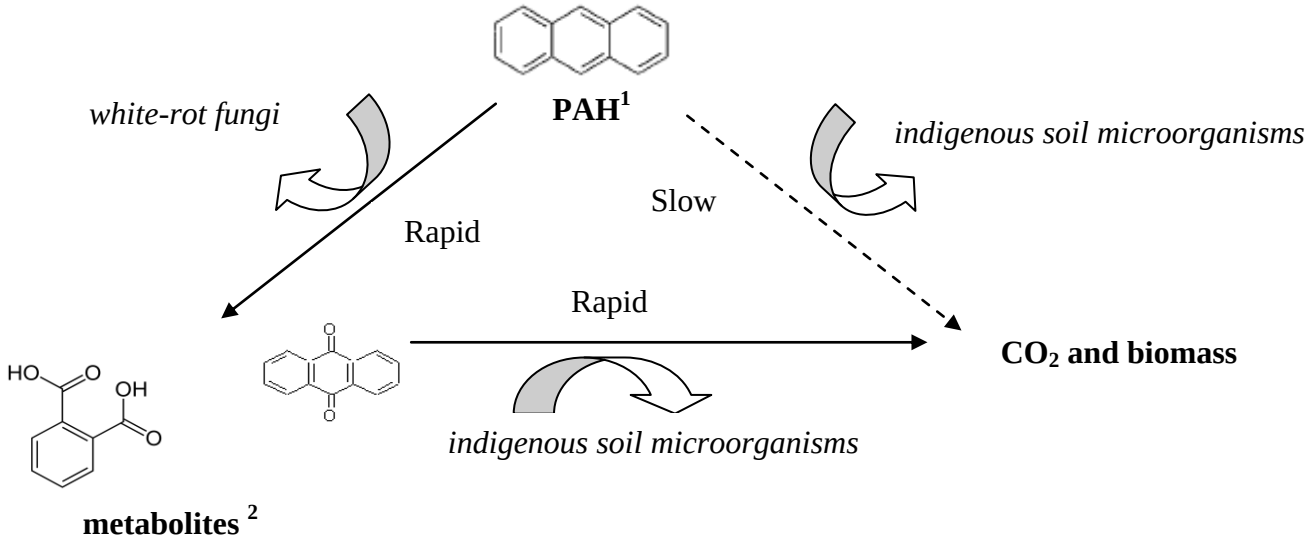


Figure 2.2 Degradation and mineralization of PAHs by the use of white-rot fungi in soil.
[1] Low water solubility, low bioavailability and highly mutagenic potential
[2] Increased water solubility and increased bioavailability (adapted from Kotterman, 1998)

It is also important to consider interactions with indigenous microbial populations. Indigenous soil bacteria can have an antagonist or synergist effect with white-rot fungi in PAHs degradation. Byss et al. (2008) determined that the efficacy of *Pleurotus ostreatus* and *Irpex lacteus* for the removal of PAHs from a creosote-contaminated soil. *P. ostreatus* was a more efficient PAH-degrading organism compared to *I. lacteus*, favoring PAHs degradation through a possible synergistic interaction with actinobacteria. Kotterman et al.

(1998) observed a positive interaction between white-rot fungi and soil microbiota in the degradation of benzo*(a)*pyrene with *Bjerkandera adusta*. Bioaugmentation with *Dichomitus squales* and *Pleurotus sp*. increased the degradation of pyrene five to 20% compared to non-bioaugmented soil (In der Wiesche et al., 1996). Bishnoi et al. (2008) evaluated the degradation of 3- and 4-ring PAHs in sterile and non-sterile soil inoculated by *Phanerochaete chrysosporium*. They found that PAHs degradation was lower in non-sterile soil. This is probably due to an effect of competition with the native microflora. Canet et al. (2001) reported that the introduction of some white-rot fungi (*Phanerochaete chrysosporium* IMI 232175, *Pleurotus ostreatus* IMI 341687, *Coriolus versicolor* IMI 210866 and Wye isolate #7) to degrade PAHs in a coal-tar contaminated soil was not successful. In fact, soil microbiota can affect fungal colonization in soil through an antagonist effect. Soil bacteria, mainly of *Pseudomonas* genera, are able to significantly inhibit the growth of *P. chrysosporium* due to the synthesis of derivates of fenazine (Radtke et al., 1994), while some fungi produce antibacterial compounds such as toxic hydroxyl radicals (Tornberg and Olsson, 2002).

2.2.3.3 Lignocellulosic materials as an energy source

Several lignocellulosic supports have been used as sources of carbon and nitrogen by white-rot fungi favoring the expression of ligninolytic enzymatic system and may confer a selective pressure against soil native microflora (Pointing et al., 2001). Lignocellulosic materials such as corn cobs (Rodriguez et al., 1998), alfalfa straw (Boyle 1995) and spent mushroom compost (Eggen 1999) have been used. Dzul-Puc et al. (2005) studied the capacity of *Phanerochaete chrysosporium* grown in soil with added sugarcane baggase and pine sawdust to remove benzo*(a)*pyrene (BaP).The BaP removal rate was faster in the fungus grown on sugarcane baggase. Mollea et al. (2005) proposed using *P. chrysosporium* supported on wheat straw as a biodegrading agent for soil that was highly contaminated with naphthalene (i.e. over 600 mg kg^{-1}). Rubilar (2007; 2010) observed that the application of wheat grain in soil followed the proliferation of microorganisms that degraded pentachlorophenol (PCP), promoting an effect that was synergic with *Anthracophyllum discolor* and *P. chrysosporium* and increasing the removal of PCP from contaminated soils.

2.2.3.4 The bioavailability of PAHs in soil

Another important aspect of enhanced PAH degradation in soil by white-rot fungi is the bioavailability of these compounds for microorganisms. Bioavailability of PAHs is generally low in soils due to their poor solubility in water and adsorption to soil particles (Meulenberg et al., 1997). As a result, it has been important to improve the availability of recalcitrant compounds for white-rot fungi and the ligninolytic system, increasing their solubility by means of the use of additives. Surfactants have been widely studied and used in the bioremediation of contaminated soil (Sobisch et al., 2000).

PAHs were extensively degraded by *P. chrysosporium* from an artificially contaminated soil (Zheng and Obbard, 2001) and a soil–slurry system in the presence of Tween 80 (Zheng and Obbard, 2002). The surfactant was catabolized without any effect on the production of ligninolytic enzymes (Zheng and Obbard, 2002). Tween 80 can transform the cell membrane structure and thereby promote the permeation of ligninolytic enzymes from the cell into the medium as well as the solubility of the contaminants (Zheng and Obbard, 2002). Moreover, the addition of Tween 80 promoted a synergistic action between indigenous microflora and the fungus. Enhanced PAHs degradation in artificially contaminated soil bioaugmented by *Pleurotus ostreatus* was observed using Tween 40, Tween 80 and Triton X-100 as surfactants (Márquez-Rocha et al., 2000). In aged soils, Tween 80 exerted a significant stimulatory effect on the PAHs degradation by *P. ostreatus* (Leonardi et al., 2007), as shown by Bogan and Lamar (1995) using Tween 80 and soybean oil on fungal degradation of PAHs. The significant concentration of polyunsaturated fatty acids in soybean oil and the presence of a monounsaturated acyl chain in Tween 80 suggests possible peroxidation reactions in the process, which enhances the degradation rate (Zheng and Obbard, 2001; Leonardi et al., 2007).

2.3 Enzymatic degradation of PAHs

Another form of PAHs biodegradation is enzymatic treatment utilizing free or immobilized enzymes, which have a minimal impact on ecosystems and low energy requirements, easy

process control and operation over a wide range of pH values, temperatures and ionic strengths (Karam and Nicell, 1997; Gianfreda et al., 1999). Whiteley and Lee (2006) reported that over 1,000 enzymes have been involved in the biodegradation of aromatic compounds. Enzymes are produced by plant root or micro-organisms (bacteria, fungi, archaea) and mainly include mono- or di-oxygenases, reductases, dehalogenases, cytochrome P450 monooxygenases, oxidoreductases and hydrolases (Gianfreda and Rao, 2004; Alcade et al., 2006). In general, these enzymes require other cellular components regenerated inside of the cells or in the presence of intact cellular membranes in the case of membrane enzymes (Torres et al., 2003). White-rot fungi enzymes have a wide substrate range and are secreted extracellularly (Torres et al., 2003). As a result, they can be used as biocatalysts in pulp and paper bleaching, wastewater treatment and soil remediation (Field et al., 1992; Karam and Nicell, 1997; Torres et al., 2003; Wu et al., 2008).

Cell-free enzymes used for the removal of xenobiotics are capable of oxidizing organic compounds to less harmful or even innocuous products without producing toxic side-products (Torres et al., 2003; Alcalde et al., 2006). The utilization of enzymes has many advantages with respect to fungi use in xenobiotics degradation, such as their catalytic power, ability to be active in presence of high concentrations of contaminants and organic solvents, much shorter treatment periods (hours), absence of delays caused by biomass latency phase, reduction in sludge volume, which also minimizes the environmental impact and easy application and control process (Karam and Nicell, 1997; Durán and Esposito, 2000; Gianfreda and Rao, 2004).

Experiments with purified cell-free enzyme extracts such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (L) have confirmed the role of ligninolytic enzymes in PAHs degradation (Table 2.3). Peroxidases need peroxide in order to be active, while laccases require O_2 (Gianfreda and Bollag, 1994). Lignin peroxidase (LiP) (EC.1.11.1) uses hydrogen peroxide to catalyze one-electron oxidations of PAHs, while manganese peroxidase (MnP) (EC.1.11.1) catalyzes the H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+} to oxidize these compounds (Gianfreda and Rao, 2004; Whiteley and Lee, 2006). Manganese independent peroxidase manganese (MiP) is a MnP characterized by its ability

to oxidize organic aromatic compounds in the absence of Mn^{2+}, as described in *Bjerkandera* sp. BOS55 (Mester and Field, 1998) and *Bjerkandera adusta* (Heinfling et al., 1998). Laccase (EC.1.10.3.2) is a copper phenoloxidase produced by plants and fungi that oxidizes PAHs to phenoxy radicals with the concomitant reduction of oxygen to water by four electrons (Chivukula and Renganathan, 1995).

The degradation process of PAHs by enzymes may be reached in much shorter periods of time with a balanced combination of all of the components, including substrates and cofactors (Mielgo et al., 2003).

2.3.1 PAH degradation by MnP

Over the past few years, MnP has received considerable attention for its potential use in the degradation of organic pollutants, particularly PAHs degradation (Baborová et al., 2006; Eibes et al., 2006). PAHs degradation by MnP was first described in *Phanerochaete chrysosporium* as a lipid peroxidation-dependent process (Moen and Hammel, 1994). An increased degradation of PAHs by free MnP from *Nematoloma frowardii* in the presence of glutathione (GSH), a mediator substance able to form reactive thioyl radicals, has been reported (Sack et al., 1997, Günther et al., 1998). Benzo*(a)*pyrene and anthracene can be mineralized by MnP from *Stropharia coronilla* (Steffen et al., 2003). MnP from *I. lacteus* was able to efficiently degrade PAHs such as phenanthrene and fluoranthene with ionization potential (IP) higher than 7.8 eV (Bogan and Lamar, 1995; Baborová et al., 2006). Complete removal of anthracene by MnP from *Bjerkandera sp.* BOS55 was achieved after a short period of 7 h under conditions that maximized the MnP-oxidative system (Eibes et al., 2006).

The kinetic and spectral studies performed by Wariishi et al. (1992) indicate that chelating organic acids (such as lactate and malonate chelate) facilitate the dissociation of Mn^{3+} from the enzyme-manganese complex, stabilizing it in aqueous solution with a relatively high redox potential (Fig. 2.3). This feature is important because the oxidation of compounds is carried out by Mn^{3+} mediator (and not by the enzyme). Furthermore, Mn^{3+} is capable of

oxidizing substrates with a high molecular weight and easily penetrates solid and liquid matrices (Mielgo et al., 2003). Mn^{3+} chelates are much smaller than proteins, can penetrate microporous barriers inaccessible to proteins and are more tolerant to protein denaturing conditions (Grabski et al., 1998).

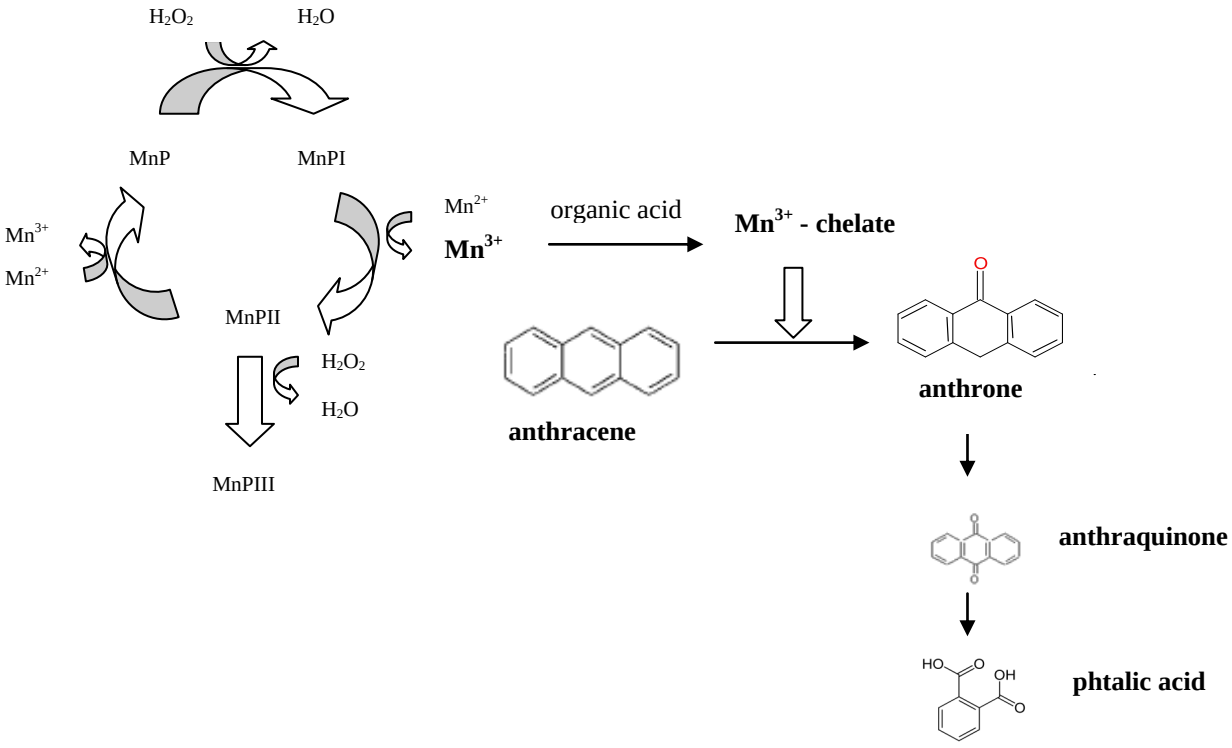


Figure 2.3 MnP catalytic cycle (adapted from Wariishi et al., 1992 ; Eibes et al., 2006).

MnP is a glycoprotein that contains one iron protoporphyrin IX prosthetic group (Fig. 2.4) (Wariishi et al., 1992). MnP has only one binding site for one Mn^{2+} near the heme and the free divalent manganese is the substrate for the enzyme Wariishi et al. (1992). Crystallographic and mutagenesis studies of MnP showed a Mn^{2+} binding site at the MnP surface, consisting of the carboxylates of aspartate 179, glutamate 35, glutamate 39 and heme propionate 6 (Gelpke et al., 2000). Arginine 177 also should be involved in Mn^{2+}

binding (Gelpke et al., 1999). The structure presents a distal region, which contains histidine, arginine, aspartate and leucine residues that are involved in the enzyme reaction with hydrogen peroxide to form compound I (Mester and Tien, 2000). The Histidine residue or the proximal region of this structure should be involved in stabilizing the Fe^{4+} state of the heme and affect only the reactivity of compound II (Mester and Tien, 2000). MnP contains five disulfide bonds (Mester and Tien, 2000) providing a high degree of rigidity to the enzyme (Banci, 1997). MnP presents calcium ions in its structure that play a key role in the stability and biological functions of the active site (Banci, 1997).

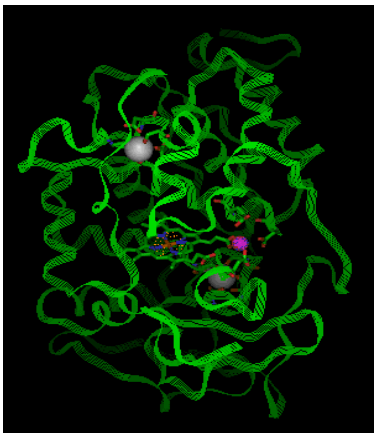


Fig. 2.4 Computer model of manganese peroxidase isolated from a *Phanerochaete chrysosporium* culture (Paszczynski et al. 1985; 1986).

MnP isoenzymes are encoded by different genes, as demonstrated by the differences in the N-terminal amino acid sequences of MnP from *P. chrysosporium* but with high structural similarity (Pease and Tien, 1992). MnP isoenzymes are differentially regulated and the isoenzymes patterns and enzyme stability depend on fungal growth conditions (Pease and Tien, 1992). Three genes (*mnp*1, *mnp*2 and *mnp*3) that codified for MnP isoenzymes have been reported for *P. chrysosporium* (Gelpke et al., 2000). MnP expression is regulated at the level of gene transcription by nitrogen, $Mn^{2+,}$ heat shock, hydrogen peroxide and other chemical stresses (Gettemy et al., 1998). It was observed that the *mnp*1, *mnp*2 and *mnp*3 promotor regions contain multiple putative consensus metal response elements (MREs) and putative heat shock elements (Gettemy et al., 1998). The results presented by Gettemy et al. (1998) demonstrated that *mnp*1 and *mnp*2 genes from *P. chrysosporium* are regulated by Mn^{2+} and nitrogen limitation, but that this relationship is not as significant for the *mnp*3

gene. In addition, *mnp*1 and *mnp*2 genes from *P. chrysosporium* are regulated by environmental conditions (stationary and agitated cultures). Gettemy et al. (1998) observed an increase in *mnp*1 transcript level in agitated cultures and a decrease in the case of *mnp*2 compared to the stationary cultures. The difference between the gene transcription levels opens a broad field of research in the area of optimizing growth conditions to favor isoenzymes expression and its possible applications on an industrial scale and xenobiotics compound degradation.

The optimal pH value for maximizing MnP activity from different strains ranges from 3 to 5 (Glenn et al., 1986; Aitken and Irvine, 1990; Steffen et al., 2002a; Makkar et al., 2001). However, Kanayama et al. (2002) reported a MnP from *Aspergillus terreus* LD-1 that was active up to pH 12.5. The temperature for maximizing MnP activity ranges from 22 to 60ºC, depending on the strain studied (Paszczynski et al., 1988; Wang et al., 2002). The reported isoelectric point is acid, lower than 4 (Johansson and Nyman 1993; Hofrichter et al., 1999; Lankinen et al., 2001). The molecular mass range of purified MnP from white-rot fungi is quite broad. Hofrichter (2002) reported MnP molecular mass from 38 kDa for *Trametes troggi* and up to 52.5 kDa for *Ceriporiopsis subvermispora*.

2.3.2 PAH degradation by laccase

Purified laccase from various strains was able to oxidize PAHs in the presence of chemical mediators such as 2,2′-azino-bis-3-ehtylbenzothiazoline-6-sulfonicacid (ABTS), hydroxybenzotriazole (HBT), syringaldazine or 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Collins et al., 1996; Johannes et al., 1996; Fabbrini et al., 2002; Torres et al., 2003; Farnet et al., 2009). Six different isoforms from laccase of *Marasmius quercophilus* strain were able to transform anthracene and benzo*(a)*pyrene, while naphthalene and phenantrene were not oxidized (Farnet et al., 2009). This result could be related to the ionization potential (IP) of PAHs that are laccases, which can oxidize PAHs with IP < 7.55 eV (Farnet et al., 2009). Efficient transformation of several PAHs was obtained using a fungal laccase in the presence of phenolic compounds related to those formed in nature

Table 2.3 Degradation of persistant organic compounds using ligninolytic enzymes from white-rot fungi.

Pollutant	Enzyme degradation (%)	White-rot fungi	References
PAHs	**Laccase**		
	Presence of pre-oxidised ABTS (6 h)		
Anthracene	80	*Marasmius quercophilus*,	Farnet et al., 2009
	Presence of p-coumaric acid and Tween 20 (24 h)		
Anthracene	95	*Pycnoporus cinnabarinus*	Cañas et al., 2007
Benzo(a)pyrene	95		
Pyrene	50		
	Presence of ABTS (240 h)	*Pleurotus ostreatus* D1	Pozdnyakova et al., 2006
Phenanthrene	72		
Anthracene	91		
Fluorene	54		
pyrene	66		
Fluoranthene	70		
Perylene	73		
Pesticides			
	Presence of ABTS, $MnSO_4$ and Tween80 (24 h)	*Nematoloma frowardii*	Pizzul et al., 2009
Gliphosate	90		
	Absence of mediators (24 h)	*Trametes villosa*	Bollag et al., 2003
4-chlorophenol	64		
2,4-dichlorophenol	96		
2,4,6-trichlorophenol	67		
Dyes			
	Absence of mediators (24 h)	*Daedalea quercina*	Baldrian, 2004a
Reactive black 5	9		
Remazol brilliant blue R	74		
Chcago sky blue 6B	65		
Reactive blue	59		
Poly B-411	59		
Trypan blue	42		
PAHs	**MnP**		
	Presence of Mn^{2+}, continuous addition of H_2O_2 (24 h)	*Bjerkandera sp. BOS55*	Eibes et al., 2006
	100		
Anthracene	> 50		
Pyrene	> 90		
Dibenzotiophene			
	In presence of $MnSO_4$, glucose, glutathione and glucose oxidase (24 h)	*Irpex lacteus*	Baborová et al., 2006
Phenanthrene	28		
Anthracene	42		
Fluoranthene	69		
Pyrene	92		
Pesticides			
	Presence of $MnSO_4$,Tween80, with or without simple addition of H_2O_2 (24 h)	*Nematoloma frowardii*	Pizzul et al., 2009
Gliphosate	100		
Dyes			
Orange IV	Presence of $MnSO_4$ and simple addition of H_2O_2 (1 h)	*Schizophyllum* sp. F17	Xiaobin et al., 2007
	30		
Reactive brilliant red K-2BP	Simple addition of H_2O_2	*Phanerochaete chrysosporium*	Yu et al., 2006
	LiP		
Anthracene	Presence of veratryl alcohol	*Phanerochaete chrysosporium*	Günther et al., 1998
Pyrene			
Reactive brilliant red K-2BP	Presence of veratryl alcohol and simple addition of H_2O_2	*Phanerochaete chrysosporium*	Yu et al., 2006
	89		

during the turnover of lignin and humus such as vanillin, acetovanillone, acetosyringone, syringaldehyde, 2,4,6- trimethylphenol, p-coumaric acid, ferulic acid, and sinapic acid (Cañas et al., 2007).

2.3.3 PAHs degradation by LiP

Extracellular crude containing LiP from *P. chrysosporium* was capable of catalyzing PAHs oxidation (Hammel et al. 1986). The purified LiP from *Nematoloma frowardii* was able to oxidize anthracene and pyrene in the presence of the mediator veratryl alcohol (Günther et al., 1998). LiP-mediated PAHs metabolism is thought to occur via a one-electron oxidation to yield quinone products (Pointing, 2001) for compounds with ionization potential (IP) ≤ 7.55 eV (Hammel et al., 1986).

2.3.4 *Anthracophyllum discolor* Sp4: a source of MnP

Several white-rot fungi have been collected in the Chilean forest in order to evaluate their ligninolytic enzyme production and capacity to degrade organic contaminants (Galeno and Agosin, 1990; Mendonça et al., 2008; Tortella et al., 2008).

Tortella et al. (2008) isolated and identified 53 strains of wood-rotting fungi from various native Chilean forests. Chilean native fungi were isolated from carpophores collected from the deadwood of native trees such as *Nothofagus obliqua* (Roble), *Laurelia sempervirens* (Laurel), *Aextoxicum punctatum* (Olivillo) and *Eucryphia cordifolia* (Ulmo). The most representative isolated genera of fungi were *Trametes* (17%), *Ganoderma* (14%), *Stereum* 12%, *Neoclitocybe* 8%, *Anthracophyllum* 6% and of *Schyzophyllum* and *Tremella* 4% (Tortella et al., 2008). Thirteen strains of isolated wood-rot fungi demonstrated a high ligninolytic activity in N-limited medium, reacting positively with Poly R-478 (Tortella et al., 2008). *Anthracophyllum discolor* Sp4 was the strain that presented the highest MnP production in liquid medium. This fungus was used in chlorophenols degradation trials, in liquid medium and in slurry phase systems and soil and was able to degrade

pentachlorophenol at elevated concentrations in 28 days (Rubilar, 2007). Rubilar et al. (2010) observed that the use of wheat grains stimulated the enzymatic activity of MnP from *A. discolor* Sp4 and favored the degradative action of pentachlorophenol in the fungi faced with the competitive effect of indigenous microflora in soil. These results seem to suggest that the native strain *Anthracophyllun discolor* Sp4, and MnP could be used in the bioremediation of polluted environments.

2.3.5 Improved efficiency of the enzyme-catalized process

One of the disadvantages associated with the use of free-cell enzymes for detoxifying xenobiotics in the environment is their short life-span in soil environments due to both non-biological and biological deactivation factors (such as adsorption on soil colloids, extreme acidity or alkalinity or protease action), which involves low operational stability (Gianfreda and Bollag, 1994). Other disadvantages are low or nill enzyme recovery and reusability or separation from the reaction products in a soluble medium (Ahn et al., 2002).

Some strategies have been used to improve the efficiency of an enzyme-catalyzed process of pollutant transformation (Table 2.4). Others have been implemented in order to improve the enzyme itself and optimize its intrinsic and inherent catalytic features. For example, researchers have generated optimized enzymes and pathways from catabolic pathway genes (Parales and Ditty, 2005). In addition, attachment of peptide extensions to the N o C-terminus of an enzyme´s polypeptide chain has been used to further improve the properties of enzymes (O´Fágáin, 2003). A site-directed mutagenesis approach has been performed through an amino acid change in the protein that the gene codes in a manner that improves enzyme function (Parales and Ditty, 2005). Alternatively, direct evolution through DNA-shuffling methods (i.e the random fragmentation of a population of mutant genes of a certain family followed by random reassembly) has been developed in order to obtain protein variants (Stemmer, 1994).

Other strategies are based on the improvement of the performance of the enzyme by acting on the conditions under which the enzyme displays its catalytic activity, thus optimizing its

intrinsic and inherent activity. They include the modification of enzymes by appropriate molecules (Efremenko et al., 2007), the use of additives or organic solvents as ameliorant of the enzyme activity (Ó´Fágáin, 2003), the use of more than one enzyme acting simultaneously or in sequence (Gullotto et al., 2008), the use of additional compounds acting as co-substrates or mediators (Cabana et al., 2007) and enzyme immobilization (Gianfreda and Bollag, 1994).

Table 2.4 Strategies for improving the efficiency of isolated microbial enzymes.

Strategies	Types	Mechanism	References
Chemical modification	- crosslinked enzyme crystals (CLECs)	Controlled precipitation of enzymes into microcrystals followed by crosslinking using bifunctional reagents to form strong covalent bond between free amino acid groups in the enzyme molecules	Roy and Abraham, 2006
	- covalent attachment of polymers	Attachment of the protein to multiple sites of a soluble polymer	Ó´Fágáin, 2003
	- surface modification	Substitution of the surface charge of protein	Ó´Fágáin, 2003
Immobilization	- Physical	Physically retaining the enzyme in the inner cavities of a polymeric matrix or a membrane by entrapment or membrane inclusion	Durán et al., 2002
	- Chemical	May reduce the activity of the enzyme and disturb the enzyme's native structure, but may provide a strong, stable enzyme attachment by adsorption, covalent bind or cross-linking	Durán et al., 2002
Stabilizing additives		Low-molecular weight additives (polyols) exert stabilizing effects by inducing preferential hydration of proteins. Useful for the protein storage.	Matulis et al., 1999; Ó´Fágáin, 2003
Organic solvents		The hydrophobic solvents are more effective than hydrophilic solvents to enhance the protein stability	
Multienzymatic system		The use of more than one enzyme acting simultaneously or in sequence	Gullotto et al., 2008
Co-substrates or cofactors		Small molecules may act as redox shuttles between the enzyme active site and the xenobiotic compound.	Cabana et al., 2007

2.3.6 Enzyme immobilization

The use of immobilized enzymes has been studied since the 1960s. Enzyme immobilization is "a process by which the degrees of freedom of movement of enzymes are restricted, completely or partially, by their binding in a support." (Taylor, 1991)

The advantages of immobilizing enzymes include increased stability in unfavorable conditions due to the protection provided against changes in pH, temperature and ionic force and protease action (Wiseman, 1985; Gianfreda and Bollag, 1994; Krajewska, 2004; Illanes et al., 2008). However, there is usually a loss of enzymatic activity due to denaturalization by heat or pH, interaction between reactive groups in the support with critical amino acids for enzymatic activity or a change in the enzyme structure (Gianfreda and Bollag, 1994). According to Gianfreda and Bollag (1994) and Worsfold (1995), the successful use of an immobilized enzyme for practical applications requires:

- a permanent, safe, inexpensive, simple and versatile immobilization method that can be easily increased in scale;
- an inexpensive support that is insoluble in water, chemically inert, mechanically stable and readily available;
- high residual activity of the immobilized enzyme;
- high stability of the immobilized enzyme for long-time storage and subsequent activity; and
- the use of non-toxic chemicals.

Many methods are available for enzyme immobilization. Most are based on chemical and physical mechanisms and present the advantages and disadvantages shown in Table 2.5. The physical mechanism (membrane inclusion) consists of physically retaining the enzyme in the inner cavities of a polymeric matrix or a membrane to avoid releasing the enzyme without preventing penetration of the substrate (Wiseman, 1985; Arroyo, 1998). This immobilization method is characterized by causing a slight disturbance in the native structure of the enzyme and its function (Durán et al., 2002). In general, chemical

immobilization methods (adsorption, covalent binding and crosslinking) tend to reduce the activity of the enzyme and may disturb the enzyme's native structure while providing strong and stable enzyme attachment (Durán et al., 2002).

2.3.7 Carriers of natural origin

Sarkar et al. (1989) used enzymes (glucose oxidase, β-D-glucosidase, acid phosphatase, tyrosinase and laccases from *Trametes versicolor*) that were were successfully immobilized in soils, bentonite and kaolinite and nearly retained their original activities. Researchers also evaluated the effect of clay content in various types of soils that where used as support and detected higher laccase activity for soils with higher clay contents. These results indicated that the clay particles should be an interesting support for enzyme immobilization. Enzyme immobilization on clay or soil provides an environment similar to the elements' natural "status" in soil (Sarkar et al., 1989).

Ahn et al. (2002) studied the potential of free and montmorillonite immobilized laccase from *Trametes villosa* to remediate polluted soil with 2,4-dichlorophenol (2,4 DCP). In soil 1 (12.8% organic matter), both free and immobilized laccase removed 100% of 2,4-DCP, whereas in soil 2 (27.4% organic matter), immobilized laccase removed more 2,4-DCP (about 95%) than free enzyme. Laccase activity lost of 23% was detected during the immobilization, though its performance was better than free laccase. Furthermore, Ahn et al. (2002) reported that montmorillonite showed higher binding capacity than kaolinite. This is probably due to the higher surface area and cation exchange capacity of montmorillonite.

Trametes versicolor laccase immobilized on supports such as kaolinite, montmorillonite and controlled-pore glass beads maintained its high activity level and did not change its kinetic properties (Gianfreda and Bollag, 1994). Montmorillonite was found to be the support with the highest binding capacity, immobilizing 71% of laccase. In addition, a removal efficiency of approximately 80% of 2,4-DCP was found under these conditions. The feasibility of using immobilized enzymes in a soil environment was evaluated by

measuring their activity in the presence of HCl-washed sand, silt loam soil and soil-sand mixtures. A decrease in enzymatic activity was correlated to an increase in the organic matter content of the soil-sand mixture (Gianfreda and Bollag, 1994). In conclusion, the authors propose using enzymes immobilized on natural supports for xenobiotics detoxification once there is a better understanding of the influence of the soil microenvironment.

Recently, nanoclay, a natural nanomaterial obtained from the clay fraction of soil, has been described as a suitable support material for enzyme immobilization (Calabi et al., 2009). Enzymes immobilized on nanoparticles have showed high mobility and activity (Wang et al., 2006). However, its use as a carrier for ligninolytic enzymes immobilization has not been explored yet.

2.3.8 Immobilized MnP application in pollutant degradation

There are very few studies of xenobiotics degradation using immobilized manganese peroxidase (Table 2.5). The first was that of Grabski et al. (1995), where a catalytically active MnP-azlactone polymer complex was prepared. Grabski et al. (1998) employed this immobilized covalently MnP purified from *Lentinula edodes* in a two-stage bioreactor for catalytic generation of chelated Mn^{3+} and subsequent oxidation of chlorophenols. Variables such as the effect of H_2O_2 and ionic strength, chelating agent's concentration, and the operational stability were studied. The immobilization procedure and the operation of this two-stage reactor system were found to be efficient in degrading about 86% of 2,4-dichlorophenol and all 2,4,6-trichlorophenol. However, Grabski et al. (1998) indicated that further experimentation is required in order to ascertain the full potential of the reactor for environmental applications.

The study published by Van Aken et al. (2000) was the first attempt at co-immobilization of MnP and glucose oxidase (Glox) on the same carrier. This co-immobilization provided an integrated system in which H_2O_2 required by MnP was produced by glucose oxidase

(Van Aken et al., 2000). This innovative study presents an efficient alternative to mineralizing several recalcitrant environmental pollutants.

Sasaki et al. (2001) used SC-26 as support material to immobilize MnP from *Phanerochaete chrysosporium*, a mesoporous material with a pore size of less than 90 Å, nearly the same as the diameter of the enzyme. The support-enzyme complex showed higher thermostability and tolerance to H_2O_2 than MnP immobilized on silica gel (50 to 300 Å pore size) (Grabski et al., 1998). The use of a two-stage reactor to immobilize MnP has allowed us to optimize the enzymatic reaction (oxidation of Mn^{2+} to Mn^{3+} by MnP) and chemical reaction (compound oxidation) under independent conditions (Grabski et al., 1998; Sasaki et al., 2001).

Edwards et al. (2002) applied immobilized laccase and MnP (from *Trametes versicolor*) to polysulphone ultrafiltration membranes for the removal of phenolic compounds from a petrochemical industrial effluent. Phenol, 2,5-dimetylphenol and 2,4-dichlorophenol were removed by immobilized enzymes.

Mielgo et al. (2003) developed a method for immobilizing MnP to be compatible with the enzymatic oxidation of persistant organic compounds. MnP of *P. chrysosporium* and *Bjerkandera sp*. BOS55 was immobilized on glutaraldehyde-agarose gels, which allowed for the evaluation of the effect of the density of activated aldehyde-agarose and ionic strength. In this study, activated glutaraldehyde-agarose gels at low ionic strength produced the best results, obtaining a fast (< 2 h) and tighter covalent attachment, increasing the resistance to H_2O_2 and obtaining long-term stability for these enzymes. These improved characteristics make their application in persistent organic compounds degradation more feasible (Mielgo et al., 2003).

Immobilized MnP is an effective alternative for the degradation of pollutants. However, no literature about its application in the bioremediation of soil contaminated with PAHs was found. Furthermore, there is a need for further study of its efficiency in PAHs degradation in soil.

Table 2.5 Advantages and disadvantages of different methodologies for enzyme immobilization

METHOD	Entrapment	Membrane Inclusion	Adsorption	Covalent binding	Cross-linking	References
Characteristics	The enzyme remains entrapped in the interstitial spaces of water-insoluble, cross-linked polymeric gels or occluded within the microcavities of a synthetic fiber	The enzyme is surrounded by permeable membranes that allow the flow of substrate and product molecules	The enzyme is bound to a support via ionic interactions, Van der Waals forces and hydrogen bridges	It is based on the activation groups of chemicals in the support so that they react with the functional groups of the enzymes, which are not essential to catalytic activity	Immobilization method by chemical bonding that uses bifunctional reactives to generate intramolecular bonds between the molecules of the enzyme	Gianfreda and Bollag,1994; Krajewska, 2004; Illanes et al., 2008
Advantages	Simple, low enzyme quantity, minimum alteration in enzyme structure, resistance of fibers to pH effect, high ionic forces, organic solvents and microbial attacks	Large contact surface between enzyme and substrate, possibility of simultaneously immobilizing several enzymes	Simple, low cost, no change in enzymatic specificity	Easy manipulation of the immobilized complexes, enzyme load remains constant after immobilization, use in continuous reactors, resistance to effect of temperature, organic solvents and/or pH when having its tertiary structure stabilized	Resistance of the complex formed to extreme pH and temperature conditions	Wiseman, 1985; Arroyo, 1998; Krajewska, 2004; Illanes et al., 2008
Disadvantages	Continuous leakage of enzyme because of wide pore-size distribution in the gel, reduced substrate accessibility to the enzyme by diffusional limitation, steric hindrance	Occasional inactivation of the enzyme during the operation of microencapsulation, high enzyme concentration required	Optimization of the variables that control adsorption (pH, ionic force, ion presence), mechanically unstable complexes, weak binding to the support. The enzyme can be easily desorbed under operating conditions.	Possible changes in the structure of the active center	Difficult reaction control, need for high quantities of enzyme, possible loss of activity due to the participation of the active center in the process of immobilization	Wiseman, 1985; Arroyo, 1998; Krajewska, 2004; Illanes et al., 2008
Preparation	Difficult	Intermediate	Simple	Difficult	Intermediate	Arroyo, 1998
Force of bonding	Medium	Weak	Medium	Strong	Weak	Arroyo, 1998
Enzymatic activity	Low	Medium – high	Medium	High	Low	Arroyo, 1998
Support regeneration	Impossible	Possible	Possible	Impossible	Impossible	Arroyo, 1998
Processing cost	Medium	Medium - high	Low	High	Medium	Arroyo, 1998
Stability	High	Medium	Low	High	High	Arroyo, 1998
Microbial resistance	Yes	Yes	No	No	Yes	Arroyo, 1998

Table 2.6 Applications of immobilized MnP in the degradation of organic pollutants.

Fungus	Application	Support	Immobilization	Treatment	Characteristics	References
Lentinula edodes	-----	Empaze polymer	Covalent binding between the carboxyl groups of the enzyme and the amino groups of the support	Two-stage bioreactor	40-50% binding efficiency	Grabski et al., 1995
Lentinula edodes	Oxidation of 2,4-DCP 2,4,6-DCP	NH_2-Empaze polymer	Covalent binding	Two-stage bioreactor	Oxidation of chlorophenols in reactor 2 by Mn^{III}-chelates generated in reactor 1	Grabski et al., 1998
Phlebia radiata	----.	Porous silica beads activated by aminoalkylethoxysilane and glutaraldehyde as coupling agent	Covalent binding co-immobilization of MnP from *Phlebia radiate* and glucose oxidase from *A. niger*	In batch	-the glucose oxidation present in the medium produces continuously and biologically H_2O_2 required by MnP - 40% loss of enzymatic activity in the immobilization process, increased K_m and stability in the presence of high H_2O_2 concentrations, pH variations and storage. - decrease thermal stability	Van Aken et al., 2000
Phanerochaete chrysosporium	Pulp effluent	SC-26 with pore size < 90Å	Adsorption	Two-stage bioreactor	- greatest enzymatic stability was obtained with FSM-16/70 medium (pore size is nearly the same diameter as that of the enzyme) - increase the thermal stability and tolerance to H_2O_2	Sasaki et al., 2001
Trametes versicolor	Petrochemical industrial effluent containing phenol, 2,5-DMP; 2,4-DCP	Polysulphone ultrafitration membrane		Reactor	Reduction in chromatographic peak of the pollutants after the enzymatic treatment.	Edwards et al., 2002
P.chrysosporium Bjerkandera sp. BOS55	----.	Glutaraldehyde-agarose gels	Covalent binding. The binding occurs between the aldehyde groups of the medium and the amino or terminal amino groups of the enzyme	In batch	- fast and tight covalent attachment - increased K_m and resistance to H_2O_2 - increase in long-term stability - MnP from *Bjerkandera* sp. BOS55 seems to be more suitable for environmental applications	Mielgo et al., 2003

3

Enhanced production of manganese peroxidase from *Anthracophyllum discolor* Sp4 and its characterization

3.1 Abstract

In this study, different growth conditions of *Antracophyllum discolor* Sp4 were evaluated. Agitation effect, addition of lignocellulosic support (wheat grains), $MnSO_4 \cdot H_2O$ as inducer and Tween 80 as surfactant were evaluated on the MnP production in Kirk medium inoculated with *A. discolor* Sp4. The enzyme was purified and characterized under the selected culture conditions. At laboratory scale, a culture system for MnP production using tubes containing glass beads was evaluated and two methods for measuring MnP activity were compared. The highest MnP production was obtained in Kirk medium supplemented with wheat grain and 0.25 mM $MnSO_4$ as inducer, incubated under static conditions at 30°C, reaching a maximum of 1,354 U l^{-1} after 13 days of incubation. This MnP production was 10 times higher than the production under basal conditions (stirring, without wheat grain and without inducer). The presence of Tween 80 (0.05% v/v) maintained a high and constant MnP activity during the incubation period. Two isoenzymes were purified (35 and 38 kDa respectively). MnP enzyme produced by *A. discolor* Sp4 presented a maximal activity in the pH range between 4.5 and 5.5, a relatively high temperature tolerance (50ºC) and a high catalytic activity for 2,6-dimethoxyphenol and hydrogen peroxide. It was possible to reproduce and even enhance MnP activity when Erlenmeyer flasks were replaced with tubes containing glass beads, favoring the interaction between *A. discolor* Sp4 and the medium for enhancing MnP production. Moreover, the MBTH/DMAB assay proved to be a suitable method for determining MnP activity in the system using tubes containing glass beads under the optimized medium conditions.

Keywords: white-rot fungi; ligninolytic enzymes; purification; lignocellulosic material

3.2 Introduction

Global population growth, urbanization and industrialization have caused environmental contamination by organic compounds. Microorganisms play an important role in removing, transforming or mineralizing xenobiotic compounds to a less toxic form (Whiteley and Lee, 2006).

White-rot fungi, which are responsible for the biodegradation of lignin in wood, present valuable ecological and metabolic characteristics that make them useful in bioremediation processes (Pointing, 2001). These microorganisms have a remarkable ability to transform and even mineralize a large spectrum of organic compounds (Rabinovich et al., 2003; Tortella et al., 2005). This is due to their characteristic enzymatic system, which is comprised of non-specific extracellular oxidative enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (L) produced during secondary metabolism (Pointing, 2001).

Anthracophyllum discolor Sp4 is a Chilean white-rot fungus isolated from decayed wood in the rain forest of southern Chile (Tortella et al., 2008). It has a potential for degradation of organic pollutants because of its high production of ligninolytic enzymes, especially MnP (Rubilar et al., 2007; Tortella et al., 2008), and can thus be used in bioremediation systems.

Optimal culture conditions are required for high enzyme yields because efficient pollutant degradation is mainly correlated with high enzymatic activities. Furthermore, practical and efficient culture systems for fungal biodegradation tests are required. An efficient culture system depends on many factors that can contribute to high enzymatic yields in white-rot fungi. Fungal MnP production can be enhanced by substrate/support, e.g. lignocellulosic materials such as poplar or fir sawdust (Giardina et al., 2000), grape seeds, barley bran and wood shavings (Moredo et al., 2003), and distilled dried grain with soluble (DDGS) (Rubilar et al., 2007). Furthermore, an enhancement of MnP production and the stimulation of enzymatic activity by white-rot fungi were observed following the addition of inducers such

as $MnSO_4$ (Gill and Arora 2003) and cosubstrates such as Tween 80 (Watanabe et al., 2000; Garon et al., 2002). The expression of MnP in fungal cultures is dependent on Mn, which regulates mnp gene transcription (Ma et al., 2004). Tween 80 can transform the cell membrane structure and thereby promote the permeation of MnP from the cell into the medium and the solubility of the contaminants (Zheng and Obbard, 2002).

Ligninolytic enzymes have been purified, characterized and used as biocatalysts in the biodegradation of pollutants. Over the past few years, a great deal of attention has been paid to the potential use of MnP in biobleaching of pulp and paper and for xenobiotic compounds degradation because of the characteristics of its catalytic cycle (Eibes et al., 2006). MnP (EC.1.11.13), a glycoprotein that contains one iron protoporphyrin IX prosthetic group, catalyzes the H_2O_2-dependent oxidation of Mn^{2+} to Mn^{3+}, which acts as a diffusible oxidizer on phenolic or non-phenolic compounds (Wariishi et al., 1992; Whiteley and Lee, 2006).

It is necessary to test the ability of *A. discolor* Sp4 to degrade various pollutants before designing bioremediation systems. Biodegradation tests require analyses of large numbers of samples over time, which can be difficult to manage in terms of space, rational use of chemicals, extraction efficiency, etc., especially using Erlenmeyer flasks as culture system. In addition, biodegradation tests normally require destructive sampling, particularly when the target compound is hydrophobic or is applied at concentrations where it is insoluble in the growth medium. A practical and simple culture technique using tubes containing glass beads has been used for the study of polycyclic aromatic hydrocarbon (PAHs) degradation by actinobacteria (Pizzul et al., 2006) and may be of interest in fungal cultures.

The purpose of this study is to evaluate the effect of Mn^{2+}, Tween80 and lignocellulosic substrate on MnP production by *A. discolor* Sp4 and to purify and characterize this enzyme. A practical culture system for MnP production using tubes containing glass beads as a replacement for Erlenmeyer flasks was evaluated. Finally, two enzymatic tests for MnP activity determination in liquid medium were compared.

3.3 Materials and methods

Chemicals

MBTH (3-methyl-2-benzothiazolinone), DMAB (3-(dimethylamino)benzoic acid), 2,6-DMP (2,6-dimethoxiphenol) (> 99.0%) was supplied by Aldrich Chemical Co., Germany. 3,4-dimethoxy-benzyl alcohol (> 95.0%) was supplied by Fluka.
All other chemicals were supplied by Merck, Oxoid, Sigma and Duchefa (Netherlands).

Microorganism

The fungal strain used was *Anthracophyllum discolor* Sp4 isolated from decayed wood in the rain forest of southern Chile (culture collection of the Environmental Biotechnology Laboratory of Universidad de La Frontera, Chile). The fungus was stored at 4ºC in glucose malt extract agar (G-MEA) slant tubes containing malt extract 30 g l^{-1}; agar 15 g l^{-1} and glucose 10 g l^{-1}. The fungus was transferred from the slant culture tubes to Petri dishes with G-MEA medium and incubated at 30ºC for 5 days before being used for inoculum preparation.

MnP production by A. discolor Sp4 in liquid medium supplemented with wheat grain

Five malt agar plugs (6 mm diameter) of active mycelia of *A. discolor* Sp4 from 5-day-old cultures on G-MEA medium were inoculated in 100 ml of Kirk medium (Tien and Kirk, 1988) in 1l Erlenmeyer flask. The Kirk medium contained the following: glucose 10 g l^{-1}, peptone 2 g l^{-1}, KH_2PO_4 2 g l^{-1}, $MgSO_4$ 0.5 g l^{-1}, $CaCl_2$ 0.1 g l^{-1}, thiamine 2 mg l^{-1} and mineral salts 10 ml l^{-1} (KH_2PO_4 2 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1}, $CaCl_2 \cdot 2H_2O$ 0.1 g l^{-1}, $MnSO_4 \cdot 5H_2O$ 50 mg l^{-1}, NaCl 10 mg l^{-1}, $FeSO_4 \cdot 7H_2O$ 1 mg l^{-1}, $CoCl_2 \cdot 6H_2O$ 1 mg l^{-1}, $ZnSO_4 \cdot 7H_2O$ 1 mg l^{-1}, $CuSO_4 \cdot 5H_2O$ 0.1 mg l^{-1}, $AlK(SO_4)_2$ 0.1 mg l^{-1}, H_3BO_3 0.1 mg l^{-1}, $NaMoO_4 \cdot 2H_2O$ 0.1 mg l^{-1}). Sterile wheat grains (32 g) were added to the medium and the fungus was grown without agitation. Cultures containing *A. discolor* Sp4 grown in Kirk medium without wheat

grain under static conditions or on a shaker at 100 rpm were used as controls. The cultures were incubated for 28 days at 30ºC. Samples were taken periodically and the MnP activity, proteolytic activity, total protein concentration and pH were ascertained.

Effect of $MnSO_4$ and/or Tween 80 on MnP production by A. discolor Sp4 grown in liquid medium supplemented with wheat grain

In order to evaluate the effect of $MnSO_4$ for MnP production, Kirk medium (100 ml) containing 0.002 mM $MnSO_4$ as trace element and supplemented with 32 g of sterile wheat grain was modified by adding various concentrations of monohydrated manganese sulphate ($MnSO_4 \cdot H_2O$) in order to obtain concentrations of 0.002, 0.25, 0.5, 1.0 and 2.0 mM of this compound. The medium was inoculated as described above and the flasks were incubated at 30ºC under static conditions for 28 days. MnP activity was determined periodically.

In order to evaluate the effect of Tween 80 on MnP production, 100 ml of Kirk medium supplemented with 32 g of sterile wheat grain and 0.25 mM of $MnSO_4 \cdot H_2O$, was modified by adding Tween 80 (0.05% v/v) and inoculated as described above. The flasks were incubated at 30ºC under static conditions for 28 days. A control flask without Tween 80 was run in parallel. MnP activity was determined periodically.

MnP purification

A. discolor Sp4 was grown under the optimized culture conditions from which MnP was subsequently purified. The mycelium was separated from the culture medium by filtration through the membrane filter Wathman (0.45 µm). The filtrate was concentrated by ultrafiltration with Amicon tubes (10 kDa) at 4ºC, and the enzyme purification was carried out using FPLC (Fast Protein Liquid Chromatograph, Amersham Pharmacia Sweden). The concentrate was applied to column Q Sepharose-1 ml, which had been equilibrated with 20 mM piperazine pH 5.0. Elution of the proteins was achieved with a flow of 1 ml min^{-1} by a gradient with 20 mM piperazine pH 5.0 and 0 – 2 M NaCl. Fractions containing the enzyme were concentrated by Amicon tubes (10 kDa) and then loaded to a GFC-100 gel filtration

column. Elution of proteins in this column was equilibrated with the same buffer with 100 mM NaCl. The gradient was isocratic and the flow was 1 ml min^{-1}. Specific activity was determined by the ratio of total activity/proteins content. Purification yield was evaluated by the ratio between the total activity for each step and the initial one. The purification fold was calculated by the ratio between specific activities at each purification step. All steps during the purification were performed at 4ºC.

Enzyme characterization

The apparent Michaelis-Menten constant K_M of the purified MnP was determined by Lineweaver-Burk plots of activity versus substrate profiles for 2,6-DMP in the range of 10 – 2,000 µM concentration and for H_2O_2 in the range of 40 – 2,400 µM concentration.

Enzymatic activity of purified MnP was determined at various pH (pH 3 – 9) and at 30ºC, after 15 min of incubation. The buffer solutions used were 150 mM sodium tartrate solution pH 3.0, 150 mM sodium malonate solution pH 4.5 and 5.5, 150 mM Tris-HCl solution pH 6.5, 7.5, 8.0 and 9.0. Enzymatic activity of purified MnP was determined at various temperatures between 25 and 70ºC, after 15 min of incubation. The activation energy (Ea) was calculated by plotting the log of activities of purified manganese peroxidase versus 1/T (in K) according to the Arrhenius equation. The value of activation energy was obtained by a computed linear regression analysis of the experimental data (Rao and Gianfreda, 2000). Enzyme thermostability was determined by incubating the purified MnP at different temperatures (40, 50, 60 and 70ºC) in sodium malonate solution (pH 4.5) for 2 h.

The molecular mass of the purified MnP was determined using SDS-PAGE electrophoresis (Laemmi, 1970) with the Protein III apparatus, Biorad. Protein bands were visualized with silver nitrate solution. The molecular mass marker used was the broad range protein molecular weight marker Promega. The molecular mass was estimated using a non-denaturizing electrophoresis (zymogram analysis) from purified MnP sample with the same molecular mass marker. Samples were applied in non-reducing denaturizing loading buffer without boiling. The gels were incubed in 50 mM sodium malonate pH 4.5 and 1 mM

$MnSO_4$ for 1 h and then stained for MnP activity with a solution containing the same solution supplemented with 1 mM de 2,6-DMP and the oxidative agent 0.4 mM H_2O_2. After the material was stained orange (the characteristic color of MnP activity), the gel was stained with Coomassie blue for better visualization of bands.

Purified MnP was observed by transmission electronic microscopy (TEM) (Jeol Jem-1200 EX 11, 120 KVolts, Camera Getan model 782, Erlangshen ES500W) in native and denaturalized state. The protein denaturalization was reached staining the enzyme with phosphotungstic acid (0.5% v/v).

Cultivation suitability of A. discolor Sp4 for MnP production in tubes containing glass beads

A. discolor Sp4 was cultivated in three culture media using two culture techniques, 100-ml Erlenmeyer flasks and 50-ml tubes containing 20 g of sterile glass beads (5 mm diameter) in Kirk medium. Erlenmeyer flasks and tubes contained 20 and 10 ml respectively of the following media: a) modified Kirk medium containing 0.25 mM $MnSO_4 \cdot H_2O$, b) modified Kirk medium containing 0.25 mM $MnSO_4 \cdot H_2O$ and sterile wheat grain (0.32 g ml^{-1}) and c) modified Kirk medium containing 0.25 mM $MnSO_4 \cdot H_2O$, sterile wheat grain (0.32 g ml^{-1}) supplemented with 0.05% v/v of Tween 80. The media were inoculated with active mycelia of *A. discolor* Sp4 from 5-day-old cultures on G-MEA medium (one plug/10 ml medium). Incubation was carried out at 30ºC under static conditions for 29 days. MnP activity in the supernatant of the fungal culture samples was monitored periodically by 2,6-DMP and MBTH/DMAB methods. All the values are the average of three analyzed samples with their standard deviation.

Statistical analysis was performed using a Student´s *t*-test for independent paired data for comparing the culture techniques on the production of MnP by *A. discolor* Sp4 growing in various media. Differences between mean values at $p \leq 0.05$ were considered to be significant.

Analyses

Enzyme assays

Manganese peroxidase activity in the supernatant of the fungal culture sample (after centrifugation for 10 min at 5,000 rpm) was determined by monitoring the oxidation of 2,6-DMP or MBTH/DMAB spectrophotometrically at 30ºC. Purified MnP was also determined by 2,6-DMP method.

2,6-DMP assay: the reaction mixture (1 ml) contained 200 µl of 250 mM sodium malonate (pH 4.5), 50 µl of 20 mM 2,6-DMP, 50 µl of 20 mM $MnSO_4 \cdot H_2O$, and 600 µl of supernatant. The reaction was initiated by adding 100 µl of 4 mM H_2O_2 and the absorbance of the colored product was measured at 468 nm and corrected for the laccase activity (Wariishi et al., 1992). One MnP activity unit (U) was defined as the amount of enzyme transforming 1 µmol 2,6-DMP per minute at pH 4.5 and 30ºC (Wariishi et al., 1992).

MBTH/DMAB assay: the reaction mixture (2 ml) contained 1460 µl of 100 mM succinate-lactate buffer (pH 4.5), 300 µl of 6.6 mM DMAB, 100 µl of 1.4 mM MBTH, 30 µl of 20 mM $MnSO_4$, and 100 µl of supernatant. The reaction was initiated by adding 10 µl of 10 mM H_2O_2 and the absorbance of the formed deep purple compound was measured at 590 nm. One unit is defined as the amount of enzyme needed to form 1 µmol of product in 1 min (Castillo et al., 1994).

Protease activity was measured using the Rowley and Bull method (1977). The supernatant of the samples was incubated with azocasein (2.5 g l^{-1}) at 37ºC for 20 minutes. The protease activity was stopped by precipitation of proteins with 2 ml of 2 M trichloroacetic acid. The precipitated proteins were eliminated by centrifugation and the change in color produced by the protease activity on the azocasein in the supernatant was measured at 400 nm. The activity was expressed in units (U) where a unit equals 1% azocasein hydrolysis in 20 min.

Protein concentration

Protein concentration in the supernatant of the fungal culture sample (after centrifugation for 10 min at 5,000 rpm) was determined using the Bradford method (Bradford 1976), with crystalline bovine serum albumin (BSA) as the protein standard. Protein content in purified MnP also was determined using the Bradford method.

The supernatant (1 ml) was added to 3 ml of Bradford reagent (Biorad) and mixed using gentle vortex mixing. The absorbance of the sample was measured at 595 nm using an ultraviolet-visible spectrophotometer against the Bradford reagent blank between 2 min and 1 h after mixing. The amount of protein was quantified by comparing the absorbance with a standard curve. All of the values are the average of three analyzed samples with their standard deviation.

3.4 Results and discussion

MnP production by A. discolor Sp4 in liquid medium supplemented with wheat grain

The addition of wheat grain to the static culture of *A. discolo*r Sp4 produced an increase in MnP activity (Fig. 3.1) compared to the controls (without wheat grain, with or without agitation). MnP activity reached the maximum value of 1,100 U l^{-1} after 13 days of growth, which is 3.8-fold higher than the control under static conditions and 8.6 times higher than the control with agitation (Fig. 3.1).

Our results are in agreement with those published in other reports in which higher ligninolytic activities were detected when lignocellulosic material was used as a substrate for fungal growth. The activity of MnP in *Pleurotus ostreatus* cultures with poplar sawdust was 8 times higher than in cultures with fir sawdust (Giardina et al., 2000) and the production of ligninolytic enzymes by *Phanerochaete chrysosporium* and *Trametes versicolor* grown with different lignocellulosic materials increased in relation to the control (without lignocellulosic

material), particularly with barley and grape residues (Moredo et al., 2003). White-rot fungi normally grow on decaying wood and forest litter. They degrade cellulose and lignin by means of their extracellular enzymatic system, which suggests that lignocellulosic substrates as energy source are suitable for their growth. The immobilization of the mycelium on the surface of wheat grains provides a greater surface area and increases mass transfer, thus improving the production of enzymes (Herpoël et al., 1999). Moreover, the fungal mycelium may penetrate the lignocellulose support, releasing additional water-soluble aromatic/phenolic substances, which in turn may induce the secretion of the ligninolytic enzymes (Kapich et al., 2004).

Low MnP activity was observed in agitated cultures. Venkatadri and Irvine (1990) indicated that the formation of mycelia pellets and O_2 limitation, shear stress of mycelia and denaturation of enzymes at high agitation speeds (100 to 200 rpm) are some explanations to this loss of activity. Also, the loss in enzyme activity is well-correlated to the degree of agitation, as described by Venkatadri and Irvine (1990) for ligninases. Therefore, static cell cultures are desirable for MnP production due to the increase in the contact area between cells and oxygen without shear stress (Ürek and Pazarlioğlu, 2007).

With the addition of wheat grain, the proteases activity was higher than the controls (without wheat grain, under static conditions or on a shaker at 100 rpm) and reached 0.20 U l^{-1} after 13 days. Under static conditions, the protease activity in the control without agitation increased 2-fold on the 13^{th} day in comparison with the agitated control, reaching a maximum value of 0.12 U l^{-1} (Fig. 3.1).

Some authors have reported that total glucose consumption by white-rot fungi in Kirk medium leads to protease production as a consequence of cell lysis, which affects ligninolytic enzyme production (Staszczak et al., 2000). In our study, MnP reached a maximum activity on day 13 followed by a significant increase in protease activity when using wheat grain (Fig. 3.1), suggesting a metabolic shift from lignin and cellulose to protein and carbohydrate degradation. In the absence of wheat grain, *A. discolor* Sp4 produced proteases (for peptone degradation) without showing a substantial MnP activity (Fig. 3.1).

In spite of higher protease production when wheat grain was used as a substrate, maximal MnP activity increased when compared with the results for the Kirk medium. This led us to consider the use of wheat grains as substrate to be beneficial for enhanced MnP production for future studies of organic pollutants degradation by *Anthracophyllum discolor* Sp4.

The protein concentration was higher than the controls in the presence of wheat grain, reaching 225 mg l^{-1} after 13 days of growth (Fig. 3.2). In the control (without wheat grains, under static conditions), the total protein concentration increased from 6 mg l^{-1} on day 6 to 40 mg l^{-1} on day 13. Under agitation, the maximum total protein concentration was 6 mg l^{-1} (Fig. 3.2).

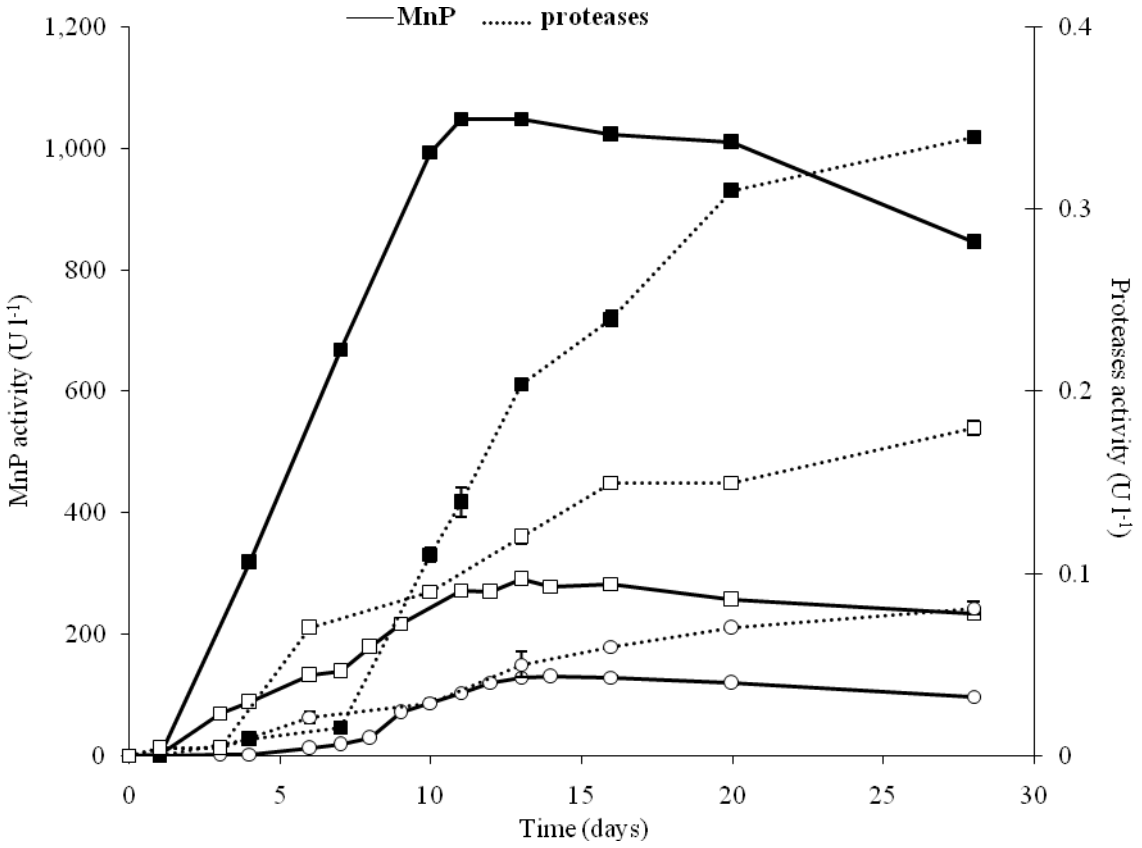


Figure 3.1 Manganese peroxidase (—) and proteases (·····) activity (U l^{-1}) in *A. discolor* Sp4 cultures growing in modified Kirk medium (■) supplemented with wheat grain and static conditions, (□) without wheat grain, under static conditions, (○) without wheat grain, on a shaker at 100 rpm.

The increase in total protein concentration (Fig. 3.2) correlated to the MnP activity (Fig. 3.1) as a response to the growth of *A. discolor* Sp4. Sato et al. (2007) have identified a large number of proteins from *Phanerochaete chrysosporium* grown on different liquid and solid substrates involved in carbohydrate, lignin and protein metabolism. Van den Wymelenberg et al. (2006) analyzed the *P. chrysosporium* genome, identifying 769 proteins within the secretosome, including cell wall-bound and endoplasmic reticulum (ER)-related proteins.

The presence of wheat grain slightly increased the pH in the culture medium from 5.2 to 5.5 (Fig. 3.2). This could be attributed to the presence of amonium in the medium, which is produced by the use of aminoacids and proteins of wheat grain (Appendix 2). The pH in the culture medium without wheat grains (with and without agitation) decreased from 5.2 to 4.2 after 28 days (Fig. 3.2). This is probably due to the secretion of organic acids, such as oxalic, formic and glyoxylic acids (Dutton et al., 1993; Dutton and Evans, 1996; Mäkelä et al., 2002). Physiological concentrations of oxalic acid have been shown to stimulate MnP activity by chelating unstable Mn^{3+} (Kuan and Tien 1993). MnP can generate H_2O_2 by oxidation of oxalic and glyoxylic acids, thus providing an endogenous source for extracellular H_2O_2 (Urzúa et al., 1998).

The pH ranges obtained in this study are adequate for white-rot fungi growth since they can generally grow at pH of between 4 and 6, with an optimum pH for enzyme production of between 4.5 and 5 (Ürek and Pazarlioğlu, 2007).

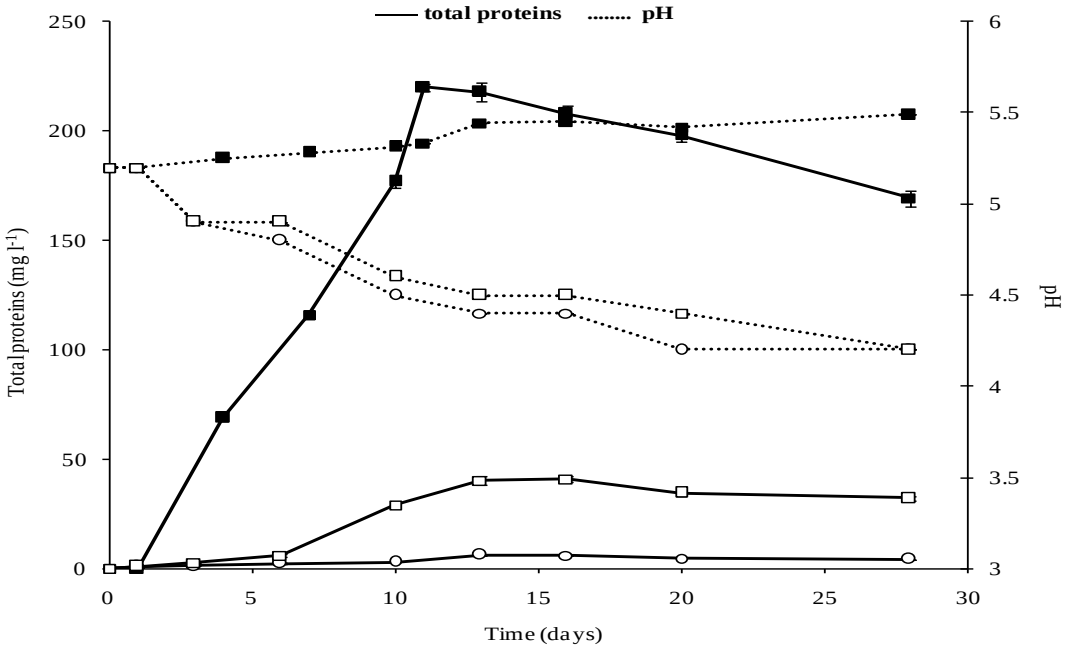


Figure 3.2 Total proteins (—) and pH (·····) in *A. discolor* Sp4 cultures growing in modified Kirk medium (■) supplemented with wheat grain, under static conditions, (□) without wheat grain, under static conditions, (○) without wheat grain, on a shaker at 100 rpm.

Effect of $MnSO_4$ and Tween 80 on MnP production by A. discolor Sp4 in liquid medium supplemented with wheat grain

The addition of 0.25 to 1 mM $MnSO_4 \cdot H_2O$ increased the production of MnP by *A. discolor* Sp4 in culture medium in presence of wheat grain (Fig. 3.3). The maximum MnP activity (1400 U l^{-1}) was obtained after 8 days of incubation with the addition of 0.25 mM of $MnSO_4 \cdot H_2O$. The highest concentration of $MnSO_4 \cdot H_2O$ (2.0 mM) added showed an inhibitory effect on the fungus growth and MnP production. In this treatment, a small mycelium growth was observed as compared to the other cultures supplemented with lower amounts of $MnSO_4 \cdot H_2O$. Moreover, the supernatant of this culture appeared turbid and darkened to brown after 8 days. This color could be attributed to the formation of a MnO_2 precipitate when Mn^{3+} is oxidized in the presence of an insufficient organic acid level in the medium (Rogalski et al., 2006). The enzymatic activity of MnP requires manganese to function. However, high concentrations of manganese can inhibit the enzyme due to the

generation of large quantities of hydrogen peroxide that inhibit the catabolic cycle of the enzyme (Wariishi et al., 1988; Bermek et al., 2002). In fact, hydrogen peroxide is known to be a product of numerous enzymatic reactions by means of aryl alcohol oxidase, glyoxal oxidase action and ligninolytic enzymes (Gómez-Toribio et al., 2001). In addition, H_2O_2 is produced by non-enzymatic reactions involving certain metals and metal complexes that have been shown to catalyze the reaction for the H_2O_2 production. Mn^{2+} reduces superoxide to H_2O_2 and is oxidized to Mn^{3+} (Schlosser and Höfer, 2002). In high concentrations of hydrogen peroxide, MnP II is converted in an inactive form (MnP III), not involved in the normal catalytic cycle (Mester and Tien, 2000). As a result, the amount of fungal biomass and the mycelia growth of the fungi are affected as well (Rothschild et al., 1999).

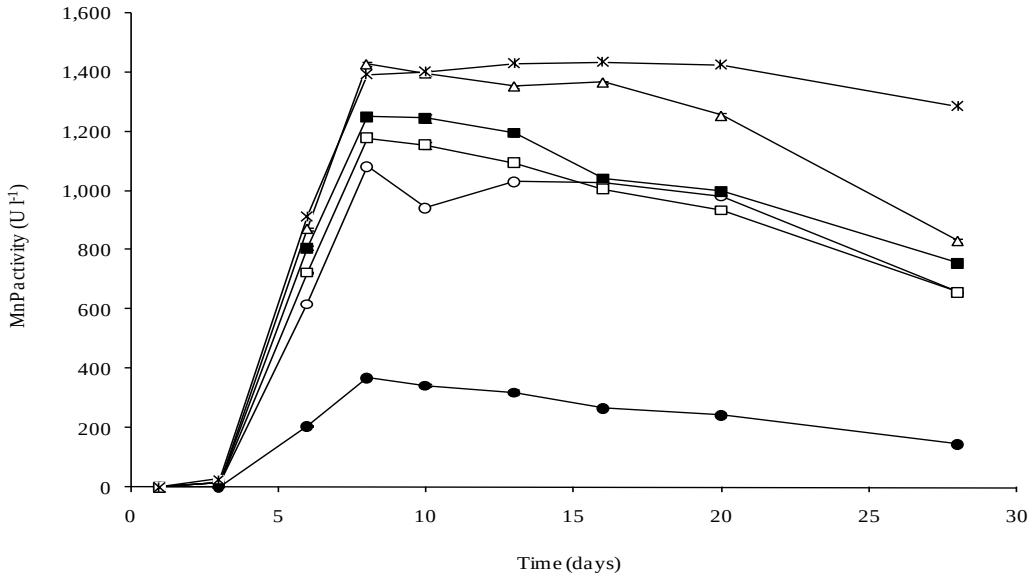


Figure 3.3 Manganese peroxidase (MnP) activity (U l^{-1}) in modified Kirk media inoculated with *A. discolor* Sp4 supplemented with wheat grain and (○) 0.002 (Δ) 0.25 (■) 0.5 (□) 1.0 (●) 2.0 mM of $MnSO_4$, (∗) 0.25 mM of $MnSO_4$ and 0.05% Tween 80, at 30°C under static conditions.

In our study, the addition of Tween 80 0.05% v/v maintained a high and constant production of the enzyme for 28 days (Fig. 3.3). Ürek and Pazarlioğlu (2005) observed that Tween 80 0.05% v/v would act as an inducer of MnP production from *P. chrysosporium*. The addition of surfactants such as Tween 80 would have a regulatory effect on the production of

ligninolytic enzymes, especially for MnP (Watanabe et al., 2000; Garon et al., 2002). Lipases may hydrolyze ester groups, releasing free fatty acids and affecting enzyme production by changing lipid metabolism (Asther et al., 1987). Surfactants also may modify the plasma membrane, altering transport of compounds in and out of the cell, or protect against mechanical inactivation of enzymes (Venkatadri and Irvine, 1990). However, a high concentration of surfactants could have an inhibiting effect on fungal growth and ligninolytic enzymes production (Zhou et al., 2007). The mechanism by which surfactants such as Tween 80 increase extracellular enzyme production has not been established.

MnP purification

MnP purification from *A. discolor* Sp4 cultivated under optimized culture conditions was carried out through two chromatographic steps: an anion-exchange chromatography using a Q-Sepharose column coupled with further gel filtration chromatography using a GFC-100 column. Table 3.1 summarizes the results obtained from the MnP purification process. MnP specific activity increased after each purification step, which suggests an increase in MnP purity. In this study, a purification yield of approximately 48% was obtained after the anion-exchange chromatography and was reduced to 5.7% after the gel filtration chromatography step (Table 3.1). Furthermore, the purification factor increased by only 0.4 units after the second chromatographic step. This indicates that a single step with anion exchange chromatography is sufficient to obtain purified MnP. After the first chromatographic step, fractions of elution volumes - 10, 10.5 and 11 ml - presented MnP activity (Fig. 3.4a) and were collected and analyzed by electrophoresis to determine the molecular mass of the protein. In the case of the second chromatographic step, two fractions (elution volumes of 5.5 and 6 ml) presented MnP activity (Fig. 3.4b), which also were collected and analyzed by electrophoresis.

MnP characterization

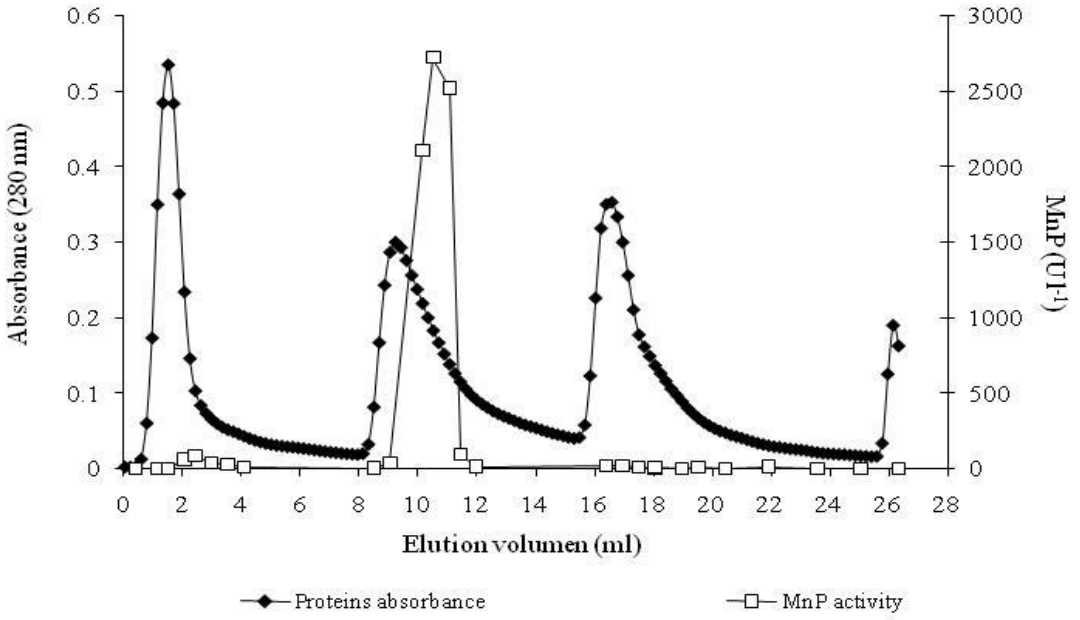
K_M value, pH and optimum temperature values were determined in order to characterize the MnP enzyme produced by *A. discolor* Sp4. The apparent K_M values of purified MnP for 2,6-

DMP and H_2O_2 were found to be 24.83 µM and 36.98 µM, respectively, indicating high affinity for both substrates. Previous research regarding kinetic parameters from *Phanerochaete chrysosporium* using 2,6-DMP as substrate shows that K_M values range from 7 to 29 µM (Palma et al., 2000, Ürek and Pazarlioğlu, 2004) depending on the operational conditions and the isoenzymes produced. They are comparable and in the same magnitude order as the results obtained for MnP enzyme from *A. discolor*. K_M values for *Bjerkandera sp.* obtained using the same substrate show a variation of between 11 and 121 µM, which can be considered a higher degradation rate than MnP enzyme from *A. discolor* Sp4. In regard to kinetic parameters considering H_2O_2 as a substrate, previous research has shown that K_M values for *Phanerochaete chrysosporium* range from 5 to 71 µM (Palma et al., 2000, Ürek and Pazarlioğlu, 2004) and arecomparable to the results obtained for MnP enzyme from *A. discolor* Sp4. K_M values for *Bjerkandera sp*. obtained using H_2O_2 show a variation of between 3 and 5 µM (Palma et al., 2000), which can be considered lower as compared with MnP enzyme from *A. discolor* Sp4.

Table 3.1 Purification process of MnP from *A. discolor* Sp4. The MnP activity in the crude extracellular fluid and after the different steps were estimated by monitoring the oxidation of 2,6-DMP spectrophotometrically. Protein content was estimated using the Bradford method.

Purification step	Total activity (U)	Total proteins (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Crude extracellular fluid	108.5	18	6.0	100	1.00
Ultrafiltration 10 kDa	102.3	9.3	11.0	94.3	1.82
Q Sepharose	52.5	2	26.2	48.3	4.35
Gel filtration GFC100	6.23	0.22	28.3	5.7	4.70

a)



b)

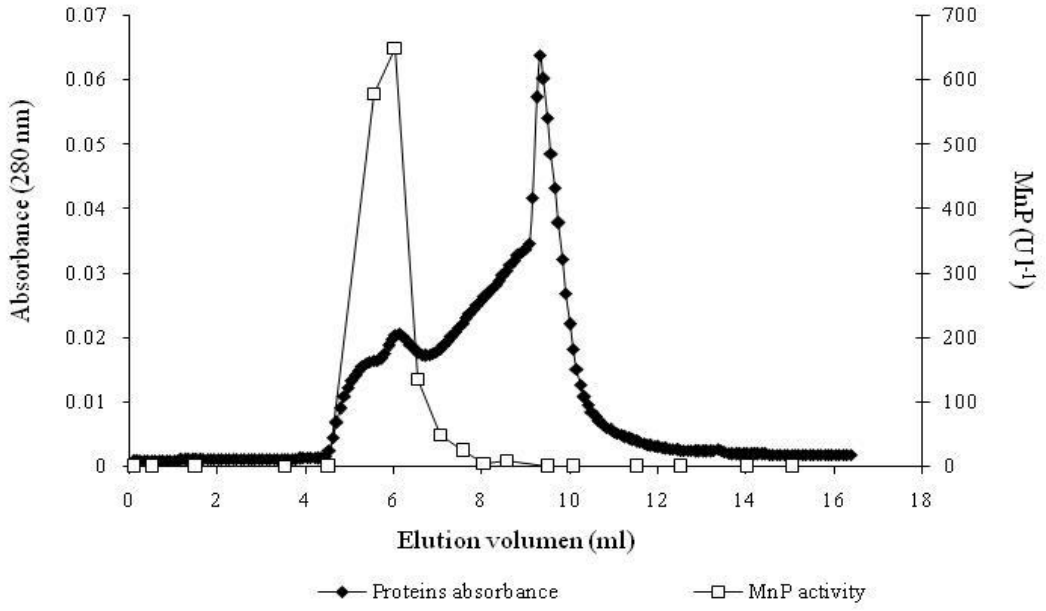


Figure 3.4 Elution profile of (○) MnP activity and (●) proteins absorption on a) Q Sepharose column and b) GFC-100 gel filtration column.

The optimum pH range was determined to be between 4.5 and 5.5, with the relative activity dropping by 20% at pH 6.5 and being inactivated at pH 9.0 (Fig. 3.5). This pH range is in agreement to the optimum pH values reported in the literature (Ürek and Pazarlioğlu 2004); nevertheless, a specific white-rot fungus (*Aspergillus terreus* LD-1) has been found to have an optimum alkaline pH range of between 11.0 and 12.5 (Kanayama et al., 2002).

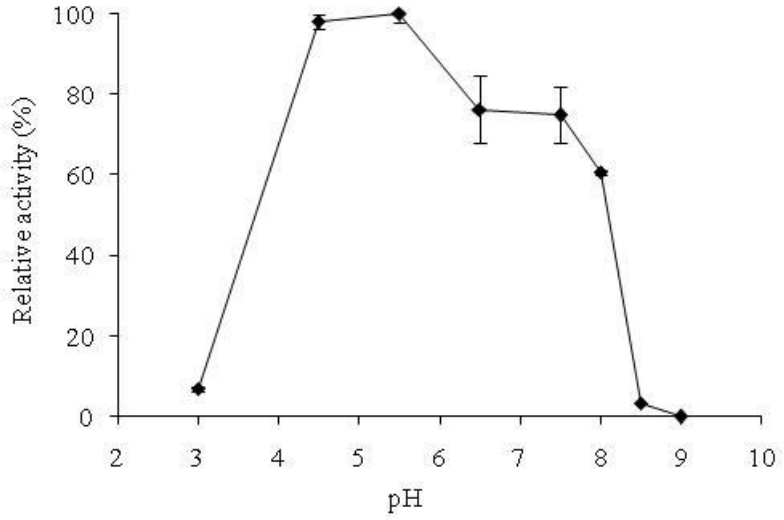


Fig. 3.5 Effect of 15 min exposure to different pHs (3 - 9) on the activity of purified MnP (■) from *A. discolor* Sp4, at 30 °C.

The temperature required to maximize MnP activity at pH 4.5 was 50ºC after 15 min of incubation, and its activity was completely lost at 70ºC (Fig. 3.6). Temperatures for maximizing MnP activity have been described as ranging from 22ºC (Paszczynski et al., 1988) to 60ºC (Wang et al., 2002), depending on the fungus species. Activation energy (Ea) of 16.5 kJ mol^{-1} was determined in the temperature range between 30°C and 50°C. This value is according to the Ea obtained for free enzymes such as invertase (19.95 kJ mol^{-1}) (Gianfreda et al., 1991) and urease from jack bean (13-15 kJ mol^{-1}) (Gianfreda et al., 1995).

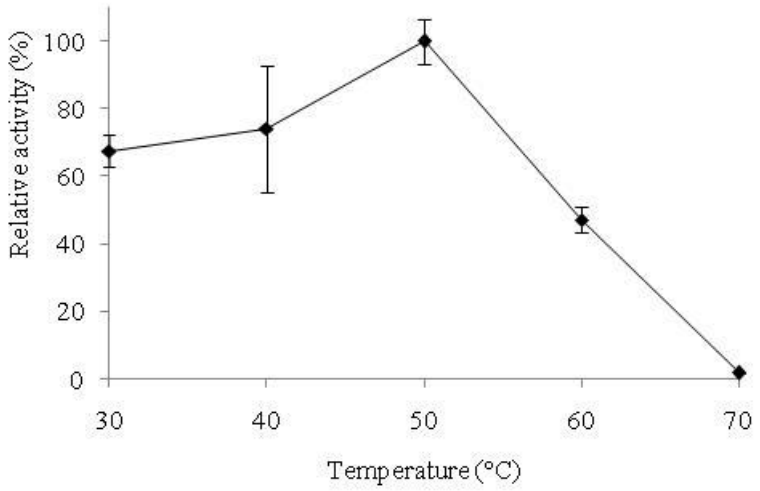


Fig. 3.6 Effect of 15 min exposure to various temperatures (30 – 70°C) at pH 4.5, on the activity of purified MnP (■) from *A. discolor* Sp4

Regarding to thermal stability, MnP showed high stability between 40 and 50ºC (Fig. 3.7). More than 95% activity was retained after 120 min of incubation at 40ºC. At 50ºC, MnP lost 30% of its activity after two hours of incubation, reaching a value of 70% of its maximal activity (Fig. 3.6). In contrast, fast deactivation occurred at 60ºC where the residual activity was 20% after 2 hours of incubation and a sudden loss of activity was observed at 70ºC where the activity decreased to zero after only 30 min of incubation. This result indicates that the enzyme could be used in the temperature range between 40 and 50°C in bioremediation processes.

Molecular mass

Two important bands were observed in the SDS-PAGE gel after purification, which suggests the presence of MnP isoenzymes (MnP_1 and MnP_2) (Fig. 3.8a). The molecular mass of MnP isoenzymes was estimated to be 35 and 38 kDa (Fig. 3.8a) via SDS-PAGE electrophoresis. The zymogram analysis of the purified enzyme sample confirmed these results, showing two isoenzymes with estimated molecular mass of 34 and 37 kDa respectively (Fig. 3.8b).

The molecular mass of the two isoenzymes was lower than those reported in the literature. The molecular mass range of MnP in white-rot fungi is quite broad. Hofrichter (2002) reported a molecular mass from 38 kDa for *Trametes troggi* up to 52.5 kDa for *Ceriporiopsis subvermispora*.

The existence of multiple MnP isoenzymes in different ligninolytic fungi has been described (Lobos et al., 1994). The differences in fungal species and strains and growth conditions (composition of culture medium, incubation time, with or without agitation) strongly affect the isoenzyme patterns produced by the different fungi (Boer et al., 2006). Purification methods and storage also can affect the relative isoenzymatic levels (Cullen and Kersten, 1996).

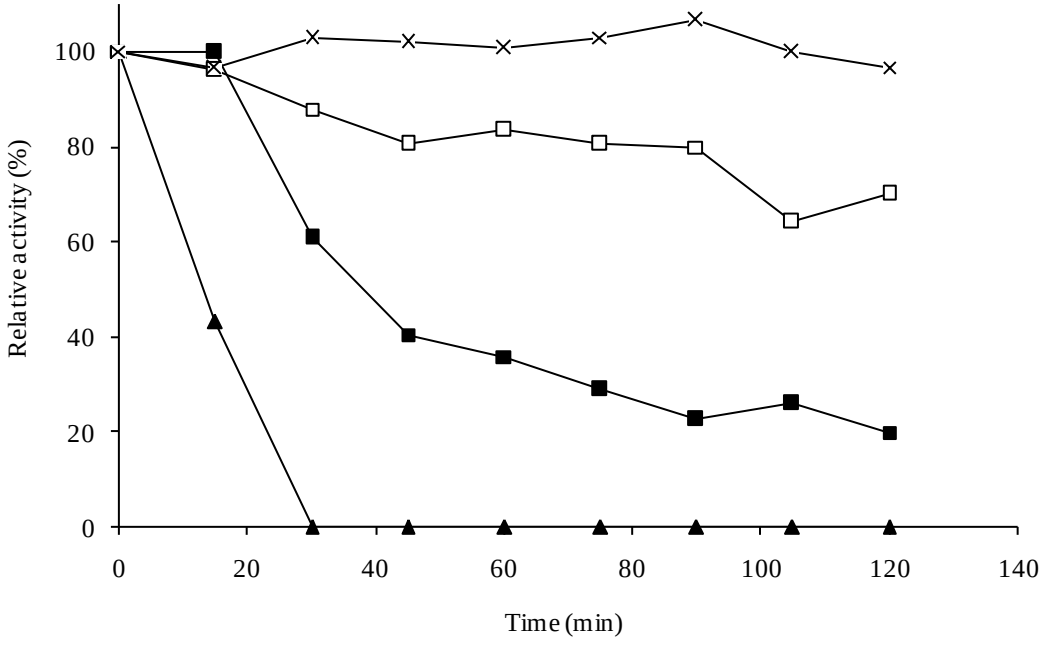
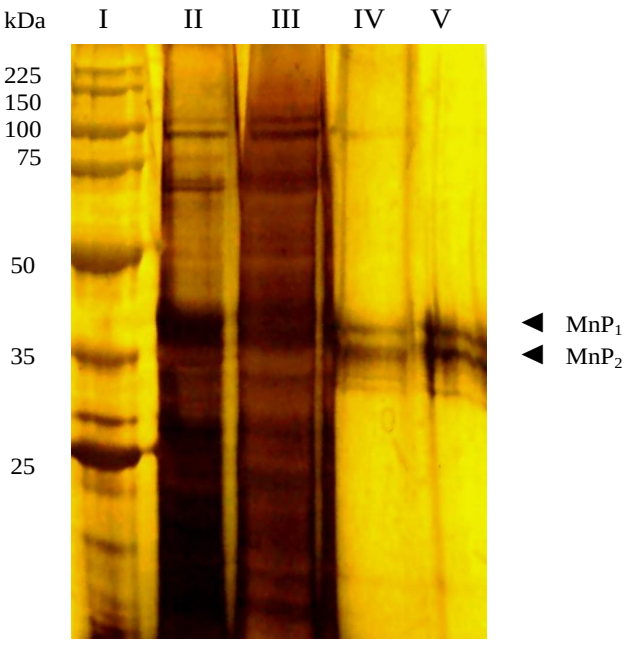
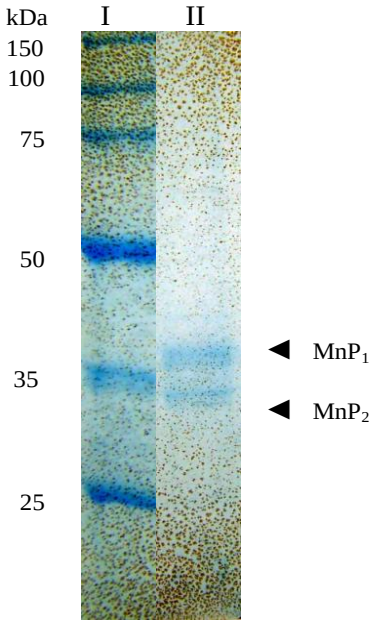


Figure 3.7 Effect of temperature on MnP activity stability during 2 h of preincubation at 40°C (x), 50°C (□), 60°C (■), 70°C (▲) at pH 4.5.



a)



b)

Figure 3.8 a) Electrophoresis SDS-PAGE of MnP from *A. discolor* Sp4 with silver nitrate staining (I) proteins standards (Promega) (II) extracellular fluid (III) concentrate (IV) after Q-Sepharose column chromatogrphy (V) after gel filtration column chromatography b) Zymogram of purified MnP (I) Proteins standards (Promega) (II) MnP isoenzymes.

Using Transmission Electronic Microscopy (TEM), it was possible to visualize the morphology of a native and denaturalized MnP isoenzyme (Figure 3.9). The morphology of native MnP is characterized by numerous dispersed filaments similar to cilia, while denaturized MnP forms aggregated filaments. Phosphotungstic acid has long been used as a protein precipitant (Silverman et al., 1969) acting as an anionic stain for the positively charged groups of protein for a better visualization of proteins by transmission electron microscopy (Fig. 3.9b).

a) b)

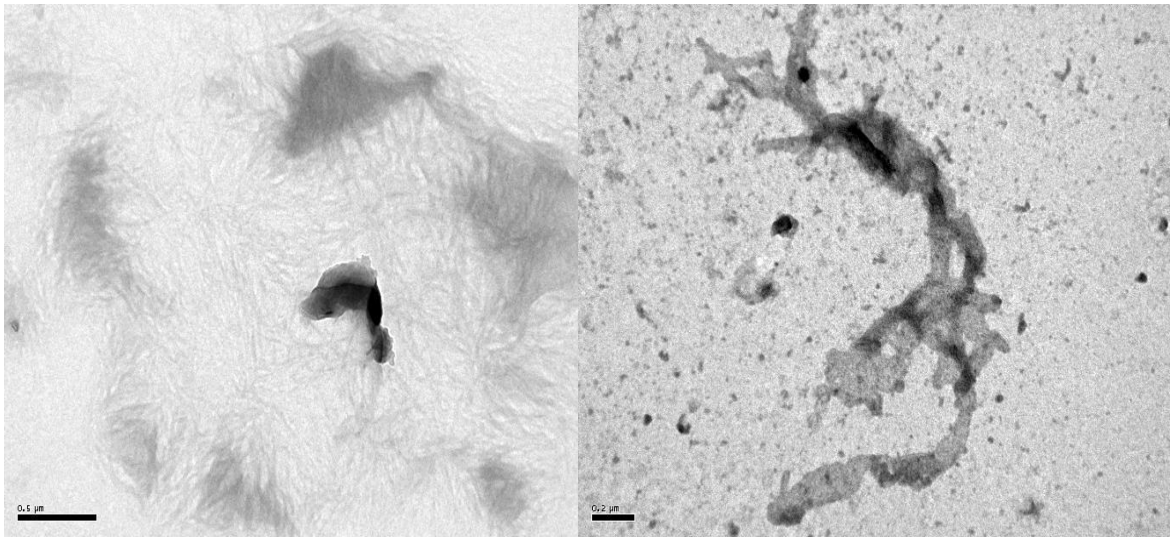


Figure 3.9 a) native and b) denaturalized purified MnP by Transmission Electronic Microscopy.

Cultivation suitability of A. discolor Sp4 for MnP production in tubes containing glass beads

A. discolor Sp4 showed significant growth in tubes containing glass beads penetrating the hyphae to the bottom of the tube and favoring interaction between the fungus and the medium for enhancing MnP production. Using this culture system, the volume of the samples was considerably reduced and each tube was used to perform the corresponding analysis without altering the assay (destructive sampling). This is relevant for

biodegradation assays over time, when it is important to carry out several experiments in parallel managing a large number of samples in terms of space.

Table 3.2 shows the MnP production from *A. discolor* Sp4 grown in Erlenmeyer flasks or tubes containing glass beads in different culture media. When comparing the MnP production on different culture media, an increase in MnP activity was observed using Kirk medium supplemented with $MnSO_4 \cdot H_2O$ and wheat grain compared to the results obtained using Kirk medium supplemented $MnSO_4 \cdot H_2O$ (without wheat grain). As shown above, the addition of wheat grain as lignocellulosic material favored the fungal growth and MnP production.

Using tubes containing glass beads, MnP activity reached the maximum value of 15.8 U l^{-1} day^{-1}. The maximal activity reached using Erlenmeyer flasks was 16.1 U l^{-1} day^{-1}. The use of the tubes containing glass beads for cultivation of *A. discolor* Sp4 did not affect MnP activity when using Kirk medium and $MnSO_4$, as the values obtained did not differ largely from those in the Erlenmeyer flasks. When using wheat grain as substrate, MnP activity in tubes was even higher than tests with Erlenmeyer flasks ($p \leq 0.05$), particularly for days 12, 15 and 19 after inoculation. MnP activity reached the maximum value (956 U l^{-1}) after 19 days, 1.7-fold higher than that which was observed in Erlenmeyer flasks after the same incubation period. In this case, *A. discolor* Sp4 covered all of the glass beads and wheat grains, which resulted in a larger contact surface area. When using Tween 80, practically no significant differences in MnP activity using either technique (tubes containing glass beads or Erlenmeyer flaks) were observed.

Pizzul et al. (2006) used the glass-beads tube technique to test PAHs degradation by actinomycetes, and the system allowed for both good microbial growth confined to the liquid medium and glass beads matrix and suitable and rapid PAH quantification. In fact, PAHs are adhered to the glass beads surface uniformly and tubes containing beads can be easily agitated and centrifuged, allowing the supernatant to be analyzed directly by gas chromatography with a PAHs recovery yield of up to 95% (Pizzul et al., 2006). Glass beads also have been used to simulate soil particles and monitor growth of the mycorrhizal fungus

Glomus intraradices (Rilling and Steinberg, 2002) and the actinobacterium *Streptomyces* (Nguyen et al., 2005). Ehlers and Rose (2005) evaluated the immobilization of three white-rot fungal cultures in trickling packed-bed reactors employing glass beads as support in biodegradation of phenol and 2,4,6-trichlorophenol-contaminated liquid. Microscopic examination showed that the glass beads were colonized by the fungal cultures. Furthermore, rapid uptake, removal and biodegradation of the compounds were obtained as a result of the large contact surface area and long exposure to lignin-degrading enzymes and mycelia (Ehlers and Rose, 2005).

Comparison of enzymatic assays

Measurements of MnP activity using the 2,6-DMP method and the MBTH/DMAB test in different culture conditions for *A. discolor* Sp4 were compared (Fig. 3.10). The research shows that the MBTH/DMAB method was more sensitive in all cases, as shown by Castillo et al. (1994). In fact, the reaction with 2,6-DMP gives a final orange color that is similar to the color of the Kirk medium supplemented with wheat grains, which interferes with the spectrophotometric reading. In contrast, the reaction with MBTH/DMAB produces a deep purple-blue color, which does not interfere with the spectrophotometric quantification. Castillo et al. (1997) compared MnP activity in extracts from straw cultures of *Phanerochaete chrysosporium* using six different substrates (MBTH/DMAB, ABTS, DMP, Guaiacol, Mn(II), Phenol Red). They showed a faster reaction using MBTH/DMAB as substrate than other assays and identified it as an appropriate method for colored samples such as culture fluids supplemented with lignocellulosic support.

Table 3.2 Manganese peroxidase (MnP) activity (U l^{-1}) in *A. discolor* Sp4 cultures grown in Erlenmeyer flasks or tubes in different culture conditions.

Time (days)	MnP activity (U l^{-1})								
	Kirk medium supplemented with $MnSO_4 \cdot H_2O$			Kirk medium supplemented with $MnSO_4 \cdot H_2O$ and wheat grain			Kirk medium supplemented with $MnSO_4 \cdot H_2O$, wheat grain and Tween 80		
	Flask	Tube	Student's t-test	Flask	Tube	Student's t-test	Flask	Tube	Student's t-test
4	2.4 ± 0.2	3.4 ± 0.2		4.5 ± 1.6	6.1 ± 1.1		3.4 ± 1.9	0.4 ± 0.2	
9	169.6 ± 18.7	25.8± 0.9	*	391.3 ± 52.2	356.9 ± 75.5		536.8 ± 85.0	319.9 ± 129.9	
12	56.7 ± 7.2	190.6 ± 2.3		425.1 ± 92.4	779.4 ± 16.1	*	629.4 ± 156.7	815.1 ± 117.6	
15	242.1 ± 27.1	143.1 ± 25.7	*	614.7 ± 134.4	934.9 ± 110.8	*	746.5 ± 225.4	769.0 ± 28.6	
19	100.9 ± 10.8	139.6 ± 5.3		546.8 ± 156.3	955.6 ± 54.4	*	696.0 ± 38.3	968.7 ± 84.4	*
29	114.9 ± 16.6	40.5 ± 19.1	*	494.6 ± 127.9	276.1 ± 81.0		413.3 ± 12.4	395.7 ± 0.4	

Values represent MnP activity mean N = 3. Asterisks (*) indicate a statistically significant difference between culture techniques at the same time ($p < 0.05$, Student's t-test)

a)

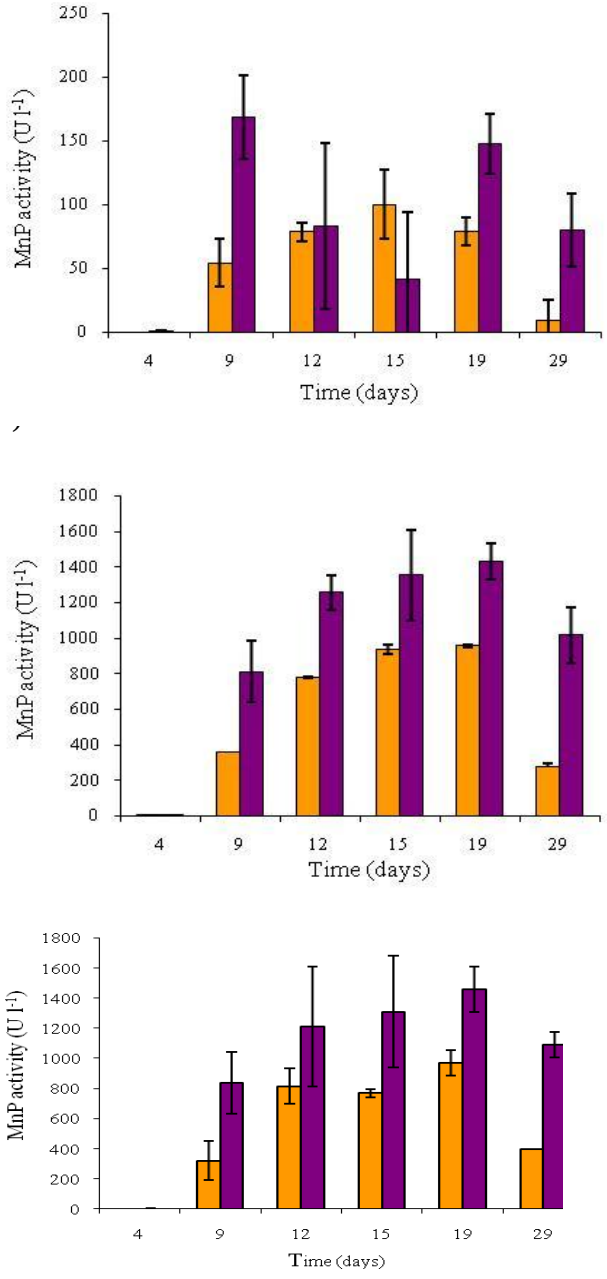


Figure 3.10 MnP activity measured by (■) 2,6-DMP and (■) MBTH/DMAB method from *A. discolor* Sp4 in tube containing glass beads grown in (a) Kirk medium with $MnSO_4 \cdot H_2O$ (b) Kirk medium with $MnSO_4 \cdot H_2O$ and wheat grains (c) Kirk medium with $MnSO_4 \cdot H_2O$, wheat grains and Tween 80.

3.5 Conclusions

Our results show that the addition of wheat grain, 0.25 mM $MnSO_4$ and 0.05% v/v Tween 80 in Kirk medium significantly increases MnP production from *A. discolor* Sp4. MnP enzyme produced by *A. discolor* Sp4 presents biochemical and kinetic properties, which could be quite advantagenous for bioremediation purposes. The simple glass-bead technique used in this study for fungal culture in liquid medium allowed for *A. discolor* Sp4 growth and MnP production at the same levels compared to the standard Erlenmeyer flasks technique. This technique reduces sample volume, simultaneously allowing for destructive sampling. Finally, the MBTH/DMAB assay was found to be more sensitive than the 2,6-DMP assay and proved to be a suitable method for determining MnP in colored samples such as culture fluids supplemented with lignocellulosic support.

3.6 Aknowledgements

This work was funded in part by FONDECYT project Nº 1050614, a doctoral scholarship from CONICYT (Chile) and the Swedish Foundation for Strategic Environmental Research (MISTRA) through funding for the Domestication of Microorganisms (DOM) Research Program and the Centre for Chemical Pesticides (CKB) at the Swedish University of Agricultural Sciences. The authors would like to thank Dr. Rodrigo Navia and Dr. María Elena Lienqueo for their suggestions.

4

Degradation of polycyclic aromatic hydrocarbons by the Chilean white-rot fungus *Anthracophyllum discolor* Sp4

4.1 Abstract

The degradation of 3 and 4-ring polycyclic aromatic hydrocarbons (PAHs) in Kirk medium by *Anthracophyllum discolor* Sp4, a white-rot fungus isolated from the forest of southern Chile, was evaluated. In addition, the removal efficiency of 3, 4 and 5-ring PAHs in contaminated soil bioaugmented with *A. discolor* Sp4 in the absence and presence of indigenous soil microorganisms was investigated. Production of lignin degrading enzymes and PAH mineralization in soil were also determined. *A. discolor* Sp4 was able to degrade PAHs simultaneously and individually in Kirk medium with the highest removal occurring in the PAH mixture, suggesting synergistic effects between PAHs or possible cometabolism. A high removal capability for phenanthrene (62%), anthracene (73%), fluoranthene (54%), pyrene (60%) and benzo*(a)*pyrene (75%) was observed in autoclaved soil inoculated with *A. discolor* Sp4 in the absence of indigenous microorganisms, associated with the production of manganese peroxidase (MnP). The metabolites found in the PAH degradation were anthraquinone, phtalic acid, 4-hidroxy-9-fluorenone, 9-fluorenone and 4,5-dihydropyrene. *A. discolor* Sp4 was able to mineralize 9% of the phenanthrene. In non-autoclaved soil, the inoculation with *A. discolor* Sp4 did not improve the removal efficiency of PAHs. Suitable conditions must be found to promote a successful fungal bioaugmentation in non-autoclaved soils. The colonization of *A. discolor* Sp4 in non-autoclaved soil may require a higher demand for substrate compared to autoclaved soil. Furthermore, its use may be limited to soils with high organic matter content (for the availability of nutrients) or will even require additional nutrients supply (biostimulation).

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Keywords: Biodegradation, *Anthracophyllum discolor* Sp4, ligninolytic enzymes, polycyclic aromatic hydrocarbons (PAHs), bioaugmentation

4.2 Introduction

Xenobiotic chemicals are continuously released into the biosphere posing a significant risk to the human health due to their toxicity and persistence in the environment. Polycyclic aromatic hydrocarbons (PAHs) from natural and/or anthropogenic sources are characterized by their teratogenic, mutagenic and carcinogenic properties (Blumer, 1976) and their persistence in the environment is related to their low aqueous solubility, vapor pressures and, high octanol/water partitioning coefficients. As consequence, PAHs have a high affinity for association with organic carbon material (humus) in soil (Alexander 1995).

Microbial degradation is one of the major processes in cleaning up PAH-contaminated environments. Among degrading microorganisms, white-rot fungi have demonstrated the ability to degrade a wide range of pollutants, including PAHs (Pointing, 2001, Hwang et al., 2007). The extracellular ligninolytic enzyme system of the white-rot fungi, consisting of peroxidases and laccases, have been directly linked to biodegradation of PAHs (Steffen et al., 2002a, Dodor et al., 2004; Eibes et al., 2006). Fungal extracellular enzymes catalyze PAH oxidation generating more polar and soluble metabolites, such as quinonas, phtalic or diphenic acid (Hwang et al., 2007). Increased polarity and water solubility are important factors for enhancement of the bioavailability of these metabolites and their mineralization by native microorganisms present in contaminated sites (Kotterman et al., 1998).

In particular, the Chilean white-rot fungus *Anthracophyllum discolor* Sp4 produces high levels of manganese peroxidase (MnP) in the presence of wheat grains as lignocellulosic support (Rubilar 2007) and to a lesser extent laccase (L) and lignin peroxidase (LiP), and is efficient in the degradation of organic pollutants such as chlorophenols and dyes (Rubilar et al., 2007; Tortella et al., 2008). Recently, bioremediation of soil contaminated with pentachlorophenol (PCP) was investigated using *A. discolor* Sp4 (Rubilar et al., 2010). The application of *A. discolor* Sp4 immobilized in wheat grains to the contaminated soil favored the spread of the fungus and a high PCP removal efficiency (70-85%) was found in comparison to that measured with the fungus as free mycelium (30-45%) (Rubilar et al., 2010).

Considering that the potential of *A. discolor* Sp4 to degrade PAHs has not been explored yet, the first purpose of this study was to evaluate the degradation by *Anthracophyllum discolor* Sp4 of 3 and 4-ring PAHs individually and simultaneously (mixture) in Kirk medium. The second objective was to investigate the 3, 4 and 5-ring PAHs removal efficiency in a contaminated soil bioaugmented with *A. discolor* Sp4 in the absence or presence of indigenous soil microorganisms, as well as the production of lignin-degrading enzymes and PAH mineralization.

4.3 Materials and methods

Microorganism and inoculum

The fungal strain used was *Anthracophyllum discolor* Sp4 (culture collection of the Environmental Biotechnology Laboratory at Universidad de La Frontera, Chile) isolated from decayed wood in the rain forest of southern Chile. This fungus was maintained at 4ºC in glucose malt extract agar (G-MEA) slants tubes containing: malt extract 30 g l^{-1}; agar 15 g l^{-1} and glucose 10 g l^{-1}. The fungus was transferred from slant culture tubes to Petri dishes with G-MEA medium and incubated at 30ºC for 7 days. One agar plug (5 mm diameter) with mycelium obtained from the colony diameter grown in G-MEA medium was used as inoculum in liquid and soil cultures, for biodegradation and mineralization assays.

Chemicals

MBTH (3-methyl-2-benzothiazolinone) and DMAB (3-(dimethylamino)benzoic acid) were supplied by Aldrich Chemical Co., Germany. 3,4-dimethoxy-benzyl alcohol (> 95%) was supplied by Fluka. Anthracene (> 96%) and phenanthrene (> 97%) were purchased from Merck (Hohenbrunn, Germany); pyrene (98%) and fluoranthene (98%) were purchased from Aldrich. Benzo*(a)*pyrene was supplied by Fluka. Labeled benzo(*a*)pyrene ([7,10-^{14}C]benzo*(a)*pyrene, specific activity, 61.0 mCi $mmol^{-1}$; radiochemical purity, 98.9%) was supplied by Amersham Biosciences (UK). Labeled phenanthrene ([9-^{14}C]phenanthrene, specific activity, 55.7 mCi $mmol^{-1}$; radiochemical purity, 98.9%) and [4,5,9,10-^{14}C]pyrene

(specific activity, 55.0 mCi $mmol^{-1}$; radiochemical purity, 97.8%) were supplied by Sigma (USA). Acetone (HPLC grade) and toluene (HPLC grade) were provided by Merck. Insta-Gel plus was supplied by Perkin-Elmer. All other chemicals were supplied by Merck, Oxoid, Sigma and Duchefa (Netherlands).

Soil

An agricultural topsoil containing 14% clay, 3% organic matter, 1.8% organic carbon, 0.11% nitrogen and with a pH in water of 6.6 was used (Pizzul et al., 2006). It was collected in Uppsala, Sweden, sieved (< 2 mm) and stored at 4°C until use.

PAH removal in liquid culture

The assay was performed using tubes with glass beads (Pizzul et al., 2006). Briefly, 20 g of glass beads (5 mm diameter) were added to 50-ml tubes and autoclaved for 20 min at 120°C. Solutions of individual PAHs (anthracene, phenanthrene, fluoranthene and pyrene) or a mixture of the four PAHs in acetone were added aseptically onto the glass beads to give a final concentration in the Kirk medium (Tien and Kirk, 1988) of 50 mg l^{-1} of each PAH. The acetone was allowed to evaporate and 10 ml Kirk medium supplemented with Tween 80 (0.05% v/v) was added into the tube and inoculated with an agar plug of *A. discolor* Sp4. Incubation was carried out at 30ºC for 28 days. Each experiment was carried out in duplicate under destructive sampling mode. The tubes were maintained at -20ºC until PAH concentration in all the treatments (individual and mixture) and ligninolytic enzyme activity (in treatments with PAHs mixture) were determined. A set of tubes with the medium inoculated with *A. discolor* Sp4 without PAHs was run in parallel to evaluate the enzymatic activities. In addition, the PAH extraction yield in each treatment without *A. discolor* Sp4 was evaluated.

PAH removal in soil

The degradation of PAHs in soil by *A. discolor* Sp4 was determined in the presence or absence of indigenous microorganisms. Ligninolytic enzymes activity in soil was also determined in the absence of indigenous microorganisms. Autoclaved (30 min at 121°C, 1 bar, three times with 24 h of interval) soil (1 kg) or non-autoclaved soil was contaminated with the mixture of 50 mg kg^{-1} of each of the following PAHs: phenanthrene, anthracene, pyrene, fluoranthene and 40 mg kg^{-1} of benzo*(a)*pyrene . The soil spiking was carried out according to Brinch et al. (2002) by treating a subsample of the soil (25%) with the PAH mixture in acetone. After evaporated acetone, the contaminated soil was mixed with the remaining soil. Contaminated autoclaved and non-autoclaved soil (10 g) was weighed into 30 ml-tubes, supplemented with Tween 80 0.05% (v/w soil) and 0.5 g of sterile wheat grains were placed above the soil. An agar plug with active mycelia of *A. discolor* Sp4 was placed on top of the wheat grains as inoculum. The tubes were incubated at 30°C. The soil water content was kept at 60% of the water-holding capacity. Contaminated soil (either autoclaved or non autoclaved soil) supplemented with Tween 80 0.05% (v/v) and 0.5 g wheat grains, without the addition of fungus was used as control for determination of PAHs content. Autoclaved soil without PAHs, supplemented with Tween 80 0.05% (v/v) and 0.5 g wheat grains and inoculated by *A. discolor* Sp4 was used as control for determining ligninolytic enzyme activity. Also, the PAH extraction yield in autoclaved soil supplemented with Tween 80 0.05% (v/v) and 0.5 g wheat grains without *A. discolor* Sp4 was evaluated. Each experiment was carried out in triplicate under destructive sampling mode. Sampling in autoclaved and non-autoclaved soil was done periodically for 60 days to determinate PAH content and the enzymatic activities were evaluated in autoclaved soil.

Mineralization studies of PAHs in soil

The degree of mineralization of ^{14}C-phenanthrene, ^{14}C-pyrene and ^{14}C-benzo*(a)*pyrene by *A. discolor* Sp4 in autoclaved soil contaminated previously with 50 mg kg^{-1} of each of the following PAHs: phenanthrene, anthracene, pyrene, fluoranthene and 40 mg kg^{-1} of benzo*(a)*pyrene was monitored for 60 days. Non-labeled CO_2 was measured during 60 days

at 30°C in autoclaved and non-autoclaved soils contaminated with PAHs and inoculated by *A. discolor* Sp4.

A portion (10 g) of contaminated autoclaved or non-autoclaved soil was weighed into 100 ml plastic vials, supplemented with Tween 80 (0.05% v/w soil) and 0.5 g sterile wheat grains and inoculated with an agar plug of *A. discolor* Sp4. The water content was adjusted to 60% of the water-holding capacity. ^{14}C-phenanthrene, ^{14}C-pyrene or ^{14}C-benzo*(a)*pyrene was added to the samples to give a total radioactivity in the soil material of about 60,000 dpm. These 100-ml plastic vials were placed in a hermetic glass jar together with two 20-ml plastic vials containing 4 ml of a 0.2 M NaOH solution and incubated at 30°C for 60 days. NaOH solution was periodically removed and respiration and mineralization determined in the respective vial. Non-inoculated soil was used as the control. The experiment was carried out in triplicate.

Mineralization was determined by collecting $^{14}CO_2$ from the degradation of the labeled PAHs in the NaOH solution and measuring in a liquid scintillation counter (Beckmann LS 600 series, USA), after mixing with 4 ml of Insta-gel Plus. Mineralization was expressed as accumulated $^{14}CO_2$ as a percentage of the initial radioactivity.

In the respiration tests, the CO_2 captured in the NaOH solution was determined by titrating the remaining alkali with 0.1 M HCl (TIM850 titration manager, Tritalab®, Radiometer Ananlytical SAS) after precipitation of the carbonate with 0.1 M $BaCl_2$. The respiration was expressed as accumulated mg CO_2 g soil^{-1}.

Analyses

Enzyme extraction

In liquid medium, 2 ml of culture was centrifuged for 10 min at 5000 rpm, and ligninolytic enzyme activity was determined in the supernatant.

In autoclaved soil, 10 g of sample was weighed in an Erlenmeyer flask and 20 ml succinate/lactate buffer 0.1 M at pH 4.5 was added. The flask was shaken at 100 rpm for 1 hour. After 10 minutes of sedimentation, 5 ml of the liquid phase were centrifuged (4000 rpm) and filtered through a 0.45 µm filter unit (Castillo and Torstensson, 2007). MnP, L and LiP activities were determined in the filtrate.

Enzymatic assay

MnP activity was determined through the MBTH/DMAB assay (Castillo et al., 1994). The reaction mixture (2 ml) contained 1,460 µl of 100 mM succinate-lactate buffer (pH 4.5), 300 µl of 6.6 mM DMAB, 100 µl of 1.4 mM MBTH, 30 µl M of 20 mM $MnSO_4$, and 100 µl of supernatant. The reaction was initiated by adding 10 µl of 10 mM H_2O_2 and the absorbance of the fdeep purple compound that formed was measured at 590 nm. One unit was defined as the amount of enzyme needed to form 1 µmol of product in 1 min (Castillo et al., 1994).
Laccase activity was also measured using the MBTH/DMAB method but H_2O_2 was omitted (Castillo et al., 1994).

LiP activity was determined according to Tien and Kirk (1988) and modified by Castillo et al. (1997). The reaction mixture (2 ml) contained 1,420 µl of 100 mM sodium-tartrate buffer pH 3.0, 400 µl of 20 mM veratryl alcohol as substrate and 100 µl of supernatant. The reaction was initiated by adding 80 µl of 10 mM H_2O_2 and the increase of absorbance was followed at 310 nm. The extinction coefficient was 0.0093 $\mu M^{-1}cm^{-1}$. One unit representing 1 µmol veratryl alcohol oxidized to veratraldehyde per minute, at pH 3.0 and 30ºC.

PAH extraction and quantification

PAHs in the liquid medium were extracted by adding 10 ml toluene to each 50 ml-tube and shaking it vigorously for 1 h. After 10 min centrifugation at 492 x *g* an aliquot of the supernatant was analyzed directly with gas chromatography-flame ionization detector (GC-FID). Metabolites were identified with gas chromatography-mass spectrometry (GC-MS).

PAHs in soil were extracted by adding 10 ml of toluene and 10 ml of 0.05 M sodium pyrophosphate to tubes containing 10 g soil and shaking them vigorously for 16 h on a shaker. The extracts were centrifuged for 10 min at 492 x *g* and an aliquot of the supernatant was analysed directly by GC-FID and GC-MS.

GC-FID analysis was performed using an HP 6890 Series GC-system equipped with a flame ionization detector and an Agilent 19091S-433 capillary column (0.25 mm inner diameter, 30 m length, 0.25 µm thickness). The oven programme was 80°C for 3 min followed by ramping at 10°C min^{-1} up to 310°C maintained for 2 min. Splitless mode was used and the injector temperature was 250°C. Quantification was performed using external standards.

The extraction yield for phenanthrene, anthracene, fluoranthene and pyrene in Kirk medium contaminated individually o simultaneously was > 95%. The extraction yield for phenanthrene, anthracene, fluoranthene, pyrene and benzo*(a)*pyrene in autoclaved soil was > 91%.

The analysis of metabolites from PAH degradation was performed using an HP 6890 Series GC-system equipped with an HP 5971 mass selective detector and an HP 19091S-433 capillary column (0.25 mm inner diameter, 30 m length, 0.25 µm thickness). The oven programme was 80°C for 4 min followed by ramping at 7°C min^{-1} up to 310°C maintained for 4 min. The injector temperature was 250°C. Quantification was performed using external standards. Metabolites identification was carried out using the Wiley 275 mass spectral library.

Kinetic rate constant determination

Kinetic rate constant (*k*) was determined using the reaction rate expression as follows (eq. 1):

$$- dC/dt = k\, C^n \qquad \text{(eq. 1)}$$

C is the concentration of PAH (mg l^{-1}), t the time (days), *k* the rate constant for chemical disappearance of PAH (days^{-1}) and n the reaction order equal 1 assuming first order kinetic as generally accepted (Alexander, 1999; Sung Hyun et al., 2009). For presenting first-order kinetics, the logarithm of the PAH remaining concentration (or logarithm of C/Co) as a function of time was plotted (Alexander, 1999).

4.4 Results

PAH removal in liquid culture

The removal efficiency of phenanthrene, anthracene, fluoranthene and pyrene by *A. discolor* Sp4 in liquid medium containing individual PAHs (anthracene, phenanthrene, fluoranthene and pyrene) or a mixture of the four PAHs in the Kirk medium after 7, 14, 21 and 28 days of incubation is shown in Table 4.1. When added individually (alone) and after 28 days of incubation the removal efficiency was in the following order: phenanthrene had the highest removal (22.6%) followed by fluoranthene (19.5%), pyrene (8.5%) and anthracene (7.0%) (Table 4.1). The same order but with a higher removal efficiency was observed when a mixture of PAHs was studied, phenanthrene (26.5%), fluoranthene (23.5%), pyrene (17.5%) and anthracene (11.3%).

The PAH removal by *A. discolor* Sp4 occurred mainly during the first 14 days as shown in Table 4.2 when the ligninolytic enzymes reached their maximal activity (Fig. 4.1). In fact, these results showed a relationship with the increase in MnP activity which reached a maximal value (832 U l^{-1}) at day 13 in the presence of PAHs mixture and was 3-fold higher than the control (without PAHs) (Fig. 4.1). The enhanced degradation of pyrene, after 14 days, may be associated with the increase of LiP, together with the simultaneous production of MnP (Fig. 4.1). Only negligible laccase activity was detected.

Table 4.1 Removal efficiency of PAHs during 28 days in Kirk medium inoculated with *A. discolor* Sp4 at 30°C. The values are means (n = 2).

	Removal efficiency (%)							
	Individual				Mixture			
	7 d	14 d	21 d	28 d	7d	14 d	21 d	28 d
Phenanthrene	5.7	17.5	17.8	22.6	13.2	23.5	24.4	26.5
Anthracene	4.2	5.3	6.8	7.0	5.3	6.3	10.1	11.3
Fluoranthene	10.1	18.0	18.3	19.5	4.4	7.1	18.8	23.5
Pyrene	0.0	0.0	0.2	8.5	2.7	6.0	12.8	17.5

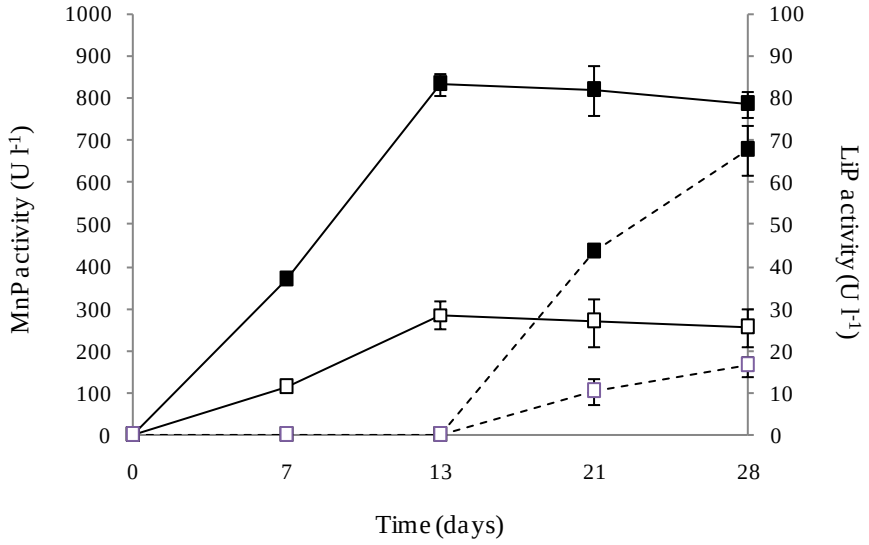


Figure 4.1 Manganese peroxidase (—) and Lignin peroxidase (·····) activity in *A. discolor* Sp4 cultures growing in Kirk medium contaminated with PAHs mixture (■) and non contaminated (□), during 28 days.

PAH removal in soils

A. discolor Sp4 was inoculated in autoclaved and non autoclaved soil contaminated with a mixture of 50 mg kg^{-1} of each of the following PAH; phenanthrene, anthracene, fluoranthene, pyrene and 40 mg kg^{-1} of benzo*(a)*pyrene.

Wheat grains were utilized as a support for the inoculum and as a vehicle for the soil colonization. *A. discolor* colonized the contaminated autoclaved soil with a dense mycelial growth over the whole period of incubation, reaching the bottom of the tubes (Fig. 4.2).

Figure 4.2 *A. discolor* Sp4 colonization in wheat grains and in autoclaved soil contaminated with 3, 4 and 5 ring-PAHs.

The content of the individual PAHs during the 60-days incubation period in inoculated and non-inoculated autoclaved soil is shown in Figure 4.3. The soil, bioaugmented with *A. discolor* Sp4, showed an enhanced PAH removal efficiency compared with the control (non-inoculated) suggesting that *A. discolor* Sp4 was able to significantly degrade the studied PAHs. An initial decrease in concentration in both inoculated and non-inoculated autoclaved soil was observed until the 14^{th} day for phenanthrene, anthracene, fluoranthene and benzo*(a)*pyrene (Fig. 2a, b, c and e, respectively). A sharp decrease in all PAH concentrations was observed between 14 and 21 days, particularly for anthracene and benzo*(a)*pyrene and between 28 and 35 days for pyrene in autoclaved soil inoculated with *A. discolor* Sp4 compared with the control (non inoculated) (Fig. 4.3). After day 21, almost all PAHs showed only a small decrease in their concentration, except for pyrene which showed this tendency after 35 days (Fig. 4.3). In the case of anthracene degradation, the formation of anthraquinone was observed; the peak of anthraquinone production was correlated with the lowest concentration level of anthracene (Fig. 4.3b).

Table 4.2 summarizes the removal efficiency of PAHs after 60 days in soil inoculated with *A. discolor* Sp4 compared with the control. In case of autoclaved soil, the removal

efficiency was higher in soil bioaugmented with *A. discolor* Sp4 compared with the control (non-inoculated).

Table 4.2 PAH removal efficiency in absence and presence of soil indigenous microorganisms inoculated and non-inoculated with *A. discolor* Sp4, after 60 days.

Compound	Removal efficiency (%)			
	autoclaved soil		non-autoclaved soil	
	non inoculated	inoculated	non inoculated	inoculated
Phenanthrene	39.5 ± 8.8	61.9 ± 1.6	98.3 ± 1.1	95.4 ± 1.8
Anthracene	27.3 ± 4.4	72.9 ± 2.0	83.1 ± 5.5	61.5 ± 9.4
Fluoranthene	27.7 ± 7.1	54.3 ± 2.5	82.5 ± 11.4	43.1± 5.7
Pyrene	24.9 ± 8.7	59.7 ± 1.7	82.5 ± 11.4	43.1± 5.7
Benzo*(a)*pyrene	28. 1 ± 6.4	75.4 ± 0.6	14.2 ± 4.4	15.8± 5.7

PAH degradation kinetic constants in soil are reported in Table 4.3. The degradation was faster for benzo*(a)*pyrene and antracene in autoclaved soil bioaugmented with *A. discolor* Sp4 in comparison with phenanthrene, pyrene and fluoranthene.

Table 4.3 PAH removal rate constant in autoclaved and non-autoclaved soil, inoculated and non-inoculated with *A. discolor* Sp4, after 60 days.

Compound	Removal rate constant k (days^{-1}) x 10^{-3}			
	autoclaved soil		non-autoclaved soil	
	non inoculated	inoculated	non inoculated	inoculated
Phenanthrene	8.5 (0.762)	18.4 (0.904)	68.0 (0.989)	51.3 (0.973)
Anthracene	5.6 (0.717)	24.7 (0.768)	28.9 (0.869)	15.9 (0.933)
Fluoranthene	5.1(0.797)	14.5 (0.933)	27.6 (0.672)	9.4 (0.931)
Pyrene	4.1 (0.923)	17.8 (0.832)	27.6 (0.828)	9.4 (0.931)
Benzo*(a)*pyrene	6.9 (0.773)	25.1(0.850)	2.6 (0.977)	2.9 (0.998)

( ) indicates the regression determining coefficients.

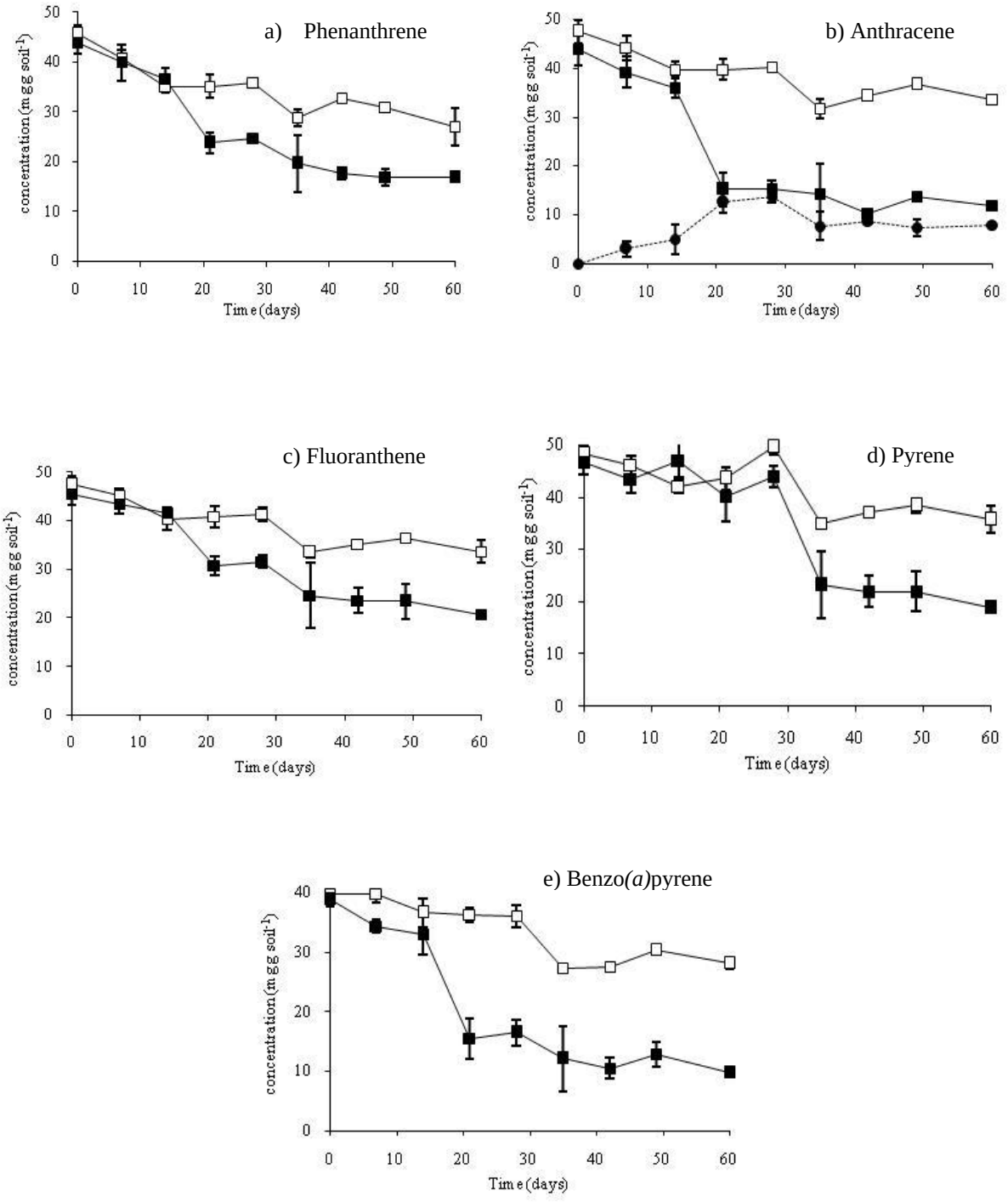


Figure 4.3 (a) phenanthrene (b) anthracene (c) fluoranthene (d) pyrene (e) benzo*(a)*pyrene concentration in autoclaved soil incubated at 30°C, inoculated with *A. discolor* Sp4 (■) and non-inoculated (□). Anthraquinone concentration (dashed line, ●) is represented in (b). The values are means ± SD (*n*=3).

The ability of *A. discolor* Sp4 to transform anthracene in autoclaved soil was confirmed through the detection of anthraquinone (Fig. 4.3b) and phtalic acid (Table 4.4). The degradation metabolites for fluoranthene were 4-hidroxy-9-fluorenone and 9-fluorenone and for pyrene it was 4,5-dihydropyrene. None of these compounds were found in the non inoculated soil. Products of the fungal metabolism were identified as chlorometoxibenzaldehyde, 3,4-dimetoxibenzaldehyde and octadecanoic acid.

A high MnP activity was observed in autoclaved soil inoculated by *A. discolor* Sp4 during 60 days (Fig. 4.4), reaching a maximum of 0.022 U g^{-1} on day 60, compared with the control (without PAHs). In non-inoculated, autoclaved soil and in the presence of PAH mixture, no significant enzymatic activity was detected. Neither laccase not lignin peroxidase were detected at any time during the experiment. When comparing the results of the enzymatic activity in inoculated autoclaved soil with and without PAHs, a higher enzymatic activity was detected in the presence of PAHs.

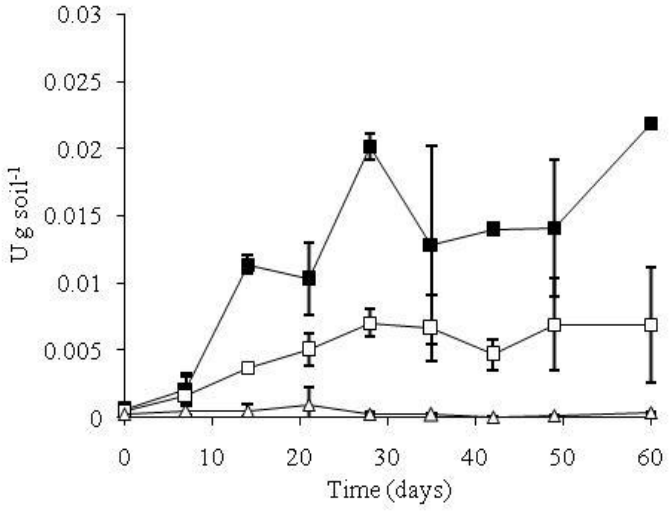


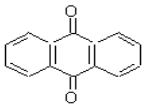
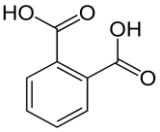
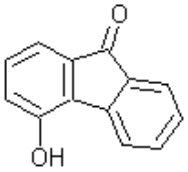
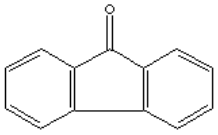
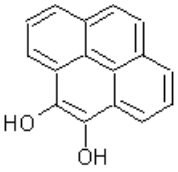
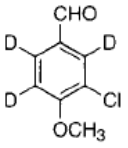
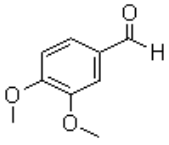
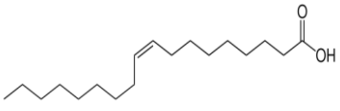
Figure 4.4 Production of MnP in autoclaved soil inoculated by *A. discolor* Sp4, in presence of PAH mixture (■), inoculated by *A. discolor* Sp4 without PAHs (□), non-inoculated, in presence of PAH mixture (Δ).

In the case of non-autoclaved soil bioaugmented with the fungus, after 30 days the removal efficiency of PAHs was similar to the non-inoculated control (data not shown). Approximately 67% of the phenanthrene, 23% of the anthracene, 14% of the fluoranthene, 14% of the pyrene and 5% of the benzo*(a)*pyrene were removed in non-autoclaved soil, both inoculated or not inoculated with *A. discolor* Sp4. In the inoculated soil, 95.4 % of the phenanthrene, 61.5% of the anthracene, 43.1% of the fluoranthene, 43.1% of the pyrene and 15.8% of the benzo*(a)*pyrene were removed after 60 days (Table 4.3), whereas 98.3% of the phenanthrene, 83.1% of the anthracene, 82.5% of the fluoranthene, 82.5% of the pyrene, 14.2% of the benzo*(a)*pyrene were removed in non-autoclaved contaminated soil without *A. discolor* Sp4. Degradation rate constants (Table 4.3) were in general lower in inoculated soil compared with the non inoculated control.

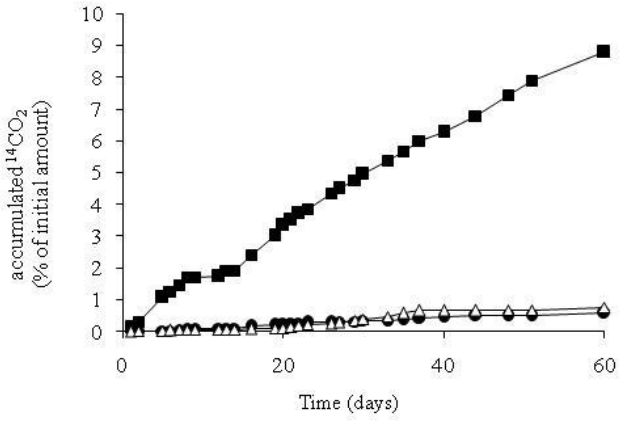
Mineralization studies of PAHs in soil

The degree of mineralization of ^{14}C-phenanthrene, ^{14}C-pyrene and ^{14}C-benzo(a)pyrene by *A. discolor* Sp4 in autoclaved soil contaminated with the PAHs mixture was monitored for 60 days. The mineralization of labeled PAHs was low, about 9% for ^{14}C-phenanthrene, 0.8% for ^{14}C pyrene and 0.6% for benzo*(a)*pyrene (Fig. 4.5a). No significant amount of ^{14}C (< 2%) was released from controls (non-inoculated autoclaved soil) during phenanthrene, pyrene and benzo*(a)*pyrene degradation (Fig. 4.5b).

Table 4.4 Found metabolites during PAH degradation in soil

Metabolite	Chemical struture	Origin
Antraquinone		Anthracene
Phtalic acid		
4-hidroxy-9-fluorenone		Fluoranthene
9-fluorenone		
4,5-dihydropyrene		Pyrene
Chlorometoxibenzaldehyde		From fungal metabolism
3,4-dimetoxibenzaldehyde		From fungal metabolism
Octadecanoic acid		From fungal metabolism

a)



(b)

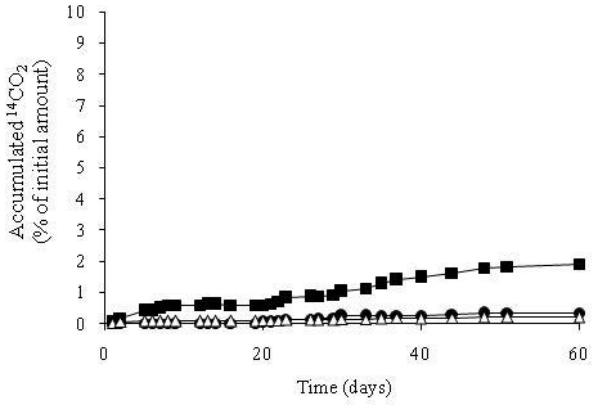


Figure 4.5 Mineralization in (a) autoclaved soil inoculated by *A. discolor* Sp4 (b) non-inoculated, contaminated with a PAH mixture and ^{14}C-phenanthrene (■),^{14}C-pyrene (□) or ^{14}C-benzo*(a)*pyrene (●).

Microbial respiration was measured for 60 days at 30°C in autoclaved and non- autoclaved contaminated soils with and without *A. discolor* Sp4. In the non-inoculated autoclaved soil very low production of CO_2 was recorded at the beginning of the experiment but the values increased over time, slowly at first and markedly after day 26. Nonetheless respiration in autoclaved non-inoculated soil was always considerably lower than in autoclaved inoculated soil (Fig. 4.6). The increase in respiration in the non-inoculated soil was attributed to the recolonization by the indigenous microflora.

The highest CO_2 production (68 mg accumulated CO_2 g $soil^{-1}$) was observed in the non-autoclaved soils where there was no appreciable difference between soil inoculated with *A. discolor* Sp4 and non-inoculated soil during the first 19 days (Fig. 4.6). Subsequently, there was a slightly higher respiration in the samples inoculated with *A. discolor* Sp4.

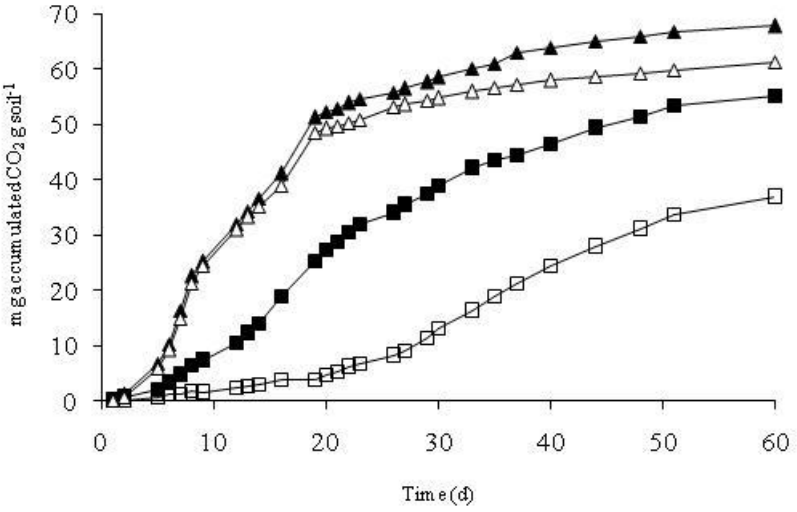


Figure 4.6 Respiration (expressed as accumulated mg CO_2 g $soil^{-1}$) in non-inoculated autoclaved soil (□), autoclaved soil inoculated by *A. discolor* Sp4 (■), non-inoculated non-autoclaved soil (Δ), non-autoclaved soil inoculated by *A. discolor* Sp4 (▲).

4.5 Discussion

PAH removal in liquid culture

Biodegradation trials at laboratory scale using a liquid medium are normally the first approach to establishing the ability of microorganisms to degrade specific pollutants.

A. discolor Sp4 was able to remove phenanthrene, anthracene, fluoranthene and pyrene in Kirk medium individually and in mixtures (Table 4.1). The removal efficiency of anthracene (11.3%) and pyrene (17.5%) in the PAH mixture in liquid medium after 28 days was higher than that of the individual compounds (7.0 and 8.5%, respectively), suggesting synergistic effects between PAHs (Bauer and Capone, 1988) or possible cometabolism (Bouchez et al.,

1995). Similar results in soil were observed by Bishnoi et al. (2008). Degradation rate for acenaphthene, anthracene, phenanthrene, fluoranthene and pyrene by *Phanerochaete chrysosporium* was found to be high when all five PAHs were present simultaneously rather than individually (Bishnoi et al., 2008). Boldrin et al. (1993) observed that fluorene, which cannot be used as a sole source of carbon, was cometabolically degraded with other PAHs as growth substrates. As mixtures of PAHs are often present in contaminated soils, cometabolic degradation or synergistic effects between PAHs could be important for bioremediation.

On the other hand, Bouchez et al. (1999) observed an inhibition phenomenon but also synergistic interactions in PAH degradation by bacterial strains. Desai et al. (2008) observed that the initial degradation rates of individual components decreased in the presence of other PAHs. Results from the mixture experiments indicated competitive inhibition interactions. Therefore, potential PAH interactions are quite numerous and complex (Desai et al., 2008), and no particular relationship can be assumed.

When comparing the results of the enzymatic activity in inoculated liquid with and without PAHs (Figure 4.1), the presence of PAHs stimulated MnP production. An important increase in MnP activity in liquid medium started on day 7 compared with the control (without PAHs), followed by a decrease of PAH content. These results suggest that MnP may be involved in PAHs degradation. In fact, MnP can oxidize (by means of the strong oxidant Mn^{3+}) PAHs with ionization potential (IP) value up to 7.8 eV such as anthracene (7.43 eV) and pyrene (7.53 eV), whereas compounds with higher IP value such as phenanthrene (8.03 eV) and fluoranthene (7.90 eV) are oxidized in the presence of cooxidants (lipids or Tween 80) (Bogan and Lamar, 1995). In addition, LiP produced and stimulated in presence of PAHs after day 14 would be also responsible for PAH degradation, particularly for anthracene and pyrene. PAHs with IP values of less than 7.55 eV are suitable substrates for direct one-electron oxidation by LiP (Hammel et al., 1986). Peroxidases and laccase expressed under nutrient-limiting conditions may be involved in PAH degradation by fungi as observed by Cajthalm et al. (2008), but there is insufficient knowledge about their combined role and interactions (Gravril et al., 2007). High complexity biodegradation mechanisms, in addition to the ligninolytic enzymes, as well as other biochemical systems,

may be responsible for PAH degradation by fungi (Novotny et al., 2004). The measurement of high activity levels of a specific enzyme (MnP in this case and LiP to a lesser extent), coupled with the removal of a pollutant (PAHs in our case) may be considered to be indicative of a relationship between enzymesactivity and degradation yield (Novotny et al., 2004; Anastasi et al., 2009).

PAH removal in soils

Upon visual examination (Fig. 4.2), *A. discolor* Sp4 showed a relevant growth in autoclaved soil, with the hyphae able to penetrate through the soil to the bottom of the tube and simultaneously adhering to the surface of the wheat grains. Soil is a matrix presenting high porosity, which promotes adequate aeration and humidity for fungal growth. Moreover, the presence of wheat grains may stimulate *A. discolor* Sp4 growth and a higher production of ligninolytic enzymes as shown by Rubilar (2007). When introduced into soil, white rot fungi may undergo an adaptation process related to exposure to an environment, which is different from wood in many respects (Baldrian, 2008). Soils generally contain fewer nutrients compared to wood, being also the bioavailability of these nutrients different in both solid matrices (Baldrian, 2008). It has been reported that the growth of white rot fungi in most soils is limited due to the availability of carbon and nitrogen (Boyle, 1995) and external substrate addition (biostimulation) is usually required (Baldrian, 2008). Lignocellulosic materials have been successfully used for introducing white-rot fungi into soil (Andersson et al., 2000; Dzul-Puc et al., 2005) promoting larger inoculum biomass and faster and more successful establishment of the fungus in the soil (Lamar et al., 1993; Leastan et al., 1996). In this sense, wheat grains were chosen as lignocellulosic substrate/support in this study.

During the first 14 days, the removal efficiency in non-inoculated and inoculated autoclaved soil was attributed to sorption into soil particles and wheat grains. An initially rapid, reversible sorption process was produced by the interaction of PAHs with soils, followed by a longer period characterized by increasingly strong interactions and leading to the recalcitrance of these compounds (Bogan et al., 2003). The adsorption of organic

compounds on soil may involve physical (van der Waals forces), hydrogen bonding, ion exchange or chemiosorption (Alexander, 1999) and can be influenced by PAH physico-chemical characteristics such as K_{oc} value and rings number; as well as soil organic matter content, pH and temperature. In this case, the soil used in this study had low organic matter content (3%) providing a favorable environment for a low PAH adsorption on soil and consequently accessibility of extracellular enzymes and microorganisms to the pollutant for its biodegradation.

Between 14 and 28 days, PAHs were significantly removed by *A. discolor* Sp4; this removal was directly correlated to an increase in MnP activity. Finally, between 28 and 60 days, the removal of all PAHs may be also attributed to the combined action of PAH adsorption on soil particles, and degradation by *A. discolor* Sp4 and by soil microflora reactivated after 26 days. As shown in the control test (Fig. 4.6), soil microflora was able to recolonize the autoclaved soil after 26 days. Some spores are able to survive under the autoclaving conditions, and these specific microorganisms are able to colonize the soil again (Tuominen et al., 1994; Andersson et al., 2000; Berns et al., 2008). Furthermore, *A. discolor* Sp4 showed biodegradation capability in autoclaved soil contaminated with PAHs; here, the degradation of benzo*(a)*pyrene was relevant. In spite of the recalcitrant characteristics of benzo*(a)*pyrene and its preference for soil particles (Bamforth and Singleton, 2005), a high removal efficiency (75.4%) of this compound was observed in autoclaved soil. Although a great diversity of organisms are capable of degrading low molecular weight PAHs such as anthracene and phenanthrene, relatively few genera have been observed to degrade high molecular weight PAHs, such as benzo*(a)*pyrene (Juhasz and Naidu, 2000), one of the most potent carcinogenic PAHs.

The PAH degradation was confirmed by the appearance of known metabolites from ligninolytic enzyme action. The appearance of anthraquinone was observed at the beginning of anthracene degradation by *A. discolor* Sp4; this compound has been reported to be the main and most stable dead-end metabolite of anthracene degradation by the enzymatic system of white-rot fungi (Cajthmal et al., 2002b), such as strains from the

genera *Bjerkandera* and *Phanerochaete* (Field et al., 1992). Phthalic acid was reported as an oxidation product of anthracene from *Bjerkandera* sp. BOS55 (Eibes et al., 2006). The degradation products for fluoranthene were 4-hidroxy-9-fluorenone and 9-fluorenone and for pyrene, it was 4,5-dihydropyrene. Other metabolites resulting from the degradation of fluoranthene by *Irpex lacteus*, such as 1,8-naphthalic anhydride and 2-formyl-acenaphthen-1-carboxylic acid methylester were found by Cajthaml et al. (2002b). In addition, trans-4,5-dihydrodiolpyrene has been reported as a degradation product of pyrene by several white- rot fungi (Bezalel et al., 1996, Heitkamp et al., 1988. Lange et al., 1994, Sack et al., 1997). During the experiment, no reported quinones intermediates of phenanthrene biodegradation were detected. Apparently, during the degradation in vivo of this study, quinones are rapidly metabolized even more. No PAH quinones production during benzo*(a)*pyrene degradation by *A. discolor* was detected in this study. The same result was observed by Field at al. (1992) evaluating the degradation potential of 12 different strains of white rot fungi such as *P. chrysosporium* BKM-F-1767, *T. versicolor* Paprican 52, *Bjerkandera adusta* CBS 595.78, *Bjerkandera sp*. Strain Bos55 and *Stereum* sp. Strain Schim 22, among others. However, benzo*(a)*pyrene 1,6-, 3,6- and/or 6,12-quinones have been detected as polar metabolites of degradation of benzo*(a)*pyrene by *C. elegans* (Cerniglia and Gibson, 1979) and *A. ochraceus* (Datta and Samanta,1988).

Chlorometoxibenzaldehyde, 3,4-dimetoxibenzaldehyde and octadecanoic acid were detected in the inoculated soil. These compounds could play vital roles in the ligninolytic enzymes system and be involved in xenobiotics degradation (Have and Teunissen, 2001). Chlorinated aromatic compounds have been detected as metabolites of basidiomycetes (De Jong and Field, 1997), biosynthesized via the phenyl-propanoid pathway. Chlorometoxibenzaldehyde acts as methyl donors and as substrates for H_2O_2-generating oxidases (De Jong and Field, 1997). 3,4-dimetoxibenzaldehyde is secreted de novo from glucose (De Jong et al., 1992), simultaneously with the extracellular ligninolytic enzymes (Silk et al., 2001), having a important role in the oxidation reactions. In presence of oxygen, p-anisylalcohol is envolved in H_2O_2 production (substrate for peroxidases) by oxidation to anisaldehyde (3,4-dimetoxibenzaldehyde) by an aryl alcohol oxidase (Silk et al., 2001). On the other hand, white-rot fungi secrete moreover unsaturated fatty acids such as octadecanoic acid, for the

xenobiotics degradation via MnP-dependent lipid peroxidation (Kapich et al., 1999, Enoki et al., 1999, Elissetche et al., 2006).

The measured amounts of $^{14}CO_2$ generated from labeled phenanthrene, pyrene and benzo*(a)*pyrene in autoclaved soil were found to be less than 15%, similar to amounts usually found in other white-rot fungi (Sanglard et al., 1986; Bezalel et al., 1996, Bogan and Lamar, 1996; Kotterman et al., 1998; Canet et al., 1999), demonstrating the low ability of this fungus to mineralize PAHs. A diverse group of white-rot fungi have the ability to oxidize PAHs, but the degree of mineralization is always limited (Kotterman et al., 1998; Juhasz and Naidu, 2000). The PAH metabolites are more polar and soluble than the parent PAHs, and thus are likely more available for degradation by other microorganisms (Kotterman et al., 1998). Due to the complexity of PAH contamination in soil, the soil microflora plays a pivotal role in minimizing the risks of accumulated PAHs metabolites. Furthermore, synergistic effects between *A. discolor* Sp4 and indigenous soil microorganisms for PAH mineralization, are desired. During the first 30 days, the removal efficiency for all PAHs was comparable between the non-autoclaved soil bioaugmented with *A. discolor* Sp4 and non-bioaugmented soil. However after 60 days, the presence of *A. discolor* Sp4 in non-autoclaved soil resulted in a less efficient removal of anthracene (83.1% vs. 61.5%) and fluoranthene and pyrene (82.5% vs. 43.1%) compared with the control, indicating possible inhibition and competition of native microbes with the fungus. *A. discolor* Sp4 may trigger some defense mechanisms which could negatively affect indigenous soil microorganisms. These mechanisms are still unresolved. Ligninolytic fungi typically react to the presence of soil microorganisms with an increase in laccase activity (Baldrian, 2004b, 2006). Eggert (1997) reported that this enzyme catalyzes the formation of antibacterial compounds in *Pycnoporus cinnabarinus,* but the direct effects of laccase on soil bacteria are not confirmed (Baldrian, 2004b). Some fungi produce toxic hydroxyl radicals in the presence of antagonistic bacteria as demonstrated by Tornberg and Olsson (2002), while their antibiotic compounds production may be another possible mechanism (Baldrian, 2008).

Most white-rot fungi utilize wheat grains, straw or wood shavings as preferred substrates (Morgan et al., 1993, Castillo et al., 2001, Rubilar, 2007). However, in this type of soil, the main factor affecting the colonization of non-autoclaved soil by *A. discolor* Sp4 was clearly the presence of indigenous soil microorganisms, which may compete with the fungus for the lignocellulosic substrate as a source of carbon and nitrogen, disabling the fungal growth. In fact, substrate limitation may occur due to competition with indigenous soil microorganisms, inhibiting lignocellulosic material decomposition by the fungus (Lang et al., 1998). Bacterial populations can decompose lignocellulosic material (Clausen, 1996) and have the ability to use it as carbon and nitrogen source (Baldrian, 2008). Adequate treatment of a lignocellulosic material may enhance its selectivity to support *A. discolor* Sp4 growth in non-autoclaved soil. In fact, when Castillo et al. (2001) used straw treated with formic acid and hot water as lignocellulosic material for *P. chrysoporium*, the effect of competition with other microorganisms diminished.

Fungal bioaugmentation clearly provides certain advantages over biostimulation such as 5 or more-ring PAHs degradation. The success of fungal bioaugmentation for enhancing removal of organic compounds may depend on the type of soil. The growth of *A. discolor* Sp4 on wheat grains producing a synergist effect with the native microflora in the degradation of organic compounds has been reached in soils with high organic matter content (18%) as shown by Rubilar (2007).

As shown in this study, the success of fungal bioaugmentation for enhancing removal of PAHs may depend on several factors. Furthermore, suitable conditions must be found to promote the growth of *A. discolor* Sp4 in soil contaminated with PAHs.

4.6 Conclusions

A. discolor Sp4, a white-rot fungus isolated from the Chilean forest, showed the ability to degrade PAHs simultaneously and individually in liquid medium. The highest removal efficiency occurred in PAH mixture, suggesting synergistic effects or cometabolism. In addition, *A. discolor* Sp4 was able to degrade phenanthrene (62%), anthracene (73%),

fluoranthene (54%), pyrene (60%) and benzo*(a)*pyrene (75%) in autoclaved soil, associated with the production of ligninolytic enzymes, mainly MnP. In particular, degradation of benzo*(a)*pyrene, one of the most potent carcinogenic PAHs, was the highest using this fungus. In non-autoclaved soil, the inoculation of *A. discolor* Sp4 did not improve the PAHs removal efficiency, and determining suitable conditions for promoting successful fungal bioaugmentation in soils requires futher study. The colonization of *A. discolor* Sp4 in non-autoclaved soil may require a higher demand for substrate compared with autoclaved soil. Furthermore, its use may be limited to soils with high organic matter content (for the availability of nutrients) or will even require additional nutrients supply (biostimulation).

4.7 Acknowledgements

This work was partially financed by FONDECYT project Nº 1050614, by a doctoral scholarship from CONICYT (Chile) and by the Swedish Foundation for Strategic Environmental Research (MISTRA) through funding for the Domestication of Microorganisms (DOM) research program and the Centre for Chemical Pesticides (CKB) at the Swedish University of Agricultural Sciences.

5

Degradation of polycyclic aromatic hydrocarbons by free and nanoclay-immobilized manganese peroxidase from *Anthracophyllum discolor* Sp4

5.1 Abstract

Manganese peroxidase (MnP) produced by *Anthracophyllum discolor* Sp4, a Chilean white rot fungus, was immobilized on nanoclay and its ability to degrade polycyclic aromatic hydrocarbons (PAHs) compared with the free enzyme was evaluated. At the same time, nanoclay characterization was performed. Nanoclay characterization by transmission electronic microscopy showed a particle average size smaller than 100 nm. The isoelectric points (IEP) of nanoclay and MnP from *A. discolor* Sp4 were 7.0 and 3.7, respectively, as determined by micro electrophoresis migration and preparative isoelectric focusing. Results indicated that 75% of the enzyme was immobilized on the nanoclay through physical adsorption. As compared to the free enzyme, immobilized MnP from *A. discolor* Sp4 achieved an improved stability to temperature and pH. The Ea value for immobilized MnP (51.9 kJ mol^{-1}) was higher than that of the free MnP (34.4 kJ mol^{-1}). The immobilized enzyme was able to degrade pyrene (> 86%), anthracene (> 65%), alone or in mixture, and to a less extent fluoranthene (< 15%) and phenanthrene (< 9%). Compared to free MnP from *A. discolor*, the enzyme immobilized on nanoclay enhanced the enzymatic transformation of anthracene in soil. Overall results indicate that nanoclay, a carrier of natural origin, is a suitable support material for MnP immobilization. In addition, immobilized MnP shows an increased stability to high temperature, pH and time storage, as well as an enhanced PAHs degradation efficiency in soil. All these characteristics may suggest the possible use of nanoclay-immobilized MnP from *A. discolor* Sp4 as a valuable option for in-situ bioremediation purposes.

Keywords: Polycyclic aromatic hydrocarbons; *Anthracophyllum discolor* Sp4; biodegradation; manganese peroxidase; immobilization; nanoclay.

5.2 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are highly toxic organic contaminants widely distributed in terrestrial and aquatic ecosystems, as products of the incomplete combustion of fossil fuels (Gibson and Subramanian, 1984; Johnsen et al., 2005). Many PAHs are mutagenic and some of them are carcinogenic, representing a considerable public health hazard (Johnsen et al., 2005). PAHs are composed of fused aromatic rings with high biochemical persistence and therefore resistant to nucleophilic attack (Johnsen et al., 2005). Their limited bioavailability is also a possible restrictive factor for microbial attack (Nikiforova et al., 2009).

Many studies using white-rot fungi to degrade PAHs have been performed (Pointing 2001; Hwang et al., 2007). Among the enzymes secreted by white rot-fungi, lignin peroxidase (LiP), laccase (L) and manganese peroxidase (MnP) were found to have a pivotal role in the degradation of PAHs (Collins et al., 1996; Steffen et al., 2002a).

In the last years, MnP has received considerable attention for its potential use in the degradation of organic pollutants, particularly PAH degradation (Baborová et al., 2006; Eibes et al., 2006). The use of MnP in industrial applications has been, however, limited by several factors, mainly the high cost and the low operational stability (Torres et al., 2003). Enzyme immobilization is a good alternative to overcome these problems. Fungal enzymes have been successfully immobilized on porous glass beads and on various clay minerals for soil pollutant degradation (Ahn et al., 2007). Alternatively, the use of nanomaterials as supports for enzyme immobilization is comparatively recent: the first study was reported in 1987 using magnetic particles as carrier (Matsunaga and Kamiya, 1987). Various nanomaterials have been used as immobilization supports (nanoparticles with surface-attached enzymes, nanofibers carrying enzymes, nanoporous matrices with entrapped enzymes and carbon nanotube-enzyme hybrid materials), varying according to their composition, form, structure and surface characteristics (Wang, 2006). Nanoparticles have the advantage over traditional support materials of providing a high surface, minimum diffusion limitation and high mass transfer (Kim et al., 2006; Wang, 2006). The observed

disadvantages have been the dispersion of nanoparticles in the reaction media, their poor and complicated recovery after the reaction, and the possible health and environmental damage from the handling of dry powders (Wang, 2006).

Allophane, one of the main constituents of the clay fraction of Southern Chilean andisols, is characterized by a high superficial area and structural stability, with a pore size between 35 to 55 Å (Parfitt, 1990; Calabi et al., 2009), and is considered a natural nanomaterial. The use of nanomaterials of natural origin, such as nanoclays, as carriers for MnP immobilization has not been explored yet.

The first objective of this study was to evaluate the potential of free MnP from *Anthracopyllum discolor* Sp4, a white-rot fungus recently isolated from southern Chile, to degrade PAHs in liquid solution. The second objective was to immobilize MnP from *A. discolor* Sp4 on a novel natural nanomaterial (nanoclay) and to determine the effect of the immobilization conditions on the properties of the biocatalyst. The stability of free and immobilized MnP was assessed at different pHs and temperatures and storage time. The last objective was to evaluate the performance of immobilized MnP in the PAH degradation in liquid medium and in sterile and non sterile soil compared with free MnP. A commercial MnP from *Nematoloma frowardii* was used as a reference both as a free enzyme and immobilized on nanoclay.

5.3 Materials and methods

Microorganism

The fungal strain *Anthracophyllum discolor* Sp4 was obtained from the culture collection of the Environmental Biotechnology Laboratory of the Universidad de La Frontera, Chile. The fungus was maintained at 4ºC in glucose malt extract agar (G-MEA) slants tubes containing: malt extract 30 g l^{-1}; agar 15 g l^{-1} and glucose 10 g l^{-1}. In this study, the fungus was transferred from slant culture tubes to Petri dishes with G-MEA medium and incubated at 30ºC for 5 to 7 days.

Chemicals

MBTH (3-methyl-2-benzothiazolinone), DMAB (3-(dimethylamino) benzoic acid), pyrene (98%) and fluoranthene (98%) were supplied by Aldrich Chemical Co., Germany. 3,4-dimethoxy-benzyl alcohol (veratryl alcohol) (> 95%) and anthraquinone were supplied by Fluka (Steinheim, Germany). Anthracene (> 96%) and phenanthrene (> 97%) were purchased from Merck (Hohenbrunn, Germany). Manganese peroxidase (EC 1.11.1.13) from *Nematoloma frowardii* was obtained from JenaBios (Jena, Germany). All other chemicals were supplied by Merck, Oxoid, Sigma and Duchefa (Netherlands).

Preparation of partially purified MnP

A plug of *A. discolor* Sp4 mycelium (5 mm diameter) grown for 7 days on G-MEA was inoculated in 100 ml of Kirk medium (Tien and Kirk, 1988) supplemented with 32 g of wheat grains as lignocellulosic substrate, 0.25 mM $MnSO_4$ as an inducer of the MnP production, and 0.05% v/v of Tween 80 as surfactant. The medium was collected after 10 days of growth at 30°C, filtered through glass microfiber filter 1.2 μm (Wathman), concentrated by ultrafiltration in an Amicon membrane (Filterpore) with a molecular weight cutoff of 30 kDa and filtered again through mixed cellulose ester filter 0.2 μm (Advantec). The filtrate was assayed for ligninolytic enzymes: laccase, manganese peroxidase and lignin peroxidase and used as partially purified MnP (14 U mg^{-1}) for following experiments. The isoelectric point (IEP) of MnP was measured by preparative isoelectric focusing (IEF) using Rotofor System (Biorad).

Enzyme assays of A. discolor Sp4 *filtrate*

MnP activity was determined by the MBTH/DMAB method (Castillo et al., 1994). The reaction mixture (2 ml) contained 1460 μl of 100 mM succinate-lactate buffer (pH 4.5), 300 μl of 6.6 mM DMAB, 100 μl of 1.4 mM MBTH, 30 μl M of 20 mM $MnSO_4$, and 100 μl of filtrate. The reaction was initiated by adding 10 μl of 10 mM H_2O_2 and the absorbance of the

formed deep purple compound was measured at 590 nm. One unit was defined as the amount of enzyme needed to form 1 μmol of product in 1 min (Castillo et al., 1994).

Laccase activity was also determined by the MBTH/DMAB method but the addition of H_2O_2 was omitted.

LiP activity was determined according to Tien and Kirk (1988) and modified by Castillo et al. (1997). The reaction mixture (2 ml) contained 1420 μl of 100 mM sodium-tartrate buffer pH 3.0, 400 μl of 20 mM veratryl alcohol as the substrate and 100 μl of filtrate. The reaction was initiated by adding 80 μl of 10 mM H_2O_2 and the increase of absorbance was followed at 310 nm. The extinction coefficient is 0.0093 $\mu M^{-1}cm^{-1}$. One unit representing 1 μmol veratryl alcohol oxidized to veratraldehyde per minute, at pH 3.0 and 30ºC.

Extraction of nanoclay

The nanoclay was obtained from an Andisol (Temuco Serie) soil from Southern Chile. Briefly, the clay fraction was separated by using a sedimentation method based on Stokes's law, with and without destruction of the organic matter. For the organic matter destruction, 20 g of soil was previously treated with 10 ml of distilled water and 10 ml of 30% hydrogen peroxide at 70°C every 2 h until no bubbling was observed. Five g of clay were suspended in 100 ml of 1 M NaCl, with agitation for 48 h. The suspension was centrifuged at 3000 rpm for 40 min and the supernatant was separated. The clay was re-suspended in 50 ml of filtered and deionized water, under moderate agitation for approximately 1 h, and then centrifuged under the previous conditions. The procedure was repeated 7 times. The recovered supernatants containing the suspended nanoclay were pooled, dialyzed until a conductivity range between 0.5 to 0.8 μS, and then concentrated. The nanoclay was characterized by transmission electronic microscopy (TEM) and its isoelectric point (IEP) was determined by micro electrophoresis migration (Calabi et al., 2008). Electrophoretic mobilities were measured with a zeta meter (ZM-77) apparatus. Diluted dispersions (0.05 g l^{-1}) were prepared in 1 mM KCl. The mobilities were averaged and the zeta potential calculated using the Helmholtz-Smoluchowski equation.

MnP immobilization on nanoclay

A portion of nanoclay (10 mg) was mixed with 250 µl of filtrate (partially purified MnP) and shaken at 200 rpm for 2 h at 30°C. The pellet was recovered by centrifugation (13000 rpm for 4 min), washed with sodium acetate buffer 50 mM pH 4.5 until no enzymatic activity was detected in the washings and dried at 30°C. Then, the pellet was re-suspended in 250 µl of 50 mM sodium acetate buffer at pH 4.5. The amount of MnP adsorbed on nanoclays was calculated by the difference between the enzymatic activity initially added and that remaining in the supernatants after washings.
Commercial MnP from *Nematoloma frowardii* was also immobilized on nanoclay according to the same procedure and run in parallel as a reference.

pH and temperature effect on immobilized MnP from A. discolor Sp4

Immobilized MnP from *A. discolor* Sp4 was incubated at different pH (pH 3.5 to 9.5) and temperature (30 to 70°C) during 1 h and then the enzymatic activity was measured as above. Free MnP was run in parallel as control.

The activation energy (Ea) was calculated by plotting the log of activities of free or immobilized MnP vs. 1/T (in °K) according to the Arrhenius equation. The value of activation energy was obtained by a computed linear regression analysis of the experimental data (Rao and Gianfreda, 2000).

Time-dependent stability

The time-dependent stability was evaluated by determining the activity of free and immobilized MnP from *A. discolor* Sp4 during 14 days at 20°C. The storage stability was evaluated by storing the free and immobilized MnP from *A. discolor* Sp4 during 6 months at 4°C. The enzyme activity was periodically measured and all experiments were performed in triplicates.

PAH degradation by free and immobilized MnP

The potential of partially purified MnP from *A. discolor* Sp4, free or immobilized on nanoclay, to degrade phenanthrene, anthracene, fluoranthene and pyrene, was evaluated in three *in vitro* assays in 10 ml-tubes containing glass beads. Glass beads (2.5 g, 2 mm diameter) were added to 10-ml tubes and autoclaved for 20 min at 120°C. Stock solutions in acetone of individual PAH or mixtures were added aseptically onto the glass beads. The acetone was allowed to evaporate.

In the first assay the degradation of individual PAH (10 mg l^{-1}) was evaluated. In the second and third assay, PAHs were added in a mixture at a concentration of 2.5 mg l^{-1} for each PAH (mixture 1) and 10 mg l^{-1} (mixture 2), respectively. The reaction mixture (1 ml) consisting of 41 mM sodium acetate buffer at pH 4.5, Tween 80 1%, v/w $MnSO_4$ 1 mM and approximately 0.15 U of free MnP was added to each tube. The reaction was initiated by the addition of 1 mM H_2O_2. In all cases, treatments with free and immobilized MnP from *N. frowardii* were run in parallel as reference. All experiments were performed in triplicates.
Buffers were autoclaved and reagents were sterilized by filtration through 0.45-μm pore size filters before use. The tubes were placed on a rotary shaker at 150 rpm and 35ºC and samples for PAHs determination were taken after 24 h and kept at -20ºC until analysis. Controls using the same reaction mixtures and containing distilled water instead of MnP were run in parallel.

Anthracene degradation in soil by free and immobilized MnP

The potential of free and immobilized MnP from *A. discolor* Sp4 to degrade anthracene in soil was evaluated and compared with immobilized MnP from *N. frowardii* (reference) in vitro assays. Anthracene degradation was followed after 24 h in spiked autoclaved soils.

An agricultural topsoil containing 14% clay, 3% organic matter, 1.8% organic carbon, 0.11% nitrogen, with a pH in water of 6.6 was used (Pizzul et al., 2006). This soil was collected in Uppsala, Sweden, sieved (< 2 mm) and stored at 4°C until use.

Autoclaved (30 min at 121°C, 1 bar, three times with 24 h of interval) and non-autoclaved soil (4 g) was weighed into 10 ml- tubes and spiked with 10 mg kg^{-1} of anthracene. The soil spiking was carried out by treating the soil with the anthracene solution in acetone. After acetone had evaporated, the spiked soil was supplemented with 0.42 ml of a solution keeping water content at 60% of the water-holding capacity. The solution consisted of 41 mM sodium acetate buffer at pH 4.5, Tween 80 1% v/w, $MnSO_4$ 1 mM and approximately 0.15 U of free or immobilized MnP. The reaction was initiated by the addition of 1 mM H_2O_2. Buffers were autoclaved and reagents were sterilized by filtration through 0.45-µm pore size filters before use. The tubes were placed on a rotary shaker at 150 rpm and 35ºC and samples were taken after 24 h incubation and kept at -20ºC until PAHs analysis. Controls using the same reaction mixture and containing distilled water instead of MnP, with and without nanoclay, were run in parallel.

PAH extraction and detection

In liquid solution PAHs were extracted by adding 1 ml of 0.05 M sodium pyrophosphate and 1 ml of toluene to each tube and shaking for 1 h. For experiments with soil, anthracene was extracted by adding 2.5 g of sterile glass beads, 4 ml of 0.05 M sodium pyrophosphate and 4 ml of toluene to each tube and shaking for 1 h. After 10 min centrifugation at 13000 rpm a sample of the supernatant was analysed directly by GC-FID. Metabolites were identified by GC-MS.

GC-FID analysis was performed using a HP 6890 Series GC-system equipped with a flame ionization detector and an Agilent 19091S-433 capillary column (0.25 mm inner diameter, 30 m length, 0.25 µm thickness). The oven program was 80°C for 3 min followed by ramping at 10°C min^{-1} up to 310°C maintained for 2 min. The mode was splitless and injector temperature was 250°C. Quantification was performed using external standards.

Analysis of metabolites was performed using a HP 6890 Series GC-system equipped with a HP 5971 mass selective detector and a HP 19091S-433 capillary column (0.25 mm inner diameter, 30 m length, 0.25 µm thickness). The oven program was 80°C for 4 min followed

by ramping at 7°C min^{-1} up to 310°C maintained for 4 min. The injector temperature was 250°C. Quantification was performed using external standards. Identification of metabolites was carried out using the Wiley 275 mass spectral library.

Statistical analysis

All PAH degradation data are the result of triplicate treatments. Data points are represented by the mean, with standard error indicated by an error bar. Data points were subjected to analysis of variance (ANOVA). Significantly different means between treatments were separated by the Tukey's test at 0.05 significance.

5.4 Results

Preparation of partially purified MnP

The *A. discolor* Sp4 filtrate showed high MnP activity (around 4000 U l^{-1}) and traces of LiP and laccase activities. This filtrate considered as partially purified MnP was used for all the following experiments. The specific activity for MnP was 14 U mg^{-1}. The IEP determined by preparative isoelectric focusing of MnP from *A. discolor* Sp4 was 3.7, similar to that of MnP from *N. frowardii* (IEP 3.2) reported in the literature (Hofrichter et al., 1999).

MnP immobilization on nanoclay

The nanoclay used for MnP immobilization was characterized by transmission electronic microscopy and showed a particle average size smaller than 100 nm (Fig. 5.1). The isoelectric points were 3.2 and 7.0 for the nanoclay with and without organic matter, respectively.

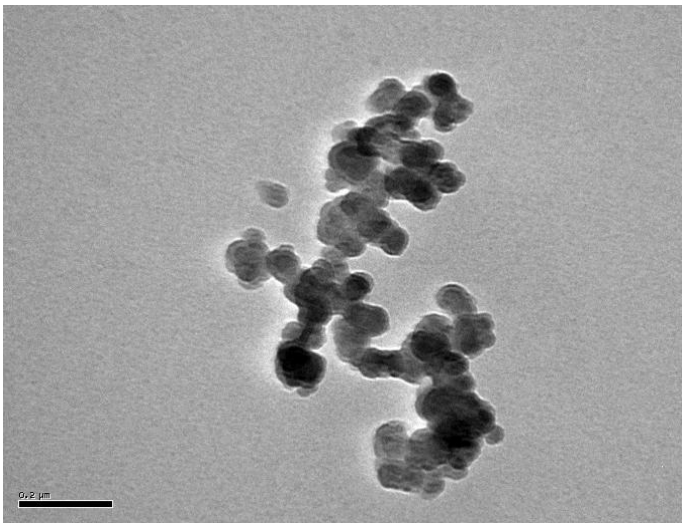


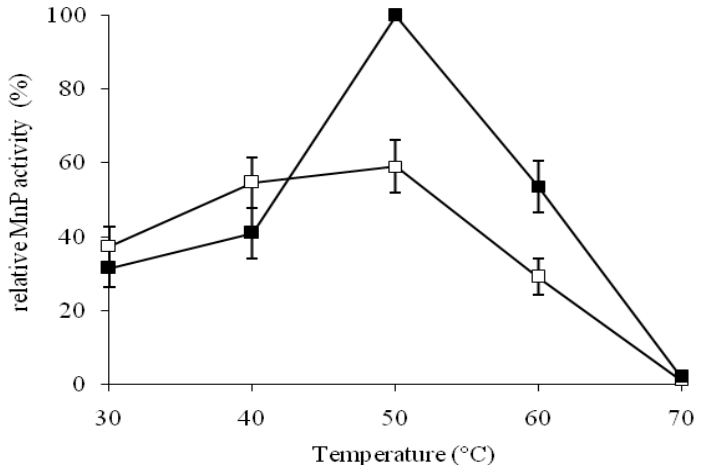
Figure 5.1 Nanoclay (without organic matter) from Andisol of the South of Chile, by Transmission Electronic Microscopy (TEM).

About 75% of MnP from *A discolor* Sp4 and 99% for MnP from *N. frowardii* were immobilized on the nanoclay without organic matter the support. However, no immobilization occurred on the nanoclay with organic matter.

pH and temperature effect on immobilized MnP

The stability of immobilized MnP from *A. discolor* Sp4 was tested at different pH and temperature and compared with the free enzyme (Fig. 5.2a and b). At 50 and 60°C, the activity of immobilized MnP was higher than that of the free MnP (Fig. 5.2a), reaching the highest difference at 50°C. While no differences in stability for immobilized and free MnP were observed in the pH range 4.5 - 8.0, immobilized MnP was stable in a broader pH range (Fig. 5.2b). The Ea value for immobilized MnP (51.9 kJ mol^{-1}) was higher than for free MnP (34.4 kJ mol^{-1}).

a)



b)

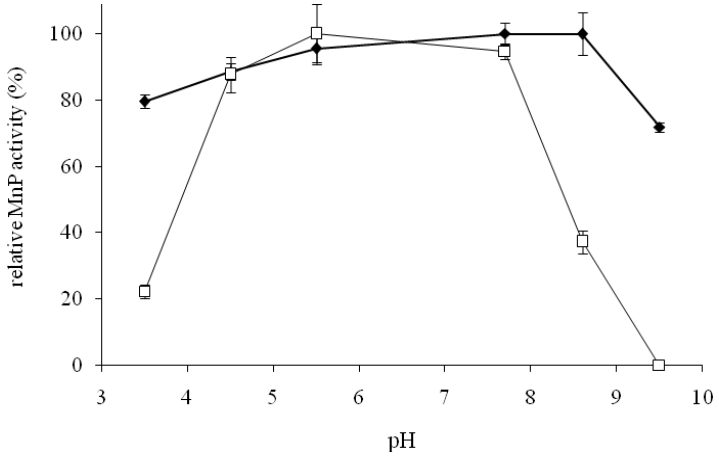


Figure 5.2 Effect of 1 h exposure to various temperatures (a) and to different pHs (b), on the activity of free (□) and immobilized MnP (■) from *A. discolor* Sp4.

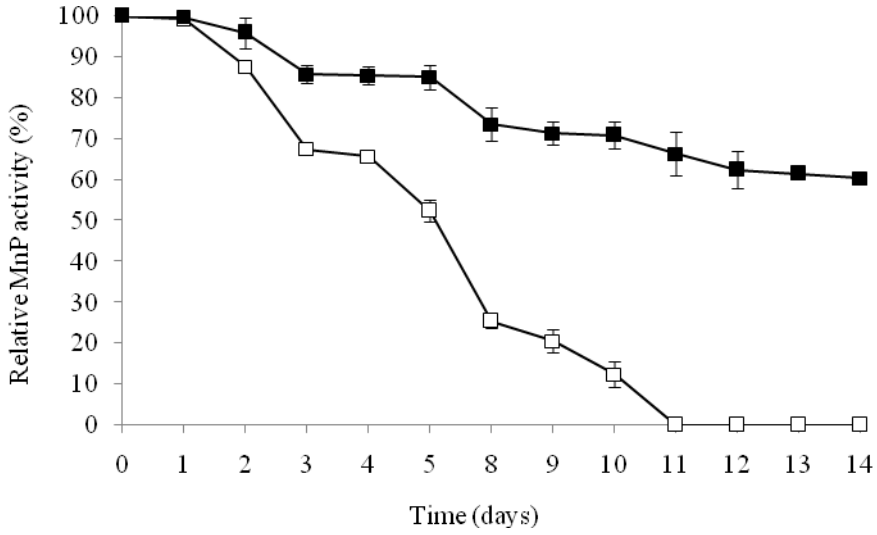
Time storage stability

Nanoclays-immobilized MnP maintained significantly higher activity during 14 days at room temperature (20°C) in comparison with the free enzyme (Fig. 5.3a). During the first 5 days, there was a 15% drop in activity and after 10 days the activity declined by 30%. At the same temperature and after the same storage time, a total loss of free enzyme activity was

observed. After 14 days of storage at 20°C, the immobilized enzyme maintained 60% of its initial activity.

After 6 months of incubation at 4°C about 20% of activity loss was measured with MnP immobilized on nanoclays; by contrast, free MnP lost 87% of its initial activity (Fig. 5.3b).

a)



b)

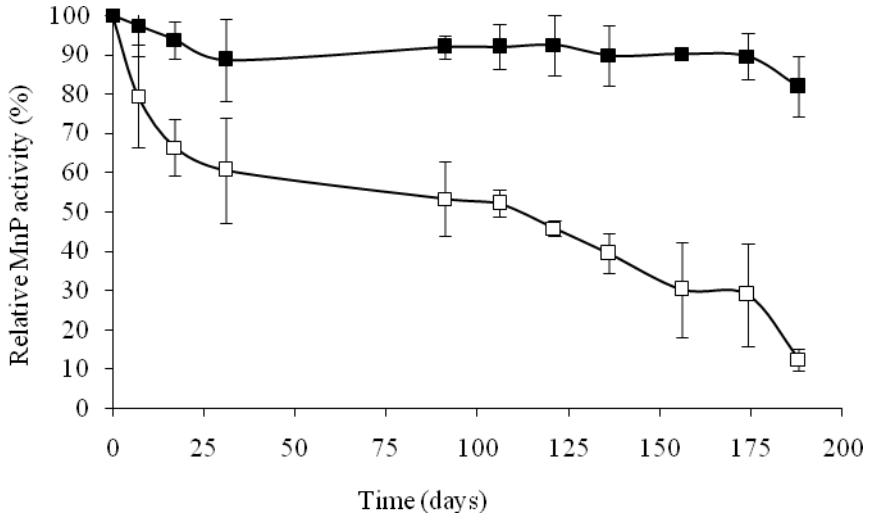


Figure 5.3 Effect of a) 14 days of storage at 20°C and b) 6 months of storage at 4°C on the activity of free (□) and immobilized MnP (■) from *A. discolor* Sp4

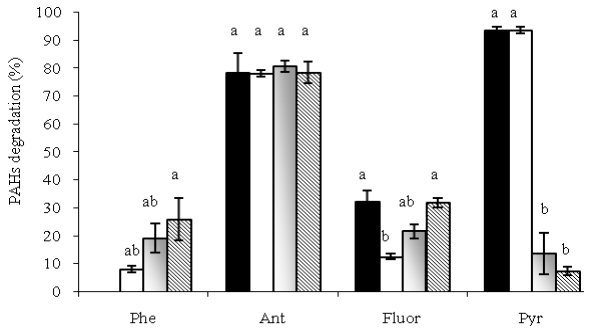
PAH degradation by free and immobilized MnP

The degradation of phenanthrene, fluoranthene, anthracene and pyrene (added alone or in mixture) was investigated using both free and immobilized MnP from *A. discolor* Sp4 or from *N. frowardii*, over a period of 24 h (Fig. 5.4). Both enzymes, free or immobilized on nanoclay, were able to degrade PAHs, but the degradation efficiency depended on the type of compound, whether it was present alone or in mixture, and on its initial concentration (Fig. 5.4). Anthracene, added alone or in a mixture, was degraded to a similar extent by both *A. discolor* Sp4 and *N. frowardii* MnP, free or immobilized on the nanoclay (Fig. 5.4). By contrast, pyrene was degraded only by MnP from *A. discolor* Sp4 and the immobilization or the presence of other PAHs in the reaction mixture did not affect the degradation efficiency.

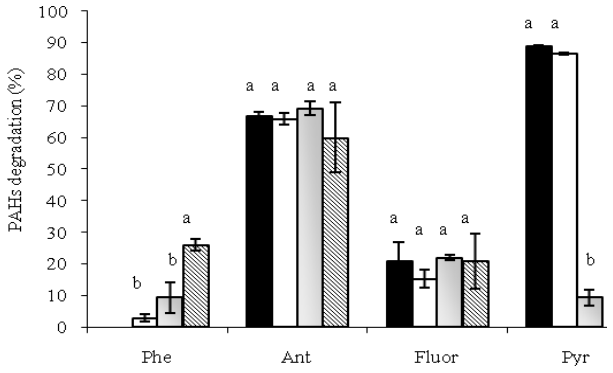
In all cases anthraquinone and 4,5-dihydropyrene were found as the main metabolites of anthracene and pyrene degradation, respectively (data not shown).

In general, a lower degradation was observed for fluoranthene in all treatments in the three assays (Fig. 5.4), with the exception a higher degradation by immobilized MnP from *N. frowardii* in the PAH mixture at the higher concentration (Fig. 5.4c).
Contrasting results were also obtained for phenanthrene. No degradation of phenanthrene occurred at all with free MnP from *A. discolor* Sp4 either alone or in mixture 1, whereas 19% transformation was measured with mixture 2. Differently, phenanthrene was not degraded in mixture 2 by MnP from free or immobilized *N. frowardii*. A detectable degradation was instead measured with the compound alone or in mixture 1.

a)



b)



c)

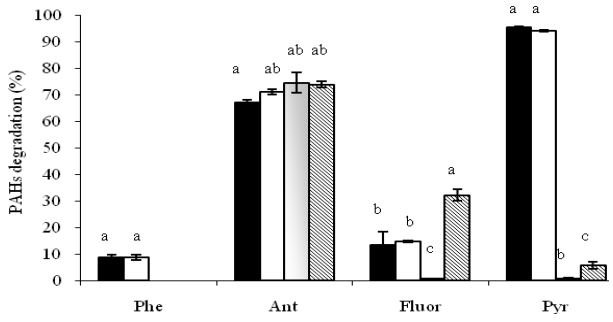


Figure 5.4 Degradation of PAHs by free (■) and immobilized MnP (□) from *A. discolor* Sp4 and by free (■) and immobilized MnP (⊟) from *N. frowardii*, at 30°C and pH 4.5. Phenanthrene (Phe), fluoranthene (Fluor), anthracene (Ant) and pyrene (Pyr) were added alone at a concentration of 10 mg l^{-1} (a), and in mixture at 2.5 mg l^{-1} (b) or 10 mg l^{-1} (c) each. Means with different letter indicate significant differences between free and immobilized MnP from *A. discolor* Sp4 and *N. frowardii*, according to the Tukey's test at 0.05 significance.

Anthracene degradation by immobilized MnP in soil

After incubation for 24 h, about 68% and 69% of anthracene was removed by immobilized MnP from *A. discolor* Sp4 in sterile and non-sterile soil respectively, significantly higher than 11% and 24% of removal reached by free MnP in the same soils (Fig. 5.5). In addition, about 90% of anthracene was removed by immobilized MnP from *N. frowardii* both in sterile and non-sterile soil. The removal of anthracene in sterile and non-sterile soil without MnP (control) was 9% and 13% respectively. Anthraquinone was identified as the major product during oxidation of anthracene by MnP from *A. discolor* Sp4 and *N. frowardii*, in sterile and non-sterile soil.

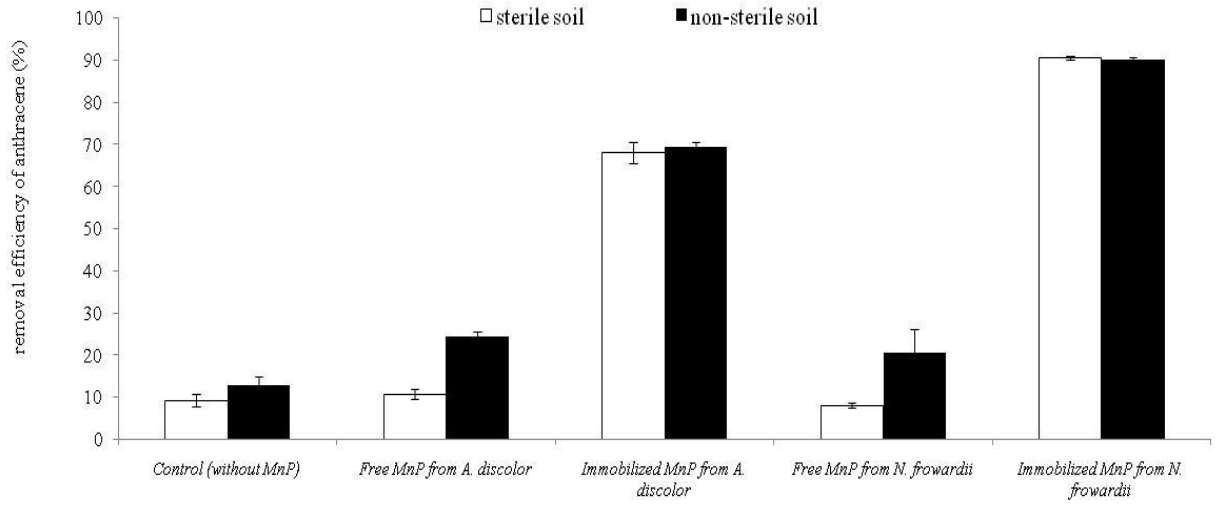


Figure 5.5 Removal efficiency of anthracene using free or immobilized MnP from *A. discolor* Sp4 or *N. frowatrdii*, in soil at 30°C.

5.4 Discussion

Previous results have demonstrated that the fungus *A. discolor* Sp4 was able to degrade PAHs and this degradation showed a positive correlation with the production of ligninolytic enzymes mainly MnP (Acevedo et al., 2009; Chapter 4). Although the important role of MnP in xenobiotics degradation has been widely demonstrated in literature (Sack et al.,

1997; Baborová et al., 2006; Steffen et al., 2007, Pizzul et al., 2009), the role of MnP from *Anthracophyllum discolor* Sp4 in PAH degradation and its use as possible biocatalyst has not been explored yet.

The use of enzymes in the degradation of organic compounds presents several advantages compared to the use of microorganisms such as their unique substrate-specificity and catalytic power, their capacity to act in the presence of many xenobiotic substances and/or under a wide range of environmental conditions, often unfavorable to active microorganisms (i.e. relatively wide temperature, pH and salinity ranges, high and low concentrations of contaminants); and their low sensitivity or susceptibility to the presence of predators and inhibitors of microbial metabolism (Gianfreda and Rao, 2004). Moreover, enzymes are able to reach substrates in pores with small dimensions, roughly 100 times smaller than bacteria (Quiquampoix et al., 2002).

In the present study we demonstrated that MnP may play an important role in the degradation of PAHs (Fig. 5.2). In fact, MnP from both investigated fungi was able to degrade the four studied PAHs. In particular, MnP from *A. discolor* Sp4 efficiently transformed after 24 h anthracene and pyrene to anthraquinone and 4,5 dihydropyrene, respectively, and to a less extent fluoranthene and phenanthrene. Production of anthraquinone was also detected using MnP from *N. frowardii*, thus confirming the participation of this enzyme in PAHs degradation (Sack et al., 1997).

As shown in Figure 5.3, MnP from *A. discolor* Sp4 was generally much efficient that MnP from *N. frowardii* in the degradation of pyrene, although their initial enzymatic activity was similar. In addition, no clear degradation pattern was found for phenanthrene for both MnP enzymes used, while a similar degradative efficiency was observed with anthracene. The different behavior shown by the two enzymes could be attributed to the different enzyme-substrate affinity of MnP from diverse strains (Palma et al., 2000). Moreover, a characteristic of this group of enzymes is their occurrence as a large family of isoenzymes (Gianfreda et al., 2002) displaying usually different kinetic parameters and regulatory properties. The higher efficiency of MnP from *A. discolor* Sp4 could be additionally

explained by either a synergism of other enzymatic activities (such as versatile peroxidase, glyoxal oxidase, aryl-alcohol oxidase, among others) or the effect of mediators (organic acids, Mn^{2+}, H_2O_2 and unsaturated fatty acids), both possibly present in the partially purified MnP extract from *A. discolor* Sp4. A higher stability of the *A. discolor* Sp4 enzymatic extract as compared to the commercial purified MnP could also be considered (Steffen et al., 2003).

Additionally, the capability of the two enzymes to degrade most of PAHs individually as well as in mixtures is a relevant result considering that in contaminated sites PAHs are usually present in different mixtures that could be degraded at high efficiency levels.

One the disadvantages associated with the use of free-cell enzymes for detoxifying xenobiotics in soil environments is their short life-span, due to both non-biological and biological deactivation factors such as adsorption on soil colloids, extreme acidity or alkalinity and protease action, which normally entails a low operational stability (Gianfreda and Bollag, 1994). Considering that enzymes become stable in soils by means of their immobilization on inorganic and/or organic soil particles, some studies have been performed using this type of natural support for enzymes immobilization and further degradation of xenobiotic compounds (Gianfreda and Bollag, 1994; Ahn et al., 2002).

In this study soil clay fraction has been selected for MnP immobilization and preferred to organic colloids because several findings have demonstrated that soil clay fraction is the most important inorganic fraction involved in the protection of organic molecules to microbial attack, assures a high enzymatic activity after immobilization (Gianfreda and Bollag, 1994), and avoid the possible inhibitory effects often shown by humic materials (as either natural and synthetic) when used for enzyme immobilization (Gianfreda and Bollag, 1994).

The nanoclay produced in this study had an estimated size of nanoparticles (< 100 nm) (Fig. 5.1) that would improve its capacity to bind enzymes, and decrease the influence of diffusion limitation. Smaller particles may have higher surface-to-volume ratios, thus

enhancing the enzyme binding capacity on their surface (Oh and Kim, 2000). Moreover, reduction of dimensions of enzyme carrier materials can generally improve the catalytic efficiency of immobilized enzymes, because the diffusional limitation for contaminant degradation may decrease (Hu et al., 2007).

No adsorption of MnP on nanoclay with organic matter was observed, probably because the IEP of MnP is too close to that of the used nanoclay. On the opposite, enzymes adsorption occurred on the nanoclay without organic matter (with an IEP of 7.0) at pH 4.5. At this pH MnP was negatively charged, whereas the organic matter-lacking nanoclay was positively charged. Therefore, electrostatic attraction was likely to be the main mechanism involved in enzyme adsorption. In general, enzymes immobilized on nanoparticles show a high mobility and activity, suggesting that the molecules are not rigidly attached to the support material (Wang, 2006). Moreover, there is a good match between the pore size of nanoclay and MnP molecular size having a stabilizing effect on the immobilized enzyme (Vamvakaki and Chaniotakis, 2007).

The higher immobilization efficiency observed for MnP from *N. frowardii* compared to MnP from *A. discolor* Sp4 may be attributed to competitive binding by the presence of other proteins and non-protein compounds in the partially purified MnP preparation (Davis and Burns, 1992). Although possible side effects and side activities (Fullbrook, 1996), the utilization of the partially purified MnP from *A. discolor* may be an interesting and practical low-cost alternative by considering the long and expensive isolation and purification procedure such as that used for MnP from *N. frowardii*.

Between 50 and 60°C, immobilized MnP from *A. discolor* Sp4 showed a higher activity compared to the free MnP. As described by Hartmeier (1988), immobilized enzymes are more stable at higher temperatures due to an enhanced rigidity achieved after the immobilization process. Enzymes stability at high temperatures is usually attributed to the prevention of conformational changes in the protein´s tertiary structure (Gianfreda et al., 2002). This particular property of immobilized enzymes enhances enzyme activity at high

temperature levels and opens a broad range of possible industrial applications (Bruins et al., 2001).

Purified free enzymes from different microorganisms show an Ea value of less than 25 kJ mol^{-1} (Wariishi et al., 1989; Gianfreda et al., 1991; Gianfreda et al., 1995). In this study, an activation energy (Ea) of 34.4 kJ mol^{-1} for the free partially purified MnP from *A. discolor* Sp4 was obtained. This Ea value indicates that partially purified MnP will need a higher energy input for performing the catalytic reaction. In addition, immobilized MnP presented an Ea value of 51.9 kJ mol^{-1}, also indicating a high energy input need for starting the enzymatic reaction. When enzymes are immobilized on solid supports, a general increase of Ea value usually occurs if modified reaction mechanisms are involved in the catalytic process (Lai and Tabatabai, 1992). However, up to date, no clear correlation between enzymes immobilization and Ea value has been presented in the literature (D´Annibale et al., 2000; Esawy and Combet-Blanc, 2006).

Immobilization of *A. discolor* MnP on nanoclay significantly altered its activity vs. pH profile. This result agrees with those of other authors, which indicated that the immobilization of enzymes on surface charged materials often leads to displacements of the pH-activity profile to either alkaline or acidic regions (Rogalski et al., 1999; David et al., 2006). Two main hypotheses have been proposed to explain the shift of the optimal pH of the catalytic activity of enzymes adsorbed on charged surfaces such as clay minerals. The first hypothesis considers that the pH in the region of the active site of the adsorbed enzyme is lower than the pH in the bulk of the solution (Quiquampoix, 2008). The second hypothesis is based on evidence of pH dependent modifications of protein conformation (Quiquampoix, 2008).

By contrast no negative effects on the catalytic efficiency of the two enzymes towards PAH degradation were observed upon their immobilization the solid support (Fig. 5.3), indicating that no conformational changes and/or no influence of the microenvironment created by the support material in the surroundings of the enzymes possibly took place (Gianfreda et al., 2002).

Compared with the free enzyme the higher stability shown by nanoclays-immobilized MnP to long-time storage at room temperature and 4°C represents an additional advantage and a prerequisite for the successful use of an immobilized enzyme for practical applications. Gianfreda and Bollag (1994) have reported similar results for acid phosphatase immobilized on montmorillonite, kaolinite and soil.

In a previous study we demonstrated PAH removal efficiency in a contaminated soil bioaugmented with *A. discolor* Sp4 (Chapter 4). The main factor affecting the colonization of soil by *A. discolor* Sp4 was clearly the presence of indigenous soil microorganisms, which may compete with the fungus for substrates disabling the fungal growth. This problem may be solved by using immobilized enzymes as shown in the present study.

As shown in Figure 5.4, immobilized MnP on nanoclays enhanced the enzymatic transformation of anthracene under the unfavorable conditions of soil after 24 h, compared to the free enzyme either from *A. discolor* Sp4 or from *N. frowardii*. After 24 h, the effect of indigenous soil microorganisms was not observed in non-sterile soil, indicating that in this period the immobilized enzyme was able to maintain its catalytic property.

Several factors including soil type (mineral and organic matter content) and physico-chemical properties (e.g. aqueous solubility, polarity, hydrophobicity, lipophilicity and molecular structure) of the contaminant determine the fate and behaviour of PAHs in the soil (Reid et al., 2000). In this sense, anthracene sorption into soil particles after 24 days was relatively low (9.3%) as shown in sterile soil without MnP, probably attributed to low organic matter content of the contaminated soil (3%) and log K_{oc} value (4.42) of anthracene. In presence of free MnP either from *A. discolor* Sp4 or from *N. frowardii*, the anthracene removal efficiency was 2-fold higher in presence of native flora compared with sterile soil indicating a possible activation of indigenous soil microorganisms. Wu et al. (2008) observed that the free laccase treatments showed no significant influence on microbial counts for actinomycetes, fungi or aromatic hydrocarbon degraders except for the bacteria. This may be due to the stimulating effect of the accompanying support matrix (dextrin) or to the enzyme itself (Wu et al., 2008).

Anthraquinone was identified as the major product during MnP oxidation of anthracene. In general, PAH-quinones are indicators of anthracene partial degradation. As they have a higher solubility and lower toxicity than their parent compounds (Torres et al., 2003), their presence suggests enzymatic remediation of PAHs-contaminated soil.

5.5 Conclusions

MnP enzyme was extracted from the white-rot fungus *Anthracophyllum discolor* Sp4, native of southern forest of Chile. In addition, the results of this study show that nanoclay, a novel natural nanomaterial, is a suitable support material for MnP immobilization. Compared with free MnP, nanoclay-immobilized MnP showed an increased stability at higher temperature, pH, and storage time. Moreover, nanoclay-immobilized MnP exhibited an enhanced PAH degradation efficiency in soil.

All these results suggest that nanoclays-immobilized MnP from *A. discolor* Sp4 could be an efficient alternative for in-situ bioremediation on a larger scale. This technology must be, however, validated in the field where the conditions vary greatly. Field studies must be performed to show that enzymatic in-situ bioremediation may be an effective and desirable alternative to current remediation strategies.

5.6. Acknowledgements

This work was partially financed by FONDECYT project Nº 1050614, by a doctoral scholarship from CONICYT (Chile) and by the Swedish Foundation for Strategic Environmental Research (MISTRA) through funding for the Domestication of Microorganisms (DOM) research program and the Centre for Chemical Pesticides (CKB) at the Swedish University of Agricultural Sciences.

6

General discussion and concluding remarks

General discussion and concluding remarks

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds consisting of at least two fused benzene rings. Environmental contamination by these compounds has been a consequence of population increase, urbanization and industrialization on a global scale. They are present in petrol and formed during the incomplete combustion of organic fuels. Although natural emissions such as forest fires and volcanic eruptions can contribute to background amounts of PAHs in the atmosphere, anthropogenic activities are the primary source of these compounds. PAHs are resistant to photolysis, chemical and biological degradation with a potentially toxic impact on human health and the environment due their mutagenic and carcinogenic potential. Due to their hydrophobicity, PAHs tend to accumulate on the soil organic matter and thus, their limited desorption from soil decreases their availability for biodegradation.

Although at global scale only few information about levels of PAHs in soil has been published, high PAHs environmental contents have been detected in Norway, Denmark, Estonia and United States. Particularly in Chile, high PAH environmental levels have been detected in mussels and sediments. Therefore, it is desirable to develop adequate technologies for their removal.

A promising strategy for environmental clean-up is the use of microorganisms for transforming or degrading organic compounds to a less harmful products. This strategy called bioremediation is accomplished by biostimulation (the addition of nutrients such as N, P and K) and/or bioaugmentation (inoculation of exogenous microorganisms with known pollutant transformation abilities) of the contaminated environment. PAH bioremediation has been already reached by using white-rot fungi and free or immobilized ligninolytic enzymes. In fact, white-rot fungi have demonstrated a great potential to degrade PAHs by their extracellular enzymatic system. However, the enzymatic characteristics of native Chilean white-rot fungi and its possible potential use for bioremediation of polluted environments with PAHs has almost not been studied. In previous study, the white-rot fungus *Anthracophyllum discolor* Sp4, was isolated from carpophores collected from the

deadwood of native trees in the Region of La Araucanía in Chile. This fungus demonstrated a high ligninolytic enzyme production in N-limited medium, mainly MnP. MnP oxidized Poly R-478 and chlorophenols. The ability of *A. discolor* Sp4 to degrade PAHs and the participation of its extracellular enzymatic system in this process has not been explored yet.

In this sense, the objective of this work was to evaluate the role of MnP produced from the recently isolated Chilean white-rot fungus *Anthracophyllum discolor* Sp4 in the degradation of PAHs and to evaluate for biotechnological applications its efficiency as a nanoclay-immobilized enzyme in the degradation of these compounds in liquid medium and soil

For this purpose, MnP was produced in high quantities and purified (Chapter 3, Paper I). A maximum of 1354 U l^{-1} of MnP after 13 days of incubation was obtained in Kirk medium supplemented with lignocellulosic material such as sterile wheat grain and inducer (0.25 mM $MnSO_4$), incubated under static conditions at 30°C. In addition, it was possible to reproduce and even enhance MnP activity when Erlenmeyer flasks were replaced by tubes containing glass beads under the optimized culture conditions. This beads-tubes system is a promising and practical method for testing bioremediation assays at laboratory scale. Two enzymatic methods for the determination of MnP activity were compared. The MBTH/DMAB assay was more sensible and suitable for colored samples in comparison with 2,6-DMP assay.

An optimized purification method for MnP from *A. discolor* Sp4 is proposed. The optimized scheme based on a two-step purification process includes a prelimary anion exchange step followed by size exclusion chromatography. However, a single step using anion exchange chromatography allows obtaining a purified MnP with a purification yield of approximately 48% and a purification factor of 4%.

The apparent K_M values of the purified MnP for 2,6-DMP and H_2O_2 were found to be 24.83 µM and 36.98 µM, respectively, indicating a high affinity for both substrates, being comparable with the kinetic parameters of *Phanerochaete chrysosporium*. MnP produced by *A. discolor* Sp4 presented a pH stability being the optimum pH range between 4.5 and 5.5.

Activation energy (Ea) of 16.5 kJ mol^{-1} was determined in the temperature range between 30°C and 50°C. In addition, the enzyme showed a relatively high temperature tolerance (50ºC) and high thermal stability between 40 and 50ºC.

In conclusion, the proposed methods for the production, isolation and purification of degradative enzymes particularly MnP from *A. discolor* Sp4 were successfully implemented and optimized in the course of this work. High enzymes production rate would be advantagenous for bioremediation applications. *A. discolor* Sp4 could be an attractive source of MnP, both as a non-purified extracellular crude or as a purified product for its use as a free or an immobilized enzyme for bioremediation purposes. In addition, applications in pulp and paper industries may improve the effectiveness of conventional bleaching processes.

In Chapter 4 (Paper II), the degradation of 3 and 4-ring PAHs in Kirk medium by *A. discolor* and the role of ligninolytic enzymes in this process were evaluated. *A. discolor* Sp4 was able to degrade phenanthrene, anthracene, fluoranthene and pyrene simultaneously and individually in Kirk medium, with the highest removal for PAH mixture, suggesting synergistic effects between PAHs or possible cometabolism. In addition, MnP and LiP produced and stimulated in the presence of PAHs would be responsible of PAH degradation.

Two different bioremediation techniques (biostimulation and bioaugmentation) of a contaminated soil with PAHs were evaluated (Chapter 4, Paper II). To achieve this objective, 3, 4 and 5-ring PAH removal by native microflora of soil and by adding *A. discolor* in presence and absence of indigenous microorganisms were evaluated. Due to its recalcitrant characteristics and its preference for non-aqueous phases and soil particles, benzo*(a)*pyrene was used as an environmental carcinogenic indicator for PAHs in soil. A high PAH removal was obtained by the action of native microflora after 60 days, biostimulated by adding wheat grains as C and N supply, water and air. 98.3% of the phenanthrene, 83.1% of the anthracene, 82.5% of the fluoranthene, 82.5% of the pyrene and 14.2% of the benzo*(a)*pyrene were removed from this soil. The presence of *A. discolor* Sp4 (bioaugmentation) caused a less efficient removal of anthracene (83.1% vs. 61.5%),

fluoranthene and pyrene (82.5% vs. 43.1%) after the same period of incubation, showing a possible inhibition and competition of native microbes with the fungus. The colonization of soil by *A. discolor* Sp4 may require a higher demand of substrates compared to the control (autoclaved soil). In absence of indigenous microorganisms, *A. discolor* Sp4 showed biodegradation capability in soil, particularly for benzo*(a)*pyrene, clearly demonstrating a positive correlation with ligninolytic enzymes production such as MnP.

Biostimulation in contaminated soil with PAHs was found to be a successful technique compared with the bioaugmentation strategy. Nevertheless, the use of *A. discolor* Sp4 under optimized conditions may be justified particularly considering its ability to degrade benzo*(a)*pyrene, a compound difficult to degrade by soil indigenous microorganisms which is normally one of the most detected and toxic PAHs in contaminated soils. There is also a clear need to better understand the microbial ecology of *A. discolor* Sp4 in highly contaminated soils before substantial progress in developing bioremediation strategies can be performed.

Another form of bioremediation is the enzymatic treatment using free or immobilized enzymes, which have a minimal impact on ecosystems, as shown in Chapter 5 and Paper III. For this purpose, MnP produced by *A. discolor* Sp4 was immobilized on nanoclay and its ability to degrade PAHs compared with the free enzyme was evaluated. The results of this study confirm that MnP from *A. discolor* Sp4 effectively plays an important role in the degradation of PAHs producing quinones as polar intermediate metabolites. The PAH metabolites observed suggest that the oxidation of the PAHs is mainly mediated by the ligninolytic enzyme system.

Enzymes naturally tend to bind to inorganic and organic soil colloids displaying an altered catalytic behavior. In fact, MnP was successfully immobilized on nanoclays through a simple adsorption process, thus conferring the enzyme an improved stability to temperature, pH and storage. In addition, the use of crude culture filtrate as a partially purified MnP constitutes an economic and efficient alternative in comparison with isolated enzymes. In general, crude culture filtrates offer additional advantages over the use of

purified enzymes: their obtention process is less expensive and the presence of proteins and other factors in the medium may stabilize crude enzymes.

Promising results were obtained with the application of nanoclay-immobilized MnP. The next step should be to implement this method on aged PAHs-contaminated soil, with low bioavailability on a larger scale.

Based on the results of this work, hypothesis 1 and 2 have been confirmed, indicating that nanoclay-immobilized MnP is a new biotechnological tool for the remediation of soil contaminated with PAHs.

However, new questions and challenges arise from this work. For instance, the presence of MnP isoenzymes could be much broader than reported here and should be studied in future research in order to test possible differences between them regarding catalytic activity and stability. On the other hand, the enhanced production of other ligninolytic enzymes such as LiP or laccase from *A. discolor* Sp4 and posterior purification could be interesting as new biocatalysts for bioremediation purposes. MnP plays an important role in the PAH degradation by *A. discolor* Sp4. However, other mechanisms behind the transformation of the PAHs by *A. discolor* Sp4 are not yet elucidated and should be also studied in further research. In addition, there is also a clear need to reveal the genetic repertoire of *A. discolor* Sp4 and to better understand the microbial ecology of *A. discolor* Sp4 in highly contaminated soils.

Summarizing, enzymatic bioremediation has become an attractive alternative as enzymes provide simpler systems compared to whole microorganisms for remediation systems. Enzymatic bioremediation is particularly suited to situations where rapid remediation is needed. In contrast to conventional bioremediation technologies (biostimulation, bioaugmentation, phytoremediation), enzyme bioremediation not depends on growth of microorganisms, and the detoxification rate is directly linked to the catalytic properties and the concentration of the applied enzyme. The main advantages of using enzymes as biocatalysts in bioremediation processes are their biodegradability and their high chemo-,

and stereo-selectivity, resulting in low by-product formation, favouring their progressive implementation. However, the high enzymes costs, low activity and/or stability under given conditions and low reaction kinetics are disadvantages. Although methods such as enzyme modification and site-directed mutagenesis have been used to stabilize enzymes, nanotechnology has provided new opportunities to improve enzyme stability and subsequent persistence in contaminated environments pushing to prices that are more competitive and suggesting a promising future for environmental biocatalysis.

7

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8

Appendix

8.1 Appendix 1

List of original papers of this thesis:

I Acevedo F., Pizzul L., Castillo MdP., Rubilar O., Lienqueo M.E., Diez M.C. (2010) Enhanced production of manganese peroxidase from *Anthracophyllum discolor* Sp4 and its characterization (to be submitted)

II Acevedo F., Pizzul L., Castillo MdP., Cuevas R., Diez M.C. (2010) Degradation of Polycyclic Aromatic Hydrocarbons by the Chilean white-rot fungus *Anthracophyllum discolor* Sp4 (submitted to Journal of Hazardous Materials)

III Acevedo F., Pizzul L., Castillo MdP., González M.E., Cea M., Gianfreda L, Diez M.C. (2010) Degradation of polycyclic aromatic hydrocarbons by free and nanoclay-immobilized manganese peroxidase from *Anthracophyllum discolor*. Chemosphere 80: 271-278

Contributions as co-author

Rubilar O, Tortella G, Cea M, **Acevedo F**, Bustamante M, Gianfreda L and Diez MC. 2010. Bioremediation of a chilean andisol contaminated with pentachlorophenol (PCP) by solid substrate cultures of white-rot fungi. Biodegradation DOI: 10.1007/s10532-010-9373-9 (in press)

Cristina Diez y **Francisca Acevedo**. 2009. Los pequeños grandes aliados. INDUAMBIENTE. 96

8.2 Appendix 2

Characterization of wheat grain and soil

(Laboratorio Suelo, Instituto de Agroindustria, Universidad de La Frontera).

Wheat grain

Parameter	Results
Dry matter (%) 105°C	88.2
Proteins (%)	11.75
Flash (%)	2.27
C (%)	54.3
C/N ratio	28.9
Mn (ppm)	39

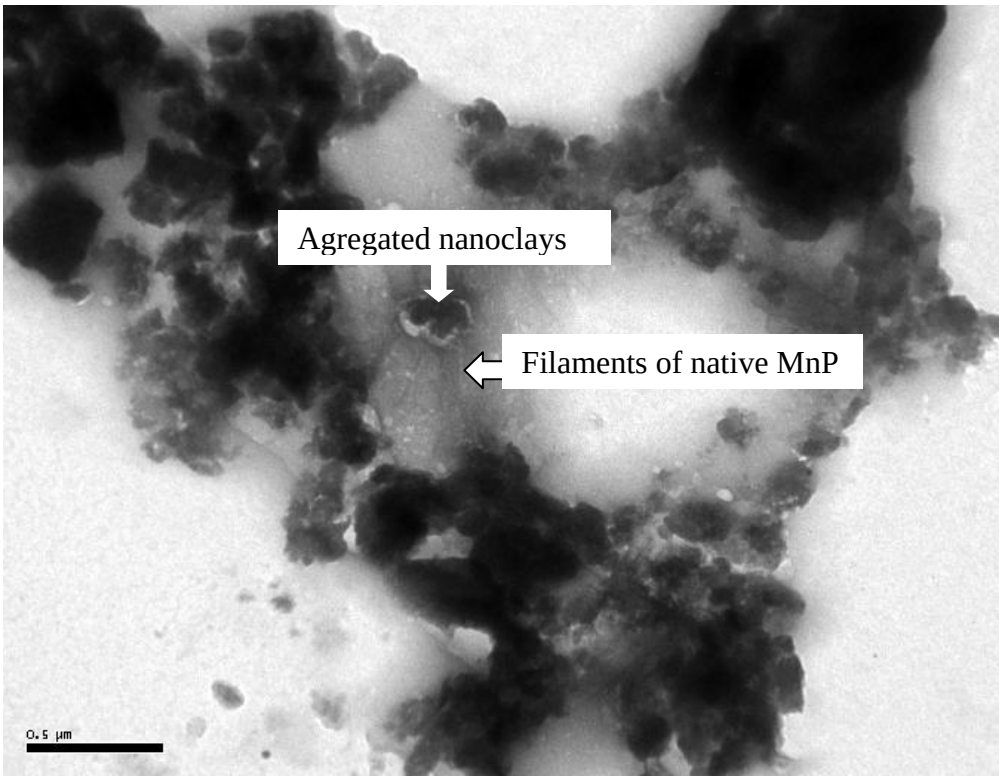
Soil

Parameter	Results
N (mg/kg)	12
P (mg/kg)	37
K (mg/kg)	66
pH (water)	6.6
Organic matter (%)	3
Clay (%)	14
Organic carbon (%)	1.8
Nitrogen (%)	0.11
K (cmol+/kg)	0.17
Na (cmol+/kg)	0.05
Ca (cmol+/kg)	6.65
Mg (cmol+/kg)	0.51
Al (cmol+/kg)	0.01
CICE	7.39

CICE: exchangeable Ca+Mg+K+Na+Al

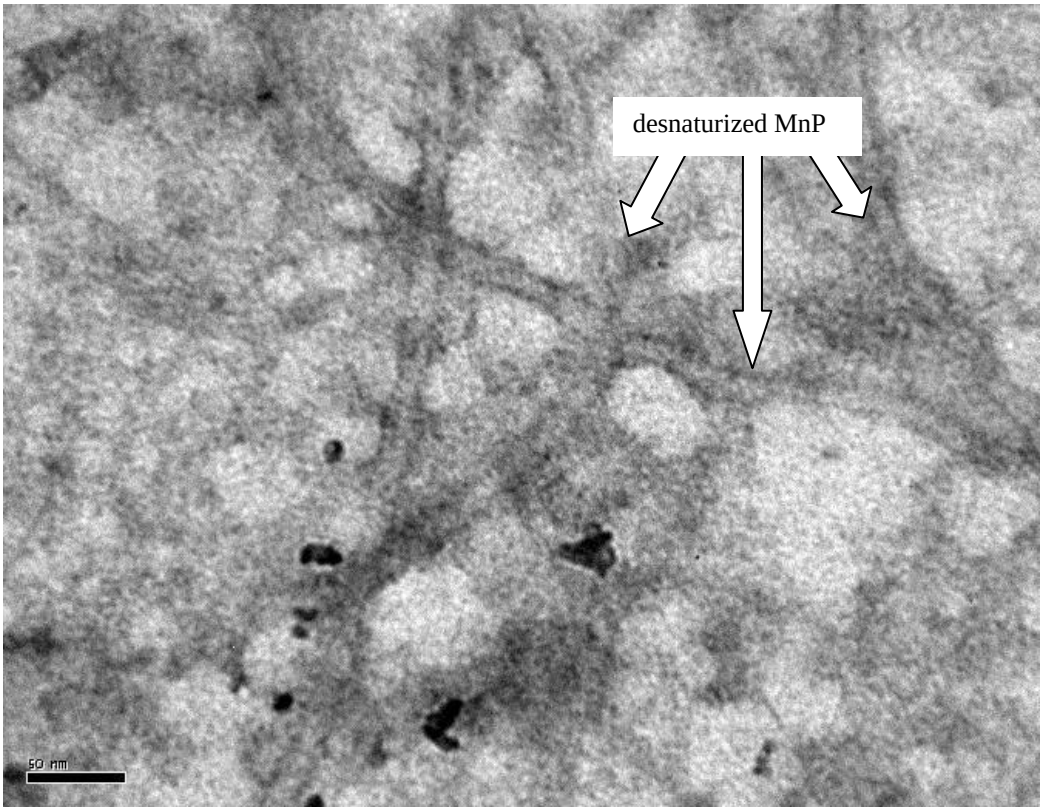
8.3 Appendix 3

Nanoclay-immobilized MnP from *A. discolor* Sp4 by Transmission Electronic Microscopy (TEM)



8.4 Appendix 4

MnP from *Nemalotoma frowardii* by Transmission Electronic Microscopy (TEM)



8.5 Appendix 5

Respiration assay

The respiration trial is set up in glass jars containing two traps of 4 ml NaOH (0.2 M) each. In the system, produced CO_2 is dissolved in NaOH and $NaCO_3$ is formed.

$$2\ NaOH + CO_2 \rightarrow Na_2CO_3 + H2O$$

When 8 ml $BaCl_2$ (0.1 M) is added to 2 ml of the samples the following reaction occurs:

$$BaCl_2 + Na_2CO_3 \rightarrow BaCO_3(s) + 2\ NaCl$$

Through titration in TIM 850 Titration Manager (adding HCl to reach a pH of 8.3) it was then possible to measure the amount NaOH that has not reacted with the $BaCl_2$. When the amount of NaOH that has not reacted is known the production of CO_2 can be calculated using a reference of fresh NaOH. The more HCl that has to be added to reach pH 8.3 the more NaOH is still left in the trap, the lower the respiration. The amount CO_2 produced per gram compost was calculated using this formula:

mg CO_2 = (nHCl(VHCl reference – VHCl sample) * $MWC0_2$* # trap)/weight of sample (g)

nHCl = 0.1M
$MWCO_2$ = 44
# traps = number of traps used (normally 2)
VHCl = volume (ml) of HCl from the titration

The respiration rate was expressed as accumulated mg CO_2 g $soil^{-1}$.