Degradation of recalcitrant biopolymers and polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi

KARI T. STEFFEN

Division of Microbiology
Department of Applied Chemistry and Microbiology
Viikki Biocenter, University of Helsinki
Finland

Academic Dissertation in Microbiology

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in the auditorium 1041 at the Viikki Biocenter (Viikinkaari 5) of the University of Helsinki on October the 28th 2003 at 12 o'clock noon.

Helsinki 2003

Supervisors: Prof. Annele Hatakka
Department of Applied Chemistry and Microbiology
University of Helsinki, Finland

Prof. Martin Hofrichter
Chair of Environmental Biotechnology
International Graduate School Zittau, Germany

Reviewers: Prof. Jim A. Field
Department of Chemical and Environmental Engineering
University of Arizona, U.S.A.

Doc. Robin Sen
Division of Microbiology, Department of Biosciences
University of Helsinki, Finland

Opponent: Prof. Yitzhak Hadar
Department of Plant Pathology and Microbiology
The Hebrew University of Jerusalem, Israel

Printed: Yliopistopaino 2003, Helsinki, Finland
Layout: Otso Koski

ISSN 1239-9469

ISBN 952-10-1051-7 printed version
ISBN 952-10-1052-5 pdf version, http://ethesis.helsinki.fi

e-mail: Kari.Steffen@Helsinki.Fi

Front cover picture: Fruiting bodies of *Stropharia rugosoannulata* G grown on a straw bale left on bare soil (photo Kari Steffen).

Urk.IV
96.16

“Es gibt keine patriotische Kunst und keine patriotische Wissenschaft. Beide gehören, wie alles hohe Gute, der ganzen Welt an, und können nur durch allgemeine freie Wechselwirkungen aller zugleich Lebenden, in steter Rücksicht auf das, was uns vom Vergangenen übrig und bekannt ist, gefördert werden.“
GOETHE

“Olen tutkijana, mikä on ankarin (ja huonopalkkaisin) ala.”
ERNESTO GUEVARA

Meinen Eltern und ihren Enkelkindern

Abstract

Litter-decomposing fungi (LDF), including agaric basidiomycetes, represent typical soil-dwellers in forests and grasslands. These microorganisms are the primary decomposers of residual plant materials in the upper most soil layer. LDF are capable of attacking all components of the lignocellulose complex, including the recalcitrant lignin polymer. Within an *in vitro* screening study for ligninolytic enzyme activities, the most active species were found in the families *Bolbitiaceae* and *Strophariaceae* (*Agrocybe praecox, Stropharia coronilla, S. rugosoannulata*). Strains of these species were assessed in mineralization experiments incorporating a ^{14}C-ring-labeled synthetic lignin (^{14}C-DHP) as substrate. These target fungi mineralized around 25% of the radiolabeled lignin to $^{14}CO_2$ within 12 weeks of incubation in a straw environment. Manganese peroxidase (MnP) was found to be the predominant extracellular ligninolytic enzyme secreted by the three fungi in liquid culture and its production was strongly enhanced in the presence of Mn^{2+} ions. Extracellular MnP was purified from liquid cultures of the LDF *A. praecox* and *S. coronilla*. Both fungi produced MnP with similar isoelectric points (p*I*) of 6.3-7.1 and a molecular mass (MW) of 41-42 kDa. Near neutral p*I* –type MnP seem to be a typical feature of LDF.

Collybia dryophila colonizing forest soil was found to decompose a natural humic acid isolated from pine-forest litter (LHA) and a synthetic ^{14}C-labeled humic acid (^{14}C-HA) prepared from [UL-^{14}C] catechol in liquid culture. Degradation resulted in the formation of polar, lower-molecular mass fulvic acids (FAs) and carbon dioxide. HA decomposition was considerably enhanced in the presence of Mn^{2+} (200 µM). As such, a strong case can be made for the role of MnP. During solid-state cultivation, *C. dryophila* released substantial amounts of water-soluble FAs (predominant MW 0.9 kDa) from insoluble litter material. The results indicate that basidiomycetes such as *C. dryophila* colonizing forest litter and soil may be involved in humus turnover by recycling high-molecular mass humic substances.

Several strains of LDF were able to partly remove PAH in a mixture of three polycyclic aromatic hydrocarbons (PAHs) (total 60 mg l^{-1}) comprising anthracene, pyrene and benzo(a)pyrene (BaP) in liquid culture. *Stropharia rugosoannulata* was the most efficient degrader, removing or transforming BaP almost completely. In the case of *S. coronilla*, the presence of Mn^{2+} led to a 20-fold increase of anthracene conversion. The effect of manganese can be attributed to the stimulation of MnP.

Stropharia coronilla was found to be capable of metabolizing and mineralizing BaP in liquid culture. Mn^{2+} supplemented at a concentration of 200 µM stimulated considerably both the conversion and mineralization of BaP. Crude and purified MnP from *S. coronilla* oxidized BaP efficiently in a cell-free reaction mixture (*in vitro*), a process, which was enhanced by the surfactant Tween 80. Clear indication was found that BaP-1,6-quinone was formed as a transient metabolite, which disappeared over the further course of the reaction. The treatment of a mixture of 16 different PAHs (EPA-PAH; total concentration 320 mg l^{-1}) with MnP resulted in concentration decreases of 10 to 100% for the individual compounds. Probably due to their lower ionization potentials, poorly bioavailable, high-molecular mass PAHs such as BaP, benzo(g,h,i)perylene and indeno(1,2,3-c,d)pyrene were converted to larger extents than low-molecular mass counterparts (e.g. phenanthrene, fluoranthene).

Taken together the data supports litter-decomposing fungi as being efficient degraders of recalcitrant organic compounds and are therefore important for the carbon cycle as well as possible soil-bioremediation applications.

Tiivistelmä (abstract in Finnish)

“Karikkeenlahottajasienet vaikeasti hajoavien biopolymeerien ja ympäristömyrkkyjen hajottajina”

Työssä tutkittiin karikkeenlahottajasienten kykyä hajottaa luonnon biopolymeerejä, kuten ligniiniä ja humusyhdisteitä, sekä orgaanisia ympäristömyrkkyjä, kuten polysyklisiä aromaattisia hiilivetyjä (PAH).

Karikkeenlahottajasienet ovat ryhmä sieniä, jotka kasvavat maassa ja maan pinnalla olevassa karike- ja humuskerroksessa. Kantasieniin kuuluvat karikkeenlahottajat muodostavat itiöemiä, jotka ovat usein vaatimattoman näköisiä, mutta niiden joukossa on myös syötäviä sieniä, kuten herkkusieni. Tutkimuksissamme on selvinyt, että kariketta hajottavat kantasienet tuottavat solunulkoisia entsyymejä, kuten mangaaniperoksidaasia ja lakkaasia, joiden avulla ne pystyvät hajottamaan ligniiniä saadakseen käyttöönsä muita hiilenlähteitä, kuten selluloosaa ja hemiselluloosaa. Nämä entsyymit ovat epäspesifisiä ja niiden aikaansaamien radikaalireaktioiden ansiosta ne rikkovat erityisesti aromattisiä rengasrakenteita. Tästä johtuen karikkeenlahottajasienet pystyvät myös hajottamaan humusyhdisteitä, esim. humushappoja, joiden tärkein lähtöaine on mm. ligniini. Samasta syystä myös PAH-yhdisteet voidaan hajottaa näiden sienten avulla.

PAH-yhdisteiden hajoamisen tutkiminen on tärkeä niiden mutageenisuuden ja syöpää aiheuttavien ominaisuuksien kannalta. Tuloksemme osoittavat selvästi, että karikkeenlahottajasienet hajottavat PAH-yhdisteitä laboratorio-olosuhteissa nesteviljelmissä, sekä pystyvät jopa mineralisoimaan syöpää aiheuttavaa bentso(a)pyreeniä (BaP). *Stropharia coronilla*n, eli nurmikaulussienen tuottama puhdistettu MnP pystyi jopa yksin hajottamaan 16 erilaista PAH-yhdistettä, joiden joukossa oli myös suurimolekyylipainoisia yhdisteitä, kuten bentso(g,h,i)peryleeni ja indeno(1,2,3,c,d)pyreeni. Lisäksi sieni mineralisoi BaP:ä.

Kariketta hajottavat kantasienet tuottavat usein mangaaniperoksidaaseja, jonka isoelektrinen piste on lähes neutraali. Tämä ominaisuus erottaa karikkeenlahottajat selvästi puunlahottajista. Tutkimuksemme tulokset viittaavat siihen, että karikkeenlahottajat eivät tuota ligniiniperoksidaasia, ja että näin ollen mangaaniperoksidaasillä on erittäin suuri merkitys aromaattisten yhdisteiden hajottamisessa, mikä korostuu mangaanin läsnäollessa, kuten maassa. Karikkeenlajottajasienet ovat sopeutuneet kasvamaan maassa ja ne näin ollen voisivat toimia saastuneen maan puhdistajina. Tulostemme mukaan karikkeenlahottajasienten merkitys maailmanlaajuisessa hiilenkierrossa voi olla merkitsevä, koska ne pystyvät hajottamaan tehokkaasti ligniiniä ja humusyhdisteitä.

TABLE OF CONTENTS

List of original publications

I Steffen KT, Hofrichter M, Hatakka A (2000) Mineralisation of ^{14}C-labelled synthetic lignin and ligninolytic enzyme activities of litter-decomposing basidiomycetous fungi. Applied Microbiology and Biotechnology, 54:819-825

II Steffen KT, Hofrichter M, Hatakka A (2002) Purification and characterization of manganese peroxidases from the litter-decomposing basidiomycetes *Agrocybe praecox* and *Stropharia coronilla*. Enzyme and Microbial Technology, 30:550-555.

III Steffen KT, Hatakka A, Hofrichter M (2002) Degradation of humic acids by the litter-decomposing basidiomycete *Collybia dryophila*. Applied and Environmental Microbiology, 68:3442-3448.

IV Steffen KT, Hatakka A, Hofrichter M (2002) Removal and mineralization of polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi. Applied Microbiology and Biotechnology, 60:212-217.

V Steffen KT, Hatakka A, Hofrichter M (2003) Degradation of benzo(a)pyrene by the litter-decomposing basidiomycete *Stropharia coronilla*: role of manganese peroxidase. Applied and Environmental Microbiology, 69:3957-3964.

The author's contribution

Kari Steffen planned and conducted the experiments, analyzed and interpreted the results, and wrote the papers. He is also the corresponding author of all five articles.

Abbreviations

AAO aryl alcohol oxidase
ABTS 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate)
BaP benzo(a)pyrene
CBQ cellobiose:quinone oxidoreductase
DHP dehydrogenation polymer (synthetic lignin)
DMF dimethyl formamide
DNA deoxyribonucleic acid
EPA Environmental Protection Agency
FA fulvic acid
FPLC fast protein liquid chromatography
GLOX glyoxal oxidase
GSH glutathione
HA humic acid
HBT hydroxybenzotriazole
HPLC high performance liquid chromatography
HPSEC high performance size exclusion chromatography
HS humic substances
IEF isoelectric focusing
IHSS International Humic Substance Society
LDF litter-decomposing fungi
LiP lignin peroxidase
LSC liquid scintillation counter
MW molecular mass
MnP manganese peroxidase
NADPH nicotineamide-adenine dinucleotide-phosphate (reduced)
PAGE polyacrylamide gel electrophoresis
PAH polycyclic aromatic hydrocarbons
p*I* isoelectric point
PTE poly diphenyl dimethyl siloxane
QTM quick turnaround method
SDS sodium dodecyl sulfate
SOM soil organic matter
TNT trinitrotoluene
UV ultraviolet

1. Introduction

1.1 Litter-decomposing fungi

Fungi that colonize soil-litter, in particular litter-decomposing fungi (LDF), include basidiomycetes and ascomycetes living in the upper most portion of the soil and in the humus layer of forests and grasslands. In general, the decomposition of litter is brought about by combined activities of bacterial, fungal and animal populations, but basidiomycetous LDF are particularly important organisms because of their production of a wide range of ligninocellulolytic enzymes (Dix and Webster 1995). Many litter-decomposing fungal species are widely distributed in northern temperate forests although not associated with any particular soil type. The presence of specific taxa varies with the type of litter available. Basidiomycetous litter-decomposers most commonly belong to the order *Agaricales*, but there are also basidiomycetes in other orders, e.g. *Boletales* and *Poriales*. Additionally many macroscopic fruiting body forming ascomycetes (e.g. *Gyromitra* spp.) can be considered as LDF in a broader sense.

Around 14 000 to 16 000 species of basidiomycetes are known (Hawksworth *et al.* 1995, Watkinson *et al.* 2000). The order of *Agaricales* comprises around 6 000 spp. Fungi in this order are commonly called mushrooms, toadstools, gill fungi, or agarics (Hawksworth *et al.* 1995). They are also referred to as being terrestrial, lignicolous, saprobic, or mycorrhizal. LDF are found in several families, e.g. *Agaricaceae* (~ 600 spp. total including *Agaricus* spp.), *Bolbitiaceae* (~ 150 spp. total including *Agrocybe* spp.), *Coprinaceae* (~720 spp. total including *Coprinus* spp.), *Strophariaceae* (~220 spp. total including *Stropharia* spp.; Fig. 1.2, 1.3, and 1.4), and *Tricholomataceae* (~150 spp. total including *Clitocybe* spp., *Collybia* spp., *Lepista* spp., *Marasmius* spp., *Mycena* spp.). The gilled wood-decayers *Pleurotus* spp. on the other hand belong to the order *Poriales* and the family *Lentinaceae* (~145 spp.). The major basidiomycetous genera which decompose litter in forests include *Clitocybe* spp., *Collybia* spp. (Fig. 1.6), *Mycena* spp., *Marasmius* spp., *Hydnum* spp., *Tricholoma* spp., and in agricultural areas (meadows e.g.) *Agaricus* spp., *Agrocybe* spp. (Fig. 1.5), *Psilocybe* spp. and *Coprinus* spp. Furthermore there are species in overlapping groups between wood-decaying and LDF including the wood-decayers *Hypholoma* spp. (*Nematoloma* spp.), *Pleurotus* spp., *Armillaria* spp., and the straw-decomposing fungi such as *Stropharia rugosoannulata*. Some species, such as *Auriscalpium vulgare*, show substrate specificity while others grow on a wide range of material, such as *Clitocybe nebularis*, *Collybia bytrycea*, or *Mycena galopus* (Dix and Webster 1995).

Though the term litter is normally associated with discarded cans, plastic wrappings, and other anthropogenic waste, in this work it is applied to plant or forest debris and other material that has a more biological origin. Thus forest litter comprises of dead leaves, needles, twigs, branches, roots, and the remains of insects, bacteria, fungi, and animals. This layer is generally present on the soil surface and can be clearly distinguished from the underlying mineral layers. From a chemical point of view this habitat consists of a diverse spectrum of carbohydrates, mainly lignocellulose and in older fractions humic substances (HS) (see also section 1.5). Plant litter is itself composed of six main categories of chemical constituents: (1) cellulose, (2) hemicellulose, (3) lignin, (4) water-soluble sugars, amino acids, and aliphatic acids, (5) ether- and alcohol-soluble constituents including fats, oils, waxes, resins, and many pigments, and (6) proteins (Satchell 1974). It is the soil-litter layer that provides a suitable habitat for LDF and it is often only 1-10 cm thick. These fungi grow over large distances in this layer to reach new substrate and their mycelium is therefore widely distributed. The mycelium can readily constitute up to 60% of the living biomass in

forest soils (Dix and Webster 1995). They often form fruiting bodies while moving forward and circles called fairy rings.

Because LDF include saprotrophic basidiomycetes, nearly all constituents of the litter are open to degradation by these fungi. The lignocellulosic complex in particular includes lignin that is attacked by a number of enzymes including manganese peroxidase (MnP) and laccase (see also section 1.3). The ability to break down lignin and cellulose enables some of the LDF to function as typical "white-rot fungi" in soil (Hofrichter 2002, see below). Thus the degradation of lignin and derived humic material can generate white-rot humus (Hintikka 1970). LDF can also produce other hydrolytic and oxidative enzymes, e.g. *Lepista nuda* produces phosphatase, protease, cellulase, β-xylosidase, β-glucosidase, and phenol oxidase (Colpaert and vanLaere 1996). LDF seem to release nitrogen during the decomposition of leaf litter (Colpaert and vanTichelen 1996) but tend to accumulate different metals and heavy metals (Rajarathnam *et al.* 1998). As such, it is clear that the impact of this fungal group is extremely important in forest and grassland ecosystems. Litter production in forests ranges from around 1.5-1.8 tons hectar^{-1} year^{-1} in Finnish birch (*Betula* spp.) stands and up to 15 tons hectar^{-1} year^{-1} in tropical rain forests (Jensen 1974). Without the activity of LDF we, and forests, would in time be buried by cast off leaves and branches. Litter is often colonized by LDF during the final stage of decay and therefore the accumulation of recalcitrant material (mainly the lignin component of litter) is minimized. This makes LDF one of the most active degraders of tree leaf litter that has major implications for recycling of carbon in soil (Dix and Webster 1995).

From an eco-physiological point of view, basidiomycetes that form macroscopic fruiting bodies can be broadly classified into wood-decaying, mycorrhiza-forming, and litter-decomposing fungi (Fig. 1.1). Wood-decomposing fungi colonizing dead or dying tree trunks and stumps utilize cellulose while modifying the hemicellulose and lignin constituents cause either brown-rot or, more commonly, white-rot via the utilization of hemicellulose and cellulose during the degradation of lignin. However, unlike mycorrhiza-forming fungi, wood-decaying fungi do not actively colonize soil. Mycorrhizal fungi form a symbiotic relationship with the roots of trees and other plants and provide them with better access to water and nutrients in return for host carbon assimilates. Until recently, they were believed not to exhibit the saprotrophic capabilities of litter-decomposing or wood-decaying fungi, although genes of ligninolytic enzymes and their expression have now been detected (Chen *et al.* 2001, Chen *et al.* 2003). Litter-decomposing fungi and mycorrhizal fungi co-exist and interact in soils.

There are, of course, overlapping habits in the three main eco-physiological groups of fungi. Some wood-decayers (e.g. *Hypholoma* spp.) are also capable of colonizing soil from bases such as wood debris, while other LDF grow on straw (e.g. *Stropharia rugosoannulata*; Fig. 1.3 and 1.4), which is usually only favored by wood-decaying fungi. Finally, there is an indication that some mycorrhizal fungi, such as *Paxillus involutus*, could be facultative mycorrhiza formers that switch between a saprotrophic and symbiotic habit and being thus able to degrade lignin to some extent (Haselwandter *et al.* 1990).

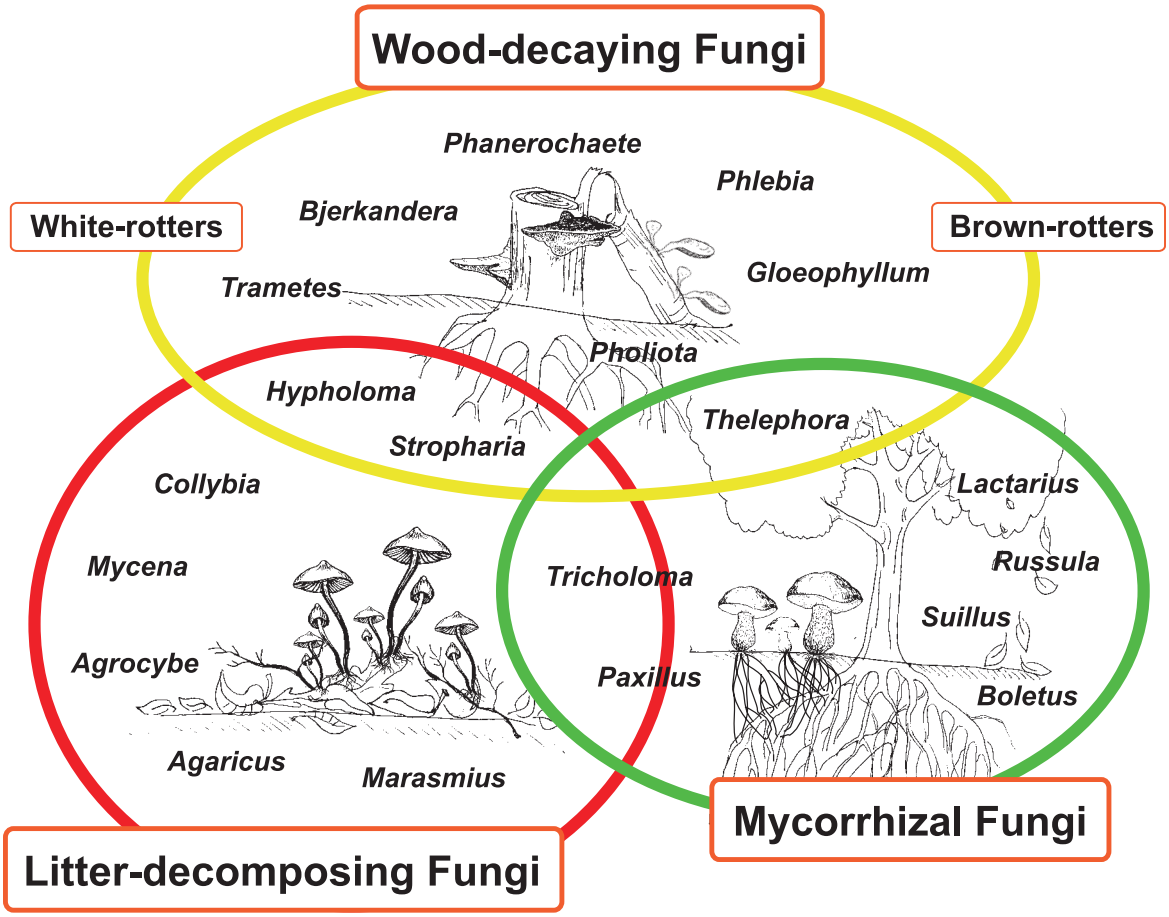


Figure 1.1: Ecophysiological division of basidiomycetous fungi into three partially overlapping groups according to their habitat and lifestyle (Steffen and Hofrichter).

Figure 1.2: Fruiting bodies of *Stropharia coronilla* (TM 47-1) grown under laboratory conditions on hemp stem residues (photo Kari Steffen).

Figure 1.3: (see text 1.4)

Figure 1.4: Stropharia rugosoannulata G (DSM 11373), a yellowish capped variant, in a young (upper picture) and mature (lower picture) state of fructification (photos Kari Steffen). The mycelium was grown on oat-straw, inoculated in June and left over winter (in Southern Finland) until the summer of the following year when fructification occurred in three waves at two week intervals.

Figure 1.5: Fruiting bodies of *Agrocybe praecox* on a leaf-litter pile in the Central Park of Helsinki, Finland (photo Kari Steffen).

Figure 1.6: *Collybia dryophila* fruiting bodies in a Southern Finnish forest in late summer (photo Kari Steffen).

In culture, LDF can remain viable for weeks or months and in nature even for decades (Watkinson *et al.* 2000). The mycelial growth of basidiomycetous LDF is initiated as a homokaryotic mycelium that arises following germination of the basidiospore. The main growth, or vegetative phase, occurs as a dikaryotic mycelium after fusion of two compatible homokaryotic mycelia (Rajarathnam *et al.* 1998). LDF display growth patterns in soil-litter that often involves connective mycelial growth that links one substrate source to another through fungal mycelial cords (rhizomorphs; Pugh 1974) consisting of hundreds of closely aggregated hyphae. Mycelial fans are developed over fresh substrates from the mycelial cord. The mycelium itself is mostly hidden in and between the growth substrate expressing small fruiting primordia from time to time, which, under favorable conditions, eventually grow into fruiting bodies and thus enter the reproductive phase (Rajarathnam *et al.* 1998). As LDF grow into soil, fungal mycelium comes into contact with different lignocellulosic materials that constitute a major component of litter, of which cellulose and hemicellulose can be utilized as a carbon source while only lignin is attacked in a co-metabolic manner.

1.2 Lignin

Wood consists of cells or fibers comprising three major constituents: cellulose, hemicellulose, and lignin that are together referred to as lignocellulose. The plant cell wall consists of several layers (secondary wall or S-layers) each of which contains all these three major components though in different amounts (Kuhad *et al.* 1997). Lignin is found in all vascular plants, a major fraction being distributed throughout the secondary walls of woody cells and also in the middle lamella between the secondary cell walls (Eriksson *et al.* 1990). Though litter also contains lignin, it is likely that the structures of litter- and wood-derived lignins are somewhat different. Whilst cellulose and hemicellulose are the supporting components of plants, lignin provides the essential rigidity and durability, especially in trees. Lignin is a natural polymer with high molecular mass of up to 100 kDa or more (Kästner 2000b) and can make up 20-30% of the lignocellulose in trees (Argyropoulos and Menachem 1997, Kuhad *et al.* 1997) there being a slightly higher content in gymnosperms (softwoods) than angiosperms (hardwoods; Eriksson *et al.* 1990). It is the most abundant aromatic carbon form and, after cellulose, the second most abundant natural organic compound on earth. Lignin is deposited as an encrusting and protecting material on the cellulose/hemicellulose matrix, and it sets up a complex and acts as a kind of glue that cements the fibrous cell walls together.

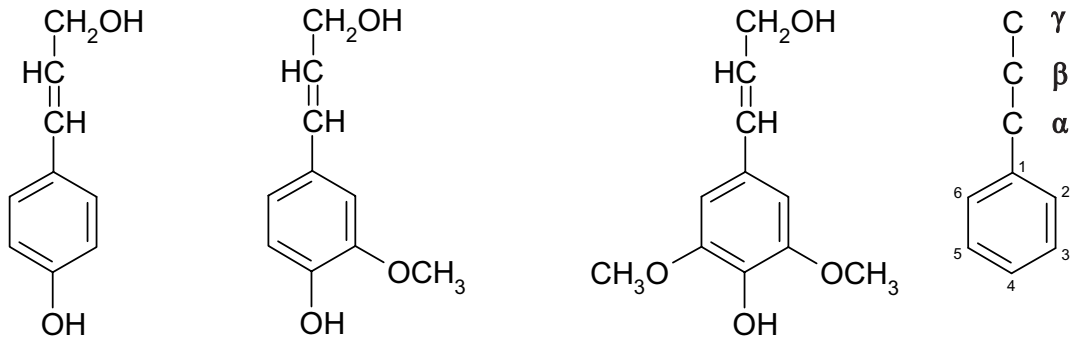


Figure 1.7: Precursors of lignin. From left to right: *p*-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol, and a model for the numeration of the carbon skeleton (obtained from Sjöström 1977).

Lignin is synthesized by higher plants from phenyl propanoid precursors by polymerization of radicals. Plant laccases are suggested to be involved in the lignification process (Monties and Fukushima 2001). Precursors are produced by plants from L-tyrosine and L-phenylalanine which are synthesized from carbohydrates by the shikimic acid metabolic pathway (Higuchi *et al.* 1977). They each consist of an aromatic ring with up to two methoxyl groups and a 3-carbon side chain designated as coumaryl, coniferyl-, and sinapyl alcohol (Fig. 1.7) and yielding the hydroxyphenol- (H-type), guaiacyl- (G-type), and syringyl subunits (S-type) of lignin structure respectively (Higuchi 1985). The ratio between syringyl and guaiacyl subgroups has been used as a comparative parameter between plant species (Monties and Fukushima 2001). Guaiacyl lignin is mainly found in softwoods (24-33% of dry biomass), guaiacyl-syringyl lignin (16-25%) in hardwoods and grasses contain guaiacyl-syringyl-*p*-hydroxyphenol lignin (< 20%; Sjöström 1977). The methylation of phenolic groups and thus the methoxyl content is recognized as an essential criterion for lignin characterization (Brown 1985). The O-methyl transferase is the key enzyme in determining the composition of lignin. Gymnosperm, angiosperm, and grass transferases catalyze different conversions leading to different precursors. This explains the occurrence of different types of lignin and relates the O-methyl transferases to the evolution of lignin.

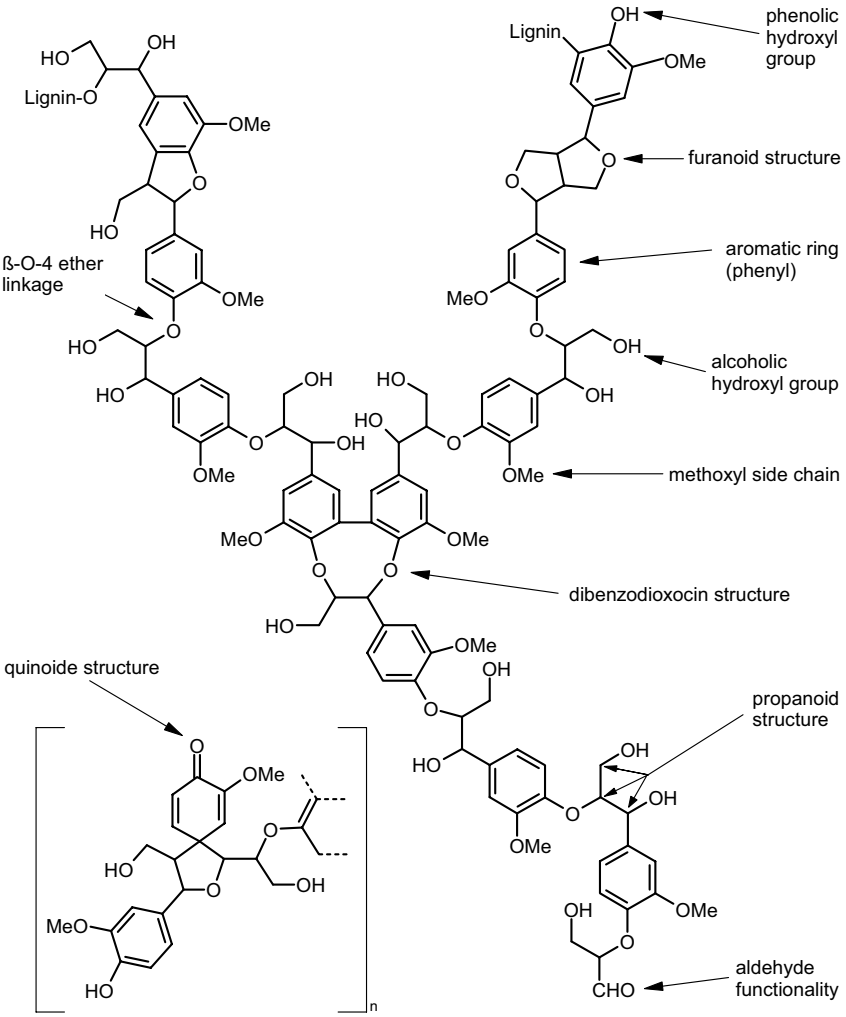


Figure 1.8: Lignin model after Brunow and coworkers (Brunow 2001) including a structure called dibenzodioxocin (Karhunen *et al.* 1995a, b).

The final step in lignin biosynthesis is brought about by peroxidase mediated dehydrogenation of the phenyl propanoid precursors producing phenoxyl radicals which yields a large, heterogeneous, and highly cross-linked polymer (Fig. 1.8; Eriksson *et al.* 1990). The phenyl propanoid units are linked together through a variety of bonds, e.g. aryl-ether, aryl-aryl, and carbon-carbon bonds (Adler 1977). Lignin differs from other natural polymers in that it has no single repeating bond (Brown 1985). The heterogeneity of this structure has been demonstrated through findings of unusual structures such as the dibenzodioxocin (Fig. 1.8) discovered recently by Brunow and coworkers (Karhunen *et al.* 1995a, Brunow 2001). Due to this unique structure, lignin is highly resistant and forms a barrier to microbial attack and degradation of wood. In general, only white-rot fungi are considered to be efficient degraders of lignin (Kirk and Farrell 1987, Griffin 1994; see section 1.4).

Synthetic lignins or dehydrogenation polymers (DHPs) were introduced in the 1970's (Haider and Trojanowski 1975, Kirk *et al.* 1975) and are widely used in biodegradation studies (e.g. Hatakka *et al.* 1983, Wood and Leatham 1983, Trojanowski *et al.* 1984, Haselwandter *et al.* 1990, Reid 1991, Hofrichter *et al.* 1999c, Tuomela *et al.* 2001, Tuomela *et al.* 2002 and more). They are accepted as generally the best available model compounds for the use in many types of experiments (Buswell and Odier 1987, Eriksson *et al.* 1990). DHPs can be produced by polymerizing phenyl propanoid precursors under laboratory conditions. Usually coniferyl alcohol is used (e. g. Wood and Leatham 1983, Hofrichter *et al.* 1999c) and the resulting guaiacyl (G-type) lignin is more recalcitrant than other natural or synthetic lignin types (Faix *et al.* 1985). Nevertheless most of the known structures of lignin are found in DHP. The use of ^{14}C-labeled precursors opens the possibilities to produce ^{14}C-labeled synthetic lignins which can be used for degradation and mineralization studies (Haider and Trojanowski 1975, Kirk *et al.* 1975). DHP can thus contain different carbon labels. As such the entire aromatic ring, the carbon side chain (commonly C-β), the methoxyl group(s) or all carbons can be labeled (uniformly labeled). In the synthesis of DHP labeled and/or non-labeled precursors are incubated together with horse-radish peroxidase and H_2O_2, with chelated Mn^{3+}, or with laccase (Trojanowski *et al.* 1984, Monties and Fukushima 2001).

1.3 Degradation of lignin by basidiomycetous fungi: the ligninolytic enzyme system

A set of enzymes preferentially produced by wood-rotting basidiomycetes are responsible for the degradation of lignin in nature. Though other microorganisms have been shown to degrade lignin to some extent (Hatakka 2001), white-rot fungi are by far the best lignin degraders. They degrade wood by a simultaneous attack of lignin and cellulose/hemicellulose or selectively degrade far more lignin than polysaccharides (Eriksson *et al.* 1990, Kuhad *et al.* 1997). The model fungus for lignin degradation is *Phanerochaete chrysosporium* (Kirk 1984) but recently certain other fungi have been thoroughly studied (*Ceriporiopsis subvermispora*, *Phlebia radiata*, *Pleurotus eryngii*; Lundell 1993, Martinez *et al.* 1994, Akthar *et al.* 1997, Hatakka 2001). Results obtained using *P. chrysosporium* identified two extracellular peroxidases that were found to be the most important enzymes involved in the degradation process. These enzymes are lignin peroxidase (LiP) and manganese peroxidase (MnP; Table 1.1). Laccase was found much earlier than MnP or LiP and its activity was assumed to be involved in lignin degradation (Leonowicz and Trojanowski 1965 referred in Leonowicz *et al.* 1999). More enzymes are expected to be found such as versatile or hybrid peroxidases, which are modifications of MnP or LiP (Mester and Field 1998, Ruiz-Duenas

et al. 2001). In addition to these, H_2O_2 producing enzymes are excreted e.g. glyoxal oxidase (GLOX) or aryl alcohol oxidase (AAO; Table 1.1). Furthermore, the ability to reduce quinones is brought about by the cellobiose:quinone oxidoreductase (CBQ). White-rot fungi excrete ligninolytic enzymes during their growth in liquid cultures, but especially on lignocellulose material. Various ligninolytic fungi produce different combinations of these enzymes, but not all of these three major enzymes are needed to degrade lignin, suggesting that there is more than one ecologically successful strategy for the degradation of lignin (Hatakka 1994, 2001). The degradation of lignin is believed to be non-specific regarding the enzyme reactions involved, which is based on the fact that radicals are involved in the attack of the aromatic moieties. Degradation can result in the formation of water soluble compounds and in mineralization, i.e. in the formation of CO_2 (Hatakka and Uusi-Rauva 1983, Dorado *et al.* 1999).

To date, little is known about the degradation of lignin by basidiomycetous fungi other than white-rot fungi. Some mycorrhizal fungi were shown to degrade lignin but the efficiency falls far behind that of white-rot fungi (Trojanowski *et al.* 1984, Haselwandter *et al.* 1990). Simple degradation studies in litter bags, on the other hand, demonstrated significant lignin loss brought about by the litter-decomposing fungus *Marasmius androsaceus* (Cox *et al.* 2001). The most studied litter-decomposing fungus, *Agaricus bisporus*, was examined in more detailed studies and its ligninolytic capabilities were shown by the mineralization of ^{14}C-synthetic lignins (Wood and Leatham 1983, Durrant *et al.* 1991). As a number of reports have been published (including results from this work, article I) confirming the production of MnP by these fungi, evidence of their ligninolytic capabilities is expected to be found in the near future.

Overall lignin degradation by white-rot fungi is believed to be a co-metabolic process requiring a carbon source other than lignin, e.g. parts of the cellulose/hemicellulose of wood are consumed. So far, no organism has been found to use macromolecular lignin as a sole carbon source (Kirk and Farrell 1987, Hatakka 2001).

Table 1.1: Extracellular ligninolytic enzymes involved in lignin degradation (modified after Hatakka 2001).

Enzyme	Cofactor	Substrate, mediator	Main effect or reaction
Lignin peroxidase, LiP	H_2O_2	Veratryl alcohol	Aromatic ring oxidized to cation radical
Manganese peroxidase, MnP	H_2O_2	Mn^{2+}, organic acids as chelators, thiols, unsaturated lipids	Mn^{2+} oxidized to Mn^{3+}; further oxidation of phenolic compounds to phenoxyl radicals
Versatile peroxidases (hybrid peroxidases)	H_2O_2	Same or similar compounds as LiP and MnP	Same effect on aromatic and phenolic compounds as LiP and MnP
Laccase, Lacc	O_2	As mediators hydroxy-benzotriazole, ABTS	Phenols are oxidized to phenoxyl radicals; mediator radicals
Glyoxal oxidase, GLOX		Glyoxal, methyl glyoxal	Glyoxal oxidized to glyoxylic acid; H_2O_2 production
Aryl alcohol oxidase, AAO		Aromatic alcohols (anisyl, veratryl alcohol)	O_2 reduced to H_2O_2
Cellobiose:quinone 1-oxidoreductase, CBQ		Cellobiose	Reduction of *o*- and *p*-quinones

1.3.1 Manganese peroxidase

Manganese peroxidase (MnP EC 1.11.1.13), which is exclusively produced by some basidiomycetes (to date 60 are known), was first discovered shortly after LiP from *Phanerochaete chrysosporium* by Kuwahara *et al.* (1984) and described by Glenn and Gold (1985). MnP is an extracellular heme containing peroxidase with a requirement for Mn^{2+} as its reducing substrate. Manganese alone can also regulate the production of MnP in *Phlebia radiata* (Moilanen *et al.* 1996). MnP oxidizes Mn^{2+} to Mn^{3+}, which then in turn oxidizes phenolic structures to phenoxyl radicals (Gold *et al.* 1989). The Mn^{3+} formed is highly reactive and complexes with chelating organic acids such as oxalate or malate (Cui and Dolphin 1990, Kishi *et al.* 1994), which are produced by the fungus (Galkin *et al.* 1998, Hofrichter *et al.* 1999b, Mäkelä *et al.* 2002). With the help of these chelators, Mn^{3+}-ions are stabilized and can diffuse into materials such as wood. The redox potential of the MnP-Mn system is lower than that of LiP and preferably oxidizes phenolic substrates (Vares 1996). The phenoxyl radicals produced can further react with the eventual release of CO_2. MnP is one of the most common lignin degrading peroxidases produced by the majority of wood-decaying fungi and by many litter-decomposing fungi (Hofrichter 2002). This extracellular enzyme is usually 40-50 kDa (max. 38-62 kDa) in mass and its p*I* varies between acidic 3 and neutral 7 being usually around 3-4 (Hofrichter 2002). A good example of a typical MnP from a white-rot fungus is the MnP2 of *Nematoloma frowardii* with a MW of 44 kDa and a p*I* of 3.2 (Schneegass *et al.* 1997).

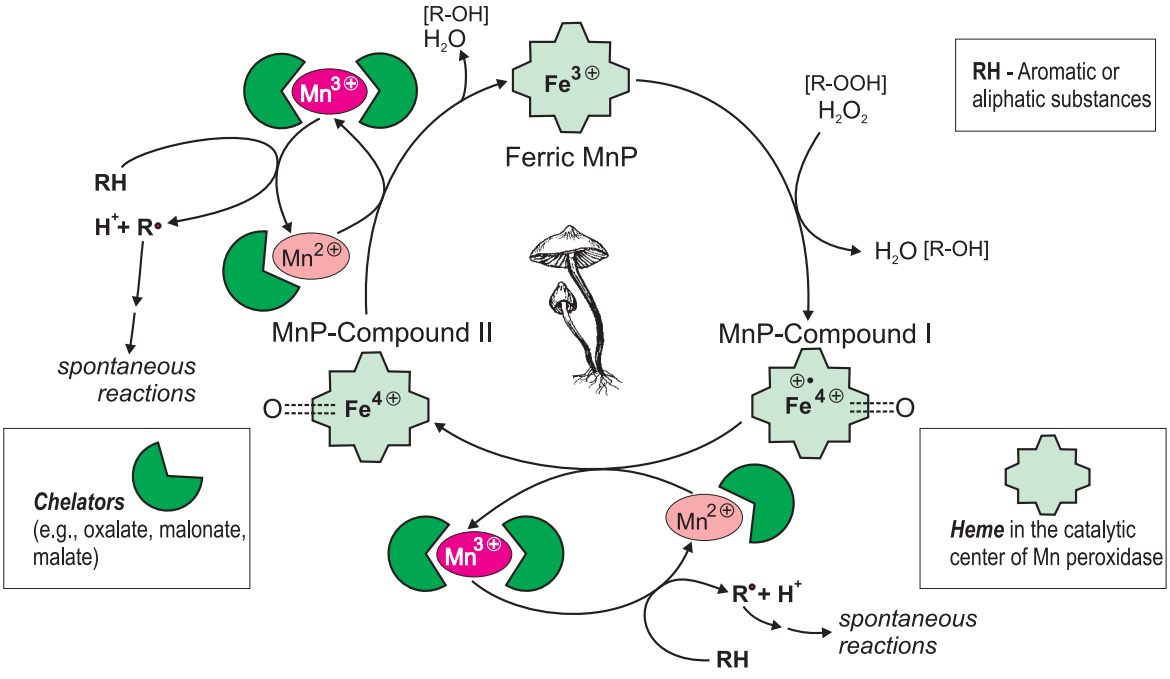


Figure 1.9: The catalytic cycle of manganese peroxidase (MnP; see text 1.3.1 for details; after Wariishi *et al.* 1988, Wariishi *et al.* 1992, Kuan *et al.* 1993, Kishi *et al.* 1994, Kirk and Cullen 1998).

The catalytic cycle of MnP (Fig. 1.9) starts with the binding of H_2O_2 to the reactive ferric enzyme. H_2O_2 is produced by the fungus using other enzymes (GLOX, AAO) or by MnP in the oxidation of glutathione (GSH), NADPH, and dihydroxy malic acid (Paszczynski *et al.* 1985). The cleavage of the oxygen-oxygen bond requires the transfer of two electrons from the heme, forming the MnP compound I. This activated state of the heme center is able to form a radical complex and to remove an electron from the Mn^{2+}-donor resulting in the

formation of a highly reactive Mn^{3+}-ion. The so formed MnP-compound II is also able to oxidize a Mn^{2+}-ion (Kishi *et al.* 1994). This step closes the cycle and the input of one H_2O_2 results in the formation of two H_2O and two Mn^{3+} (chelated; Wariishi *et al.* 1992). This Mn^{3+} or chelated Mn^{3+} is in turn able to oxidize various monomeric and dimeric phenols, as well as carboxylic acids, thiols and unsaturated fatty acids forming radicals thereof (Hofrichter 2002). Forrester *et al.* (1988) even showed that suitably chelated Mn^{3+} was able to oxidize lignin model compounds in absence of the enzyme.

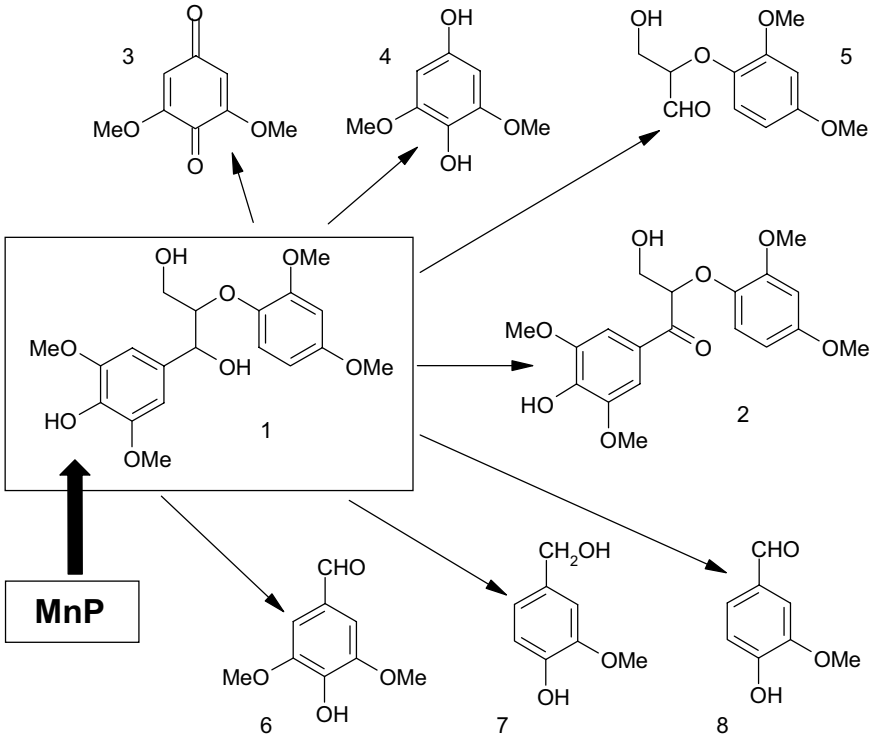


Figure 1.10: Compounds produced from the oxidation of a phenolic lignin model dimer (1) by MnP (obtained from Hofrichter 2002). (2) keto form of 1; (3) para-quinone; (4) dihydroxybenzene; (5) hydroxypropanal; (6) hydroxybenzaldehyde; (7) hydroxybenzyl alcohol and (8) benzaldehyde.

The catalytic cycle of MnP is very similar to that of LiP differing only in that compound II is readily reduced by Mn^{2+} to its native form (Wariishi *et al.* 1989). The phenoxyl radicals formed subsequently cleave Cα-Cβ (see Fig. 1.7) or alkyl-phenyl bonds causing depolymerization to smaller intermediates including quinones and hydroxylquinones (Kuhad *et al.* 1997). The oxidation of a phenolic lignin model by MnP demonstrates that the formation of different monomers is possible (Fig. 1.10). Non-phenolic compounds can be oxidized by MnP only in the presence of oxygen and GSH or unsaturated fatty acids (Fig. 1.11).

Purified or crude MnP has been used in cell-free systems (*in vitro*) and shown to oxidize not only lignin (Hofrichter *et al.* 1999a, Hofrichter *et al.* 2001), chlorolignins (Lackner *et al.* 1991), and synthetic lignin compounds (Wariishi *et al.* 1991, Hofrichter *et al.* 1999c), but also HS from brown coal (Hofrichter and Fritsche 1997b, Ziegenhagen and Hofrichter 1998), and HS synthesized from catechol (Hofrichter *et al.* 1998b), nylon (Deguchi *et al.* 1998), PAH (Bogan and Lamar 1996, Bogan *et al.* 1996, Sack *et al.* 1997b, Günther *et al.* 1998), chlorophenols (Hofrichter *et al.* 1998a), nitroaromatic compounds (Valli *et al.* 1992, Hofrichter *et al.* 1998a, Scheibner and Hofrichter 1998, Van Aken *et al.* 1999, Van Aken *et al.* 2000) and arsenic-containing warfare agents (Fritsche *et al.* 2000).

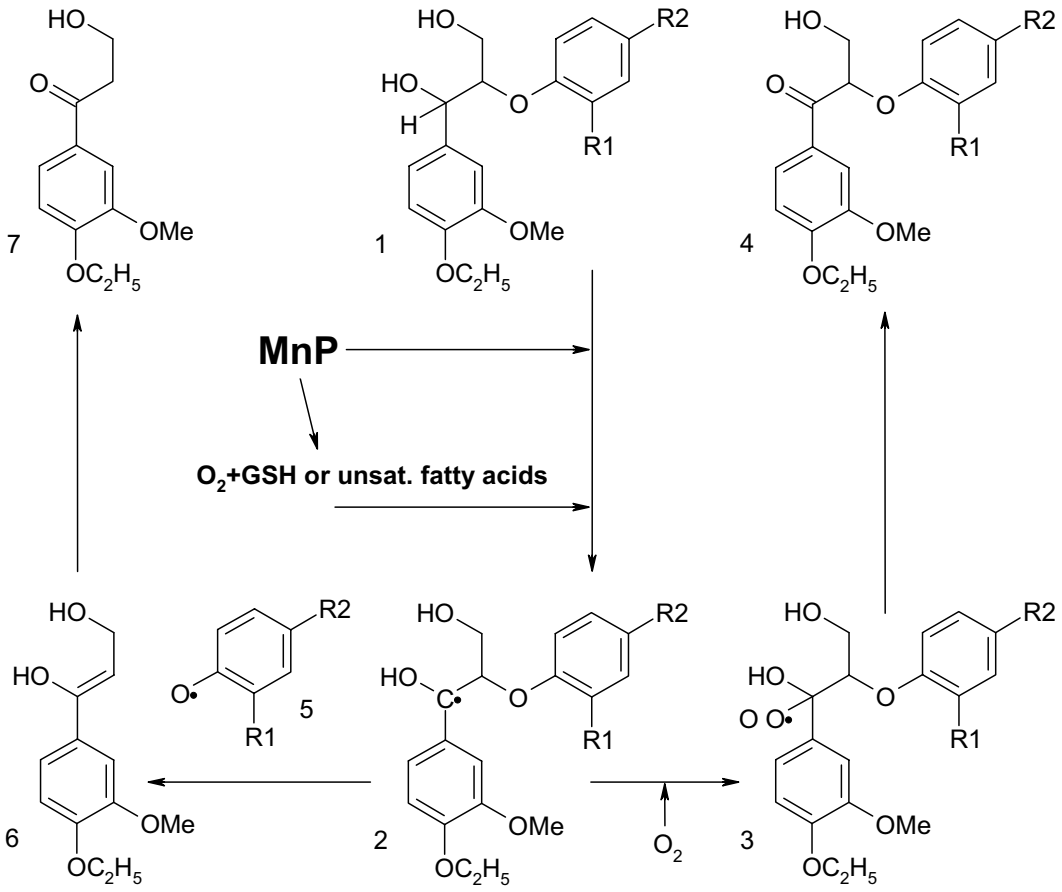


Figure 1.11: Proposed scheme for the oxidation of a non-phenolic *β*-O-4 lignin model dimer (1) by MnP in the presence of glutathione (GSH) or unsaturated fatty acids (obtained from Hofrichter 2002). (2) benzyl radical; (3) peroxyl radical; (4) keto form; (5) phenoxyl radical; (6) hydroxypropane derivative and (7) keto form of 6.

To date, there is limited knowledge on MnP production in basidiomycetes other than white-rot. An MnP gene was recently detected in a mycorrhizal fungus (*Cortinarius rotundisporus*) but no activity was detected (Chen *et al.* 2001). Some reports on the production of MnP by LDF are available. The best known litter-decomposing fungus, *Agaricus bisporus*, produces MnP (Bonnen *et al.* 1994, Lankinen *et al.* 2001) as well as the coprophilic species *Paneolus sphinctrinus* (Heinzkill *et al.* 1998) and the oak leave degrading fungus *Marasmius quercophilus* (Tagger *et al.* 1998). Thus new information on the production of MnP by LDF has been added to the literature as a result of this work (I, II and III).

1.3.2 Laccase

Laccase (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) is a copper-containing phenol oxidase which does not require H_2O_2 but uses molecular oxygen (Thurston 1994). The enzyme is produced by higher plants and fungi, but is also found in molds, black yeasts, and some bacteria (Bollag and Leonowicz 1984, Thurston 1994, Yaropolov *et al.* 1994, Mayer and Staples 2002, Claus 2003). As in the case of MnP laccases prefer lignin compounds with a free phenolic group and likewise form phenoxyl radicals. Laccases reduce O_2 to H_2O in oxidizing phenolic substrates via a one-electron reaction creating a free

radical, which can be likened to a carbon-centered cation radical formed in a MnP reaction (Kersten *et al.* 1990). However, in the presence of suitable mediators such as ABTS (2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate)) or HBT (hydroxybenzotriazole), laccase is able to oxidize certain non-phenolic compounds and veratryl alcohol (Bourbonnais and Paice 1990, Eggert *et al.* 1996, Call and Mücke 1997, Collins and Dobson 1997). Laccase is produced by most white-rot fungi (Hatakka 1994) but normally not by *Phanerochaete chrysosporium* (Kirk and Farrell 1987). The molecular mass for laccases of basidiomycetes varies between 50 and 70 kDa which is usually smaller than that of plant laccases (Thurston 1994, Yaropolov *et al.* 1994) and the acidic p*I* ranges between 3-4 (Hatakka 1994). It is also found to be involved in both the polymerization as well as in the degradation of lignin (Eriksson *et al.* 1990). For certain fungi, laccase might be essential for lignin degradation as shown for *Pycnoporus cinnabarinus* (Eggert *et al.* 1997), which is believed to use mediators such as 3-hydroxyanthrilate to oxidize non-phenolic substrates (Eggert *et al.* 1996).

Some LDF are known to produce laccase including the MnP forming species *Paneolus sphinctrinus*, *Marasmius quercophilus*, and *Agaricus bisporus* (Leontievsky *et al.* 1997, Heinzkill *et al.* 1998, Tagger *et al.* 1998, Dedeyan *et al.* 2000). Some publications indicate the production of phenol oxidases, most probably laccases, by *Coprinus* spp., *Lepista nuda*, and *Clitocybe nebularis* (Heinzkill *et al.* 1998, Soponsathien 1998, Morisaki *et al.* 2001).

1.3.3 Other ligninolytic enzymes

One of the best known ligninolytic enzymes is lignin peroxidase (ligninase; LiP; EC 1.11.1.14) which was discovered a little earlier than MnP (reviewed by Kirk and Farrell 1987, Kirk and Cullen 1998). This enzyme has been found in some wood-rotting species such as *Phanerochaete chrysosporium* (Glenn *et al.* 1983, Tien and Kirk 1983), *Phlebia radiata* (Niku-Paavola *et al.* 1988), and *Trametes versicolor* (Dodson *et al.* 1987). LiP is an extracellular heme containing peroxidase which is dependent on H_2O_2, and has an unusually high redox potential and low optimum pH (Gold and Alic 1993), typically showing little specificity towards substrates and degrades a variety of lignin related and other compounds (Barr and Aust 1994). It preferably oxidizes methoxylated aromatic rings without a free phenolic group, such as the model compound dimethoxybenzene (Kersten *et al.* 1990). Thus the cleavage of Cα-Cβ bonds are catalyzed preferentially in dimeric non-phenolic lignin model compounds (Kuhad *et al.* 1997). LiP oxidizes target substrates by two one-electron oxidation steps with intermediate cation radical formation. Several studies have indicated the involvement of LiP in the degradation of xenobiotics (Haemmerli *et al.* 1986, Hammel *et al.* 1986, Sanglard *et al.* 1986, Hammel and Tardone 1988, Male *et al.* 1995). So far, no LiP has been found in litter-decomposing fungi.

White-rot fungi possess a variety of different oxidative enzymes, which are capable of generating H_2O_2, required by peroxidases, through the oxidation of different substrates. Glyoxal oxidase (GLOX; EC 1.2.3.5) and aryl alcohol oxidase (AAO; EC 1.1.3.7) are both extracellular enzymes first described by Kersten and Kirk (1987), Waldner *et al.* (1988), and Muheim *et al.* (1990). They use either glyoxal or aromatic alcohols as their substrate (Hatakka 2001). In particular AAO is involved in the selective degradation of lignin by *Pleurotus* species (Martinez *et al.* 1994). Furthermore, intracellular enzymes that produce H_2O_2, such as glucose oxidase and pyranose oxidase, can be formed by white-rot fungi (Volc *et al.* 2001). Enzymes which produce H_2O_2 have as yet not been described in LDF.

1.4 Humic substances

Humic substances (HS) are natural non-living organic materials widely distributed in soils, as well as aquatic environments, including natural waters, marine and lake sediments, and are incorporated into peat and brown-coals (and other deposits) that represent a major part of the soil organic matter (SOM; Stevenson 1994, Fakoussa and Hofrichter 1999, Frimmel 2001, Senesi and Loffredo 2001; Fig. 1.12). These yellow and brownish colored, colloidal substances may constitute up to 30% of soil (Kästner 2000b) and comprise a heterogeneous mixture of relatively high molecular mass compounds (MW between 0.5 – 20 kDa, ocasionally up to 100 kDa) with aliphatic and aromatic structures (Stevenson 1994, Kästner and Hofrichter 2001). HS are commonly classified into humin, humic acids (HAs), and fulvic acids (FAs) by their solubility in alkali and acid (Senesi and Loffredo 2001). They are formed during the humification process when molecules originating from fragments of decaying biomass are coupled. Thus phenols and amino acids are oxidized and polymerized to HS. This formation includes a random condensation and polymerization of free radicals released through autolytic oxidative enzymes from dead plant and microbial cells, as well as extracellular enzymes of bacterial and fungal origin. Lignin and its transformation products are important parent materials providing HS with aromatic building blocks (Stevenson 1994, Shevchenko and Bailey 1996).

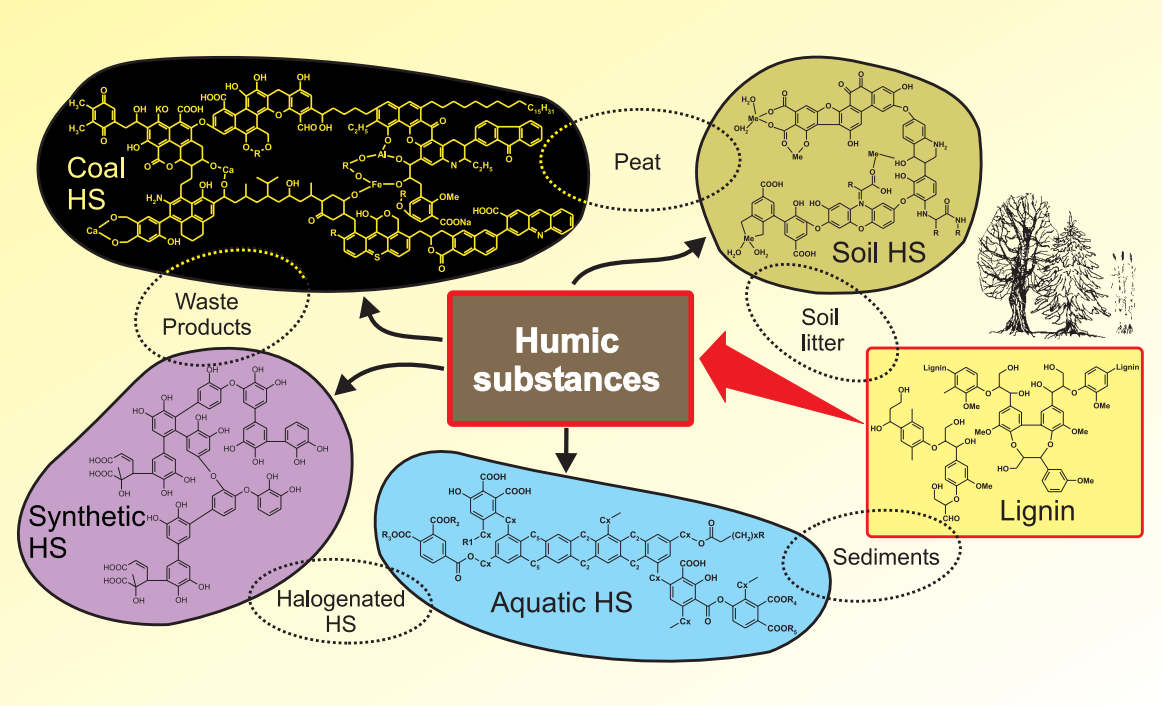


Figure 1.12: Models of humic acids (HAs) in the environment and their parent source of lignin (Figure courtesy of M. Hofrichter).

HS are essential for soil fertility and act as a source of growth promoting substrates for plants and other soil organisms (Kästner 2000b). They have an impact on the water holding capacity, ion exchange capacity, water and gas permeability, and degree of soil particle aggregation. They are also efficient sorbents, both for polar cationic organic molecules and lipophilic substrates (Kästner 2000b). Additionally HS contain large amounts of Fe in ferroheme or porphyrine structures derived from mainly microbial enzymes such as cytochromes or peroxidases (Stevenson 1994, Kästner 2000b).

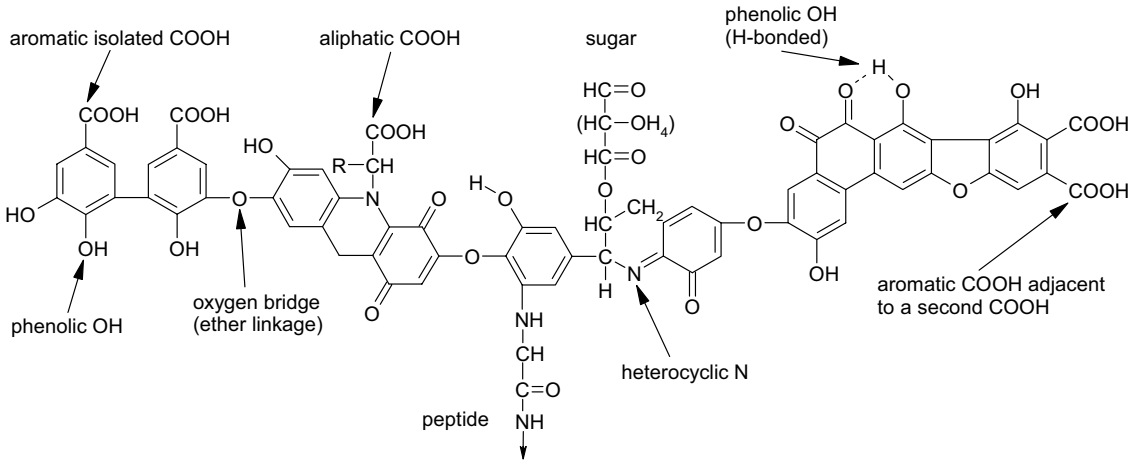


Figure 1.13: A model for a soil-humic acid (adapted from Stevenson 1994).

1.4.1 Occurrence and structure of humic substances

Both humin and humic acids (HAs) represent high molecular mass aromatic moieties of the SOM. Humin comprises the non NaOH dispersible fraction of SOM and is composed of FAs and HAs in addition to non-soluble plant and microbial constituents, such as undecomposed cellulose, ligniferrous materials, microbial cell walls, and some charcoal (Senesi and Loffredo 2001). The molecular mass is assumed to be similar to that of HAs. These dark-brown high molecular mass HAs can be extracted as sodium salts (Na humates) with NaOH from soil, litter, or low-rank coal (= lignite, brown coal; Hofrichter and Fakoussa 2001, Klein *et al.* 2001) and precipitate at pH 2 (Senesi and Loffredo 2001). Their molecular masses ranges from 1.4 to 100 kDa (Paul and Clark 1989, Kästner 2000b). HAs contain aromatic rings, nitrogen in cyclic forms and in peptide chains (Fig. 1.13) and are formed by the polycondensation of similar but non-identical constituents so that no two humic substances are identical in composition (Kästner 2000b). Over time, several hypothesis of humification and thus of the structure of HS have been developed including the lignin-protein, sugar-amine, and polyphenol theory (Shevchenko and Bailey 1996, Senesi and Loffredo 2001). The names of these hypotheses clearly imply that different carbon substances are involved in the formation of HS. Thus reactions such as the demethylation of lignin and the formation of hydroquinones as well as the Maillard reaction (Fig. 1.14) are involved in the formation of larger building blocks of HS. These polyaromatic and non-polyaromatic building blocks are held together by ether linkages, cyclic nitrogen, and hydrogen bonding (Fig. 1.13), and contain about 57% carbon plus 4% nitrogen (Paul and Clark 1989). The functional groups are primarily carboxyl groups, phenolic hydroxyl groups, alcoholic hydroxyl, and small amounts of ketonic oxygen (Paul and Clark 1989).

Besides natural HS, a large variety of HS can be synthesized and are often used as model compounds in degradation or polymerization studies (Kästner and Hofrichter 2001). Auto-oxidation processes or enzymatic oxidation through laccases or peroxidases are used to initiate the condensation of macromolecules with properties of HS. Phenols are the main compounds transformed to radicals, and they tend to form macromolecules mainly coupled by carbon-carbon or ether bonds. Preparation of these compounds is not difficult and radioactive labeled compounds can easily be incorporated (see also Fig. 3.2). Nevertheless, synthetic HS are humic like substances but not necessarily comparable to natural HS.

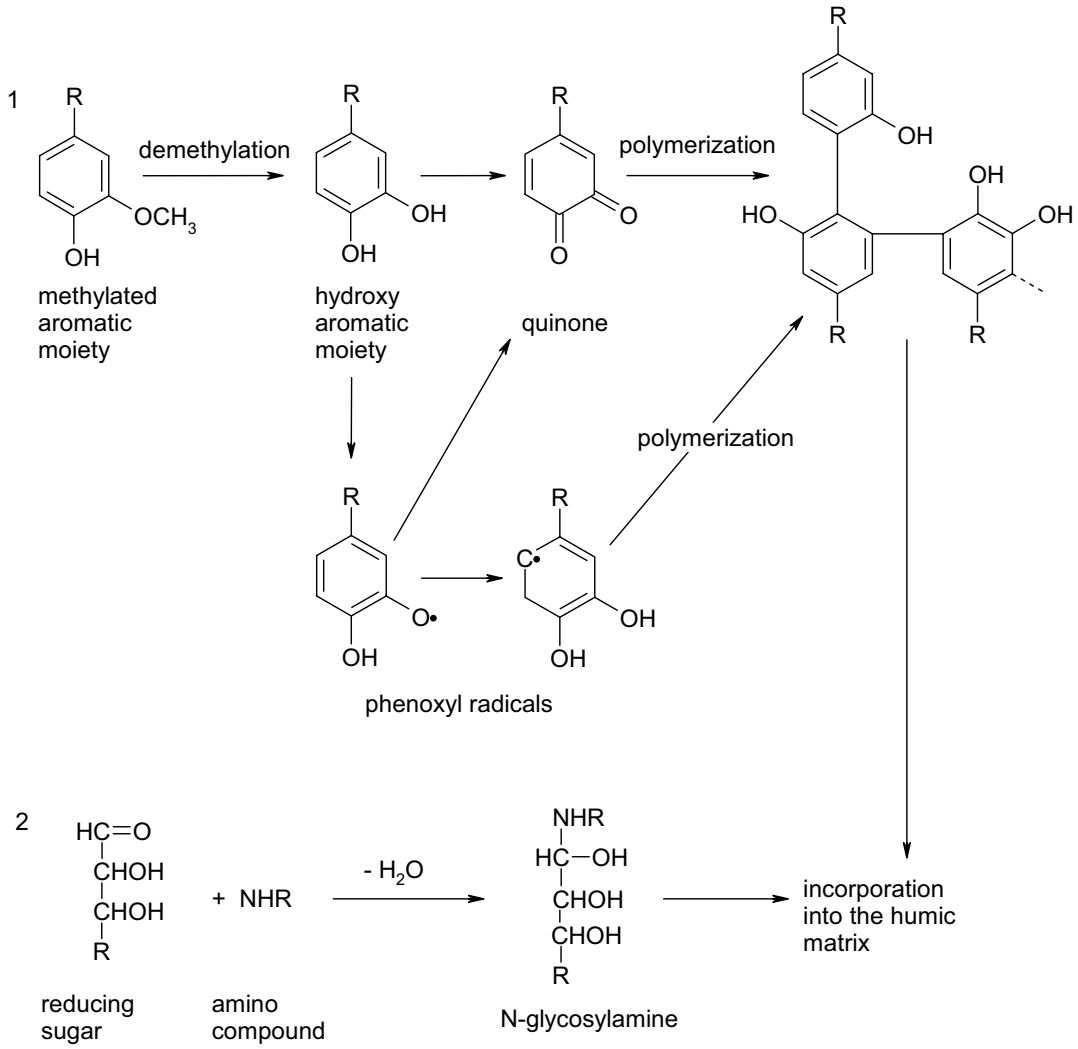


Figure 1.14: Reactions involved in the formation of humic substances (HS). (1) Demethylation of lignin, formation of phenoxyl radicals and quinones, and polymerization to larger building blocks (modified after Field 2001); (2) Maillard reaction to incorporate sugars and amino compounds into HS (modified after Stevenson 1994).

1.4.2 Degradation of humic substances

Different microorganisms are able to degrade HS and HAs to some extent. Limitations are the aging effect on HS and their large molecular size (Kästner and Hofrichter 2001). Thus, large molecules (> 0.6 kDa) or aggregates are not expected to be taken up by microbial cells or localize close to active sites of enzymes. It is therefore more likely that chemically or enzymatically generated radical reactions will take place causing the degradation of these molecules (Kästner and Hofrichter 2001). In fact, the same types of enzymes, which can polymerize HS under certain conditions, are also responsible for their degradation. Degradation can be monitored using several photometric, gravimetric, and ^{14}C-methods (Senesi and Loffredo 2001; see also methods used in III). An easy and accurate method is to use ^{14}C-labeled model compounds and to detect $^{14}CO_2$ (Blondeau 1989, Hofrichter *et al.* 1998b, Wunderwald *et al.* 2000). It should be stressed that synthetic HS or HAs differ from those present in nature and the mineralization of synthetic compounds does not reflect the entire process in nature. The application of synthetic HS is however one of the best methods currently available to explain the mineralization of HS. Natural derived HAs can

additionally be used in degradation studies, e.g. when they are extracted using the IHSS (International Humic Substance Society) methodology (Senesi and Loffredo 2001), and analyzed by HPSEC (high pressure size exclusion chromatography).

Certain bacteria are able to degrade or decolorize HAs, including actinomycetes such as *Streptomyces* spp., or other bacteria, e.g. *Pseudomonas* spp. (Kästner and Hofrichter 2001). The decolorization is brought about either by cell surface enzymes, where bacteria are able to bind to HS, or by extracellular non-selective enzymes (Adhi *et al.* 1989). In most cases degradation occurs co-metabolically and hydrolysable carbohydrates often serve as carbon sources (Gramss *et al.* 1999c). Several molds are also reported to degrade HAs and some were found to produce phenol oxidases (e.g. *Chaetomium* sp., *Fusarium* spp., *Penicillium* spp.; Rodriguez *et al.* 1996, Chefetz *et al.* 1998, Regalado *et al.* 1999). Yet the most efficient degraders of HS are found among the basidiomycetes, especially among the white-rot fungi, which has been realized already in the 1960's (Hurst *et al.* 1962).

Microbial degradation of HS and in particular HAs is of utmost importance to drive humus turn-over that is essential in maintaining the global carbon cycle (Haider 1998). HA degradation has been studied by several authors using ligninolytic white-rot fungi (Hurst *et al.* 1962, Blondeau 1989, Dehorter and Blondeau 1992, Dehorter *et al.* 1992, Hofrichter and Fritsche 1997a, Willmann and Fakoussa 1997a). The reason for using white-rot fungi lies in their ability to efficiently degrade lignin, which is one of the main parent materials of HAs (Shevchenko and Bailey 1996). White-rot fungi such as *Trametes versicolor* and *Phanerochaete chrysosporium* were successfully used to degrade HAs in a co-metabolical process (Blondeau 1989, Dehorter and Blondeau 1992), but it remains doubtful whether they are involved to a large extent in HA degradation in nature because they are mainly restricted to wood and do not compete well in soil environments (Kästner and Hofrichter 2001). Nevertheless, they degrade HAs and form lower molecular mass FAs and CO_2. Extracellular peroxidase activities were found to correlate with HA degradation. HAs were shown to elicit the expression of lignin degrading peroxidases, which are known to play an important role in the degradation of HAs (Haider and Martin 1988, Dehorter and Blondeau 1992). However, HAs together with FAs can, under certain conditions, have an inhibitory effect on peroxidases and laccases (Sarkar and Bollag 1987, Ralph and Catcheside 1994). In a comparison between *P. chrysosporium* and *T. versicolor* the latter was found to be more effective at degrading HAs (Dehorter and Blondeau 1992) and it was suggested that MnP had a more important role than LiP in the degradation process. *In vitro* studies confirmed that MnP was able to depolymerize and mineralize HAs (Dehorter and Blondeau 1993, Hofrichter *et al.* 1998b, Wunderwald *et al.* 2000) and thus underlines the importance of this enzyme.

The main product of HA disintegration are FAs. These can be extracted by NaOH but are still acid soluble at pH 2 and have smaller molecular mass than HAs ranging from 0.5 – 2.1 kDa (Kästner 2000b; 1.0 – 30 kDa; Paul and Clark 1989). FAs are composed of a series of highly oxidized aromatic rings with a large number of side chains (Paul and Clark 1989) containing benzene carboxylic acids and phenolic acids (Fig. 1.15). They are typically held together by hydrogen and ionic bonding, as well as van der Waals' forces.

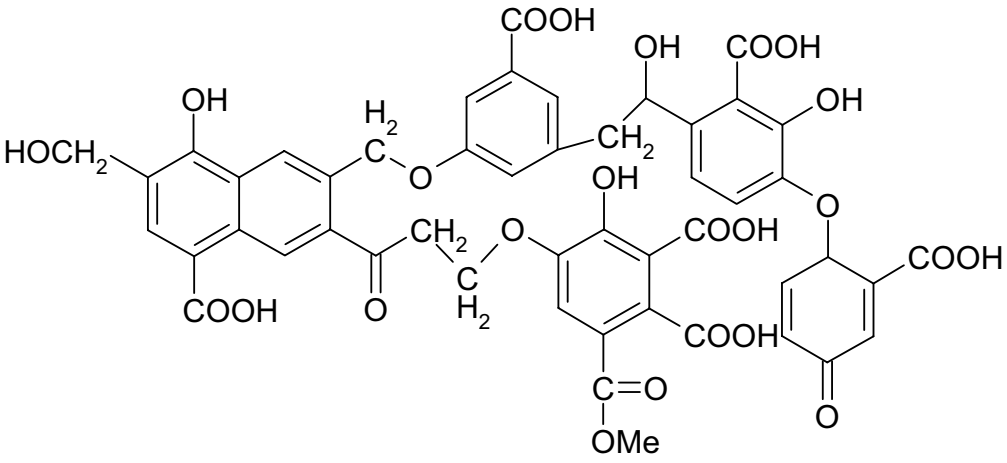


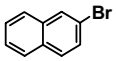
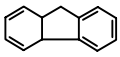
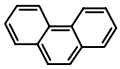
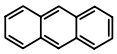
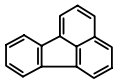
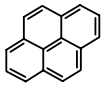
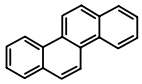
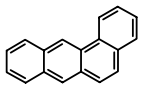
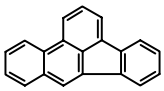
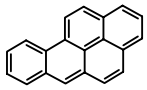
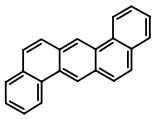
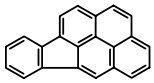
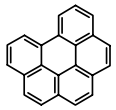
Figure 1.15 : Proposed structure of a fulvic acid (FA; after Langford *et al.* 1983).

1.5 Polycyclic aromatic hydrocarbons (PAH)

PAH are ubiquitous environmental pollutants derived from various man made and natural resources (Wilson and Jones 1993, Kästner 2000a). They are formed during pyrolysis and incomplete combustion of biological material and organic compounds (Blumer 1976). PAH are present at various concentrations in coal tar, petroleum, and oil based fuels (Ramdahl 1985). Thus they can be found in soils from gas works (Saraswathy and Hallberg 2002), carbochemical plants, power plants using fossil fuels, and traditionally from coke production sites (Wilson and Jones 1993). Filling stations and other facilities handling fossil fuel, e.g. oil storage facilities or loading stations especially in harbors, are susceptible to spillage and thus the soil or aquatic area can be contaminated. PAH are also formed "naturally" during forest fires or through volcanic activities.

Several hundred PAH compounds are known (Kästner 2000a). They consist of two or more fused benzene rings in linear, angular, or cluster arrangements (Blumer 1976). By definition they contain only carbon and hydrogen, although in a broader sense heterocyclic PAH containing N, S and O atoms are also considered to be PAH (Kästner 2000a). Because of their hydrophobic properties they tend to adsorb to surfaces in aquatic environments (Cerniglia and Heitkamp 1989) or to dust and soil particles, which can be evenly distributed through the air (Kästner 2000a). PAH water solubility and thus bioavailability decreases with an increase in molecular mass (Wilson and Jones 1993). Large PAH with four or more rings are not only poorly bioavailable and recalcitrant to microbial degradation, but are also more carcinogenic and mutagenic than smaller counterparts (Cerniglia and Heitkamp 1989, Cerniglia 1992, 1993). Due to their genotoxicity, 16 PAH were listed by the U.S. Environmental Protection Agency (EPA) as priority pollutants which should be monitored in aquatic and terrestrial ecosystems (Table 1.2). The ubiquitous occurrence of these carcinogenic PAH represent an obvious health risk and public concern as to their fate and in the removal of these compounds from the environment is on the increase.

Table 1.2: 16 EPA-PAH in order of appearance when detected with a gas chromatograph (GC) and a PTE column (poly diphenyl dimethyl siloxane; EPA-PAH as sold by Supleco, Belfonte, Pa.)

PAH		Characteristics					
		MW, g/mol (1)	Water solubility mg/l (1, 4)	Ionization potential eV (1, 2)	Relative cancer potency (1)	Toxicity equivalent factor (1)	Genotoxicity (3)
Naphthalene		128.19	31.0	8.12 ± 0.02			-
Acenaphthylene		152.20	16.1	8.22 ± 0.04		0.001	
2-Bromo-naphthalene*		(208.09)					
Acenaphthene		152.21	3.80	7.68 ± 0.05		0.001	Ames
Fluorene		166.22	1.90	7.88 ± 0.05		0.001	-
Phenanthrene		178.23	4.57	7.90 8.03		0.001	-
Anthracene		178.23	0.045	7.44 ± 0.06		0.010	-
Fluoranthene		202.26	0.26	7.9 ± 0.1		0.001	Ames, weak carcinogen
Pyrene		202.26	0.132	7.43 ± 0.01 7.53		0.001	Ames, UDS, SCE
Chrysene		228.29	0.0006	7.60 ± 0.03 7.21	0.0044	0.010	Ames, SCE, CA
Benzo(a)anthracene		228.29	0.011	7.53 ± 0.30	0.145	0.100	Ames, CA, UDS, SCE, carcinogen
Benzo(b) fluoranthene		252.31	0.0015	7.70	0.167	0.100	
Benzo(a)pyrene		252.31	0.0038	7.10 7.21	1.000	1.000	Ames, CA, UDS, DA, SCE, carcinogen
Dibenzo(a,h)anthracene		278.35	0.0006	7.38 ± 0.02	1.11	5.000	Ames, CA, DNA damage
Indeno(1,2,3-c,d) pyrene		276.33	0.062			0.100	Ames
Benzo(g,h,i)perylene		268.35	0.00026	7.31		0.010	

Symbols are (DA) DNA adducts, (SCE) sister chromatid exchange, (CA) chromosomal aberrations, (Ames) *Salmonella typhimurium* reversion assay, (UDS) unscheduled DNA synthesis, (-) non genotoxic

1 (Dabestani and Ivanov 1999); 2 (Bogan and Lamar 1995); 3 (Cerniglia and Heitkamp 1989, Cerniglia 1992); 4 (Kästner 2000a)

* 2-bromo-naphthalene is not on the EPA list and the 16th EPA not included is benzo(k)fluoranthene

Human exposure can occur by inhalation (smoke, contaminated air), through the uptake of contaminated food (e.g. burned or barbequed food), or through direct exposure to contaminated soil or water (Kästner 2000a). Although many PAH have been identified as probable human carcinogens, PAH such as benzo(a)pyrene (BaP) are appropriately termed precarcinogens. BaP can be metabolically activated to arene oxides, a reaction which is catalyzed by multiple forms of cytochrome P-450 monooxygenase enzymes (Cerniglia 1984). The activation of BaP in mammals requires the further oxidation to 7,8-dihydrodiol-9,10-epoxide (Fig. 1.16), which is the ultimate carcinogen (Sutherland *et al.* 1995). This compound is able to directly bind to the DNA with serious consequences to DNA replication and transcription (Fig. 1.16; Cerniglia 1984, Barry *et al.* 1996).

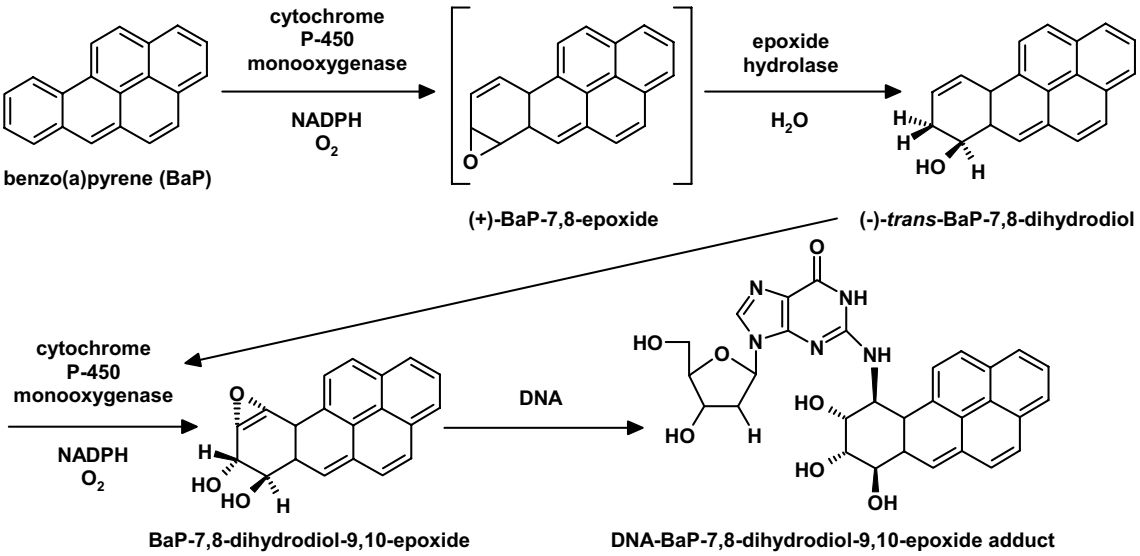


Figure 1.16: Activation of the pre-cancerogen benzo(a)pyrene (BaP) through cytochrome P-450 monooxygenase and the further transformation to the ultimate carcinogen 7,8-dihydrodiol-9,10-epoxide, a DNA binding entity (Cerniglia 1984, Barry *et al.* 1996).

1.5.1 Degradation of PAH

The degradation of PAH depend on their physical and chemical properties, concentrations, rates of diffusion in soils and water, as well as their bioavailability (Sutherland *et al.* 1995). Recalcitrance to microbial degradation increases directly with molecular mass and decreases with water solubility (Cerniglia 1992; see also Table 1.2). The microbial conversion of PAH has been well documented in numerous reviews, particularly those of Cerniglia and coworkers (Cerniglia 1984, Cerniglia and Heitkamp 1989, Cerniglia 1992, 1993, Sutherland *et al.* 1995, Cerniglia 1997). The main finding was that low molecular mass PAH (e.g. naphthalene, anthracene, phenanthrene) are readily degraded by a number of aerobic bacteria, which utilize PAH as a carbon source. BaP or other high molecular mass PAH are usually co-metabolically oxidized by a restricted number of bacterial species such as *Mycobacterium* spp. or *Sphingomonas* spp. (Schneider *et al.* 1996, Ye *et al.* 1996). To date, bacteria have not been isolated which are able to use BaP as the sole carbon source (Juhasz and Naidu 2000).

PAH are oxidized by cytochrome P-450 monooxygenases to arene oxides (Fig. 1.17). A non-enzymatic rearrangement can convert them to phenols or they are further hydrolyzed to *trans*-dihydrodiols. Certain bacteria and algae possess dioxygenases which can further

oxidize aromatic hydrocarbons to *cis*-dihydrodiols. This degradation pathway continues with a dehydration to dihydroxy-PAH, which are subject to ring cleavage through different fission pathways, resulting in the formation of organic acids (e.g. succinic, pyruvic, fumaric, or acetic acid; Wilson and Jones 1993) and can end in the complete metabolization of the compound. An unusual pathway of PAH degradation operates in white-rot fungi; these fungi produce a set of ligninolytic enzymes (Hatakka 2001) which are able to attack the aromatic structure by forming PAH-quinones. Further attack by enzyme generated radicals will eventually lead to ring fission and to the release of CO_2.

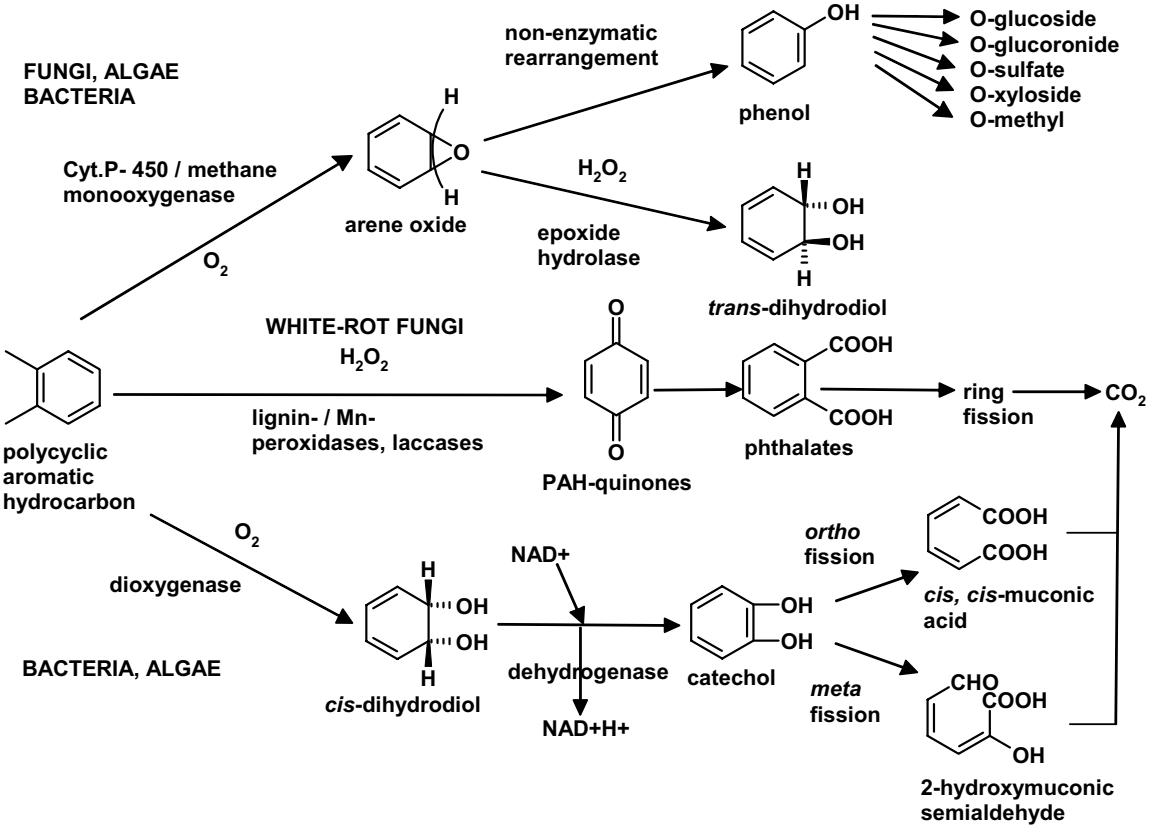


Figure 1.17: Different strategies used by microorganisms to attack the aromatic structure of PAH (modified after Cerniglia 1984, 1989, 1992, 1993, 1997 and Sutherland *et al*. 1995).

1.5.2 Degradation of PAH by fungi

PAH, including the genotoxic and recalcitrant BaP, have been shown to be metabolized by several molds (deuteromycetes and zygomycetes), including *Aspergillus ochrachae* (Datta and Samanta 1988), *Cunninghamella elegans* (Cerniglia and Gibson 1979), and *Penicillium* spp. (Kapoor and Lin 1984). These fungi use cytochrome P-450 monooxygenase enzymes to attack PAH. These enzyme systems readily metabolize low molecular mass PAH but are also found to be involved in the degradation of high molecular mass PAH. Whereas some molds were able to use pyrene as a sole carbon source (Saraswathy and Hallberg 2002), higher molecular mass PAH are only degraded co-metabolically. This is an additional reason why PAH with four or more rings are considered to be highly recalcitrant and more resistant to microbial degradation (Cerniglia 1992).

PAH are especially subject to non-specific oxidation by radicals produced by the ligninolytic enzymes of white-rot fungi. Soon after the discovery of LiP and MnP from

P. chrysosporium it was shown that this fungus was able to degrade several xenobiotic compounds including high molecular weight PAH (Bumpus *et al.* 1985). The involvement of LiP and MnP in the degradation process was demonstrated soon after (Haemmerli *et al.* 1986, Hammel *et al.* 1986, Sanglard *et al.* 1986) but cytochrome P-450 mediated oxidation was observed as well (Dhawale *et al.* 1992). The involvement of cytochrome P-450 monooxygenase has also been shown in other white-rot fungi (Sutherland *et al.* 1995, Bezalel *et al.* 1997). Nevertheless, several studies demonstrated that PAH conversion correlates with the activity of ligninolytic enzymes (Field *et al.* 1992, Sack *et al.* 1997a). Numerous studies show that white-rot fungi are efficient degraders of PAH, including species such as *P. chrysosporium* (Bumpus 1989), *Pleurotus* sp. (Bezalel *et al.* 1997, Wolter *et al.* 1997), *Bjerkandera* sp. (Kotterman *et al.* 1998), and *Trametes versicolor* (Morgan *et al.* 1991). It is now generally accepted that ligninolytic enzymes are involved in PAH degradation by white-rot fungi (Hammel *et al.* 1986, Kästner 2000a, Pointing 2001). MnP is implicated as the key enzyme in the degradation process (Moen and Hammel 1994, Bogan and Lamar 1996, Bogan *et al.* 1996, Collins and Dobson 1996, Sack *et al.* 1997b). *In vitro* studies proved that MnP directly attacks PAH and is able to catalyze total mineralization (Sack *et al.* 1997b). The reactions are similar to those found in lignin degradation. Thus, PAH breakdown occurs via the formation of free radicals (Kirk and Farrell 1987, Hatakka 1994, 2001, Hofrichter 2002), a process called “enzymatic combustion”.

Little is known about the degradation of PAH by LDF. A few litter-decomposing strains were shown to oxidize PAH in liquid cultures (Sack and Günther 1993, Lange *et al.* 1996, Wunch *et al.* 1997, Gramss *et al.* 1999a). One particular species, *Marasmiellus troyanus*, was even able to mineralize BaP (Wunch *et al.* 1999). Indications of the involvement of ligninolytic enzymes were found by Gramss *et al.* (1999a). Substantial new knowledge on the degradation of PAH and the involvement of MnP from LDF has been obtained in the present study (articles IV and V).

2. Objectives of the study

2.1 Background

The topic of this work originally arose from the idea of using fungi for soil bioremediation. In the middle of the 1990’s it was known that white-rot fungi were able to degrade different recalcitrant organic compounds via the activities of ligninolytic enzymes that were identified as being responsible for the breakdown of organopollutants.

Nevertheless, the application of white-rot fungi in soil clean-up has been problematic, because the most efficient degraders showed poor competitive growth in the presence of indigenous soil micro-flora. A limited number of white-rot basidiomycetes have the ability to compete and grow into the soil and break down contaminants. However, it was obvious that studies have been restricted to only a few species and that there remained great potential in the, as yet, untested LDF. In addition to the degradation potential the ability to grow in soil is now regarded as a major factor for the successful application of a given fungus in soil bioremediation. A simple idea was born, to look for fungi which are natural soil dwellers. When focusing on basidiomycetes there were only two choices: mycorrhizal fungi or soil inhabiting saprotrophs. Mycorrhizal basidiomycetes have been examined for a long time. The lack of non specific oxidative activity however makes the use of these fungi for bioremediation inefficient. On the other hand, soil-litter inhabiting species are soil borne saprophytes and there was already promising evidence that they possess an enzyme arsenal similar to white-rot fungi for the utilization of the lignocellulose contained in litter.

These species are called litter-decomposing fungi (LDF). Thus, it seemed only logical to investigate members of this fungal community for their ability to efficiently degrade the target marker compounds of choice.

To randomly select one or two strains for soil bioremediation without resorting to pre-screening studies was regarded as too uncertain an approach since the biodegradative capability of LDF was poorly described at that time. The limited amount of data on the degradative capabilities of LDF from earlier results of other research groups provided only hints that had to serve as a basis to start the research described in this thesis. At that time (end of 1998), only Gramss *et al.* (1998), Wunch *et al.* (1997) and coworkers had reported oxidative enzyme activities and degradation of PAH by LDF. The lack of knowledge in this area was therefore quite obvious. Wunch *et al.* (1997) and Scheibner *et al.* (1997) implied that LDF could have some degradative abilities towards aromatic compounds and the general view was that LDF were able to degrade all components of litter.

A research plan was formulated to reveal the degrading capabilities of this particular fungal group. Furthermore, it was evident that the research, which would lead to a successful application *in situ*, would be too ambitious for a single dissertation thesis. Therefore we focused primarily on the physiology and biochemistry of LDF grown in liquid cultures. A simpler and more controlled environment, together with easier applicable analyses should produce results which could be used in future as a basis for soil-bioremediation studies. Moreover, the lack of basic knowledge about LDF made it necessary to carry out more basic research, for example to find out more about their enzymes. Compared with other fungi, including molds and yeasts, research on LDF has largely been neglected. Thus the aim was to characterize lignin and xenobiotic degrading abilities of selected litter-decomposing fungi.

2.2 Aims of the study

The following aims were set in the form of questions which we hoped to get answers for:

1. **Are litter-decomposing fungi able to degrade lignin and to what extent?**
2. **What kind of ligninolytic enzymes do they use to accomplish this?**

After a first successful set of screening tests and enzyme activity measurements we found reason to extend our goals. Manganese peroxidase (MnP) seemed now to be an important enzyme in a large number of the species tested, so this enzyme in particular was included in a number of further aims:

3. **Is MnP a key enzyme in the degradation process of lignin by litter-decomposing fungi?**
4. **What are the main characteristics of this enzyme?**
5. **Are litter-decomposing fungi involved in the degradation of other recalcitrant materials in the litter such as humic acids and is MnP involved in the degradation process?**
6. **Concerning the bioremediation of contaminated soils, are litter-decomposing fungi generally able to degrade aromatic pollutants such as PAH, and is MnP involved in the degradation process?**

3. Material and methods

3.1 Fungi

All fungal strains used in the experiments are listed in Table 4.1 (see Appendix for trivial names). All strains designated with the letter "K" were isolated by the author from Finnish forests or grasslands and are deposited at the Culture Collection of the Department of Applied Chemistry and Microbiology at the University of Helsinki (Finland). In addition, other strains were obtained from the German Collection of Microorganisms in Braunschweig (DSMZ, Germany) or from the former Culture Collection of the Institute of Microbiology at the University of Jena (Germany), which is in part continued at the International Graduate School in Zittau (Germany). Information on the culture conditions are published in all articles (I-V).

The following LDF were studied in more detail: *Agrocybe praecox* (I, II, IV), *Collybia dryophila* (III), *Stropharia coronilla* (I, II, IV, V), and *Stropharia rugosoannulata* (I, IV). *Collybia dryophila* was not originally part of the original screening (I) and was later chosen (III) because of abundance of this species and its ecological impact in the environment.

Agrocybe praecox (Pers.: Fr.) Fayod (Fig. 1.5) is a typical medium sized agaric (fruiting body 4-9 cm hight, cap 3-6 cm broad) with a cream cap, light brown gills and a ring on the stem. It occurs on soil among grass, sometimes on rotten straw, in thickets and woodland edges. Fruiting bodies are produced in summer and autumn and the fungus is widely distributed across Europe and Northern America.

Collybia dryophila (Bull.: Fr.) Kummer (formerly *Gymnopus dryophilus*; Fig. 1.6) is a smaller agaric with a pale tan cap, whitish gills, and stem flushed tan. The species is found in small groups on soil and leaf litter under broad-leaf and coniferous trees. Fruiting bodies appear from spring to winter with a peak in summer and autumn and the fungus is very common in Europe and Northern America.

Stropharia coronilla (Bull.: Fr.) Quelet (Fig. 1.2) is a smaller agaric with a pale-yellow cap, white stem with a ring, and pale lilac-grey gills. It grows solitary among grasses on pastures and meadows fruiting in spring-summer time. The species is infrequently found in Northern America and Europe.

Stropharia rugosoannulata Farlow (Fig. 1.3 and 1.4) is a large sized (cap up to 20 cm broad) fleshy agaric with a brownish or yellow cap, robust white stem with a ring, and violet-brown gills. The fungus prefers to grow on straw or rotting straw but forms fruiting bodies often when it comes in contact with soil. Fruiting bodies occur in summer and autumn. *Stropharia rugosoannulata* is a commercially cultivated mushroom in several European countries.

3.2 Schematic outline of the study

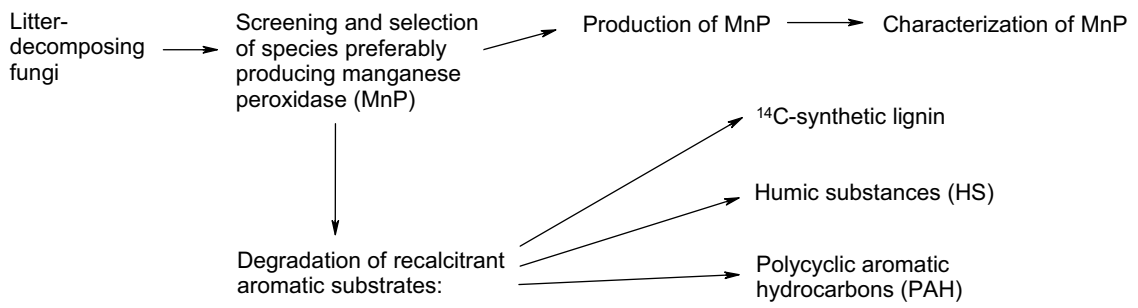


Figure 3.1: Schematic outline of the experimental work of this thesis.

3.3 Chemicals

Source details concerning the chemicals used are listed in the published articles (I-V). The synthetic ^{14}C-ring-labeled lignin (^{14}C-DHP; dehydrogenation polymer) with a molecular mass of 4-10 kDa was polymerized from ^{14}C-ring-labeled coniferyl alcohol (I). This DHP was produced and distributed to several researchers by Ander and Eriksson, STFI, Stockholm, Sweden and Odier and Heckman, INRA, Paris, France (Eriksson *et al.* 1990, p. 232). The ^{14}C-*β*-labeled DHP with an average molecular size of 3.1 kDa was synthesized by Brunow *et al.* (1998).

The ^{14}C-labeled humic acid (^{14}C-HA) was synthesized by spontaneous oxidative polymerization of [U-^{14}C] catechol in an alkaline solution (III; Fig. 3.2). Unlabeled HA was either prepared from unlabeled catechol or extracted from forest litter (III; Fig. 3.2).

Unlabeled PAH compounds were obtained from Sigma at the highest available purity. [7,10-^{14}C]-BaP was obtained from Amersham Buchler, Braunschweig, Germany. A QTM (quick turnaround method) PAH mix containing 16 different EPA-PAHs was obtained from Supleco, Bellefonte, PA.

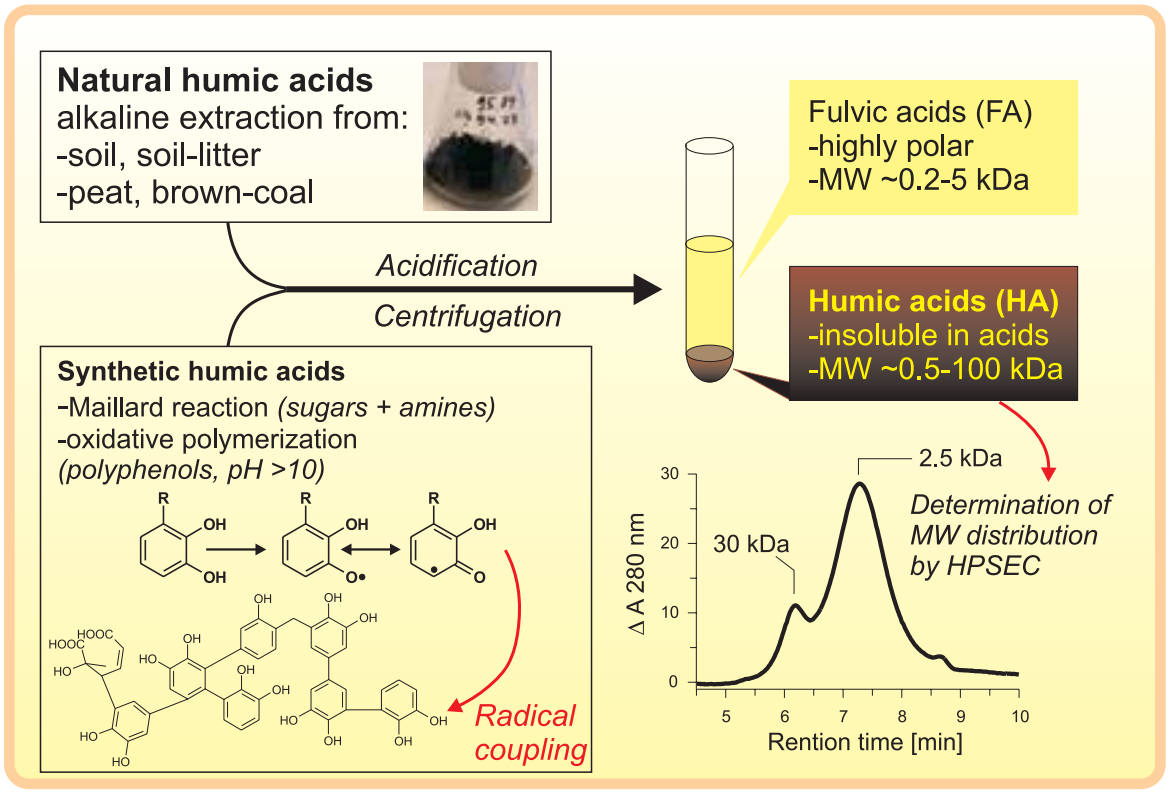


Figure 3.2: Preparation of synthetic HA from catechol, extraction and preparation of natural HA from soil-litter.

3.4 Experimental setup and methods

The experimental setup as well as the methods used are described in detail in the published articles I-V and summarized in Table 3.1. The number of fungal strains used in the experiments was higher than that in the published results. These additional strains were mainly white-rot fungi and were used for comparison (see section 4. results). The conditions and setup of the experiments for the additional data are thus identical to those described in articles I, II, and IV.

Table 3.1: Methods used in this dissertation study

Method	Published in article
Isolation of fungi using selective agar media	III
Agar plate screening tests with ABTS, HA, and Mn	I
Liquid cultivation of fungi	I – V
Solid-state cultivation of fungi	I (and additional data)
Preparation of ^{14}C-labeled HA	III
Experiments with ^{14}C-labeled compounds	I, III - V
- liquid scintillation counting (LSC)	I, III - V
- extraction of ^{14}C-labeled compounds	I, III - V
- combustion of ^{14}C-labeled material	I, III - V
Enzyme purification	II, III
- ultrafiltration	II, III
- fast protein liquid chromatography (FPLC)	II, III
- protein concentration (Bradford)	II, III
- SDS-PAGE	II, III
- isoelectric focusing (IEF)	II, III
Spectrophotometric measurement of enzyme activities (enzyme assays)	I – V
Extraction of PAH	IV, V
High performance size exclusion chromatography (HPSEC)	III
High performance liquid chromatography (HPLC)	III – V

Conversion and mineralization experiments were performed in non-agitated liquid or solid-state straw cultures. Unpublished data for *Collybia dryophila* was obtained using solid-state cultures on autoclaved pine-spruce needles litter from a mixed coniferous forest. Fungal cultures used in mineralization studies were maintained in gas tight flasks and flushed weekly with O_2 (Fig. 3.3).

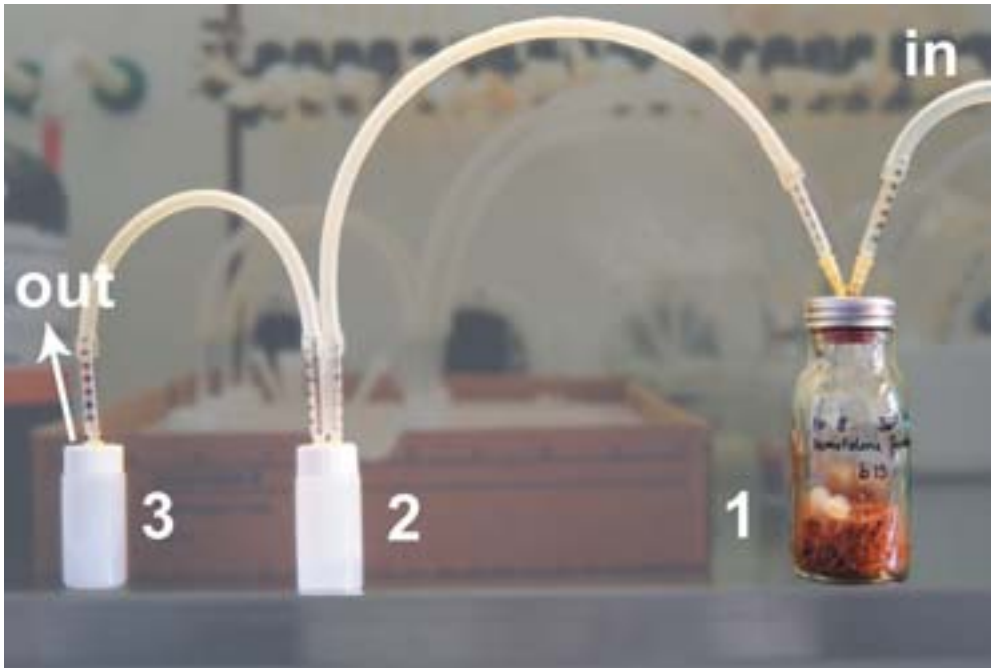


Figure 3.3: Experimental setup for the flushing of cultures flasks during a mineralization experiment. (1) culture flask (2) volatile organic compound trap with Optifluor® (3) CO_2 trap with Optifluor® and Carbosorb®. The procedure is described in articles I, III, IV and V.

4. Results

4.1 Degradation of synthetic lignin (I)

In order to evaluate the ligninolytic capability of LDF an agar-plate screening was performed with different species of LDF and, for comparison, with white-rot species. The production of extracellular oxidoreductases was monitored using ABTS containing agar plates. The formation of the dark-green ABTS cation radical indicated a positive result e.g. the production of oxidoreductases (Fig. 4.1 left). Nearly all strains tested positive including, as expected, all white-rot strains (Table 4.1). The bleaching of dark brown HA agar plates (Fig. 4.1 middle), as well as the formation of MnO_2 spots on Mn supplemented agar plates were followed (Fig. 4.1 right). Positive reactions on all plates were less common among litter-decomposing strains compared to those among white-rot strains (Table 4.1). The most active strains that produced positive results on all plates in the agar-plate screening were used for lignin mineralization studies. Of the 13 strains exhibiting degradative activity three were chosen for detailed investigation in the mineralization studies.

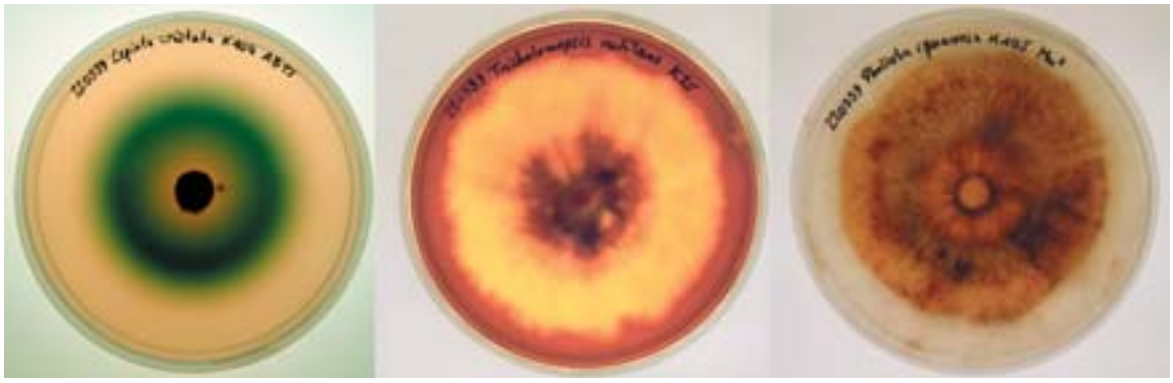


Figure 4.1: Photos of positive ager plate results; from left to right: ABTS with *Lepiota cristata* (LDF), HA with *Tricholomopsis rutilans* (WR), Mn with *Pholiota squarrosa* (WR)

These fungi were *Agrocybe praecox*, *Stropharia coronilla*, *S. rugosoannulata*, and as a representative of a non-HA bleaching fungus *Stropharia semiglobata*. Three of the four selected litter-decomposing species were able to mineralize up to 25% of synthetic lignin added within 12 weeks (Fig. 4.2 B). White-rot fungi were more efficient degrading up to 60% under identical conditions (Fig. 4.2 A). Interestingly, the coprophilic fungus *S. semiglobata*, which lacks MnP, was only able to mineralize synthetic lignin by 5%. Thus these data emphasize that white-rot fungi may be more efficient in degrading lignin than LDF. However, unpublished results with *Collybia dryophila* showed that the rate of mineralization is tightly dependent on the substrate used. Thus, *C. dryophila* was able to mineralize 40% of the β-labeled DHP in 12 weeks on coniferous litter (Fig. 4.2 C) demonstrating the improved effectiveness of LDF on this substrate as compared to straw. All in all, MnP obviously plays a key role in the mineralization process, since all fungi showing higher mineralization capability produced this peroxidase.

Table 4.1: Agar-plate screenings assessing the abilities of litter-decomposing and white-rot fungi to oxidize ABTS, to degrade humic acids, and to oxidize manganese. (+) positive and (-) negative result. This table contains additional data not included in Table 1 published in article I. (For trivial names see Appendix).

Fungus	Strain	Family	Decay	ABTS oxidation *	Humic acid bleaching #	Mn^{2+} oxidation §
Agrocybe dura	K102	*Bolbitiaceae*	L	+	+	+
Agrocybe praecox	TM 70.84	*Bolbitiaceae*	L	+	+	+
Bjerkandera adusta	b1	*Poriaceae*	w-r	+	+	+
Calocybe indica	x28	*Tricholomataceae*	w-r	+	+	+
Clitocybe clavipes	K71	*Tricholomataceae*	L	+	-	-
Clitocybe dealbata	K197	*Tricholomataceae*	L	+	-	+
Clitocybe gibba	K32	*Tricholomataceae*	L	+	+	+
Clitocybe lignatilis	K82	*Tricholomataceae*	L	+	-	-
Clitocybe metachroa	K213	*Tricholomataceae*	L	+	+	+
Clitocybe odora	TM 3	*Tricholomataceae*	L	+	-	-
Clitocybe sp.	K28	*Tricholomataceae*	L	+	+	-
Clitopilus prunulus	K200	*Entolomataceae*	L	+	-	-
Collybia dryophila	K209	*Tricholomataceae*	L	+	+	+
Collybia dryophila	K220	*Tricholomataceae*	L	+	+	+
Collybia sp.	K190i	*Tricholomataceae*	L	+	-	+
Coprinus atramentarius	K119	*Coprinaceae*	L	+	-	-
Coprinus comatus	K131	*Coprinaceae*	L	+	-	-
Dermocybe amiantinum	K189	*Cortinariaceae*	L	+	-	-
Galerina marginata	K96	*Cortinariaceae*	L	+	+	-
Gymnopilus junonius	K89	*Cortinariaceae*	L	+	+	+
Hygrophoropsis aurantiaca	K123	*Paxillaceae*	L	-	-	-
Hypholoma capnoides	K87	*Strophariaceae*	w-r	+	+	+
Hypholoma marginatum	K92	*Strophariaceae*	L	+	+	-
Kuehneromyces mutabilis	K3	*Strophariaceae*	w-r	+	+	+
Lepiota cristata	K104	*Agaricaceae*	L	+	-	-
Lepista nebularis	K103	*Tricholomataceae*	L	+	+	-
Lepista nuda	K110	*Tricholomataceae*	L	+	-	-
Lyophyllum decastes	K80	*Tricholomataceae*	L	+	-	-
Lyophyllum ulmarium	K144	*Tricholomataceae*	L	+	-	-
Marasmius alliaceus	TM SW1	*Tricholomataceae*	L	+	+	-
Marasmius scorodonius	TM SW2	*Tricholomataceae*	L	+	-	-
Mycena amicata	K91	*Tricholomataceae*	L	+	+	-
Mycena epipterygia	K72	*Tricholomataceae*	L	+	+	-
Mycena polygramma	K191	*Tricholomataceae*	L	+	-	-
Mycena sp.	K215	*Tricholomataceae*	L	+	-	+
Nematoloma frowardii	DSM 11239	*Strophariaceae*	w-r	+	+	+
Omphalina epichysium	K95	*Tricholomataceae*	L	-	-	-
Panellus mitis	K83	*Tricholomataceae*	w-r	+	+	+
Pholiota nameko	Ho1	*Strophariaceae*	w-r	+	+	+
Pholiota squarrosa	K105	*Strophariaceae*	w-r	+	+	+
Phaeolepiota aurea	K111	*Agaricaceae*	L	+	-	-
Pleurotus ostreatus	336	*Lentinaceae*	w-r	+	+	+
Pleurotus pulmonarius	P14	*Lentinaceae*	w-r	+	+	+
Pleurotus sapidus	P2	*Lentinaceae*	w-r	+	+	+
Pleurotus P1 Florida	DSM 11191	*Lentinaceae*	w-r	+	+	+
Stropharia aeruginosa	K47	*Strophariaceae*	L	+	+	+
Stropharia coronilla	TM 47-1	*Strophariaceae*	L	+	+	+
Stropharia cubensis	TM SW3	*Strophariaceae*	L	+	+	+
Stropharia hornemannii	K122	*Strophariaceae*	L	+	+	+
Stropharia rugosoannulata	DSM 11372	*Strophariaceae*	L	+	+	+
Stropharia rugosoannulata	DSM 11373	*Strophariaceae*	L	+	+	+
Stropharia semiglobata	K79	*Strophariaceae*	L	+	-	-
Trametes hirsuta	K21A	*Poriaceae*	w-r	+	+	+
Tricholomopsis rutilans	K25	*Tricholomataceae*	w-r	+	+	+

* formation of dark-green rings around the mycelium; # bleaching of dark-brown humic acids
§ formation of black flecks of MnO_2 ; L: litter-decomposing fungus; w-r: white-rot fungus

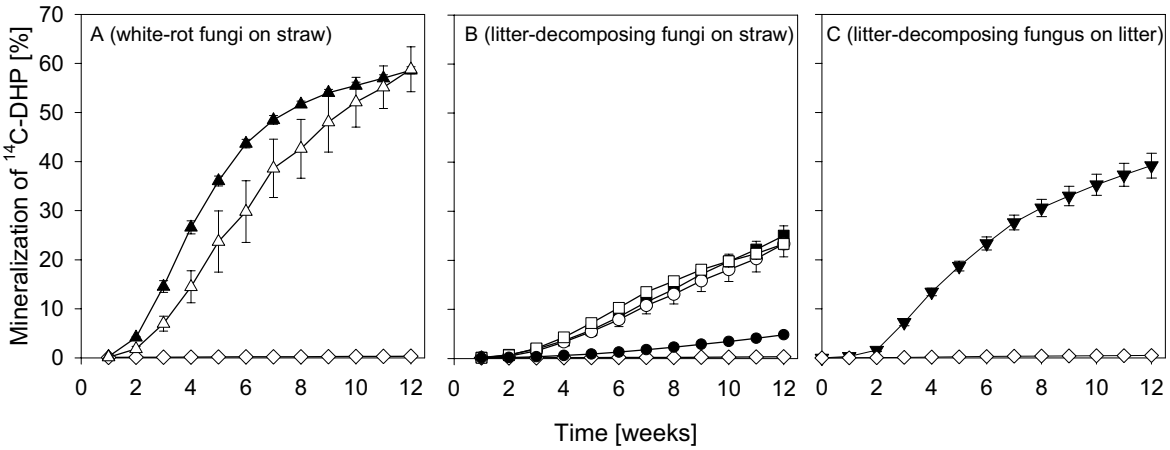


Figure 4.2: Release of $^{14}CO_2$ from ^{14}C-ring labeled synthetic lignin (39 000 dpm/flask) during the growth of wood-decaying fungi (A) and different LDF (B) on wheat straw as well as from a β^{14}C-labeled DHP on coniferous litter (C). *N. frowardii* (filled triangles), *Pleurotus* P1 Florida (empty triangles), *S. rugosoannulata* (empty squares), *A. praecox* (filled squares), *S. coronilla* (empty circles), *S. semiglobata* (filled circles), uninoculated control (empty diamonds); *C. dryophila* (upside down filled triangles). Data published in article I and unpublished data.

4.2 Characteristics of ligninolytic enzymes from litter-decomposing fungi (II and III)

Extracellular MnP and laccase were purified from liquid cultures of four LDF (Table 4.2). Details of three strains have been published in articles II and III. Key MnP and laccase data from *Stropharia rugosoannulata* B is presented in Figure 4.3. The molecular mass (MW) of MnPs produced by these fungi ranged only from 41-44 kDa but they differed clearly in their p*I*s ranging from as low as 3.2 up to 7.1 (Table 4.2). This broad range was also observed for another species, namely *Stropharia coronilla*, which produced different MnPs with p*I*s between 3.5 and 7.1. Interestingly, most of the MnPs were produced only in the presence of Mn, although *S. coronilla* produced a partly constitutive MnP, which even appeared in Mn-free medium. However, trace amounts of Mn were probably present, due to the difficulty in producing Mn-free media, which may have been sufficient to trigger MnP production.

Table 4.2: Molecular masses (MWs) and isoelectric points (p*I*s) of MnP and laccase isoenzymes from three different LDF (published in II and III with additional data*; n.d. not determined).

Fungus	Enzyme	MW [kDa]	p*I*	Published in
Agrocybe praecox TM 70.84	MnP 1	42	6.3-7.0	II
	MnP 2	42	6.3-6.7	II
	laccase	66	4.0	II
Collybia dryophila K 209	MnP 1	44	4.7	III
	laccase	n.d.	n.d.	-
Stropharia coronilla TM 47-1	MnP 1	41	6.3-7.1	II
	MnP 2	41	3.5 and 3.7	II
	MnP 3	43	5.1	II
	laccase	67	4.4	II
Stropharia rugosoannulata B*	MnP 1	41	3.2	unpublished
	MnP 2	43	3.2	unpublished
	laccase	67	3.3-3.4	unpublished

All strains used for the characterization of ligninolytic enzymes also produced laccases to some extent (Table 4.2). As with MnP the MW showed very limited variation (66-67 kDa) but only acidic p*I*s were observed (3.3-4.4).

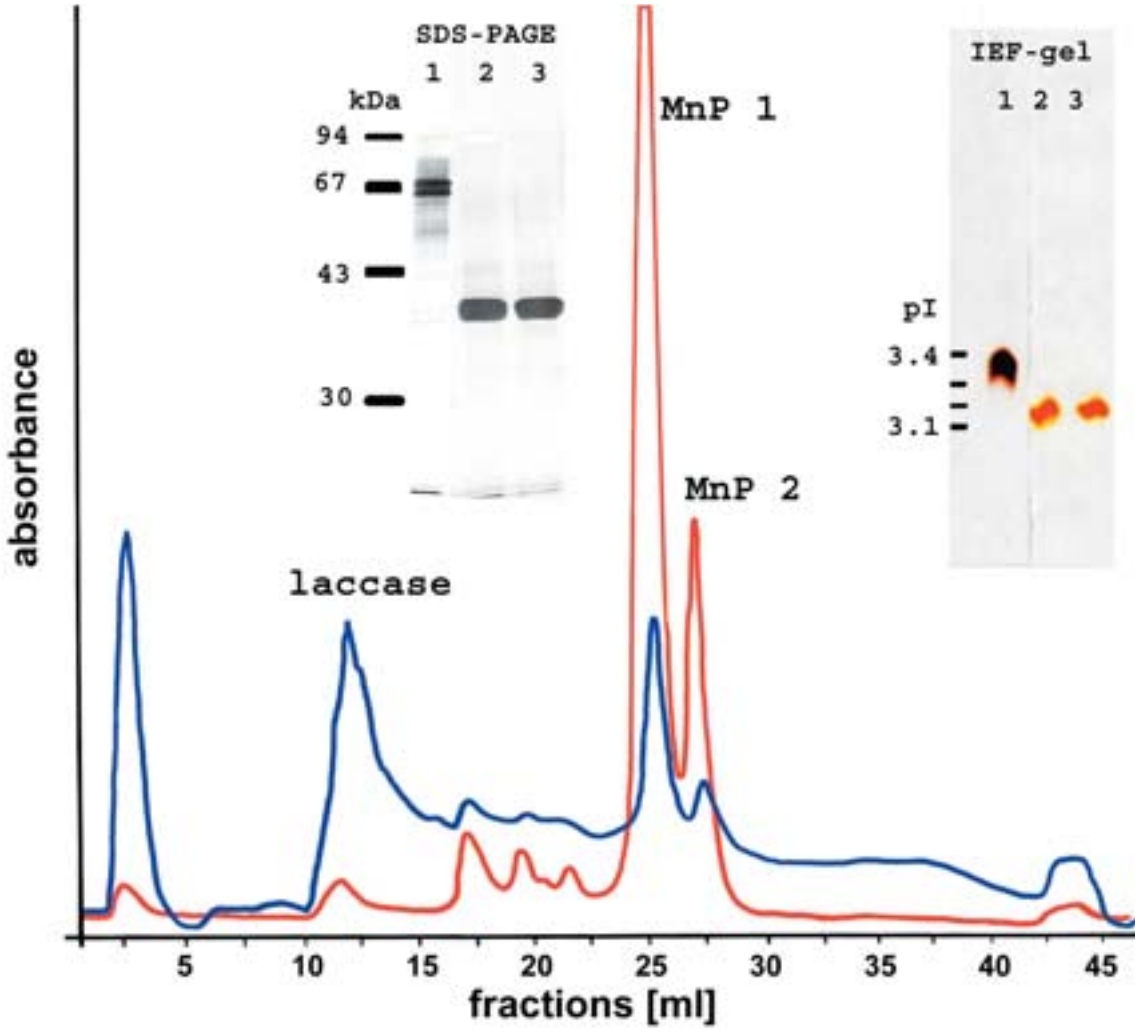


Figure 4.3: Anion exchange chromatography of proteins from cultures of *S. rugosoannulata* B in Mn supplemented cultures (FPLC with Mono-Q column and NaAc gradient 10 mM-1M pH 6.5). Red line: absorbance at 405 nm; blue line absorbance at 280 nm; gel lanes (1) laccase, (2) MnP1, (3) MnP2. Note: arbitrary absorbance units.

4.3 Degradation of humic acids (HA) by *Collybia dryophila* (III)

The litter-decomposing fungus *Collybia dryophila* converted HAs extracted from litter (75% in Mn supplemented cultures; Fig. 4.4 A) mostly to lower-molecular mass FAs (Fig. 4.4 B). A large part of the HAs was mineralized (Fig. 4.5 A). The addition of Mn^{2+} enhanced the mineralization in liquid cultures (Fig. 4.5 A). Over time HA was degraded to the same extent in autoclaved pine-birch forest litter (Fig. 4.5 B). Here, however, the addition of Mn^{2+} had no effect on the mineralization. This may be due to the high Mn content, which was as high as 260 mg/kg soil in our samples.

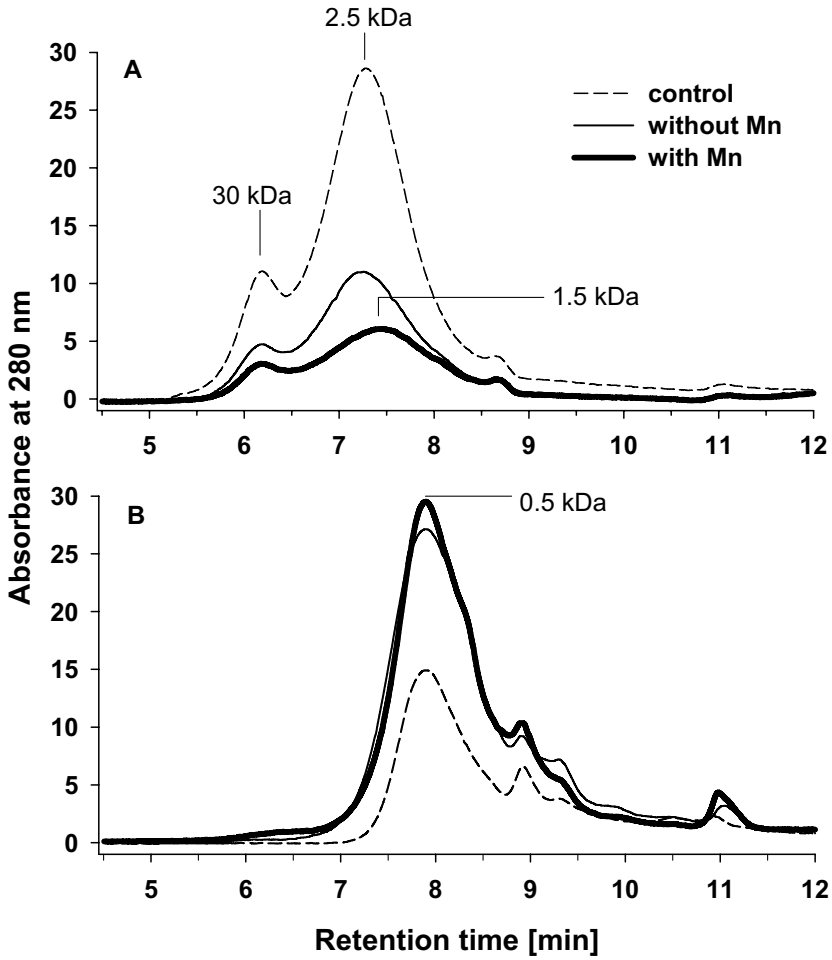


Figure 4.4: HPSEC elution profiles of HAs, extracted from litter, recovered from liquid cultures of *Collybia dryophila* grown in the presence or absence of Mn^{2+} (A) and of the respective FAs formed (B). Fungal cultures supplemented with 200 µM $MnCl_2$ (bold lines), fungal cultures without Mn^{2+} (thin lines), and controls without fungus (dotted lines). Data published in article III.

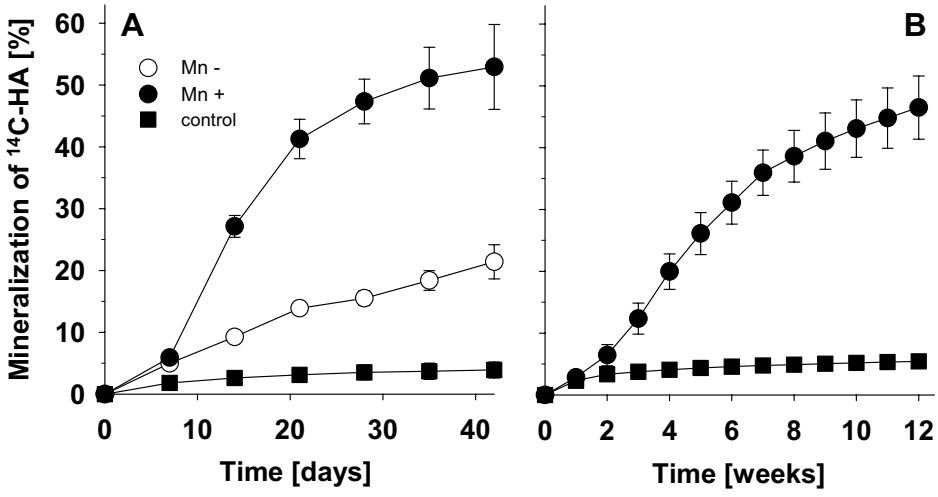


Figure 4.5: Mineralization of ^{14}C-HA by *Collybia dryophila* in liquid culture (A) and in sterilized birch-pine forest litter (B). Fungus in Mn^{2+}-supplemented medium (closed circles); fungus in Mn-free medium (A; open circles) and without Mn^{2+} addition (B; open circles); control without fungus (squares) (A published in III). Data points represent means of three parallels with standard deviations.

4.4 Degradation of PAH (IV and V)

All strains of LDF used in the PAH degradation studies, as well as white-rot fungi, were able to convert different PAH to some extent (Fig. 4.6). In general, MnP producing fungi converted PAH more effectively than those species, e.g. *Clitocybe odora* or *Lepista nuda*, which only produced laccase (enzyme data not shown). The impact of intracellular enzyme activities was not monitored although they might have had a role in the conversion process. Results obtained in Mn^{2+} free cultures could partly be attributed to intracellular activities. The addition of Mn^{2+} to the cultures enhanced the conversion of anthracene and benzo(a)pyrene. The effect was less pronounced for pyrene. Furthermore, Mn^{2+} had more impact on the conversion of PAH by LDF than by white-rot fungi.

LDF of the genus *Stropharia* were able to mineralize BaP in liquid culture and on straw (Fig. 4.7). In liquid cultures Mn^{2+} supplementation enhanced mineralization considerably. Only negligible amounts of BaP were mineralized in Mn^{2+} free cultures indicating that MnP played a crucial role in the mineralization of BaP. This process was faster in liquid cultures than in solid-state straw cultures possibly due to the different bioavailability of BaP.

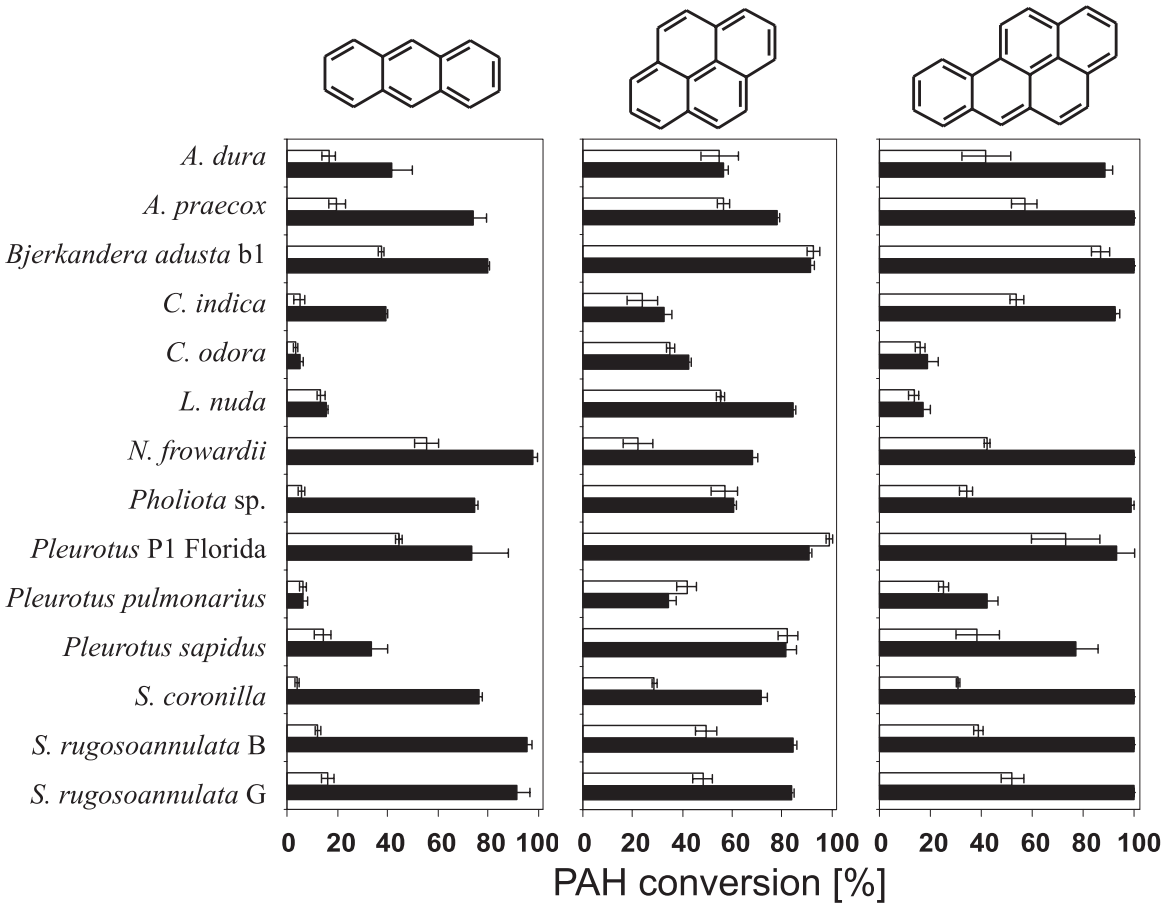


Figure 4.6: Removal of anthracene, pyrene, and BaP from liquid cultures of different LDF and white-rot fungi after six weeks of incubation. Mn^{2+}-supplemented (200 µM) cultures (dark columns), Mn-free cultures (white columns). The columns represent means of three replicates with standard deviation in % normalized against the recovered PAH from controls. Parts of the data published in article IV.

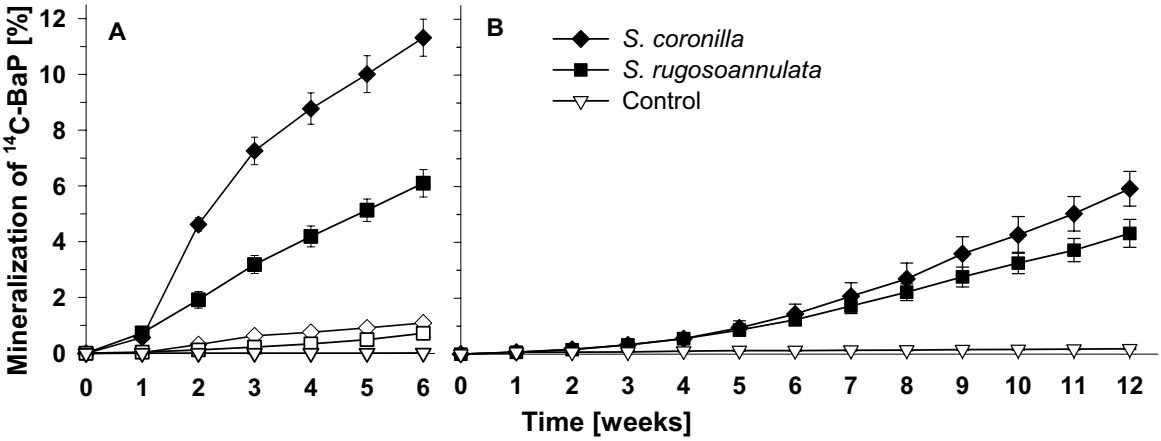


Figure 4.7: Release of $^{14}CO_2$ from ^{14}C-7,10 labeled BaP (349 000 dpm/flask) during the growth of *S. rugosoannulata* and *S. coronilla* in liquid culture supplemented with Mn^{2+} (filled symbols) or without Mn^{2+} (empty symbols) (A). Time course of $^{14}CO_2$ release from ^{14}C-7,10 labeled BaP (39 000 dpm/flask) during the growth on wheat straw of the LDF *S. coronilla* (diamonds) and *S. rugosoannulata* (squares)(B). The data points represent means of three replicates with standard deviation. Data published in articles IV and V.

4.5 Degradation of BaP by *Stropharia coronilla* (V)

Stropharia coronilla in particular showed a high PAH degradation potential (Fig. 4.6) and was able to mineralize BaP in liquid and straw cultures (Fig. 4.7). The role of MnP in these processes was evaluated using crude or purified MnP 1 from this fungus. Both oxidized BaP efficiently in a cell-free reaction mixture (*in vitro*; Fig. 4.8). BaP-quinone was identified as one of the transient metabolites but it was further degraded in the reaction.

MnP 1 of *S. coronilla* was also able to convert 15 out of the 16 different PAH substances tested (naphthalene evaporated too quickly; Fig. 4.9). Interestingly, some smaller PAH such as phenanthrene and fluoranthene were only partially converted while high molecular mass PAH such as indeno(1,2,3-c,d)pyrene and benzo(g,h,I)perylene were converted completely.

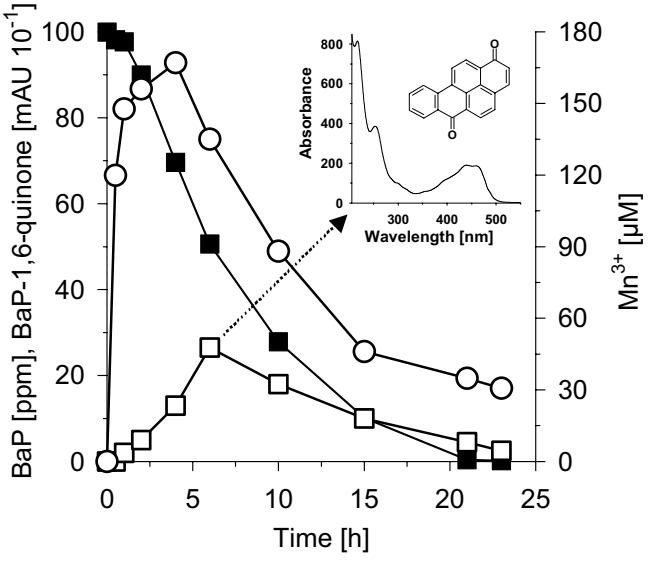


Figure 4.8: Conversion of BaP (closed squares, 100 mg liter^{-1}), formation of Mn^{3+}-ions (circles) and BaP-1,6-quinone (open squares; the inset shows its UV spectrum) in a sodium malonate buffered, cell free reaction mixture (total volume 200 ml) containing 1 U ml^{-1} of purified MnP 1 from *S. coronilla.* The enzymatic reaction was performed under continuous stirring at 37°C in the dark. Data published in article V.

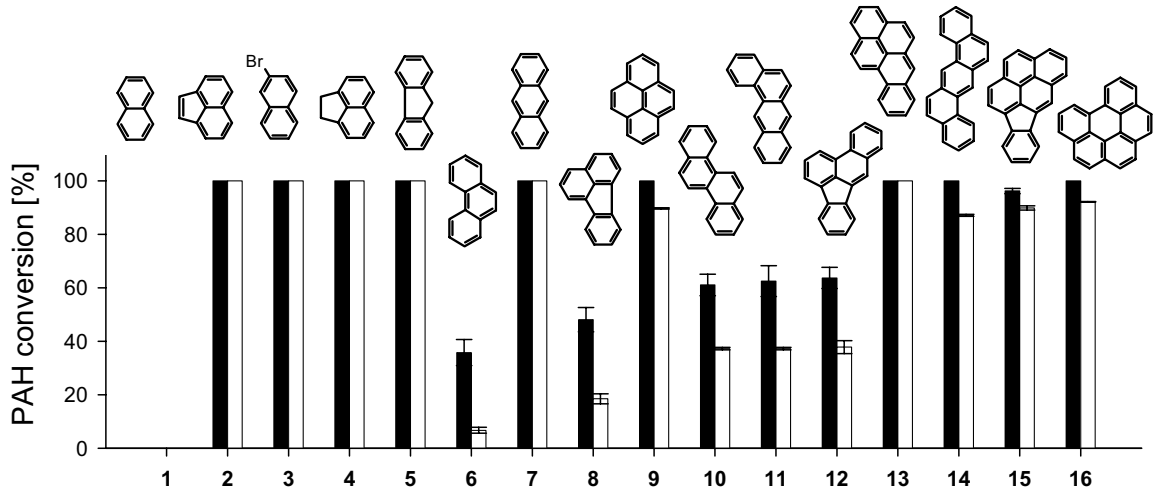


Figure 4.9: Conversion of 16 EPA-PAH by crude MnP (dark columns) and purified MnP 1 (white columns) of *S. coronilla* with an addition of Tween 80 (to both experiments) *in vitro*. For the nomenclature of PAH see Table 1.2. No data was available for naphthalene (1) because it evaporated too quickly. Results are means from three replicates in % normalized against the recovered PAH from controls. Data published in article V.

5. Discussion

5.1 Degradation of synthetic lignin

The data published in article I clearly demonstrate that certain LDF are able to degrade and even mineralize synthetic lignin. Furthermore, the involvement of MnP in the degradation process is substantiated in the observations that the most active species produced extracellular radical generating enzymes, of which MnP predominated. Additionally, species lacking MnP, such as *Stropharia semiglobata*, were found to be poorer degraders of lignin. This supports the assumption that MnP might play a key role in the degradation and, particularly, in the mineralization of lignin by LDF.

Screening tests with LDF and white-rot fungi using agar plates with different indicator substances proved useful tools for the evaluation of oxidative activities of basidiomycetous fungi (I). Similar tests have been used successfully for the selection of humic acid- and coal-depolymerizing fungi (Hofrichter and Fritsche 1996, Hofrichter *et al.* 1997). Spot tests have been used to identify oxidative enzyme activities in ectomycorrhizal, wood, and litter-decaying fungi (Gramss *et al.* 1998) and revealed that LDF test positive for laccase and peroxidase activities. Our screening data correlate well with the enzyme activities and identified mineralization rates (I). They also concur with the results obtained in another screening test, in which the mineralization of an aromatic compound, TNT (trinitrotoluene) was examined (Scheibner *et al.* 1997). This work indicated that LDF were also able to degrade aromatic compounds other than lignin and showed that *Stropharia* species, especially *S. rugosoannulata*, were among the most efficient degraders.

The degradation of lignin by LDF has only been studied by a few researchers. In most cases the loss of lignin was monitored in litter samples supporting inoculant fungal growth. Nevertheless, a clear indication of lignin degradation by LDF has been obtained. Species such as *Collybia* spp. or *Mycena* spp. were able to bring about a 25% decrease in lignin content (Tanesaka *et al.* 1993) and *Marasmius androsaceus* decreased the lignin content of litter bags by about 20% (Cox *et al.* 2001). A single litter-decomposing fungus has been subject to thorough investigation, namely *Agaricus bisporus*, the white button mushroom. The reason for this is the interest of the food industry as *A. bisporus* is the most produced and consumed mushroom in the world. In similar lignin degradation studies involving *A. bisporus*, depending on the label in the synthetic lignin (uniformly or side chain label), the fungus was able to mineralize from as little as approx. 2% in two weeks (Wood and Leatham 1983) to as much as 35% in 80 days (Durrant *et al.* 1991) of the lignin model compounds. It is not surprising that this fungus produces MnP and laccase (Bonnen *et al.* 1994, Leontievsky *et al.* 1997, Lankinen *et al.* 2001) and thus fits into our findings that a litter-decomposing fungus producing the right set of ligninolytic enzymes is able to efficiently degrade lignin.

The central importance of ligninolytic enzymes, particularly that of MnP, in lignin degradation is clear, as summarized in several reviews (Hatakka 1994, 2001, Hofrichter 2002). MnP isolated from the most studied wood-decaying fungus *Phanerochaete chrysosporium* was able to depolymerize lignin (Wariishi *et al.* 1991) as well as several phenolic lignin model compounds *in vitro* (Wariishi *et al.* 1989). Straw- and synthetic lignin was depolymerized and mineralized by MnP of the wood-decaying fungus *Nematoloma frowardii* (Hofrichter *et al.* 1999a, Hofrichter *et al.* 1999c) and even milled pine wood could be converted by MnP of *Phlebia radiata in vitro* (Hofrichter *et al.* 2001). In all these studies purified or crude MnP from wood-decaying fungi were used but similar enzyme preparations from LDF were not tested. Comparing both groups of fungi, it is obvious that the latter degrade lignin at a slower rate when cultured on a medium such as straw, which

favors wood-decaying fungi (I). However, purified MnP from a LDF has not yet been used in *in vitro* studies together with a lignin model compound to obtain comparative activity data with counterpart MnP from wood-decaying fungi.

Efficiency differences are, however, not too surprising since wood-decaying fungi are specialized in degrading lignin in wood, their preferred habitat. Litter-decomposing fungi are most likely generalists when compared to white-rot fungi. We could expect that LDF are able to attack a wider range of degradable compounds than white-rot fungi since these fungi are faced with a larger variety of organic compounds in soil-litter than white-rot fungi in wood. The comparison of lignin mineralization however is possible with the results of the work published in article I and other publications. Thus LDF were found to mineralize up to 25% of the DHP whereas wood-decaying fungi such as *N. frowardii* or *Pleurotus* sp. were more than twice as efficient in mineralizing up to 60% (I). Similar and even higher mineralization was obtained in other studies where *Phlebia radiata* or *Phanerochaete chrysosporium* mineralized 71% and 57% respectively (Hatakka and Uusi-Rauva 1983, Leatham 1986). When cultured on an appropriate substrate LDF such as *Collybia dryophila* are able to degrade lignin more efficiently (unpublished results, see Fig. 4.2). If fungi lack ligninolytic activities or express only one of the ligninolytic enzymes (e.g. only laccase), lignin degradation activity remains compromised compared to that of species producing MnP. Less than 5% mineralization was found in fungi that only produce laccase, e.g. *Stropharia semiglobata* (5%; I) or mycorrhizal fungi (max. 4.7%; Trojanowski *et al.* 1984). Lignin mineralization brought about by bacteria (*Streptomyces* spp.) or deuteromycetes is in turn very limited (Hatakka 2001) but might be important in microbial consortia degrading lignin.

From this it can be concluded that white-rot, litter-decomposing, and mycorrhiza forming fungi can be eco-physiologically grouped not only on the basis of their habitat and lifestyle, but also on their ability to degrade lignin. This grouping would place white-rot fungi as efficient, litter-decomposing fungi as moderate, and mycorrhiza as limited or non-lignin degraders (exceptions and overlaps certainly occur).

5.2 The ligninolytic enzyme system of litter-decomposing fungi

All the published articles indicate that several LDF produce MnP and laccase (I-V), and no LiP activities were found in any of the strain tested. Efficient degraders of lignin (I), HA (III), or PAH (IV and V) all produce both laccase and MnP, and MnP was found to be a key enzyme in the degradation of recalcitrant aromatic compounds (I, III-V). The addition of Mn^{2+} to liquid cultures enhanced the production and activity of MnP (I-V).

Though limited data concerning ligninolytic enzymes of LDF are available it seems that the combination of laccase and MnP is not uncommon. The most investigated litter-decomposing fungus *Agaricus bisporus* produces both enzymes (Bonnen *et al.* 1994, Leontievsky *et al.* 1997, Lankinen *et al.* 2001). In addition, *Mycena galopus* (Ghosh *et al.* 2003) and the coprophilic fungus *Paneolus sphinctrinus* (Heinzkill *et al.* 1998) were also reported to produce laccase and MnP. The study of Heinzkill *et al*. (1998) showed also that several *Coprinus* spp. produced laccase and another type of peroxidase. Interestingly, the characteristics (p*I* and MW) were in the same range as that found in our study (II). MnPs of white-rot fungi usually have a p*I* around 3.5 and a MW of 45 kDa (Hofrichter 2002). Most of the MnP of LDF fall into the same range. The MnP of *Agaricus bisporus* has p*I*s of 3.25, 3.3 and 3.5 (Bonnen *et al.* 1994, Lankinen *et al.* 2001) with MWs of 40 kDa which are in the same range of the MnPs from *Stropharia coronilla* and *S. rugosoannulata*. Though

some LDF (*Agrocybe praecox*, *S. coronilla*, and *P. sphinctrinus*) seem to produce a different kind of MnP with similar MW, but much higher p*I* (between 6.3 and 7.2; II, Heinzkill *et al.* 1998). The production of MnP with a p*I* close to neutral seems to be a distinct feature for some LDF when compared to wood-decaying fungi. A missing MnP of this kind in *S. rugosoannulata* implies that this straw colonizing fungus might be more closely related to some wood-decayers than to "true" litter-decomposers. Thus, data of MnP p*I* could provide protein-level support for phylogenetic trees where MnP sequence data has been used. A recent relevant example could be to explain MnP amino-acid sequence variations in spruce, pine or fir specific inter-sterility groups of *Heterobasidion* spp. (Maijala *et al.* 2003).

Manganese can lead to the expression of high p*I* MnP (II) in LDF as well as directly enhance the activity. The positive response to Mn^{2+} addition at least in combination with chelating buffers is already known from MnP producing white-rot fungi (Moilanen *et al.* 1996, Fu *et al.* 1997, Vares and Hatakka 1997). Mn^{2+} clearly increased the production and activity of MnP in liquid cultures of *Agrocybe praecox* and *Stropharia* spp. (I, II, IV, and V). Since Mn is abundant in both wood and soil (Young and Guinn 1966, Blanchette 1984, Barceloux 1999) both ecophysiological groups are able to use Mn in the degradation process of lignocellulose. Furthermore, basidiomycetous fungi produce organic acids such as oxalate or malate (Hofrichter *et al.* 1999b, Hatakka 2001, Mäkelä *et al.* 2002), which work as chelators and stabilize Mn^{3+} ions (Cui and Dolphin 1990, Kishi *et al.* 1994) formed by MnP. It is likely that LDF produce organic acids as well and indications have also been found in our studies (V).

In contrast to MnP, differences between the laccases of wood-decaying and LDF are not so obvious. Laccase is most commonly found with MWs greater than 60 kDa and narrow p*I*s ranging between 3 and 4.5. *Agaricus bisporus*, *Coprinus* spp., *Marasmius quercophilus*, *Paneolus* spp., and *Stropharia rugosoannulata* produced laccases with p*I*s of 3.5-3.6 and MWs of 60-66 kDa (Leontievsky *et al.* 1997, Heinzkill *et al.* 1998, Dedeyan *et al.* 2000, Schlosser and Hofer 2002). Laccases found in this study had MWs ranging from 66-67 kDa with slightly higher p*I*s from 3.3 to 4.4 (II). The p*I*s and MWs of laccases of wood-decaying fungi range from 2.9-5.2 and 53-64 kDa, respectively (Hatakka 1994) and are thus in the same range as those of LDF.

The apparent lack of LiP suggests that MnP is the key enzyme involved in the litter degradation processes of LDF. Though laccase is possibly more common among these fungi, a lack of MnP is most likely to go along with far lower detectable degradation ability. Interestingly, the group of MnP and laccase producing white-rot fungi are found to be the most efficient degraders of lignin (Hatakka 1994) implying that some LDF could be equally efficient in their own habitat.

5.3 Degradation of synthetic and natural humic acids

Collybia dryophila, a common litter-decomposing fungus found in European and North American forests (Phillips 1991, Jordan 1995), decomposed natural HAs and synthetic HAs (prepared from ^{14}C-catechol) in liquid cultures (III). The degradation resulted in the formation of lower molecular mass FAs and carbon dioxide. To our best knowledge comparable studies using LDF appear to have not been reported. As observed in lignin degradation with other LDF (I), HA decomposition was considerably enhanced when Mn^{2+} was present (III). The growth of *C. dryophila* on sterilized pine-spruce forest litter released substantial amounts of FAs from water insoluble material. Due to the high content of Mn in soil-litter (260 mg/kg) an addition of Mn^{2+} did not have any effect on the degradation of

HAs. The high Mn content of litter may select for MnP in LDF as opposed to LiP in white-rot fungi.

Hurst *et al*. (1962) have already shown that several white-rot fungi species (*Trametes* spp., *Hypholoma fasciculare*) were able to degrade HAs from podzol soil. Studies with efficient HA degraders involved mostly white-rot fungi. Different species were used for the degradation of low-rank-coal (brown-coal) derived HAs. Thus several investigations showed that HAs from brown-coal were degraded (decolorized) by *Phanerochaete chrysosporium* (Ralph and Catcheside 1994), *Trametes versicolor* (Fakoussa and Frost 1999), *Nematoloma frowardii* (Hofrichter and Fritsche 1997a), and by an unidentified strain of the order *Agaricales* (Willmann and Fakoussa 1997a). One important finding was that ligninolytic enzymes were involved in the degradation process. All three major enzymes, MnP, LiP, and laccase, were suggested to be involved (Hofrichter and Fritsche 1997a, Willmann and Fakoussa 1997b, Fakoussa and Frost 1999, Ralph and Catcheside 1999). Our recent findings show that *C. dryophila* produces MnP and laccase as well as efficiently degrades ^{14}C-labeled HA and natural HAs (Table 4.1; III). This provides additional support for the role of these enzymes in LDF.

The use of ^{14}C-labeled HA made it possible to carry out a more detailed analysis of the degradation, conversion, and mineralization of HA. Earlier studies using ^{14}C-labeled melanoidins provided evidence that *P. chrysosporium*, as well as *T. versicolor* were able to degrade and mineralize these specific HAs (Blondeau 1989, Dehorter and Blondeau 1992, 1993). Haider and Martin (1988) used ^{14}C-HAs extracted from ^{14}C-wheat straw to monitor the mineralization by *P. chrysosporium*. These studies also suggested that ligninolytic enzymes were involved in the mineralization which was also observed in our study (III). A more recalcitrant synthetic HA polymerized from ^{14}C-catechol was used to prove that MnP was able to mineralize HAs and to form lower molecular mass products thereof (Hofrichter *et al.* 1998b). Additionally, *in vitro* studies with HAs obtained from low-rank-coal and MnP from *Clitocybula dusenii* demonstrated that FA was formed from HA (Ziegenhagen and Hofrichter 1998). *Collybia dryophila* showed enhanced decolorization of HAs and mineralization of ^{14}C-HAs in Mn supplemented media while MnP activity was high (III). According to the results obtained with MnP preparations from white-rot fungi it is most likely that MnP is also responsible for the HA degradation and mineralization by *C. dryophila*. Very few investigations with LDF have yet been performed to support this. Nevertheless, the straw-decomposing fungus *Stropharia rugosoannulata* was found to decolorize HAs derived from brow-coal on agar plates (Hofrichter and Fritsche 1996) and alkaline humic extracts in surface cultures (Gramss *et al.* 1999c). Gramss *et al.* (1999c) also showed that other "true" LDF were able to degrade these compounds but it seemed that they were only half as efficient as white-rot fungi or *S. rugosoannulata*.

Whether or not laccase is involved in the degradation process still needs to be clarified. Laccase production has been observed during HA degradation or is known to occur in fungi able to degrade HAs (Fakoussa and Frost 1999, Scheel *et al.* 1999) but it is also responsible for the polymerization and formation of HAs in compost (Chefetz *et al.* 1998). It might be more important as to whether or not LiP or MnP is produced, in addition to laccase. The humification effect of laccase was brought about by the cellulotytic filamentous fungus *Chaetomium thermophilum*, which apparently does not produce any MnP (Chefetz *et al.* 1998). On the other hand, *Collybia dryophila* produced considerably lower amounts of laccase compared to MnP suggesting that a polymerization effect of laccase is overruled by MnP degradation activity.

Thus, LDF are certainly involved in the degradation of HS and HAs in soil-litter and therefore play an important role in the recycling of soil organic matter (SOM). The known recalcitrance of HS and lignin fragments in soil-litter argues for a significant contribution being made by litter-decomposing fungi to the global carbon cycle.

5.4 Degradation of PAH

Our results demonstrate that LDF are able to attack anthracene, pyrene, and BaP in liquid cultures. Several species were even able to completely convert the compounds when supplemented with Mn^{2+} (IV). Several strains were able to mineralize BaP in liquid cultures and in solid-state straw cultures (IV, V). Comparable data has been reported for white-rot fungi but little is available for LDF. The addition of Mn enhanced the conversion and mineralization for those species producing MnP indicating its important role in the degradation process. Finally, crude and purified MnP was used *in vitro* to convert and mineralize BaP, as well as to convert 16 EPA-PAH in the presence the unsaturated fatty acid derivative Tween 80 (V). To date, only MnP from white-rot fungi has been used in *in vitro* studies.

As previously observed in lignin and HA degradation studies, white-rot fungi have also been used intensively for PAH degradation studies. Several genera, including *Bjerkandera*, *Phanerochaete*, *Pleurotus*, and *Trametes*, were reported to degrade PAH (Kästner 2000a, Pointing 2001). Not surprisingly, work carried out with *P. chrysosporium* was the first to show the oxidation and mineralization of PAH, in particular BaP (Bumpus *et al.* 1985, Sanglard *et al.* 1986, Bumpus 1989, Field *et al.* 1992, Bogan and Lamar 1996). Other species of white-rot fungi such as *Trametes versicolor*, *Bjerkandera* sp, and *Pleurotus* spp. were found to be capable of PAH degradation as well (Field *et al.* 1992, Collins and Dobson 1996, Wolter *et al.* 1997). The results obtained with *Phanerochaete chrysosporium* demonstrated the involvement and importance of ligninolytic enzymes in the degradation process (Bumpus *et al.* 1985, Bogan and Lamar 1996) as demonstrated in our work with LDF. LiP was first found to be involved in the initial oxidation reaction (Haemmerli *et al.* 1986, Hammel *et al.* 1986, Sanglard *et al.* 1986, Bogan *et al.* 1996) but has not yet been found in LDF. The impact of the more widely distributed MnP was shown in the oxidation of phenanthrene and fluorene in *Trametes versicolor* cultures with high MnP activity (Collins and Dobson 1996). Proof of the conversion and mineralization abilities of MnP was obtained when MnP crude preparations from *Nematoloma frowardii* degraded PAH *in vitro* (Sack *et al.* 1997b). Several PAH were even mineralized to some extend (2.5-7.3%), including BaP (4%). Our results show that LDF efficiently degrade PAH compounds though it seems that white-rot fungi are more effective (IV). Furthermore, the degradation of PAH by LDF, as well as white-rot fungi, could be correlated to the extracellular activity of MnP (IV, V). Though not monitored, MnP was most likely to be active during the mineralization of BaP in straw cultures as observed with *Pleurotus* sp. during pyrene mineralization on straw (Lang *et al.* 1996). Efficient mineralization of PAH therefore might be a sign of the production of MnP or LiP. For LDF this points to the importance of MnP as the key enzyme of the PAH mineralization process, since they have not been shown to produce LiP.

A few screening studies have evaluated the potential of LDF to degrade PAH (Martens and Zadrazil 1998, Gramss *et al.* 1999a, Gramss *et al.* 1999b) or other compounds such as TNT (Scheibner *et al.* 1997). Among the litter-decomposers tested *Agrocybe praecox* and *Stropharia rugosoannulata* were reported to be the best PAH degraders in this group. We have now confirmed these results in our more detailed studies (IV). Interestingly, the

litter-decomposer *Marasmiellus troyanus* was used in detailed BaP degradation studies and found to convert and mineralize BaP (Wunch *et al.* 1997, Wunch *et al.* 1999). However, no oxidative enzyme activities were observed at that time. In our studies, the central importance of MnP in the degradation process was clearly identified (IV, V). As with the *in vitro* studies performed with MnP from *Nematoloma frowardii* (Sack *et al.* 1997b), MnP 1 from *Stropharia coronilla* was able to convert BaP completely in 24 hours with some mineralization (1.4%, V). The influence of the unsaturated fatty acid derivative Tween 80 was obvious. Kotterman *et al*. (1998) reported that Tween 80 kept BaP dispersed in liquid cultures of *Bjerkandera* sp. and prolonged enhanced oxidation rates. In our *in vitro* case study, Tween 80 enabled MnP 1 linked degradation of PAH, which otherwise cannot be directly oxidized due to their high ionization potential (Bogan and Lamar 1995, Bogan *et al.* 1996). For the first time we could show that even poorly bioavailable high molecular mass PAH, such as benzo(g,h,i)perylene, were converted by MnP.

The impact of Mn on the activity of MnP and on the degradation and mineralization of PAH in LDF is obvious. Though it seems that Mn has a greater influence on the degradation for LDF than for white-rot fungi (Fig. 4.6), Mn had only limited impact on the degradation of anthracene or the polymeric dye Poly-R-478 for *Bjerkandera* sp. BOS55 (Kotterman *et al.* 1996). This might be explained by the different set of enzymes produced by *Bjerkandera* sp., which are LiP, MnP, and hybrid (versatile) peroxidase (Mester and Field 1998, ten Have *et al.* 1998). LDF, however, seem to produce only MnP and laccase but no LiP (I, II). Thus Mn induces MnP in both, white-rot and litter-decomposing fungi, but decreases LiP activity and the production of aryl metabolites in e.g. *Bjerkandera* sp. (Mester *et al.* 1995, Mester *et al.* 1997) while having possibly little or no effect on versatile peroxidases at all. Consequently the degradative capability will only increase in LDF because of the positive effect of Mn on MnP and there might be only a slight increase in degradation activity in white-rot fungi producing MnP and LiP, because LiP activity will be reduced while, at the same time, MnP activity will be increased which produces an equalizing effect. Similar data was obtained in our studies (Fig. 4.6) especially with *Pleurotus* spp. A positive effect of Mn on other enzymes, such as Cytochrome P-450 monooxygenase, cannot be ruled out.

Taken together, our own studies and those of several other research groups allow us to propose a hypothetical scheme for the mineralization of BaP by the MnP-Mn^{3+} complex that explains the reactions which occur during the oxidative degradation of the BaP molecule (Fig. 5.1). BaP possesses an appropriate redox potential (~7.1 eV) that allows a direct attack by reactive Mn^{3+}-chelate complexes formed by MnP. Simultaneously, organic acids (e.g. oxalate, malonate, malate) act as chelators and are oxidized in the presence of oxygen (O_2) to peroxyl and other radicals including superoxide. These free radicals may initiate and drive forward the following reactions leading to the stepwise oxidation of BaP. After an initial one-electron abstraction of the BaP molecule, an aryl-cation radical may be formed (2), which tends to react with water to give an unstable hydroxyl compound (dien radical) (3). The latter spontaneously releases a proton and an electron, and forms a BaP-ol (4). The phenolic group of this metabolite is a good target for chelated Mn^{3+} which further oxidizes the phenolic moiety into a phenoxyl radical (5). Different mesomeric forms of such a phenoxyl radical can be postulated, among others a carbon centered radical (6) (Gierer 1997). Depending on the subsequent reactions either with oxygen or superoxide, a quinone (8; via a BaP-peroxyl radical 7) and then BaP-diol (9) (Haemmerli *et al.* 1986), or an ether peroxide (10) may be formed. The unstable ether peroxide may undergo spontaneous ring fission leading to the formation of a free carboxylic group (11) (Gierer 1997). This carboxylic group can be split off by Mn^{3+} resulting in the release of CO_2, and the remaining

carbon-centered radical can react further with O_2 and free radicals to give more CO_2 and/or low-molecular mass acids such as formate.

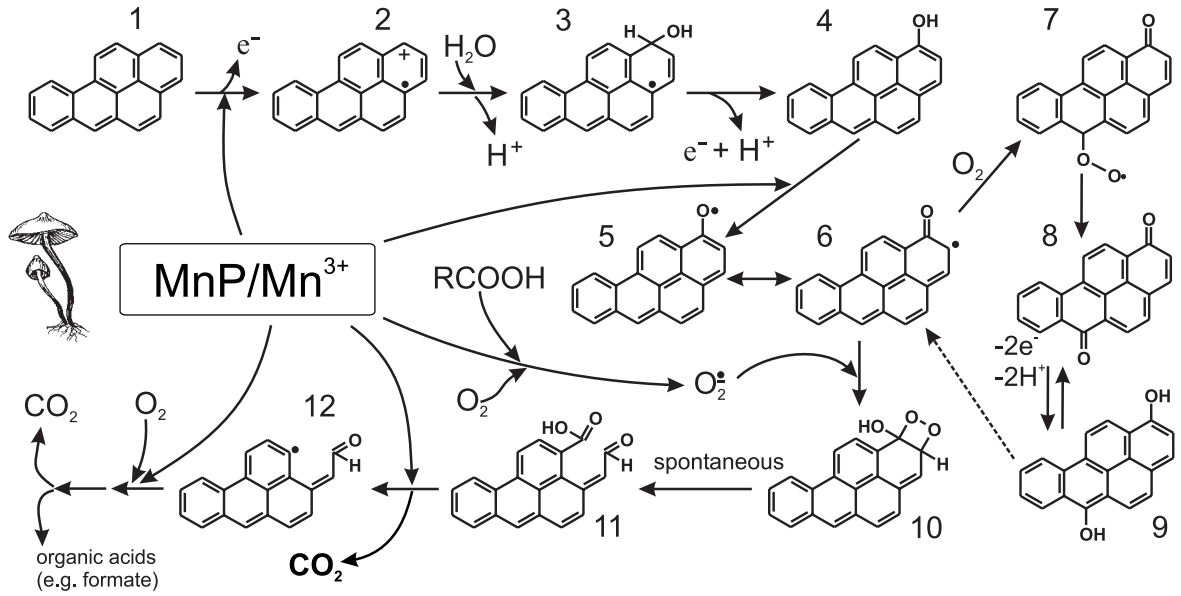


Fig: 5.1: Hypothetical scheme for the mineralization of BaP by the MnP system (After Steffen and Hofrichter and including findings from different research groups cited in the text).

Litter-decomposing fungi produce relatively low amounts of laccase during the degradation of PAH (IV, V). The impact of laccase seems to be much smaller under the tested conditions than that of MnP. Nevertheless, laccase has been shown to be involved in the degradation of different PAH, including BaP (Collins *et al.* 1996, Johannes *et al.* 1996, Pickard *et al.* 1999). The degradation usually required a mediator substance which was added, or produced by the fungus. These natural mediators could be phenols, anilins, or 4-hydroxybenzoic acid (Johannes and Majcherczyk 2000). Synthetic mediators such as 1-hydroxybenzotriazole (HBT) or ABTS were used in several experiments (Collins and Dobson 1996, Johannes *et al.* 1996, Böhmer *et al.* 1998, Pickard *et al.* 1999, Johannes and Majcherczyk 2000). It is clear that further work is needed for a conclusive evaluation of laccase involvement in PAH degradation by LDF.

In addition to MnP, LiP, and laccase, cytochrome P-450 monooxygenase plays a role in PAH breakdown. The filamentous fungus *Cunninghamella elegans* converted BaP to dihydrodiol and other metabolites indicating an initiation through cytochrome P-450 monooxygenase (Cerniglia and Gibson 1979). Similar reactions were observed using the mold *Aspergillus ochraceus* (Datta and Samanta 1988). Furthermore, the white-rot fungus *Pleurotus pulmonarius* was also found to use this enzyme for BaP oxidation (Masaphy *et al.* 1995). However metabolites from cytochrome P-450 monooxygenase catalyzed reactions could not be observed in our studies, which indicated the predominance of ligninolytic enzymes, in this case MnP, in the process. Nevertheless, the involvement of cytochrome P-450 monooxygenase cannot be ruled out in the degradation of certain PAH. This enzyme might be especially involved in the degradation of lower molecular weight compounds in LDF that needs further investigation. *Phanerochaete chrysosporium* was found to use monooxygenase to degrade phenanthrene (Sutherland *et al.* 1991). Findings demonstrated that MnP was not essential for the conversion (Dhawale *et al.* 1992) but LiP

could be involved (Hammel *et al.* 1992). Similar observations were reported in another white-rot fungus, the oyster mushroom *Pleurotus ostreatus*, indicating the involvement of cytochrome P-450 monooxygenase in the degradation of phenanthrene (Bezalel *et al.* 1996b, Bezalel *et al.* 1997) as well as pyrene, anthracene, and fluorene (Bezalel *et al.* 1996a). As in BaP degradation, the formation of a dihydrodiol implied the involvement of this enzyme mechanism (Bezalel *et al.* 1996a). In our own studies quinones, but not dihydrodiols, were found underlining the more profound role of MnP than of cytochrome P-450 monooxygenase in the degradation process of PAH by LDF.

5.5 Future perspectives

Litter-decomposing fungi are able to degrade various recalcitrant aromatic substrates (I-V) and they naturally inhabit the soil-litter layer of forests and grasslands. These properties demand that more ecophysiological research is carried out on LDF. Not only could these fungi be used for bioremediation studies but also their true ecological roles could be evaluated using these and other techniques.

Since evidence for the direct involvement of ligninolytic enzymes of LDF in the degradation of lignin, especially litter-lignin, is still missing, future studies should include a demonstration of the lignin breakdown initiated by MnP and laccase of these fungi. *In vitro* studies with purified or crude enzyme preparations and the use of different native or synthetic lignin preparations, such as DHP or dimers, could reveal the impact on and efficiency of these enzymes in the degradation process. The results could be directly compared to those obtained with wood-decaying fungi and would contribute important knowledge on the role of LDF in the degradation of recalcitrant aromatic substances in nature. Similarly, HS should also be evaluated in the same type of degradation studies. During the course of this work several new ideas were formulated to investigate the fate of MnP in litter, e.g. the half-life and the activity of MnP in litter as well as the actual degradation and fate of lignin and HA in the soil-litter layer.

To date, a number of white-rot fungi have been tested for bioremediation of contaminated soil. Among them were *Phanerochaete chrysosporium* and *Pleurotus ostreatus* (Morgan *et al.* 1993, Eggen and Majcherczyk 1998, Martens *et al.* 1999, Baldrian *et al.* 2000). PAH such as anthracene, phenanthrene, pyrene, benz(a)anthracene, and BaP were degraded and partly mineralized. Interestingly, ligninolytic enzyme activities of MnP and laccase (Novotný *et al.* 1999) and, in case of *Irpex lacteus*, LiP (Novotný *et al.* 2000) could be assayed. Thus, ligninolytic enzymes do play a role in soil bioremediation, but the use of white-rot fungi seems to be limited due to their often poor growth into soil populated by competitive indigenous microorganisms. The use of MnP producing fungi, which are true soil-dwellers, i. e. litter-decomposing fungi, could provide a new arsenal of fungi for the use in soil bioremediation studies.

The field of bioremediation would only be one benefactor of further studies with LDF. A general lack of knowledge on the ecology and the interaction of LDF with their environment is apparent. Further research is required to improve the understanding of the role of LDF in humus turnover in natural habitats such as forests and pastures. In particular their extracellular ligninolytic enzymes need to be studied with regard to the degradation and transformation of all fractions of litter and HS. The interaction with ascomycetes and mycorrhizal fungi as well as bacteria should be evaluated in order to understand the role of LDF in the carbon and nutrient transfer in soil. An encouraging start has been made by Björn Lindahl in his thesis “Nutrient cycling in boreal forest – a mycological perspective” (Lindahl

2001). He evaluated the phosphorous translocation between mycelia of the wood-decaying fungus *Hypholoma fasciculare* to mycorrhizal fungi and associated pine seedlings grown in soil. Recently, Cairney and Meharg (2002) reviewed this subject and high-lightened the extent of interaction between fungal and other microbial groups and their likely influence on carbon and nutrient cycling that still has to be determined. The use of true soil-dwelling saprophytic fungi in these kinds of studies is still lacking and would contribute a great deal to the understanding of fungal communication below-ground. Phylogenetic comparisons of ligninolytic genes from different fungal groups could give an insight to their evolution (Maijala *et al.* 2003), and *in situ* analysis of the expression of these genes in soil could explain the role of these groups and relationships between them.

6. Key findings and conclusions

The following conclusions summarize the main findings of this work in relation to the original aims (see Figure 6.1 for a summary of MnP activity in the soil-litter environment):

1. **Certain litter-decomposing fungi exhibit high degradative capabilities and are able to efficiently mineralize synthetic lignin (23-25% in 12 weeks).**
2. **The most active strains produce manganese peroxidase (MnP) and laccase. Fungi producing MnP are the most efficient degraders of aromatic substances.**
3. **MnP and laccase, but not LiP, are expressed by several litter-decomposing fungi. Mn^{2+} enhanced the degradation of aromatic compounds and it is therefore concluded that MnP is the key enzyme in the degradation process.**
4. **MnPs of litter-decomposing fungi have similar characteristics and resemble those of white-rot fungi. However, the production of MnP with a near neutral p*I* implies that there are differences between MnPs of these two ecophysiological groups of fungi. Near neutral MnPs might be a characteristic of litter-decomposing fungi.**
5. **The degradation of HS including HAs and modified lignin fragments is a key function of litter-decomposing fungi. They are able to degrade HAs to CO_2 and polar, low molecular weight FAs and thus may contribute to the recycling of organic carbon. Mn^{2+} enhanced the degradation in liquid culture further indicating the importance of MnP in the degradation process.**
6. **Litter-decomposing fungi are able to degrade a variety of PAH in liquid culture and to mineralize BaP in liquid and solid-state straw cultures. Crude or purified MnP extracts were able to mineralize and degrade BaP as well as to convert at least 15 different PAH *in vitro* including high molecular weight PAH such as benzo(g,h,i)perylene and indeno(1,2,3-c,d)pyrene. Hence MnP is considered to be the key enzyme in the degradation of PAH by litter-decomposing fungi.**

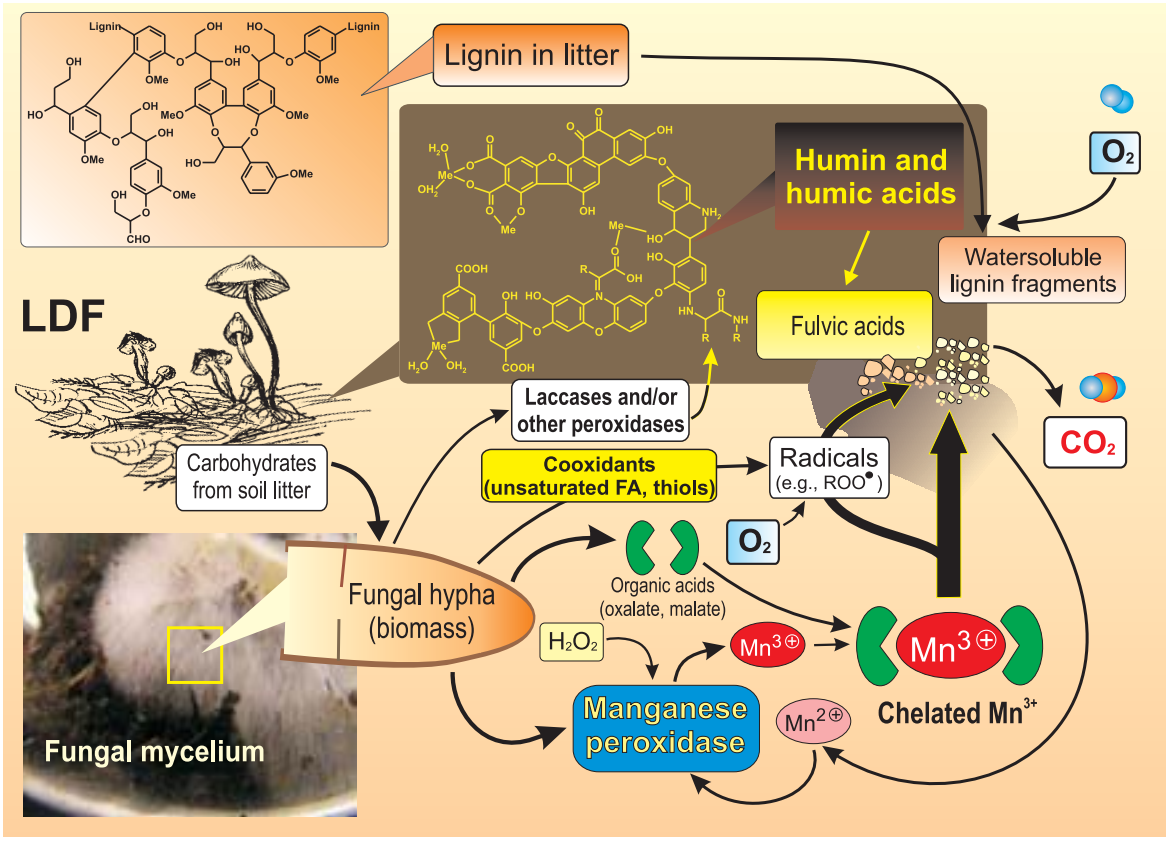


Figure 6.1: Summary of the possible breakdown of lignin and humic substances (HS) by manganese peroxidase (MnP) of litter-decomposing fungi after Hofrichter (2001) with modifications.

The degradation of lignin and HS (and possibly PAH) in the soil-litter layer by LDF is a co-metabolical process driven by carbon from cellulose and hemicelluloses, which is part of the residual lignocellulose in litter (Fig. 6.1). H_2O_2 required for the peroxidase activity is produced by other fungal enzymes as well as Mn^{3+} chelating organic acids such as oxalate or malate. Co-oxidants, such as unsaturated lipids or thiols, as well as O_2, are used in the formation of radicals. Extracellular MnP produced by LDF generates highly reactive Mn^{3+} ions from Mn^{2+} ions present in soil, litter, or wood. Phenolic moieties of lignin or HS are directly attacked by Mn^{3+} and co-oxidants may form reactive radicals that lead to the destruction of the aromatic structures. CO_2, lignin fragments, and FAs are released. In addition to humification it is assumed that, because of their aromatic structure, PAH will undergo the same type of destruction in soil.

7. Acknowledgements

This work was carried out at the Division of Microbiology, Department of Applied Chemistry and Microbiology, in the Biocenter of the University of Helsinki. The major part of this study was connected to two projects (39906 and 52063) funded by the Academy of Finland especially the project "Bioconversion of recalcitrant soil organic matter by litter-decomposing basidiomycetous fungi (52063)" which was awarded to my supervisor Prof. Martin Hofrichter. I am very grateful to him for all the knowledge he shared with me and for all the methods he taught me. I admire his knowledge in the field and look up to him as my master.

I was very lucky to work in Prof. Annele Hatakka's group "Environmental Biotechnology and Biotechnology of Renewable Natural Resources (the Lignin Group)". She was my second supervisor and co-author of the articles. I thank her for giving me the opportunity to work in her group and laboratory and for accepting me as her doctoral student.

In addition to the funding of the Academy project, I want to thank the Graduate School of Environmental Science and Technology (EnSTe) for accepting me as a student and thus giving me the convenience of a salary during the time of my Ph.D. work.

I want to thank Prof. Jim Field and Doc Robin Sen for reviewing my thesis and for their valuable comments.

My warmest thanks go to my colleagues in our research group for providing a pleasant working environment: Aila, Beata, Kristiina, Marja, Miia, Outi, Pauliina, Pekka, Petri, Sari, Taina, Terhi, and to our former group members Alex, Kent, Mika, PekkO, and Yu-Cheng. Several trainees helped me in our lab during my Ph.D. work and Elsa, Inga, Jarmo, Ralf, Sanna, Suvi, and Sven are acknowledged.

I am grateful to Kaj-Roger Hurme for his help and Antti Uusi-Rauva for providing the facilities of the isotopic laboratory.

All colleagues at the Division of Microbiology and our Centre of Excellence are thanked for providing a nice working environment where a scientist can ask for help and get some. Especially German, Kaarina, Leo, Leone, Marko, Mirja, Per, Riitta, Timo, and Zewdu, as well as our secretaries Leena and Hannele are acknowledged.

I thank Fred Gates for proof-reading my thesis and my brother OOz for the layout of this book.

Finally I wish to thank my family and friends for their participation in my life outside the work. I owe my deepest gratitude to my parents Auli and Manfred for supporting me throughout all my studies at the university and Malla and Henning for taking care of my son Mats for several weeks so that I could finish my work.

My greatest thanks belong to my beloved wife for not only taking care of our son but organizing a great deal of daily life and "the party".

Helsinki, October 2003

8. References

Adhi, T. P., Korus, R. A., and Crawford, D. L. (1989) Production of major extracellular enzymes during lignocellulose degradation by two streptomycetes in agitated submerged culture. Appl. Environ. Microbiol., 55:1165-1168.

Adler, E. (1977) Lignin Chemistry - Past, Present and Future. Wood Sci. Technol., 11:169-218.

Akthar, M., Blanchette, R. A., and Kirk, T. K. (1997) Fungal delignification and biomechanical pulping of wood. In K. E. Eriksson (ed) *Advances in Biochemical Engineering Biotechnology*, Springer Verlag, Germany. 159-195.

Argyropoulos, D. S. and Menachem, S. B. (1997) Lignin. In K. E. Eriksson (ed) *Advances in Biochemical Engineering Biotechnology*, Springer-Verlag, Germany. 127-158.

Baldrian, P., in der Wiesche, C., Gabriel, J., Nerud, F., and Zadrazil, F. (2000) Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. Appl. Environ. Microbiol., 66:2471-2478.

Barceloux, D. G. (1999) Manganese. J. Toxicol. Clinical Toxicol., 37:293-307.

Barr, D. P. and Aust, S. D. (1994) Mechanisms white-rot fungi use to degrade pollutants. Environ. Sci. Technol., 28:78-87.

Barry, J. P., Norwood, C., and Vouros, P. (1996) Detection and identification of benzo[a]pyrene diol epoxide adducts to DNA utilizing capillary electrophoresis-electrospray mass spectrometry. Anal. Chem., 68:1432-1438.

Bezalel, L., Hadar, Y., and Cerniglia, C. E. (1997) Enzymatic mechanisms involved in phenanthrene degradation by the white-rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol., 63:2495-2501.

Bezalel, L., Hadar, Y., Fu, P. P., Freeman, J. P., and Cerniglia, C. E. (1996a) Initial oxidation products in the metabolism of pyrene, anthracene, fluorene and dibenzothiophene by white-rot fungus *Pleurotus ostreatus.* Appl. Environ. Microbiol., 62:2554-2559.

Bezalel, L., Hadar, Y., Fu, P. P., Freeman, J. P., and Cerniglia, C. E. (1996b) Metabolism of phenanthrene by the white rot fungus *Pleurotus ostreatus*. Appl. Environ. Microbiol., 62:2547-2553.

Blanchette, R. A. (1984) Manganese accumulation in wood decayed by white rot fungi. Phytopathology, 74:725-730.

Blondeau, R. (1989) Biodegradation of natural and synthetic humic acids by the white-rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol., 55:1282-1285.

Blumer, M. (1976) Polycyclic aromatic compounds in nature. Scientific American, 234: 35-45.

Bogan, B. W. and Lamar, R. T. (1995) One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol., 61:2631-2635.

Bogan, B. W. and Lamar, R. T. (1996) Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. Appl. Environ. Microbiol., 62:1597-1603.

Bogan, B. W., Lamar, R. T., and Hammel, K. E. (1996) Fluorene oxidation in vivo by *Phanerochaete chrysosporium* and in vitro during manganese peroxidase-dependent lipid peroxidation. Appl. Environ. Microbiol., 62:1788-1792.

Böhmer, S., Messner, K., and Srebotnik, E. (1998) Oxidation of phenanthrene by a fungal laccase in the presence of 1-hydroxybenzotriazole and unsaturated lipids. Biochem. Biophys. Res. Comm., 244:233-238.

Bollag, J. M. and Leonowicz, A. (1984) Comparative studies of extracellular fungal laccases. Appl. Environ. Microbiol., 48:849-854.

Bonnen, A. M., Anton, L. H., and Orth, A. B. (1994) Lignin-degrading enzymes of the commercial button mushroom *Agaricus bisporus*. Appl. Environ. Microbiol., 60:960-965.

Bourbonnais, R. and Paice, M. G. (1990) Oxidation of nonphenolic substrates - an expanded role for laccase in lignin biodegradation. FEBS Lett., 267:99-102.

Brown, A. (1985) Review of lignin biomass. J. Appl. Biochem., 7:371-387.

Brunow, G. (2001) Methods to reveal the structure of lignin. In M. Hofrichter and A. Steinbüchel (eds) *Biopolymers*, Vol. 1, Wiley-VCH, Weinheim, Germany. 89-116.

Brunow, G., Raiskila, S., and Sipilä, J. (1998) The incorporation of 3,4-dichloroaniline, a pesticide metabolite, into dehydrogenation polymers of coniferyl alcohol (DHPs). Acta Chemica Scandinavica, 52:1338-1342.

Bumpus, J. A. (1989) Biodegradation of polycyclic hydrocarbons by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol., 55:154-158.

Bumpus, J. A., Tien, M., Wright, D., and Aust, S. D. (1985) Oxidation of persistent environmental pollutants by a white-rot fungus. Science, 228:1434-1436.

Buswell, J. A. and Odier, E. (1987) Lignin biodegradation. CRC Crit. Rev. Biotechnol., 6: 1-60.

Cairney, J. W. G. and Meharg, A. A. (2002) Interactions between ectomycorrhizal fungi and soil saprotrophs: implications for decomposition of organic matter in soils and degradation of organic pollutants in the rhizosphere. Can. J. Botany, 80:803-809.

Call, H. P. and Mücke, I. (1997) History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym$^{(R)}$-process). J. Biotechnol., 53: 163-202.

Cerniglia, C. E. (1984) Microbial metabolism of polycyclic aromatic hydrocarbons. Adv. Appl. Microbiol., 30:31-71.

Cerniglia, C. E. (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation, 3:351-368.

Cerniglia, C. E. (1993) Biodegradation of polycyclic aromatic hydrocarbons. Curr. Opin. Biotechnol., 4:331-338.

Cerniglia, C. E. (1997) Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. J. Industr. Microbiol. Biotechnol., 19: 324-333.

Cerniglia, C. E. and Gibson, D. T. (1979) Oxidation of benzo(a)pyrene by the filamentous fungus *Cunninghamella elegans*. J. Biol. Chem., 254:12174-12180.

Cerniglia, C. E. and Heitkamp, M. A. (1989) Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment. In U. Varanasi (ed) *Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment*, CRC Press, Inc., Boca Raton, Florida. 41-68.

Chefetz, B., Chen, Y., and Hadar, Y. (1998) Purification and characterization of laccase from *Chaetomium thermophilium* and its role in humification. Appl. Environ. Microbiol., 64:3175-3179.

Chen, D. M., Bastias, B. A., Taylor, A. F. S., and Cairney, J. W. G. (2003) Identification of laccase-like genes in ectomycorrhizal basidiomycetes and transcriptional regulation by nitrogen in *Piloderma byssinum*. New Phytologist, 157:547-554.

Chen, D. M., Taylor, A. F. S., Burke, R. M., and Cairney, J. W. G. (2001) Identification of genes for lignin peroxidases and manganese peroxidases in ectomycorrhizal fungi. New Phytologist, 152:151-158.

Claus, H. (2003) Laccases and their occurrence in prokaryotes. Arch. Microbiol., 179:145-150.

Collins, P. J. and Dobson, A. D. W. (1996) Oxidation of fluorene and phenanthrene by Mn(II) dependent peroxidase activity in whole cultures of *Trametes (Coriolus) versicolor*. Biotechnol. Lett., 18:801-804.

Collins, P. J. and Dobson, A. D. W. (1997) Regulation of laccase gene transcription in *Trametes versicolor*. Appl. Environ. Microbiol., 63:3444-3450.

Collins, P. J., Kotterman, M. J., Field, J. A., and Dobson, A. D. W. (1996) Oxidation of anthracene and benzo(a)pyrene by laccases from *Trametes versicolor*. Appl. Environ. Microbiol., 62:4563-4567.

Colpaert, J. V. and vanLaere, A. (1996) A comparison of the extracellular enzyme activities of two ectomycorrhizal and a leaf-saprotrophic basidiomycete colonizing beech leaf litter. New Phytologist, 134:133-141.

Colpaert, J. V. and vanTichelen, K. K. (1996) Decomposition, nitrogen and phosphorus mineralization from beech leaf litter colonized by ectomycorrhizal or litter-decomposing basidiomycetes. New Phytologist, 134:123-132.

Cox, P., Wilkinson, S. P., and Anderson, J. M. (2001) Effects of fungal inocula on the decomposition of lignin and structural polysaccharides in *Pinus sylvestris* litter. Biol. Fertil. Soils., 33:246-251.

Cui, F. and Dolphin, D. (1990) The role of manganese in model systems related to lignin biodegradation. Holzforschung, 44:279-283.

Dabestani, R. and Ivanov, N. (1999) Invited review: A compilation of physical, spectroscopic and photophysical properties of polycyclic aromatic hydrocarbons. Photochem. Photobiol., 70:10-34.

Datta, D. and Samanta, T. B. (1988) Effect of inducers on metabolism of benzo(a)pyrene in vivo and in vitro: analysis by high pressure liquid chromatography. Biochem. Biophys. Res. Commun., 155:493-502.

Dedeyan, B., Klonowska, A., Tagger, S., Tron, T., Iacazio, G., Gil, G., and Le Petit, J. (2000) Biochemical and molecular characterization of a laccase from *Marasmius quercophilus*. Appl. Environ. Microbiol., 66:925-929.

Deguchi, T., Kitaoka, Y., Kakezawa, M., and Nishida, T. (1998) Purification and characterization of a nylon-degrading enzyme. Appl. Environ. Microbiol., 64:1366-1371.

Dehorter, B. and Blondeau, R. (1992) Extracellular enzyme activities during humic acid degradation by the white rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor*. FEMS Microbiol. Lett., 94:209-215.

Dehorter, B. and Blondeau, R. (1993) Isolation of an extracellular Mn-dependent enzyme mineralizing melanoidins from the white-rot fungus *Trametes versicolor*. FEMS Microbiol. Lett., 109:117-122.

Dehorter, B., Kontchou, C. Y., and Blondeau, R. (1992) ^{13}C-NMR spectroscopic

analysis of soil humic acids recovered after incubation with some white rot fungi and actinomycetes. Soil Biol. Biochem., 24:667-673.

Dhawale, S. W., Dhawale, S. S., and Dean-Ross, D. (1992) Degradation of phenanthrene by *Phanerochaete chrysosporium* occurs under ligninolytic as well as nonligninolytic conditions. Appl. Environ. Microbiol., 58:3000-3006.

Dix, N. J. and Webster, J. (1995) Fungal ecology. Chapman & Hall, London, UK.

Dodson, P. J., Evans, C. S., Harvey, P. J., and Palmer, J. M. (1987) Production and properties of an extracellular peroxidase from *Coriolus versicolor* which catalyzes C-alpha C-beta cleavage in a lignin model-compound. FEMS Microbiol. Lett., 42:17-22.

Dorado, J., Almendros, G., Camarero, S., Martinez, A. T., Vares, T., and Hatakka, A. (1999) Transformation of wheat straw in the course of solid-state fermentation by four ligninolytic basidiomycetes. Enzyme Microb. Technol., 25:605-612.

Durrant, A. J., Wood, D. A., and Cain, R. B. (1991) Lignocellulose biodegradation by *Agaricus bisporus* during solid substrate fermentation. J. Gen. Microbiol., 137:751-755.

Eggen, T. and Majcherczyk, A. (1998) Removal of polycyclic aromatic hydrocarbons (PAH) in contaminated soil by white-rot fungus *Pleurotus ostreatus*. Int. Biodet. Biodegr., 41:111-117.

Eggert, C., Temp, U., Dean, J. F., and Eriksson, K. E. (1996) A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. FEBS Lett., 391:144-148.

Eggert, C., Temp, U., and Eriksson, K. E. (1997) Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*. FEBS Lett., 407:89-92.

Eriksson, K. E., Blanchette, R. A., and Ander, P. (1990) Microbial and Enzymatic Degradation of Wood and Wood Components. Springer Verlag, New York, NY.

Faix, O., Mozuch, M. D., and Kirk, T. K. (1985) Degradation of gymnosperm (guaiacyl) vs angiosperm (syringyl/guaiacyl) lignins by *Phanerochaete chrysosporium*. Holzforschung, 39:203-208.

Fakoussa, R. M. and Frost, P. J. (1999) In vivo-decolorization of coal-derived humic acids by laccase-excreting fungus *Trametes versicolor*. Appl. Microbiol. Biotechnol., 52: 60-65.

Fakoussa, R. M. and Hofrichter, M. (1999) Biotechnology and microbiology of coal degradation. Appl. Microbiol. Biotechnol., 52:25-40.

Field, J. A. (2001) Recalcitrance as a catalyst for new developments. Water Sci. Technol., 44:33-40.

Field, J. A., de Jong, E., Feijoo Costa, G., and de Bont, J. A. (1992) Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi. Appl. Environ. Microbiol., 58:2219-2226.

Forrester, I. T., Grabski, A. C., Burgess, R. R., and Leatham, G. F. (1988) Manganese, Mn-dependent peroxidase, and the biodegradation of lignin. Biophys. Biochem. Res. Comm., 157:992-999.

Frimmel, F. H. (2001) Aquatic humic substances. In M. Hofrichter and A. Steinbüchel (eds) *Lignin, humic substances and coal*, Vol. 1, Wiley-VCH, Weinheim, Germany. 301-324.

Fritsche, W., Scheibner, K., Herre, A., and Hofrichter, M. (2000) Fungal degradation of explosives: TNT and related nitroaromatic compounds. In J. C. Spain, J. B. Hughes and H.-J. Knackmuss (eds) *Biodegradation of Nitroaromatic Compounds and explosives*, Lewis Publishers, Washington, D.C., 213-237.

Fu, S. Y., Yu, H.-S., and Buswell, J. A. (1997) Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by *Pleurotus sajor-caju*. FEMS Microbiol. Lett., 147:133-137.

Galkin, S., Vares, T., Kalsi, M., and Hatakka, A. (1998) Production of organic acids by different white-rot fungi as detected using capillary zone electrophoresis. Biotechnol. Techniques, 12:267-271.

Ghosh, A., Frankland, J. C., Thurston, C. F., and Robinson, C. H. (2003) Enzyme production by *Mycena galopus* mycelium in artificial media and in *Picea sitchensis* F_1 horizon litter. Mycol. Res., 107:996-1008.

Gierer, J. (1997) Formation and involvement of superoxide ($O_2^{\cdot}$-/$HO_2^{\cdot}$) and hydroxyl ($OH^{\cdot}$) radicals in TCF bleaching processes: a review. Holzforschung, 51:34-46.

Glenn, J. K. and Gold, M. H. (1985) Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys., 242:329-341.

Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M., and Gold, M. H. (1983) An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Comm., 114:1077-1083.

Gold, M. H. and Alic, M. (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev., 57:605-622.

Gold, M. H., Wariishi, H., and Valli, K. (1989) Extracellular peroxidases involved in lignin degradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. In J. R. Whitaker and P. E. Sonnet (eds) *Biocatalysis in Agricultural Biotechnology*, Vol. ACS Symp. Ser. No. 389, The American Chemical Society, Washington, DC. 128-140.

Gramss, G., Günther, T., and Fritsche, W. (1998) Spot tests for oxidative enzymes in ectomycorrhizal, wood-, and litter decaying fungi. Mycol. Res., 102:67-72.

Gramss, G., Kirsche, B., Voigt, K.-D., Günther, T., and Fritsche, W. (1999a) Conversion rates of five polycyclic aromatic hydrocarbons in liquid cultures of fifty-eight fungi and the concomitant production of oxidative enzymes. Mycol. Res., 103:1009-1018.

Gramss, G., Voigt, K. D., and Kirsche, B. (1999b) Degradation of polycyclic aromatic hydrocarbons with three to seven aromatic rings by higher fungi in sterile and unsterile soils. Biodegradation, 10:51-62.

Gramss, G., Ziegenhagen, D., and Sorge, S. (1999c) Degradation of soil humic extract by wood- and soil-associated fungi, bacteria, and commercial enzymes. Microbial Ecology, 37:140-151.

Griffin, D. H. (1994) Fungal Physiology. Wiley-Liss., New York.

Günther, T., Sack, U., Hofrichter, M., and Lätz, M. (1998) Oxidation of PAH and PAH-derivatives by fungal and plant oxidoreductases. J. Bas. Microbiol., 38:113-122.

Haemmerli, S. D., Leisola, M. S. A., Sanglard, D., and Fiechter, A. (1986) Oxidation of benzo(a)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. J. Biol. Chem., 261:6900-6903.

Haider, K. (1998) Physical and chemical stabilisation mechanisms of RSOM. Mittl. Dtsch. Bodenkundl. Ges., 87:119-132.

Haider, K. and Trojanowski, J. (1975) Decomposition of specifically ^{14}C-labeled phenols and dehydropolymers of coniferyl alcohol as models for lignin degradation by soft and white rot fungi. Arch. Microbiol., 105:33-41.

Haider, K. M. and Martin, J. P. (1988) Mineralization of ^{14}C-labeled humic acids and of humic acid bound ^{14}C xenobiotics by *Phanerochaete chrysosporium*. Soil Biol. Biochem., 20:425-429.

Hammel, K. E., Gai, W. Z., Green, B., and Moen, M. A. (1992) Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol., 58:1832-1838.

Hammel, K. E., Kalyanaraman, B., and Kirk, T. K. (1986) Oxidation of polycyclic aromatic hydrocarbons and dibenzo[p]-dioxins by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem., 261:16948-16952.

Hammel, K. E. and Tardone, P. J. (1988) The oxidative 4-dechlorination of polychlorinated phenols is catalyzed by extracellular fungal lignin peroxidases. Biochem., 27:6563-6568.

Haselwandter, K., Bobleter, O., and Read, D. J. (1990) Degradation of ^{14}C-labelled lignin and dehydropolymer of coniferyl alcohol by ericoid and ectomycorrhizal fungi. Arch. Microbiol., 153:352-354.

Hatakka, A. (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev., 13:125-135.

Hatakka, A. (2001) Biodegradation of lignin. In M. Hofrichter and A. Steinbüchel (eds) *Lignin, Humic Substances and Coal*, Vol. 1, Wiley-VCH, Weinheim, Germany. 129-180.

Hatakka, A., Buswell, J. A., Pirhonen, T. I., and Uusi-Rauva, A. K. (1983) Degradation of ^{14}C-labelled lignins by white-rot fungi. In T. Higuchi, H. Chang and T. K. Kirk (eds) *Recent Advances in Lignin Biodegradation Research*, Uni Publishers Co., Ltd., Tokyo, Japan. 176-187.

Hatakka, A. I. and Uusi-Rauva, A. K. (1983) Degradation of ^{14}C-labeled poplar wood lignin by selected white-rot fungi. Europ. J. Appl. Microbiol. Biotechnol., 17:235-242.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C., and Pegler, D. N. (1995) Ainsworth & Bisby´s Dictionary of the Fungi. 8 Edition, University Press, Cambridge, U.K.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P., and Anke, T. (1998) Characterization of laccases and peroxidases from wood-rotting fungi (family *Coprinaceae*). Appl. Environ. Microbiol., 64:1601-1606.

Higuchi, T. (1985) Biosynthesis of lignin. In T. Higuchi (ed) *Biosynthesis and Biodegradation of Wood Components*, Academic Press, San Diego. 141-160.

Higuchi, T., Shimada, M., Nakatsubo, F., and Tanahashi, M. (1977) Differences in biosyntheses of guaiacyl and syringyl lignins in woods. Wood Sci. Technol., 11:153-167.

Hintikka, V. (1970) Studies on white-rot humus formed by higher fungi in forest soils. Communicationes Instituti Forestalis Fenniae, 69:2.

Hofrichter, M. (2002) Review: Lignin conversion by manganese peroxidase (MnP). Enzyme Microbiol. Technol., 30:454-466.

Hofrichter, M., Bublitz, F., and Fritsche, W. (1997) Fungal attack on coal: II. Solubilization of low-rank coal by filamentous fungi. Fuel Process. Technol., 52:55-64.

Hofrichter, M. and Fakoussa, R. (2001) Microbial degradation and modification of coal. In A. Steinbüchel and M. Hofrichter (eds) *Lignin, Humic Substances and Coal*, Vol. 1, Wiley-VCH, Weinheim, Germany. 393-427.

Hofrichter, M. and Fritsche, W. (1996) Depolymerization of low-rank coal by extracellular fungal enzyme systems. I. Screening for low-rank-coal-depolymerizing activities. Appl. Microbiol. Biotechnol., 46:220-225.

Hofrichter, M. and Fritsche, W. (1997a) Depolymerization of low-rank coal by extracellular fungal enzyme systems. II. The ligninolytic enzymes of the coal-humic-acid-depolymerizing fungus *Nematoloma frowardii* b19. Appl. Microbiol. Biotechnol., 47:419-424.

Hofrichter, M. and Fritsche, W. (1997b) Depolymerization of low-rank coal by extracellular fungal enzyme systems. III. In vitro depolymerization of coal humic acids by a crude preparation of manganese peroxidase from the white-rot fungus *Nematoloma frowardii* b19. Appl. Microbiol. Biotechnol., 47:566-571.

Hofrichter, M., Lundell, T., and Hatakka, A. (2001) Conversion of milled pine wood by manganese peroxidase from *Phlebia radiata*. Appl. Environ. Microbiol., 67:4588-4593.

Hofrichter, M., Scheibner, K., Bublitz, F., Schneegaß, I., Ziegenhagen, D., Martens, R., and Fritsche, W. (1999a) Depolymerization of straw lignin by manganese peroxidase from *Nematoloma frowardii* is accompanied by release of carbon dioxide. Holzforschung, 53:161-166.

Hofrichter, M., Scheibner, K., Schneegass, I., and Fritsche, W. (1998a) Enzymatic combustion of aromatic and aliphatic compounds by manganese peroxidase from *Nematoloma frowardii*. Appl. Environ. Microbiol., 64:399-404.

Hofrichter, M., Scheibner, K., Schneegaß, I., Ziegenhagen, D., and Fritsche, W. (1998b) Mineralization of synthetic humic substances by manganese peroxidase from the white-rot fungus *Nematoloma frowardii*. Appl. Microbiol. Biotechnol., 49:584-588.

Hofrichter, M., Vares, T., Kalsi, M., Galkin, S., Scheibner, K., Fritsche, W., and Hatakka, A. (1999b) Production of manganese peroxidase and organic acids and mineralization of ^{14}C-labelled lignin (^{14}C-DHP) during solid-state fermentation of wheat straw with the white-rot fungus *Nematoloma frowardii*. Appl. Environ. Microbiol., 65:1864-1870.

Hofrichter, M., Vares, T., Scheibner, K., Galkin, S., Sipilä, J., and Hatakka, A. (1999c) Mineralization and solubilization of synthetic lignin by manganese peroxidases from *Nematoloma frowardii* and *Phlebia radiata*. J. Biotechnol., 67:217-228.

Hurst, H. M., Burges, A., and Latter, P. (1962) Some aspects of the biochemistry of humic acid decomposition by fungi. Phytochem., 1:227-231.

Jensen, V. (1974) Decomposition of angiosperm tree leaf litter. In C. H. Dickinson and G. J. F. Pugh (eds) *Biology of Plant Litter Decomposition*, Vol. 1, Academic Press, London, U.K., 241.

Johannes, C. and Majcherczyk, A. (2000) Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl. Environ. Microbiol., 66:524-528.

Johannes, C., Majcherczyk, A., and Hüttermann, A. (1996) Degradation of anthracene by laccase of *Trametes versicolor* in the presence of different mediator compounds. Appl. Microbiol. Biotechnol., 46:313-317.

Jordan, M. (1995) Encoclypedia of Fungi in Britain and Europe. David & Charles, Newton Abbot, Devon, U.K.

Juhasz, A. L. and Naidu, R. (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: A review of the microbial degradation of benzo[a]pyrene. Int. Biodeterior. Biodegr., 45:57-88.

Kapoor, M. and Lin, W. S. (1984) Studies on the induction of aryl hydrocarbon (benzo[a]pyrene) hydroxylase in *Neurospora crassa*, and its suppression by sodium selenite. Xenobiotica, 14:903-915.

Karhunen, P., Rummakko, P., Sipilä, J., Brunow, G., and Kilpeläinen, I. (1995a) Dibenzodioxocins - a novel type of linkage in softwood lignins. Tetrahedron Lett., 36: 169-170.

Karhunen, P., Rummakko, P., Sipilä, J., Brunow, G., and Kilpeläinen, I. (1995b) The formation of dibenzodioxocin structures by oxidative coupling - a model reaction for lignin biosynthesis. Tetrahedron Lett., 36:4501-4504.

Kästner, M. (2000a) Degradation of aromatic and polyaromatic compounds. In H.-J. Rehm and G. Reed (eds) *Biotechnology*, Vol. 11b, Wiley-VCH, Weinheim, Germany. 211-239.

Kästner, M. (2000b) "Humification" Process or Formation of Refractory Soil Organic Matter. In J. Klein (ed) *Environmental Processes II*, Vol. 11b, 2 nd Edition Wiley-VCH, Weinheim, Germany. 89-125.

Kästner, M. and Hofrichter, M. (2001) Biodegradation of humic substances. In M. Hofrichter and A. Steinbüchel (eds) *Biopolymers. Lignin, Humic Substances and Coal*, Vol. 1, Wiley-VCH, Weinheim, Germany. 349-378.

Kersten, P. J., Kalyanaraman, B., Hammel, K. E., Reinhammar, B., and Kirk, T. K. (1990) Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. Biochem. J., 268:475-480.

Kersten, P. J. and Kirk, T. K. (1987) Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. J. Bacteriol., 169:2195-2201.

Kirk, T. K. (1984) Degradation of lignin. In D. T. Gibson (ed) *Microbial Degradation of Organic Compounds*, Marcel Dekker, New York, NY. 399-437.

Kirk, T. K., Connors, W. J., Bleam, R. D., Hackett, W. F., and Zeikus, J. G. (1975) Preparation and microbial decomposition of synthetic [^{14}C]-lignins. Proc. Nat. Acad. Sci. USA, 72:2515-2519.

Kirk, T. K. and Cullen, D. (1998) Enzymology and molecular genetics of wood degradation by white-rot fungi. In R. A. Young and M. Akthar (eds) *Environmentally Friendly Technologies for the Pulp and Paper Industry*, John Wiley & Sons, New York, NY. 273-307.

Kirk, T. K. and Farrell, R. L. (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol., 41:465-505.

Kishi, K., Wariishi, H., Marquez, L., Dunford, H. B., and Gold, M. H. (1994) Mechanism of manganese peroxidase compound II reduction. Effect of organic acid chelators and pH. Biochemistry, 33:8694-8701.

Klein, J., Fakoussa, R., Hölker, U., Hofrichter, M., Schmiers, H., Sinder, C., and Steinbüchel, A. (2001) Biotechnology of Coal. In H.-J. Rehm (ed) *Special Processes*, Vol. 10, Wiley-VCH, Weinheim, Germany. 153-189.

Kotterman, M. J., Vis, E. H., and Field, J. A. (1998) Successive mineralization and detoxification of benzo[a]pyrene by the white rot fungus *Bjerkandera* sp. strain BOS55 and indigenous microflora. Appl. Environ. Microbiol., 64:2853-2858.

Kotterman, M. J. J., Wasseveld, R. A., and Field, J. A. (1996) Hydrogen peroxide production as a limiting factor in xenobiotic compound oxidation by nitrogen-sufficient cultures of *Bjerkandera* sp. strain BOS55 overproducing peroxidases. Appl. Environ. Microbiol., 62:880-885.

Kuan, I. C., Johnson, K. A., and Tien, M. (1993) Kinetic analysis of manganese peroxidase - the reaction with manganese complexes. J. Biol. Chem., 268:20064-20070.

Kuhad, R. C., Singh, A., and Eriksson, K. E. (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. In K. E. Eriksson (ed) *Advances in Biochemical Engineering Biotechnology*, Springer-Verlag, Germany. 46-125.

Kuwahara, M., Glenn, J. K., Morgan, M. A., and Gold, M. H. (1984) Separation and characterization of 2 extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett., 169:247-250.

Lackner, R., Srebotnik, E., and Messner, K. (1991) Oxidative degradation of high molecular weight chlorolignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Comm., 178:1092-1098.

Lang, E., Nerud, F., Novotná, E., Zadrazil, F., and Martens, R. (1996) Production of ligninolytic exoenzymes and ^{14}C-pyrene mineralization by *Pleurotus* sp. in lignocellulose substrate. Folia Microbiol., 41:489-493.

Lange, B., Kremer, S., Sterner, O., and Anke, H. (1996) Metabolism of pyrene by basidiomycetous fungi of the genera *Crinipellis*, *Marasmius*, and *Marasmiellus*. Can. J. Microbiol., 42:1179-1183.

Langford, C. H., Gamble, D. S., Underdown, A. W., and Lee, S. (1983) Interaction of metal ions with a well characterized fulvic acid. In R. F. Christman and E. T. Gjessing (eds) *Aquatic and Terrestrial Humic Materials*, Ann Arbor Science, Ann Arbor, MI. 219-237.

Lankinen, V. P., Bonnen, A. M., Anton, L. H., Wood, D. A., Kalkkinen, N., Hatakka, A., and Thurston, C. F. (2001) Characteristics and N-terminal amino acid sequence of manganese peroxidase from solid substrate cultures of *Agaricus bisporus*. Appl. Microbiol. Biotechnol., 55:170-176.

Leatham, G. F. (1986) The ligninolytic activities of *Lentinus edodes* and *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol., 24:51-58.

Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtas-Wasilewska, M., Cho, N. S., Hofrichter, M., and Rogalski, J. (1999) Biodegradation of lignin by white rot fungi. Fungal Genet. Biol., 27:175-185.

Leonowicz, A. and Trojanowski, J. (1965) Exoenzymes in fungi degrading lignin. I. *Pholiota mutabilis*. Acta Microbiologica Polonica, 14:55-61.

Leontievsky, A. A., Vares, T., Lankinen, P., Shergill, J. K., Pozdnyakova, N. N., Myasoedova, N. M., Kalkkinen, N., Golovleva, L. A., Cammack, R., Thurston, C. F., and Hatakka, A. (1997) Blue and yellow laccases of ligninolytic fungi. FEMS Microbiol. Lett., 156:9-14.

Lindahl, B. (2001) Nutrient cycling in boreal forests - a mycological perspective. Vol. Silvestria 214, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Lundell, T. (1993) Ligninolytic System of the White-rot Fungus *Phlebia radiata*: Lignin Model Compound Studies. University of Helsinki, Helsinki, Finland.

Maijala, P., Harrington, T. C., and Raudaskoski, M. (2003) A peroxidase gene family and gene trees in *Heterobasidion* and related genera. Mycologia, 95:209-221.

Mäkelä, M., Galkin, S., Hatakka, A., and Lundell, T. (2002) Production of organic acids and oxalate decarboxylase in lignin-degrading white rot fungi. Enzyme Microb. Technol., 30:542-549.

Male, K. B., Brown, R. S., and Luong, J. H. T. (1995) Enzymatic oxidation of water-soluble cyclodextrin-polynuclear aromatic hydrocarbon inclusion complexes, using lignin peroxidase. Enzyme Microb. Technol., 17:607-614.

Martens, R., Wolter, M., Bahadir, M., and Zadrazil, F. (1999) Mineralization of ^{14}C-labelled highly-condensed polycyclic aromatic hydrocarbons in soils by *Pleurotus* sp. Florida. Soil Biol. Biochem., 31:1893-1899.

Martens, R. and Zadrazil, F. (1998) Screening of white-rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil. Folia Microbiol., 43:97-103.

Martinez, A. T., Camarero, S., Guillen, F., Gutierrez, A., Munoz, C., Varela, E., Martinez, M. J., Barrasa, J. M., Ruel, K., and Pelayo, J. M. (1994) Progress in biopulping of non-woody materials - chemical, enzymatic and ultrastructural aspects of wheat-straw delignification with ligninolytic fungi from the genus *Pleurotus*. FEMS Microbiol. Rev., 13:265-274.

Masaphy, S., Levanon, D., Henis, Y., Venkateswarlu, K., and Kelly, S. L. (1995) Microsomal and cytosolic cytochrome P450 mediated benzo(a)pyrene hydroxylation in *Pleurotus pulmonarius*. Biotechnol. Lett., 17:969-974.

Masaphy, S., Levanon, D., Henis, Y., Venkateswarlu, K., and Kelly, S. L. (1996) Evidence for cytochrome P-450 and P-450-mediated benzo(a)pyrene hydroxylation in the white-rot fungus *Phanerochaete chrysosporium*. FEMS Microbiol. Lett., 135:51-55.

Mayer, A. M. and Staples, R. C. (2002) Laccase: new functions for an old enzyme. Phytochem., 60:551-565.

Mester, T., de Jong, E., and Field, J. A. (1995) Manganese regulation of veratryl alcohol in white-rot fungi and its indirect effect on lignin peroxidase. Appl. Environ. Microbiol, 61:1881-1887.

Mester, T. and Field, J. A. (1998) Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. J. Biol. Chem., 273:15412-15417.

Mester, T., Swarts, H. J., Romero I Solé, S., de Bont, J. A. M., and Field, J. A. (1997) Stimulation of aryl metabolite production in the basidiomycete *Bjerkandera* sp. strain BOS55 with biosynthetic precursors and lignin degradation products. Appl. Environ. Microbiol, 63:1987-1994.

Moen, M. A. and Hammel, K. E. (1994) Lipid peroxidation by the manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. Appl. Environ. Microbiol., 60:1956-1961.

Moilanen, A. M., Lundell, T., Vares, T., and Hatakka, A. (1996) Manganese and malonate are individual regulators for the production of lignin and manganese peroxidase isozymes and in the degradation of lignin by *Phlebia radiata*. Appl. Microbiol. Biotechnol., 45: 792-799.

Monties, B. and Fukushima, Y. (2001) Occurence, function and biosynthesis of lignins. In M. Hofrichter and A. Steinbüchel (eds) *Biopolymers*, Vol. 1, Wiley-VCH, Weinheim, Germany. 1-64.

Morgan, P., Lee, S. A., Lewis, S. T., Sheppard, A. N., and Watkinson, R. J. (1993) Growth and biodegradation by white-rot fungi inoculated into soil. Soil Biol. Biochem., 25:279-287.

Morgan, P., Lewis, S. T., and Watkinson, R. J. (1991) Comparison of abilities of white-rot fungi to mineralize selected xenobiotic compounds. Appl. Microbiol. Biotechnol., 34: 693-696.

Morisaki, K., Fushimi, T. G., Kaneko, S., Kusakabe, I., and Kobayashi, H. (2001) Screening for phenoloxidases from edible mushrooms. Bioscience Biotechnology and Biochemistry, 65:2334-2336.

Muheim, A., Waldner, R., Leisola, M. S. A., and Fiechter, A. (1990) An extracellular aryl-alcohol oxidase from the white-rot fungus *Bjerkandera adusta*. Enzyme Microb. Technol., 12:204-209.

Niku-Paavola, M. L., Karhunen, E., Salola, P., and Raunio, V. (1988) Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem. J., 254:877-883.

Novotný, C., Erbanová, P., Cajthaml, T., Rothschild, N., Dosoretz, C., and Sasek, V. (2000) *Irpex lacteus*, a white rot fungus applicable to water and soil bioremediation. Appl. Microbiol. Biotechnol., 54:850-853.

Novotný, C., Erbanová, P., Sasek, V., Kubátová, A., Cajthaml, T., Lang, E., Krahl, J., and Zadrazil, F. (1999) Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. Biodegradation, 10:159-168.

Paszczynski, A., Huynh, V. B., and Crawford, R. (1985) Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett., 29:37-41.

Paul, E. A. and Clark, F. E. (1989) Soil Microbiology and Biochemistry. Academic Press, Inc., San Diego, CA.

Phillips, R. (1991) Mushrooms of North America. Brown & Company Inc., New York, N.Y.

Pickard, M. A., Roman, R., Tinoco, R., and Vazquez-Duhalt, R. (1999) Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. Appl. Environ. Microbiol., 65:3805-3809.

Pointing, S. B. (2001) Feasibility of bioremediation by white-rot fungi. Appl. Microbiol. Biotechnol., 57:20-33.

Pugh, G. J. F. (1974) Terrestrial fungi. In C. H. Dickinson and G. J. F. Pugh (eds) *Biology of Plant Litter Decomposition*, Vol. 2, Academic Press, London, U.K., 246-775.

Rajarathnam, S., Shashirekha, M. N. J., and Bano, Z. (1998) Biodegradative and biosynthetic capacities of mushrooms: Present and future strategies. Crit. Rev. Biotechnol., 18:91-236.

Ralph, J. P. and Catcheside, D. E. A. (1994) Decolorization and depolymerization of solubilized low-rank coal by the white-rot basidiomycete *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol., 42:536-542.

Ralph, J. P. and Catcheside, D. E. A. (1999) Transformation of macromolecules from a brown coal by lignin peroxidase. Appl. Microbiol. Biotechnol., 52:70-77.

Ramdahl, T. (1985) PAH emissions from combustion of biomass. In A. Bjørseth and T. Ramdahl (eds) *Handbook of Polycyclic Aromatic Hydrocarbons*, Marcel Dekker, N.Y., 61-85.

Regalado, V., Perestelo, F., Rodriguez, A., Carnicero, A., Sosa, F. J., De la Fuente, G., and Falcon, M. A. (1999) Activated oxygen species and two extracellular enzymes: laccase and aryl-alcohol oxidase, novel for the lignin-degrading fungus *Fusarium proliferatum*. Appl. Microbiol. Biotechnol., 51:388-390.

Reid, I. D. (1991) Intermediates and products of synthetic lignin (dehydrogenative polymerizate) degradation by *Phlebia tremellosa*. Appl. Environ. Microbiol., 57:2834-2840.

Rodriguez, A., Falcon, M. A., Carnicero, A., Perestelo, F., DelaFuente, G., and Trojanowski, J. (1996) Laccase activities of *Penicillium chrysogenum* in relation to lignin degradation. Appl. Microbiol. Biotechnol., 45:399-403.

Ruiz-Duenas, F. J., Camarero, S., Perez-Boada, M., Martinez, M. J., and Martinez, A. T. (2001) A new versatile peroxidase from *Pleurotus*. Biochem. Soc. Trans., 29:116-122.

Sack, U. and Günther, T. (1993) Metabolism of PAH by fungi and correlation with extracellular enzymatic activities. J. Bas. Microbiol., 33:269-277.

Sack, U., Heinze, T. M., Deck, J., Cerniglia, C. E., Martens, R., Zadrazil, F., and Fritsche, W. (1997a) Comparison of phenanthrene and pyrene degradation by different wood-decaying fungi. Appl. Environ. Microbiol., 63:3919-3925.

Sack, U., Hofrichter, M., and Fritsche, W. (1997b) Degradation of polycyclic aromatic hydrocarbons by manganese peroxidase of *Nematoloma frowardii*. FEMS Microbiol. Lett., 152:227-234.

Sanglard, D., Leisola, M. S. A., and Fiechter, A. (1986) Role of extracellular ligninases in biodegradation of benzo(a)pyrene by *Phanerochaete chrysosporium*. Enzyme Microb. Technol., 8:209-212.

Saraswathy, A. and Hallberg, R. (2002) Degradation of pyrene by indigenous fungi from a former gasworks site. FEMS Microbiol. Lett., 210:227-232.

Sarkar, J. M. and Bollag, J. M. (1987) Inhibitory effect of humic and fulvic acids on oxidoreductases as measured by the coupling of 2,4-dichlorophenol to humic substances. Sci. Tot. Environ., 62:367-377.

Satchell, J. E. (1974) Litter-interface of animate/inanimate matter. In C. H. Dickinson and G. J. F. Pugh (eds) *Biology of Plant Litter Decomposition*, Vol. 1, Academic Press, London, U.K., 241.

Scheel, T., Holker, U., Ludwig, S., and Hofer, M. (1999) Evidence for and expression of a laccase gene in three basidiomycetes degrading humic acids. Appl. Microbiol. Biotechnol., 52:66-69.

Scheibner, K. and Hofrichter, M. (1998) Conversion of aminonitrotoluenes by fungal manganese peroxidase. J. Bas. Microbiol., 38:51-59.

Scheibner, K., Hofrichter, M., Herre, A., Michels, J., and Fritsche, W. (1997) Screening for fungi intensively mineralizing 2,4,6-trinitrotoluene. Appl. Microbiol. Biotechnol., 47: 452-457.

Schlosser, D. and Hofer, C. (2002) Laccase-catalyzed oxidation of Mn^{2+} in the presence of natural Mn^{3+} chelators as a novel source of extracellular H_2O_2 production and its impact on manganese peroxidase. Appl. Environ. Microbiol., 68:3514-3521.

Schneegass, I., Hofrichter, M., Scheibner, K., and Fritsche, W. (1997) Purification of the main manganese peroxidase isoenzyme MnP2 from the white-rot fungus *Nematoloma frowardii* b19. Appl. Microbiol. Biotechnol., 48:602-605.

Schneider, J., Grosser, R., Jayasimhulu, K., Xue, W., and Warshawsky, D. (1996) Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. Appl. Environ. Microbiol., 62:13-19.

Senesi, N. and Loffredo, E. (2001) Soil humic substances. In M. Hofrichter and A. Steinbüchel (eds) *Lignin, Humic Substances and Coal*, Vol. 1, Wiley-VCH, Weinheim, Germany. 247-299.

Shevchenko, S. M. and Bailey, G. W. (1996) Life after death: Lignin-humic relationships reexamined. Crit. Rev. Environ. Sci. Technol., 26:95-153.

Sjöström, E. (1977) Puukemia. 4 Edition, Otakustantamo, Helsinki, Finland.

Soponsathien, S. (1998) Some characteristics of ammonia fungi 1. In relation to their ligninolytic enzyme activities. J. Gen. Appl. Microbiol., 44:337-345.

Stevenson, F. J. (1994) Humus chemistry. Genesis, composition, reactions. 2 Edition, John Wiley Sons, New York, N.Y.

Sutherland, J. B., Rafii, F., Khan, A. A., and Cerniglia, C. E. (1995) Mechanisms of polycyclic aromatic hydrocarbon degradation. In L. Young and C. E. Cerniglia (eds) *Microbial Transformation and Degradation of Toxic Organic Chemicals*, Wiley-Liss, New York, N.Y., 269-306.

Sutherland, J. B., Selby, A. L., Freeman, J. P., Evans, F. E., and Cerniglia, C. E. (1991) Metabolism of phenanthrene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol., 57:3310-3316.

Tagger, S., Perissol, C., Gil, G., Vogt, G., and Le Petit, J. (1998) Phenoloxidases of the white-rot fungus *Marasmius quercophilus* isolated from an evergreen oak litter (*Quercus ilex* L.). Enzyme Microb. Technol., 23:372-379.

Tanesaka, E., Masuda, H., and Kinugawa, K. (1993) Wood degrading ability of basidiomycetes that are wood decomposers, litter decomposers, or mycorrhizal symbionts. Mycologia, 85:347-354.

ten Have, R., Hartmans, S., Teunissen, P. J., and Field, J. A. (1998) Purification and characterization of two lignin peroxidase isozymes produced by *Bjerkandera* sp. strain BOS55. FEBS Lett., 422:391-394.

Thurston, C. F. (1994) The structure and function of fungal laccases. Microbiology, 140: 19-26.

Tien, M. and Kirk, T. K. (1983) Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. Science, 221:661-662.

Trojanowski, J., Haider, K., and Hüttermann, A. (1984) Decomposition of ^{14}C-labeled lignin, holocellulose and lignocellulose by mycorrhizal fungi. Arch. Microbiol., 139:202-206.

Tuomela, M., Hatakka, A., Raiskila, S., Vikman, M., and Itävaara, M. (2001) Biodegradation of radiolabelled synthetic lignin (^{14}C-DHP) and mechanical pulp in a compost environment. Appl. Microbiol. Biotechnol., 55:492-499.

Tuomela, M., Oivanen, P., and Hatakka, A. (2002) Degradation of synthetic ^{14}C-lignin by various white-rot fungi in soil. Soil Biol. Biochem., 34:1613-1620.

Valli, K., Brock, B. J., Joshi, D. K., and Gold, M. H. (1992) Degradation of 2,4-dinitrotoluene by the lignin-degrading fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol., 58:221-228.

Van Aken, B., Cameron, M. D., Stahl, J. D., Plumat, A., Naveau, H., Aust, S. D., and Agathos, S. N. (2000) Glutathione-mediated mineralization of C^{14}-labeled 2-amino-4,6-dinitrotoluene by manganese-dependent peroxidase H5 from the white-rot fungus *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol., 54:659-664.

Van Aken, B., Hofrichter, M., Scheibner, K., Hatakka, A. I., Naveau, H., and Agathos, S. N. (1999) Transformation and mineralization of 2,4,6-trinitrotoluene (TNT) by manganese peroxidase from the white-rot basidiomycete *Phlebia radiata*. Biodegradation, 10:83-91.

Vares, T. (1996) Ligninolytic enzymes and lignin-degrading activity of taxonomically different white-rot fungi. PhD Thesis. Vol. 44, Dep. of Appl. Chem. and Microbiol., University of Helsinki, Finland.

Vares, T. and Hatakka, A. (1997) Lignin-degrading activity and ligninolytic enzymes of different white-rot fungi: effects of manganese and malonate. Can. J. Bot., 75:61-71.

Volc, J., Kubatova, E., Daniel, G., Sedmera, P., and Haltrich, D. (2001) Screening of basidiomycete fungi for the quinone-dependent sugar C-2/C-3 oxidoreductase, pyranose dehydrogenase, and properties of the enzyme from *Macrolepiota rhacodes*. Arch. Microbiol., 176:178-186.

Waldner, R., Leisola, M. S. A., and Fiechter, A. (1988) Comparison of ligninolytic activities of selected white-rot fungi. Appl. Microbiol. Biotechnol., 29:400-407.

Wariishi, H., Akileswaran, L., and Gold, M. H. (1988) Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: spectral characterization of the oxidized states and the catalytic cycle. Biochem., 27:5365-5370.

Wariishi, H., Valli, K., and Gold, M. H. (1991) *In vitro* depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun., 176:269-275.

Wariishi, H., Valli, K., and Gold, M. H. (1992) Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelators. J. Biol. Chem., 267:23688-23695.

Wariishi, H., Valli, K., Renganathan, V., and Gold, M. H. (1989) Thiol-mediated oxidation of nonphenolic lignin model compounds by manganese peroxidase of *Phanerochaete chrysosporium*. J. Biol. Chem., 264:14185-14191.

Watkinson, S. C., Carlile, M. C., and Gooday, G. W. (2000) The Fungi. Academic Press Limited, London, U.K.

Willmann, G. and Fakoussa, R. M. (1997a) Biological bleaching of water-soluble coal macromolecules by a basidiomycete strain. Appl. Microbiol. Biotechnol., 47:95-101.

Willmann, G. and Fakoussa, R. M. (1997b) Extracellular oxidative enzymes of coal-attacking fungi. Fuel Processing Technology, 52:27-41.

Wilson, S. C. and Jones, K. C. (1993) Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. Environ. Poll., 81:229-249.

Wolter, M., Zadrazil, F., Martens, R., and Bahadir, M. (1997) Degradation of eight highly condensed polycyclic aromatic hydrocarbons by *Pleurotus* sp. Florida in solid wheat straw substrate. Appl. Microbiol. Biotechnol., 48:398-404.

Wood, D. A. and Leatham, G. F. (1983) Lignocellulose degradation during the life cycle of *Agaricus bisporus*. FEMS Microbiol. Lett., 20:421-424.

Wunch, K. G., Alworth, W. L., and Bennett, J. W. (1999) Mineralization of benzo[a]pyrene by *Marasmiellus troyanus*, a mushroom isolated from a toxic waste site. Microbiol. Res., 154:75-79.

Wunch, K. G., Feibelman, T., and Bennett, J. W. (1997) Screening for fungi capable of removing benzo(a)pyrene in culture. Appl. Microbiol. Biotechnol., 47:620-624.

Wunderwald, U., Kreisel, G., Braun, M., Schulz, M., Jäger, C., and Hofrichter, M. (2000) Formation and degradation of a synthetic humic acid derived from 3-fluorocatechol. Appl. Microbiol. Biotechnol., 53:441-446.

Yaropolov, A. I., Skorobogat'ko, O. V., Vartanov, S. S., and Varfolomeyev, S. D. (1994) Laccase: properties, catalytic mechanisms, and applicability. Appl. Biochem. Biotechnol., 49:257-280.

Ye, D. Y., Siddiqi, M. A., Maccubbin, A. E., Kumar, S., and Sikka, H. C. (1996) Degradation of polynuclear aromatic hydrocarbons by *Sphingomonas paucimobilis*. Environ. Sci. Technol., 30:136-142.

Young, H. E. and Guinn, V. P. (1966) Chemical elements in complete mature trees of 7 species in Maine. Tappi, 49:190-197.

Ziegenhagen, D. and Hofrichter, M. (1998) Degradation of humic acids by manganese peroxidase from the white-rot fungus *Clitocybula dusenii*. J. Bas. Microbiol., 38:289-299.

Appendix: Trivial names of some basidiomycetous fungi

Table 8.1: Trivial, common or nick names of some litter-decomposing (L) and white-rot fungi (w-r) in four different languages (obtained from: http://users.quista.net/sjgall/Nicknames.htm.; Suomen ja Pohjolan Sienet, Ryman S. & Holmåsen I., WSOY, ISBN 951-0-14286-7; Handbuch Pilze, Gerhardt E., BSV, ISBN 3-405-14737-9).

Fungus	Decay	English	Finnish	Swedish	German
Agaricus bisporus	L	white button mushroom	herkkusieni	champinjon	Champignon
Agrocybe dura	L	-	-piennarsieni	-åkerskivling	Rissiger Ackerling
Agrocybe praecox	L	-	kesäpiennarsieni	tidig åkerskivling	Voreilender Ackerling
Bjerkandera adusta	w-r	-	tuhkakääpä	svedticka	Angebrannter Rauchporling
Calocybe indica	w-r	-	-kaunolakki	-musseron	- Schönkopf
Clitocybe clavipes	L	club foot	nuijamalikka	mörk trattskivling	Keulenfuß-Trichterling
Clitocybe dealbata	L	-	myrkkymalikka	gifttrattskivling	Feldtrichterling
Clitocybe gibba	L	common funnel cap	suppilomalikka	sommartrattskivling	Ockerbrauner Trichterling
Clitocybe lignatilis	L	-	jauhovinokas	mjölmussling	-
Clitocybe metachroa	L	-	harmaamalikka	grå trattskivling	-
Clitocybe odora	L	aniseed toadstool	vihertuoksumalikka	grön trattskivling	Grüner Anistrichterling
Clitopilus prunulus	L	the miller	jauhosieni	mjölskivling	Mehlräsling
Collybia dryophila	L	russet tough-shank	kalpeajuurekas	blek nagelskivling	Waldfreund-Rübling
Coprinus atramentarius	L	common ink-cap	harmaamustesieni	grå bläcksvamp	Grauer Tintling
Coprinus comatus	L	shaggy ink-cap	suomumustesieni	fjällig bläcksvamp	Schopf-Tintling
Galerina marginata	L	-	myrkkynääpikkä	gifthätting	Nadelholz-Häubling
Gymnopilus junonius	L	-	isokarvaslakki	ringbitterskivling	- Flämmling
Hygrophoropsis aurantiaca	L	false chantarelle	valevahvero	falsk (narr-) kantarell	Falscher Pfifferling
Hypholoma capnoides	w-r	-	kuusilahokka	rökslöjskivling	Rauchgrauer Schwefelkopf
Hypholoma marginatum	L	-	parvilahokka	kantslöjskivling	Geselliger Schwefelkopf
Kuehneromyces mutabilis	w-r	-	koivunkantosieni	föränderlig tofsskivling	Stockschwämmchen
Irpex lacteus	w-r	-	maitohampikka	slingerpicka	? Spaltporling
Lepiota cristata	L	crested lepiota	puistoukonsieni	fjällskivling	Stinkschirmling
Lepista nebularis	L	clouded agaric	härmämalikka	pudrad trattskivling	Nebelgrauer Röteltrichterling
Lepista nuda	L	wood blewit	sinivalmuska	blåmusseron	Violetter Rötelritterling
Lyophyllum decastes	L	fried chicken mushroom	tummatupaskynsikäs	mörk tuvskivling	Brauner Rasling
Lyophyllum ulmarium	L	-	runkokynsikäs	almmussling	-
Marasmius alliaceus	L	-	pyökkinahikas	stor lökbroskskivling	Saitenstielige Knoblauch-schwindling
Marasmius quercophilus	L	-	etelänahikas	ekbroskskivling	-
Marasmius scorodonius	L	-	laukkanahikas	lökbroskskivling	Echter Knoblauchschwindling
Mycena amicata	L	-	nukkajalkahiippo	fjunhätta	-
Mycena epipterygia	L	yellow stemmed mycena	keltajalkahiippo	flåhätta	Dehnbarer Helmling
Mycena polygramma	L	-	tinahiippo	silverhätta	Rillstieliger Helmling
Nematoloma frowardii	w-r	-	-lahokka	-slöjskivling	-
Omphalina epichysium	L	-	harmaanapalakki	grånavling	? Nabeling
Panellus mitis	w-r	kidney-shaped pleurotus	pikkuvinokas	vintermussling	Milder Zwergknäueling
Phlebia radiata	w-r	-	rusorypykkä	ribbgrynna	Orangefarbener Kammpilz
Pholiota nameko	w-r	-	-helokka	-tofsskivling	-
Pholiota squarrosa	w-r	shaggy pholiota	pörhösuomuhelokka	fjällig tofsskivling	Sparriger Schüppling
Phaeolepiota aurea	L	-	kultasieni	guldtofsskivling	Glimmer-Schüppling
Pleurotus ostreatus	w-r	oyster mushroom	osterivinokas	ostronmussling	Austernseitling
Pleurotus pulmonarius	w-r	-	koivuvinokas	blek ostronmussling	-Seitling
Stropharia aeruginosa	L	verdigris agaric	viherkaulussieni	ärggrön kragskivling	Grünspanträuschling
Stropharia coronilla	L	-	nurmikaulussieni	veckad kragskivling	Krönchen-Träuschling
Stropharia cubensis	L	golden cap	-kaulussieni	-	Cubensis Mexikaner
Stropharia hornemannii	L	-	isokaulussieni	stor kragskivling	-
Stropharia rugosoannulata	L	king stropharia	viljelykaulussieni	jätte kragskivling	Riesenträuschling
Stropharia semiglobata	L	dung roundhead	lantakaulussieni	gul kragskivling	Halbkugeliger Träuschling
Trametes hirsuta	w-r	hairy stereum	karvavyökääpä	borstticka	Striegelige Tramete
Trametes versicolor	w-r	turkey tail	silkkivyökääpä	sidenticka	Schmetterlings-Tramete
Tricholomopsis rutilans	w-r	plums and custard	purppuravalmuska	prickmusseron	Rötlicher Holzritterling