University of Alberta

Polycyclic Aromatic Hydrocarbon Metabolism by the White-Rot Fungus *Bjerkandera adusta* and Oxidation by Manganese Peroxidase

by

Yuxin Wang

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Yuxin Wang

Yuxin Wang
2823, 104 Street
Edmonton, AB
Canada T6J 4C7

Date: Sept. 3, 2002

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Polycyclic Aromatic Hydrocarbon Metabolism by the White-Rot Fungus *Bjerkandera adusta* and Oxidation by Manganese Peroxidase by Yuxin Wang in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and Biotechnology.

Dr. M. A. Pickard
(Supervisor)

Dr. M. R. Gray

Dr. P. M. Fedorak

Dr. M. Palcic

Dr. R. L. Crawford
(External Examiner)

Date 3 September 2002

Abstract

Polycyclic aromatic hydrocarbons are typically found in creosote, oils, tars and sites contaminated with theses products: they are produced by incomplete combustion of organic carbon compounds. They are toxic pollutants and the research project was to investigate their metabolism as a means of bioremediation. White-rot fungi and their extracellular enzymes, which have the ability to degrade the naturally occurring polymer lignin, have been evaluated for their potential to degrade polycyclic aromatic hydrocarbons. We have shown that two *Bjerkandera adusta* strains from the University of Alberta Microfungus Collection and Herbarium (UAMH) 8258 and 7308 are active in polycyclic aromatic hydrocarbon degradation.

Grown under ligninolytic conditions, *B. adusta* UAMH 8258 and 7308 produce only manganese peroxidase, and under optimal conditions, *B. adusta* UAMH 8258 and 7308 can produce high levels of manganese peroxidase in both shake flasks and stirred tank reactors, indicating that these two strains are good candidates for large scale enzyme production.

Manganese peroxidase from *B. adusta* 8258 was purified and characterized. The enzyme exhibited both Mn(II)-dependent and Mn(II)-independent activity, different from manganese peroxidase from *Phanerochaete chrysosporium*. The purified enzyme was modified with cyanuric chloride-activated methoxypolyethylene glycol and the modified enzyme showed greater resistance to denaturation by hydrogen peroxide, presence of organic solvents, high temperature and low pH.

Polycyclic aromatic hydrocarbon oxidation was studied using whole cells and purified manganese peroxidase from *B. adusta* UAMH 8258. Polycyclic aromatic

hydrocarbon oxidation by the purified enzyme was reduced by the presence of Mn(II) and the inhibition kinetics were shown to be partially noncompetitive. The substrates oxidized by manganese peroxidase were anthracene, and its methyl derivatives, pyrene, and benzo[a]pyrene: polycyclic aromatic hydrocarbons with ionization potentials of 7.43 eV or lower. The polycyclic aromatic hydrocarbon metabolites of the Mn(II)-independent reaction were identified as the corresponding quinones. The chemically modified enzyme had an enhanced oxidation rate compared with the native enzyme. Polycyclic aromatic hydrocarbon metabolism by cultures of *B. adusta* UAMH 8258 showed no clear differences between ligninolytic and nonligninolytic conditions, indicating the involvement of both intracellular and extracellular enzymes. Although *B. adusta* UAMH 8258 was able to cometabolize all the tested polycyclic aromatic hydrocarbons, only limited mineralization occurred.

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List of Abbreviations

ABTS	2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
ATCC	American Type Culture Collection
BF	Bran flakes
GC	Gas chromatography
DMP	2,6-dimethoxyphenol
FPLC	Fast protein liquid chromatography
GYM	Glucose-malt-yeast extract
HBT	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IP	Ionization potential
LiP	Lignin peroxidase
MALDI-TOF	Matrix-assisted laser desorption ionization time-of -flight
MnP	Manganese peroxidase
MS	Mass spectrametry
OB	Oat bran
PAGE	Polyacryamid gel electrophoresis
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl
PEG	Polyethylene glycol
PEI	Polyethyleneimine

RB	Rice bran
Rz	Reinheitzahl
SBM	Standard basal medium
SDS	Sodium dodecyl sulfate
STR	Stirred tank reactor
UAMH	University of Alberta Microfungus Collection and Herbarium
VA	Veratryl alcohol
WB	Wheat bran

1. Introduction

1.1 Project background

The research described in this thesis originated from observations that white-rot fungi are the most active degraders of the complex aromatic plant polymer lignin in natural environments and the ability of these fungi to metabolize a large variety of xenobiotics, including polycyclic aromatic hydrocarbons (PAHs), chlorinated phenols, dyes, munitions, pesticides, and other compounds of environmental concern.

Early reports by Bumpus *et al.* (1985) and Eaton (1985) indicating that the white-rot fungus *Phanerochaete chrysosporium* degrades dioxins, polychlorinated biphenyls (PCBs) and other chloroorganics, propelled white-rot fungi into the forefront of bioremediation research. *P. chrysosporium* is the most extensively studied of the ligninolytic white-rot fungi in degradation of environmental pollutants (Aust 1990; Hammel *et al.* 1992).

Early studies on PAHs degradation by white-rot fungi were mainly carried out with *P. chrysosporium.* Bumpus (1987) showed that at least 22 PAHs, including all of the most abundant PAHs in anthracene oil, undergo 70-100% breakdown in 27 days in *P. chrysosporium* cultures. Kennes and Lema (1994) reported the degradation by *P. chrysosporium* of a mixture of naphthalene, phenanthrene, anthracene, *p*-cresol, pentachlorophenol and phenol, which represent major components of creosotes. Further studies by this group demonstrated that some PAHs, such as benzo[a]pyrene, benzo[a]anthracene, pyrene and perylene are directly oxidized by lignin peroxidase (LiP) from *P. chrysosporium* to quinone-type products, whereas other PAHs, such as

benzo[e]pyrene, benzo[c]phenanthrene, phenanthrene, chrysene, and naphthalene do not serve as LiP substrates, but are degraded by *P. chrysosporium* cultures.

The extracellular lignin-degrading enzyme systems of *P. chrysosporium* and other white-rot fungi are proposed to be involved in xenobiotic degradation. Lignin peroxidase, manganese peroxidase (MnP) and laccase are key components of lignin-degrading systems. Lignin peroxidase (EC 1.11.1.7) is an extracellular glycosylated heme protein that catalyzes H_2O_2-dependent one-electron oxidation of a variety of lignin-related aromatic substrates, resulting in formation of aryl cation radicals which undergo various of non-enzymatic reactions yielding a multiplicity of end products (Kirk and Farrell 1987). Manganese peroxidase (EC 1.11.1.13) is an extracellular glycosylated heme protein that catalyzes the H_2O_2-dependent oxidation of Mn(II) to Mn(III), Mn(III) mediating the oxidation of a variety of phenolic substrates (Gold and Alic 1993). Laccase (EC 1.10.3.2) is a copper-containing oxidase that utilizes molecular oxygen as an oxidant and also oxidizes phenolic substrates to phenoxy radicals (Thurston 1994). Laccase can also oxidize non-phenolic aromatics in the presence of compounds such as 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Hatakka 1994).

Enzymatic oxidation of PAHs has been reported with purified preparations of LiP from *P. chrysosporium* (Hammel *et al.* 1986; Vazquez-Duhalt *et al.* 1994), MnP from *P. chrysosporium* (Bogan and Lamar 1995; Bogan *et al.* 1996) and laccase (Majcherczyk *et al.* 1998; Pickard *et al.* 1999b; Johannes and Majcherczyk 2000). Not all PAHs are substrates for these lignin-degrading enzymes. A correlation has been found between the ionization potential of PAHs and the specific activity of MnP

and LiP from *P. chrysosporium*. A threshold value of ionization potential has been observed for each enzyme: LiP oxidizes PAHs with ionization potentials ≤ 7.55 eV (Vazquez-Duhalt *et al.* 1994), whereas MnP oxidizes PAHs with ionization potentials up to 8.2 eV (Bogan and Lamar 1995; Bogan *et al.* 1996). No correlation has been found between PAH oxidation and ionization potential with laccases from *Trametes versicolor* (Majcherczyk *et al.* 1998) or *Coriolopsis gallica* (Pickard *et al.* 1999b).

Several other white-rot fungal strains were evaluated for their pollutant-degrading abilities. Notable differences among fungi with regard to the extent of pollutant mineralization and transformation ability, as well as the nature of pollutant-derived metabolites, have been demonstrated. Many strains have been found to metabolism PAHs more effectively than *P. chrysosporium*. For example, *T. versicolor* and *Chrysosporium lignorum* are better than *P. chrysosporium* in mineralizing phenanthrene (Morgan *et al.* 1991). *Pleurotus ostreatus, T. versicolor* and *Coriolopsis polyzona* have been shown to oxidize anthracene without accumulation of anthraquinone, which has been found as the dead end product of anthracene metabolism with *P. chrysosporium* (Vyas *et al.* 1994). Bogan and Lamar (1996) found that various PAHs were degraded faster and more extensively by *Phanerochaete laevis* than by *P. chrysosporium.* The comparison of *in vivo* metabolism of anthracene, pyrene, and phenanthrene by different white-rot fungi has shown that two *Bjerkandera adusta* strains from the University of Alberta Microfungus Collection and Herbrium (UAMH) 8258 and 7308 are able to degrade these three PAHs to a higher level than *P. chrysosporium* (Pickard *et al.* 1999b).

The lignin-degrading enzymes of *P. chrysosporium* have been well studied, and include six LiP isoenzymes, and four MnP isoenzymes (Farrell *et al.* 1989). Recent studies have shown that *Pleurotus eryngii,* an active lignin-degrading organism, produces a peroxidase different from *P. chrysosporium* MnP in that it can both efficiently oxidizes Mn(II) to Mn(III), and oxidizes a variety of aromatic and nonaromatic substrates without Mn(II) addition (Martinez *et al.* 1996). The two less well-characterized *B. adusta* strains UAMH 7308 and 8258, which have been shown to have high PAH degradation abilities, produce only MnP under defined conditions. The role of this enzyme in PAH metabolism is of interest.

In the study of PAH degradation by ligninolytic enzymes, low substrate solubility is an important issue. Water-miscible organic solvents can be added to increase substrate availability in aqueous-based enzyme reactions. This is possible as extracellular fungal oxidative enzymes, such as LiP and MnP are glycoproteins with inherent resistance to low solvent concentrations. Enzyme catalysis in organic solvents has received considerable attention and tremendous progress in the improvement of catalyst properties through chemical modifications has been made in recent years (Khmelnitsky and Rich 1999; Klibanov 2001). Chemically modified LiP has been produced with enhanced ability in the oxidation of aromatic nitrogen-containing compounds (Vazquez-Duhalt *et al.* 1995). Enhanced stability in organic solvents has been observed for chemically modified LiP (Yoshida *et al.* 1996; Wang *et al.* 1999).

The objectives of this study were to 1) find an organism suitable for large-scale MnP production; 2) isolate and characterize this MnP; 3) chemically modify this

MnP; 4) use the purified- and chemically-modified enzymes to study PAH oxidation and finally 5) study *in vivo* PAH metabolism and mineralization to determine to what extant the enzyme could be responsible for overall PAH metabolism.

1.2 Literature review

1.2.1 Lignin biodegradation

Wood and plant materials are composed mainly of three structural polymers which include cellulose, hemicellulose and lignin. Approximately 45% of the weight of wood is cellulose, with hemicellulose making up 20% for soft wood or 25-30% for hard wood, while lignin comprises 15-36% of the total lignocellulosic materials in wood. A typical lignin contains 10-20% phenolic hydroxyl groups (Lin and Dence 1992) providing increased rigidity to the plant cell wall and also protects plants from pathogenic organisms (Higuchi 1990).

Although wood was known to be degraded by some organisms, particularly fungi (Kirk and Farrell 1987), substantial progress in lignin biodegradation research did not commence until a better understanding of the lignin structure was gained in the 1960s. During the 1970s, assays for lignin biodegradation using lignin model compounds and ^{14}C-labelled lignins were developed (Crawford 1981). This section comprises a brief review of lignin structure, the microorganisms involved in lignin degradation and lignin degradation by white-rot fungi.

1.2.1.1 Lignin structure

Lignin is a complex, heterogeneous and random phenylpropanoid polymer synthesized from three substituted cinnamyl alcohols: *p*-coumaryl, coniferyl, and sinapyl alcohols (Fig. I-1). Free radical condensation of these alcohols, initiated by plant cell wall peroxidases, results in the formation of a heterogeneous, amorphous, optically inactive, random, and highly branched polymer lignin (Fig. I-1).

1.2.1.2 Lignin-degrading microorganisms

Most wood-decaying microorganisms degrade wood polysaccharides by means of hydrolases, such as cellulases and xylanases. Many organisms are able to chemically modify lignin but only few can actually degrade lignin to CO_2 and H_2O owing to its complex and heterogenous structure. Thus, the biodegradation of lignin is accomplished by special groups of some bacteria and fungi (Kirk and Shimada 1985; Kirk and Farrell 1987).

1.2.1.2.1 Lignin-degrading fungi

Wood-decaying fungi are classified according to the wood polymer they degrade and the decay pattern. Three general classification categories are used: brown rot, soft-rot and white-rot fungi (Winkelmann 1992).

Brown-rot fungi decompose mainly cellulose and hemicellulose while leaving the lignin intact (Eriksson 1990). Brown-rot fungi predominantly attack softwood, and belong to the *Basidiomycetes* group. Analysis of brown-rotted lignin has shown that brown-rot fungi cause lignin modification including a decreased methoxy content

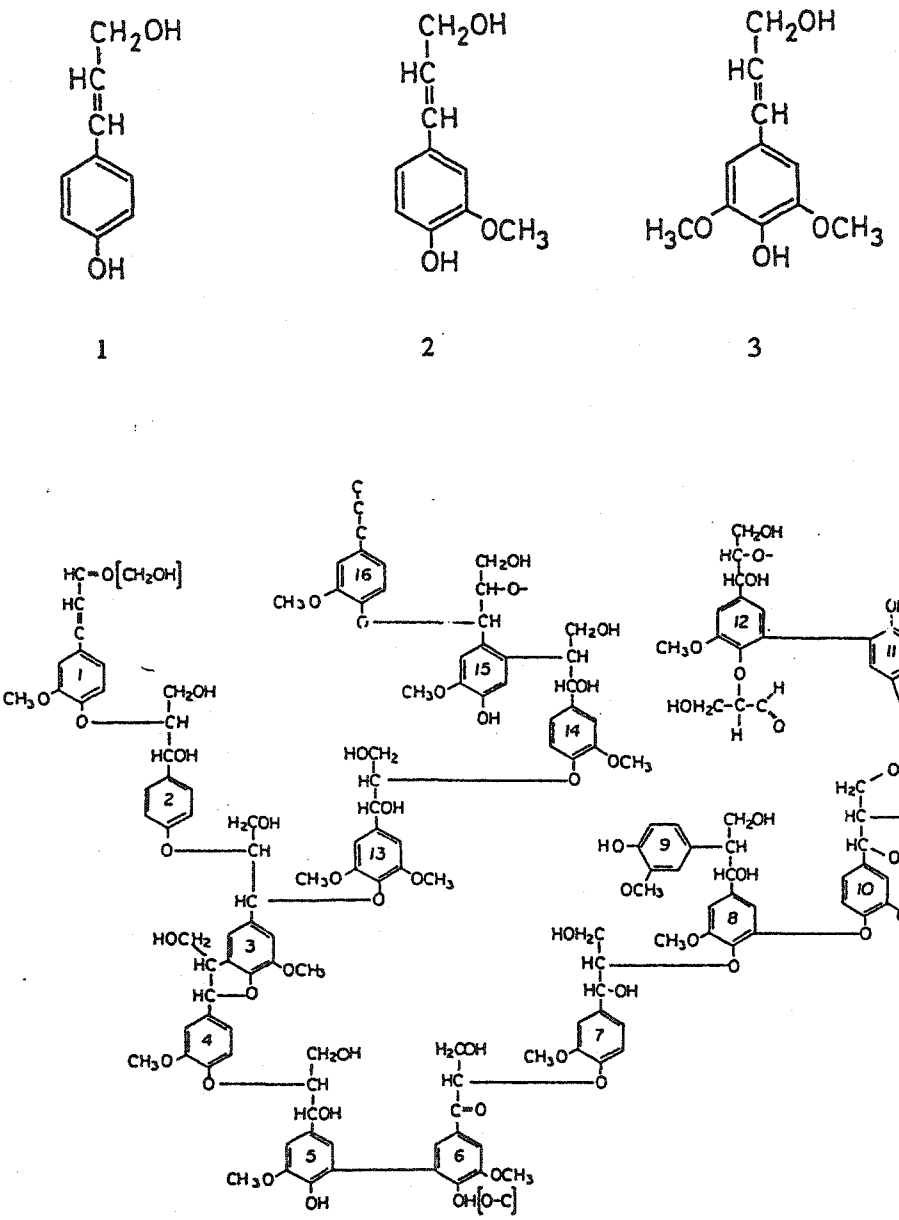

Fig. I-1. Lignin precursors: (1) *p*-coumaryl, (2) coniferyl, and (3) sinapyl alcohols and structure of spruce lignin (Adler, 1977).

(Crawford 1981). The dark brown color is caused by the generation of quinones and conjugated carbonyl groups on the decayed lignin.

Soft-rot fungi degrade cellulose and hemicellulose in wood and belong to the *Ascomycotina* and *Deuteromycotina* group. Soft-rot fungi preferably attack hardwood, whereas it has been shown that soft-rot fungi are poor at degrading synthetic lignin, they can rapidly mineralize lignin-related phenolic compounds (Ander *et al.* 1994). The lignin biodegradation capacity of these fungi and the mechanisms involved has not been well studied due to difficulties with laboratory cultivation.

White-rot fungi are able to degrade lignin extensively and primarily decompose hardwood. The name "white-rot" comes from the observation that these organisms degrade cell wall lignin leaving the colorless cellulose. They are also able to degrade cellulose and hemicellulose (Winkelmann 1992). White-rot fungi are the only microorganisms shown to be able to degrade lignin totally and most white-rot fungi belong to the *Basidiomycetes* group.

The biochemistry of lignin biodegradation has mainly been elucidated from studies using white-rot fungi, in particular *P. chrysosporium*. Elemental and functional group analyses of lignin decayed by *P. chrysosporium* suggest that fungal degradation of lignin is an oxidative process (Kirk and Chang 1974, Crawford 1981). Product analysis of released fragments indicates that degradation occurs mainly through C_{α}-C_{β} cleavage of propyl side chains and through aryl-ether cleavage followed by side chains modification, and aromatic ring fission (Kirk and Chang 1974, 1975; Chen *et al.* 1982; Chen and Chang 1985; Higuchi 1990; Archibald *et al.*

1997; Kirk and Cullen 1998). Detailed studies of lignin degradation of lignin by white-rot fungi are difficult owing to the complex and heterogeneous structure of lignin.

1.2.1.2.2 Lignin biodegradation by other microorganisms

Although lignin biodegradation research has concentrated mainly on white-rot fungi, it is clear that several bacterial species are able to degrade lignin. *Actinomycetes* are filamentous bacteria, which are found in soil and composts where lignocellulose is decomposed. Several reports provide evidence that species belonging to the genus *Streptomyces* are able to degrade lignin (Crawford 1981). Other lignin-degrading *Actinomycetes* include *Thermomonospora mesophila, Actinomadura* and *Micromonospora,* with *Streptomyces* exhibiting the highest lignin-degradation ability (Winkelmann 1992). Lignin-degradation by bacteria is however, generally slower than by white-rot fungi. Most of the enzymes involved in bacterial lignin degradation are intracellular. Interestingly, peroxidases possibly involved in lignin degradation have been discovered in several *Streptomyces* (Ramachandra *et al.* 1988; Adhi *et al.* 1989) and a peroxidase-like enzyme secreted by *Streptomyces viridosporus* has been characterized (Ramachandra *et al.* 1988). This suggests that actinomycetous bacteria may utilize a ligninolytic system related to that of white-rot fungi.

For several decades, it has been known that various fungi can degrade lignin and secrete oxidative enzymes suspected to be involved in lignin degradation. However, the mechanisms by which these fungi degrade lignin were not understood. Until the elucidation of lignin structure and the development of methods in lignin chemistry in the 1960s, little progress was made. In the early 1980s, a major breakthrough in understanding the mechanism of lignin degradation was achieved. Failure to identify any fungal enzyme capable of depolymerizing lignin led Hall *et al.* (1980) to propose that lignin decay was not the result of an enzyme action upon lignin, but that a reactive oxygen species, produced extracellularly, could be responsible for the degradation. Forney *et al.* (1982) and Kutsuki and Gold (1982) showed that the ligninolytic activity was related to H_2O_2 production by the fungus. Then three laboratories independently reported the discovery of ligninase, a H_2O_2-dependent enzyme (Glenn *et al.* 1983; Shimada and Higuchi 1983; Tien and Kirk 1983). This enzyme is now referred to as LiP. This enzyme can oxidize a range of lignin model compounds (Glenn *et al.* 1983). Later, some of the peroxidase activity detected in the extracellular fluid of *P. chrysosporium* was later found to be dependent on Mn(II), leading to the discovery of MnP (Glenn and Gold 1985; Paszczynski *et al.* 1986). These peroxidases initiate free radical reactions to oxidize other chemicals and are considered to be the core of the lignin-degrading system of white-rot fungi (Cameron *et al.* 2000). Although there is no detectable laccase produced in *P. chrysosporium,* laccase is produced by many ligninolytic white-rot fungi and it is an important enzyme involved in lignin degradation (Reinhammar 1984).

1.2.1.3.1 Laccase

Laccase (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) is a copper-containing oxidase (blue copper oxidase) (Reinhammar 1984) and is produced by many white-rot fungi and by higher plants. Laccase catalyzes the 1-electron oxidation of a variety of phenolic substrates, reducing oxygen to water by four electrons. Laccases are typically 60-80 kDa and can be monomeric, dimeric or tetrameric glycoproteins with a carbohydrate content of 11-25% (Thurston 1994). Sequencing of fungal laccase gene sequences has demonstrated that most are between 520 and 550 amino acids. Most have acidic isoelectric points (Kirk and Farrell 1987). *T. versicolor* laccase contains four copper atoms at the active site with three structural types: 1, 2 and 3. The single type 1 copper is responsible for the visible blue color of the enzyme. The two type 3 and one type 2 copper atoms are found in a trinuclear cluster slightly removed from the type 1 copper center. The laccase catalytic cycle includes three steps: 1) the type 1 copper is reduced by the substrate being oxidized, followed by 2) transfer of the gained electron from the type 1 copper to the trinuclear cluster of type 2 and 3 coppers, and finally 3) oxygen reduction to water at the trinuclear cluster (Gianfreda *et al.* 1999).

Early observations demonstrated that most laccases cannot oxidize non-phenolic lignin related compounds (Kirk *et al.* 1968; Wariishi *et al.* 1987). Later, Bourbonnais and Paice (1990) noted that in the presence of Remazol Blue and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), laccase from *T. versicolor* could oxidize veratryl alcohol and non-phenolic lignin moieties whereas alone it

could not. Many further compounds have since been studied for mediating ability. Each successful mediator has been found to be a substrate of laccase (Call and Mucke 1997). The most effective mediators to date are typically N-heterocyclics with N-OH groups; included in this cohort are ABTS and 1-hydroxybenzotriazole (HBT). These mediator systems have been used to expand the range of PAHs (Pickard *et al.* 1999b; Johannes and Majcherczyk 2000), industrial dyes (Reyes *et al.* 1999), lignin and lignin derivatives (Bourbonnais *et al.* 1995) oxidized by laccase. Further, industrial delignification systems for the pulp and paper industry have been designed (Call and Mucke 1997).

1.2.1.3.2 Lignin peroxidase and manganese peroxidase

Although many basidiomycetous white-rot fungi secrete laccase, *P. chrysosporium* produces no detectable laccase activity under commonly used ligninolytic culture conditions. Ten extracellular peroxidase iosenzymes have been purified from cultures of *P. chrysosporium,* according to their order of elution from an anion exchange columns, named H1-H10. H1, H2, H6, H7, H8 and H10 were identified as LiP isoenzymes, and H3, H4, H5 and H9 as MnP isoenzymes. Both LiP and MnP are able to oxidize lignin, lignin derivatives, and a variety of lignin model compounds (Hammel et al 1985; Kirk and Farrell 1987; Gold *et al.* 1989; Wariishi *et al.* 1989; Wariishi *et al.* 1991). Lignin peroxidase and MnP from *P. chrysosporium* share 43% amino acid sequence identity and similar heme environments (Poulos *et al.* 1993). Both LiP and MnP have a classical peroxidase catalytic mechanism (Fig. I-2).

Lignin peroxidases have molecular masses of approximately 40 kDa, are glycosylated, and have acidic isoelectric points and pH optima. They contain a single ferric protoporphyrin IX heme moiety and function via a typical peroxidase catalytic cycle (Fig. I-2). Lignin peroxidase is oxidized by H_2O_2 to a 2-electon-deficient intermediate termed Compound I, which returns to its resting state by performing two 1-electron oxidations of donor substrates, the 1-electron deficient intermediate is termed Compound II (Kirk and Cullen 1998). Lignin peroxidase is capable of oxidizing various phenolic and non-phenolic substrates with a calculated ionization potential (IP), a measure of the ease of abstracting an electron from the highest occupied molecular orbital, of up to 9.0 eV (ten Have and Teunissen 2001). Lignin peroxidase has been shown to oxidize fully methylated lignin and lignin model compounds as well as various PAHs (Hammel *et al.* 1985; Buswell and Odier 1987; Vazquez-Duhalt *et al.* 1994). The ligninase-catalyzed oxidation of lignin substructures begins with the abstraction of 1-electron from the aromatic ring of the donor substrate; the resulting aryl cation radical then reacts both as a radical and as a cation, forming a variety of degradation fragments (Hammel *et al.* 1985). Among the oxidation reactions catalyzed by LiP are the cleavage of C_α-C_β and aryl C_α bonds, aromatic ring opening, and demethylation (Buswell and Odier 1987).

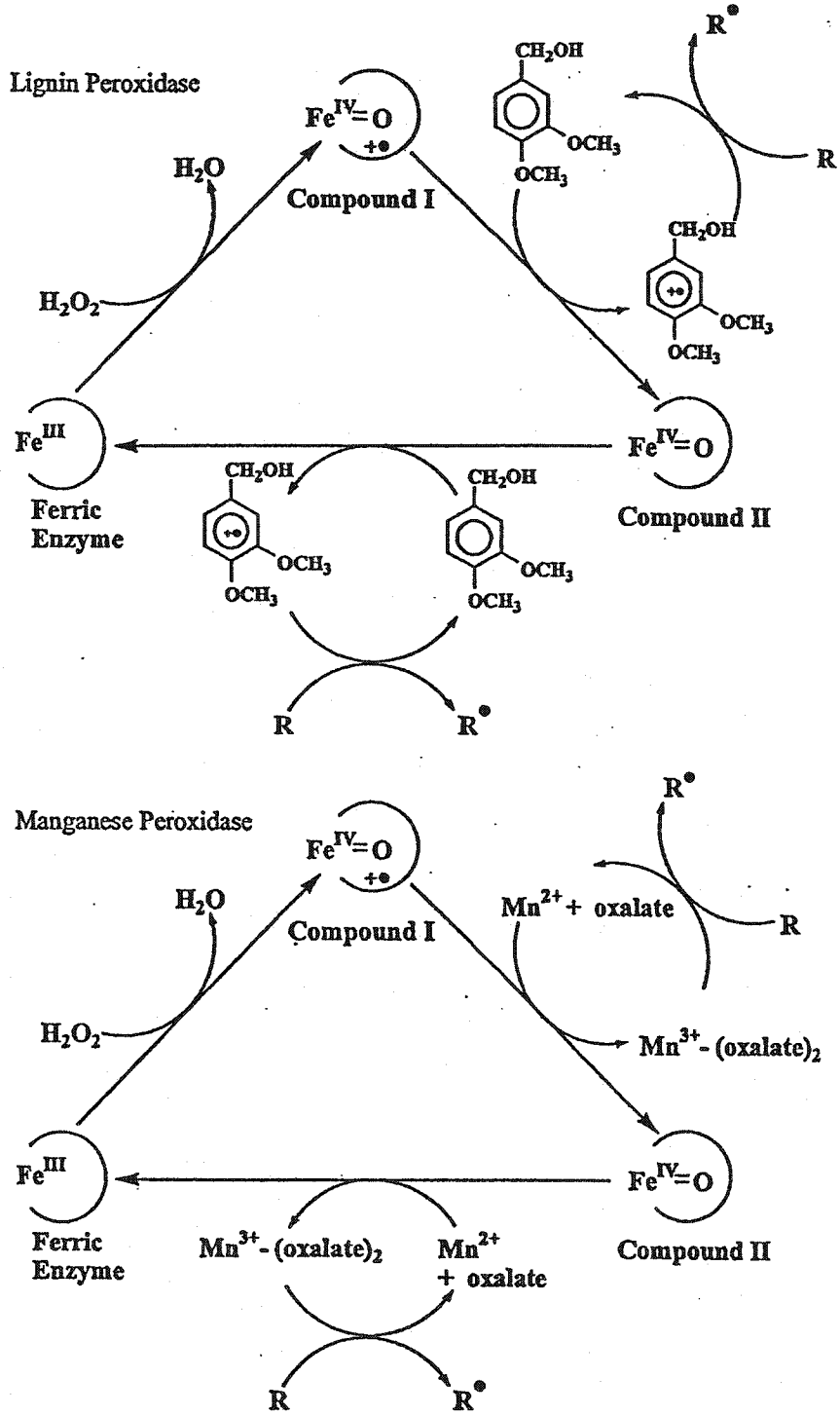

Fig. I-2. Catalysis and mediation by lignin peroxidase and manganses peroxidase (Cameron *et al.* 2000)

With respect to mediators, one secondary metabolite, veratryl alcohol, which is synthesized and secreted by *P. chrysosporium* has been the focus of many studies. Veratryl alcohol is an excellent substrate for LiP and enhances the oxidation of otherwise poor or terminal LiP substrates (Hammel *et al.* 1985). The major roles of veratryl alcohol have been summarized by ten Have and Teunissen (2001): 1) veratryl alcohol may act as a mediator in electron-transfer reactions; 2) veratryl alcohol is a good substrate for Compound II, and is therefore, essential for completing the catalytic cycle of LiP during the oxidation of terminal substrates; 3) veratryl alcohol prevents the H_2O_2-dependent inactivation of LiP by reducing Compound II back to native LiP. Only a few compounds, such as 3,4-dimethoxytoluene, 1,4-dimethoxybenzene, and 3,4,5-trimethoxybenzyl alcohol, have been found to replace the function of veratryl alcohol as a cofactor of LiP (ten Have and Teunissen 2001).

The main difference between LiP and MnP is in the nature of the substrates. Lignin peroxidase oxidizes non-phenolic aromatics generating aryl π-cation radicals (Buswell and Odier 1987; Kirk and Farrell 1987; Gold *et al.* 1989). In contrast, the primary reducing substrate for MnP is the Mn(II) ion rather than a phenol, producing highly reactive Mn(III) which, chelated with organic acids for stability, oxidizes various phenolic compounds (Glenn and Gold 1985; Wariishi *et al.* 1992; Kuan and Tien 1993). The difference in the substrate-binding sites in LiP and MnP are most likely responsible for the functional differences between these homologous enzymes. The Mn(II)-binding site, formed by the carboxylate oxygen atoms of two glutamates (E35, E39), an aspartate (D179), a heme propionate oxygen, and two water oxygen

atoms, is unique to MnP (Sundaramoorthy *et al.* 1994). Substrate oxidation by LiP has been studied for more than 15 years, but the location of binding sites remains elusive. A broad range of LiP substrates, which include small aromatic molecules and bulky substrates, such as lignin and ferrocytochrome *c* suggested that at least one substrate-binding site should be on the surface of the enzyme and include aromatic or other nonpolar amino acid residues to interact with substrates of various structure (Du *et al.* 1992).

1.2.1.3.3 Hydrogen peroxide generating enzymes

Both LiP and MnP require an extracellular source of H_2O_2 for catalysis. White-rot fungi produce a variety of intracellular and extracellular H_2O_2 generating enzymes which can provide H_2O_2 for peroxidase activity. The intracellular enzymes include glucose-1-oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) (Kelly and Reddy 1986), pyranose-2-oxidase (pyranose:oxygen 2-oxidorductase, EC 1.1.3.10) (Eriksson *et al.* 1986), and methanol oxidase (alcohol oxidase, EC 1.1.3.13) (Nishida and Eriksson 1987). The preferred substrate for glucose-1-oxidase is glucose, pyranose-2-oxidase oxidizes glucose and xylose (Eriksson *et al.* 1990) and methanol oxidase reacts with primary alcohols and unsaturated alcohols (Nishida and Eriksson 1987).

The extracellular enzymes include glyoxal oxidase (EC 1.2.3.5) and aryl-alcohol oxidase (EC 1.1.3.7) (Bourbonnais and Paice 1992). Glyoxal oxidase oxidizes glyoxal, methylglyoxal, and several other α-hydroxy carbonyl and dicarbonyl compounds coupled to the reduction of O_2 to H_2O_2 (Kirk and Cullen 1998). Aryl

alcohol oxidase oxidizes various non-phenolic aromatic alcohols to the corresponding aldehydes via a 2-electron oxidation with concomitant reduction of O_2 to H_2O_2 (Bourbonnais and Paice 1992).

1.2.1.3.4 Reductases

Various quinone intermediates are generated during lignin biodegradation. White-rot fungi are able to reduce these quinones to the corresponding phenols by intracellular and extracellular enzymes. An intracellular quinone-reducing enzyme, NAD(P)H:quinone oxidoreductase (EC 1.6.99.2) has been characterized (Buswell *et al.* 1987). This enzyme is expressed under both primary and secondary metabolic conditions, suggesting that regulation is independent of LiP and MnP (Brock *et al.* 1995).

Among the extracellular quinone reducing enzymes, cellobiose:quinone oxidoreductase (EC 1.1.5.1) and cellobiose dehydrogenase (EC 1.1.99.18) have been purified and extensively characterized from several white-rot fungi (Westermark and Eriksson 1974). One important function of cellobiose:quinone oxidoreductase and cellobiose dehydrogenase is the reduction of phenoxy or cation radicals generated by peroxidases, thus preventing lignin repolymerization. Another proposed role of cellobiose dehydrogenase is the generation of hydroxyl radicals through a Fenton's reaction in the presence of H_2O_2 (Ander 1994).

1.2.2 Manganese peroxidase

1.2.2.1 General properties

1.2.2.1.1 Physical properties of MnP

Manganese peroxidase (EC 1.11.1.13) was first isolated in Gold's lab from *P. chrysosporium* (Kuwahara *et al.* 1984). It contains one iron protoporphyrin IX prosthetic group, is a glycoprotein of molecular mass 45-47 kDa, and exists a series of isoenzymes with isoelectric points ranging from 3.2-4.9 (Glenn and Gold 1985; Gold and Alic 1993). Detailed electron paramagnetic resonance, nuclear magnetic resonance, and resonance raman spectral studies of LiP and MnP have also demonstrated that the native forms of these enzymes exists as ferric, high-spin, pentacoordinate heme proteins with the protein ligated to the heme iron through a proximal histidine residue, similar to HRP (Kirk and Farrell 1987; Gold *et al.* 1989). The sequences of MnP (*mnp*) complementary DNA (cDNA) (Gold and Alic 1993) and genomic clones (*mnp 1* and *mnp2)* (Gold and Alic 1993) encoding two *P. chrysosporium* MnP isoenzymes have been determined. The crystal structures of both LiP and MnP have been reported and also confirm that the heme environments of MnP and LiP are similar to those of other plant and fungal peroxidases (Sundaramoorthy *et al.* 1994).

1.2.2.1.2 Catalytic properties of MnP

The mechanism of MnP catalysis has been studied in detail (Wariishi *et al.* 1988). It was shown in Fig. I-2 that the ferric MnP native enzyme donated 2 electrons to hydrogen peroxide, and its heme is oxidized to an oxo-iron(IV) porphyrin-based π-

free radical (Valderrama *et al.* 2002). This form of enzyme is named Compound I. In the next step, Compound I reacts with a Mn(II), oxidizing it to Mn(III), and is converted to Compound II. Compound II can be reduced to the native enzyme by oxidizing another Mn(II) ion to Mn(III). Transient-state kinetics has shown that Compound I of MnP can be reduced by both Mn(II) and phenolic compounds, whereas only Mn(II) reduces Compound II (Wariishi *et al* 1988). Mn(III) in turn, oxidizes organic substrates such as lignin substructure model compounds (Tuor *et al.* 1992), synthetic lignin (Wariishi *et al.* 1991; Bao *et al.* 1994), and aromatic pollutants (Valli and Gold 1991; Joshi and Gold 1993). The manganese ion acts as a redox mediator rather than an enzyme-binding activator (Glenn *et al.* 1986). To oxidize other compounds, Mn(III) has to be sufficiently stable and also able to dissociate from the active site of the enzyme. These requirements are satisfied by organic acid chelators such as carboxylic acids (oxalate, malonate and lactate), which are also secreted by the fungus (Wariishi *et al.* 1992). Even in the absence of the enzyme, suitably chelated Mn(III) ions act as ligninolytic agents and oxidize veratryl alcohol, lignin and lignin model compounds (Forrester *et al.* 1988).

1.2.2.1.2.1 Mn(II) binding site

The unique ability of MnP to oxidize Mn(II) to Mn(III) strongly indicates that MnP has at least one Mn(II) binding and oxidation site. The nature of the Mn(II) binding site in MnP has been under investigation for several years. Although Mn(II) oxidation at heme edge had been suggested (Harris *et al.* 1991), a Mn(II)-binding site near the internal heme propionate (involving MnP1 E35, E39 and D179) was first

postulated in theoretical (Johnson *et al.* 1994) and crystal models (Sundaramoorthy *et al.* 1994) and then confirmed by site-directed mutagenesis and x-ray diffraction of MnP/Mn(II) complexes (Sundaramoorthy *et al.* 1997).

1.2.2.1.2.2 Oxidation of non-phenolic lignin model compounds

Manganese peroxidase can oxidize phenolic lignin substructures and various phenols but is normally unable to oxidize non-phenolic lignins. However, recent studies have shown that in the presence of mediators non-phenolic lignin substructures can also be degraded. Wariishi *et al.* (1989b) found that manganese peroxidase could efficiently oxidize veratryl alcohol, anisyl alcohol, and benzyl alcohol when glutathione was included in reaction mixture. They demonstrated that Mn(III) oxidizes the thiol of glutathione to a thiyl radical, which in turn extracts a hydrogen from the alcohol substrate to form the corresponding aldehyde. In a study by Bao *et al.* (1994), it was shown that, in the presence of Tween 80, MnP and Mn(II) could oxidize a β-O-4 lignin model compound without addition of H_2O_2. They suggested a mechanism called "MnP mediated lipid peroxidation" by which lipid peroxy-radicals are generated. These radicals easily extract hydrogens from substrates. The same mechanism of non-phenolic lignin degradation was demonstrated in *Ceriporiopsis subvermispora* cultures in which MnP, but not LiP was produced (Jensen *et al.* 1996). In the studies of PAH oxidation by ligninolytic enzymes from white-rot fungi, some PAHs, which are not substrates of MnP and LiP, can be oxidized via MnP-mediated lipid peroxidation (Moen and Hammel 1994; Bogan and Lamar 1996).

1.2.2.1.2.3 *Pleurotus* and *Bjerkandera* manganese peroxidases

Lignin peroxidase and MnP were first described in *P. chrysosporium* and the main distinction between these two enzymes was their natural substrates. Later, active lignin-degrading strains of *Pleurotus* and *Bjerkandera* were shown to produce a peroxidase, different from *P. chrysosporium* LiP and MnP, that could both oxidize Mn(II) to Mn(III), and catalyze Mn(II)-independent reactions on aromatic substrates (Martinez *et al.* 1996). The peroxidase from *Pleurotus* first described as a "Mn-oxidizing peroxidase exhibiting Mn-independent activity" (Martinez and Martinez 1996). "Versatile peroxidase" was used by the Martinez group to describe a similar peroxidase from *Pleurotus eryngii* (Martinez 2002); and "novel manganese-lignin peroxidase (MnLiP) hybrid enzyme" was used to describe the peroxidase from *Bjerkandera* sp. BOS55 (Mester and Field 1998).

Other peroxidases that can oxidize both Mn(II) and aromatic compounds have been isolated from *Pleurotus pulmonarius* (Camarero *et al.* 1996), *Pleurotus ostreatus* (Sarkar *et al.* 1997), and *Bjerkandera adusta* (Heinfling *et al.* 1998). These peroxidases are able to oxidize Mn(II) to Mn(III) (as MnP does), degrade the lignin model dimer veratrylglycerol-β-guaiacyl ether (used to demonstrate LiP ligninolytic ability) yielding veratraldehyde, and oxidize veratryl alcohol and *p*-dimethoxybenzene to veratraldehyde and *p*-benzoquinone respectively (as reported for LiP) with lower affinity (Heinfling *et al.* 1998; Caramelo *et al.* 1999). They also oxidize hydroquinones and substituted phenols that are substrates for plant peroxidases. The pH optima for oxidation of Mn(II) (pH 5) and aromatic compounds

and dyes (pH 3) differ, being similar to those of optimal MnP and LiP activity (Heinfling *et al.* 1998).

Mn(II)-binding sites exist in *Pleurotus, Bjerkandera* and *P. chrysosporium* MnP, but no such a site exists in *P. chrysosporium* LiP, which lacks the ability to oxidize Mn(II) efficiently. A Mn(II)-binding site has been created in *P. chrysosporium* LiPH8 by site-directed mutagenesis and the variant obtained is able to oxidize both veratryl alcohol and Mn(II) (Mester and Tien 2001). The three acidic residues (Asp179, Glu35, Glu39) involved in Mn(II)-binding are conserved in *P. chrysosporium* and *T. versicolor* MnP, and in all *Pleurotus* peroxidases.

The aromatic substrate oxidation site in *P. chrysosporium* LiP has been studied and a long-range electron transfer pathway has been proposed. An exposed tryptophan (W171) was confirmed to be involved in veratryl alcohol oxidation by tryptophan bromination and site-directed mutagenesis (Blodig *et al.* 1998). A similar pathway has been identified in *P. eryngii* MnP and this tryptophan is conserved in all LiP sequences, as well as in *Pleurotus* peroxidases (Martinez 2002). Indeed, different substrate oxidation sites in *P. eryngii* MnP appear to exist as suggested by non-competitive inhibition between oxidation of Mn(II) and high redox-potential dyes (Ruiz-Duenas *et al.* 2001).

1.2.2.2 Manganese peroxidase production

1.2.2.2.1 Manganese peroxidase production by white-rot fungi

Manganese peroxidase was first isolated and characterized in the well studied white-rot fungus *P. chrysosporium* (Glenn and Gold 1985). Some white-rot fungi

produce only MnP, whereas others produce combinations of MnP, LiP and laccase (Table I-1).

Manganese peroxidase production studies have been conducted in many ligninolytic fungi and different levels of enzyme are produced by different strains, even within the same species. Culture conditions and medium composition play a major role in the level of enzyme expression (Pickard *et al.* 1999a). Some white-rot fungi that produce high levels of ligninolytic enzymes have been considered as good candidates for large-scale MnP production: *Bjerkandera* sp. BOS55 produced peak titres of MnP (up to 1.25 U/ml) in stationary culture (Mester and Field 1997). Another prodigious MnP producer is *Nematoloma frowardii*, which in solid-state wheat straw fermentations produced peak titres of MnP up to 5.5 U/ml based on the water content of the straw (Hofrichter *et al.* 1999). Large-scale MnP production was performed in an airlift bioreactor in which *P. chrysosporium* I-1512, an MnP hypersecretory strain, was immobilized on a stainless steel mesh. These production levels were 6.6 U/ml in a 100-L bioreactor with glycerol as carbon source, veratryl alcohol as inducer and a pure oxygen feed (Herpoel *et al.* 1999). The production of MnP and LiP by *P. chrysosporium* in a new solid-state bioreactor (Immersion Bioreactor) resulted in MnP and LiP activities of 0.99 U/ml and 0.36 U/ml, respectively (Rivela *et al.* 2000). An agaric basidiomycete *Clitocybula dusenii* was used to produce MnP up to 3 U/ml by immobilization of mycelium on cellulose and polypropylene (Ziegenhagen and Hofrichter 2000).

Table I-1. Some MnP-producing white-rot fungi

White-rot fungus	Ligninolytic enzymes
P. chrysosporium (Paszczynski *et al.* 1988)	MnP, LiP
T. versicolor (Paice *et al.* 1993)	MnP, laccase
Ceriporiopsis subvermispora (Urzua *et al.* 1998)	MnP, laccase
Cyathus stercoreus (Sethuraman *et al.* 1999)	MnP, laccase
Phlebia radiata (van Aken *et al.* 1999)	MnP, LiP, laccase
Nematoloma frowardii (Schneegaß *et al.* 1997; Hofrichter *et al.* 1999)	MnP, LiP, laccase
Pleurotus eryngii, P. ostreatus, P. pulmonarius (Caramelo *et al.* 1999)	MnP, laccase
Bjerkandera sp. BOS55 (Mester and Field 1997)	MnP
B. adusta UAMH 8258 and 7308 (Wang *et al.* 2002)	MnP

1.2.2.2.2 Recombinant MnP expression system

Recombinant peroxidase production is important in biotechnological applications. Both homologous and heterologous expression in prokaryotic and eukaryotic systems have been reported for ligninolytic peroxidases.

The production of active recombinant LiP or MnP using prokaryotic *Escherichia coli* expression system enables rapid high-yield protein production. However, these systems are strongly limited by the low yield of *in vitro* folding necessary to incorporate the heme into the protein recovered from protein inclusion bodies. The reconstitution of LiP or MnP expressed in *E. coli* using similar methods to those described for HRP yields only a small amount of active enzyme (<1% of total protein) (Smith *et al.* 1990).

Eukaryotic expression systems, unlike prokaryotic systems, can produce fully active enzymes which allow for *in vivo* incorporation of heme and some protein glycosylation, for example, many eukaryotic proteins requiring post-translational modifications have been successfully produced in insect tissue cultures using baculovirus transfer vectors (Smith *et al.* 1983; Miller 1988). A cDNA clone encoding LiP isoenzyme H8 has been used to express protein which is glycosylated, contains heme, and is capable of oxidizing iodide and veratryl alcohol (Johnson and Li 1991). However, the yields of LiP and MnP in these systems are low. The baculovirus system is also relatively expensive and thus may not be suitable for large-scale enzyme production. In addition, these recombinant enzymes often are produced in multiple forms which complicates purification (Johnson and Li 1991). A cDNA encoding MnP isozyme H4 has been expressed in *P. chrysosporium* to produce an

extracellular antibody-reactive and heme-containing MnP which has a molecular weight similar to that of wild-type MnP. This enzyme is active and dependent on both Mn(II) and H_2O_2 for activity (Pease *et al.* 1991).

Heterologous expression of ligninolytic enzymes in fungal hosts has been reported. For example, *Aspergillus oryzae* expression of *P. chrysosporium* MnP (Stewart *et al.* 1996), *Emericella nidulans* expression of *P. eryngii* MnP (Ruiz-Duenas *et al.* 1999b) and *Aspergillus niger* expression of *P. chrysosporium* LiP (Aifa *et al.* 1999) and MnP (Conesa *et al.* 2000). Although *Aspergillus* hosts are currently used for the industrial production of many enzymes, the yields for ligninolytic peroxidases are comparatively low. Coexpression with folding accessory proteins is being investigated to increase the yield of heterologous peroxidase production. Further identification and optimization of the heterologous expression systems of ligninolytic peroxidases are necessary for large-scale enzyme production (Martinez 2002).

1.2.2.2.3 Regulation of MnP genes

Several regulatory elements have been described in the promoter regions of *P. chrysosporium* MnP and LiP, and *P. eryngii* MnP. Putative regulatory elements in these promoters include xenobiotic response elements and elements involved in cAMP-mediated response in LiP genes, heat shock and metal response elements in MnP genes (Brown *et al.* 1991; Lobos *et al.* 1998). The transcriptional regulation of ligninolytic peroxidases in *P. chrysosporium* shows that LiP levels are dependent on nitrogen limitation, and that MnP expression is regulated by Mn(II), H_2O_2, chemical

agents, oxygen concentration, and heat shock (in nitrogen-limited cultures) (Brown *et al.* 1993; Li *et al.* 1995).

Regulation of MnP gene expression in *Pleurotus* species appears to be different from that in *P. chrysosporium.* Ligninolytic peroxidases of *Pleurotus* are hardly produced in nitrogen-limited media, but strongly produced in peptone-containing media or in solid-state lignocellulose cultures (Caramelo *et al.* 1999). High levels of MnP production in nitrogen-rich media by *B. adusta* have been reported (Wang *et al.* 2001). In addition to the nitrogen source, it has been shown that the transcription level of *P. eryngii* MnP is controlled by Mn(II) and oxidative stress. In this case, the hydroxyl radical could be a stronger inducer than H_2O_2 (Ruiz-Duenas *et al.* 1999). Once again, in contrast to *P. chrysosporium,* MnP levels are lowered by the addition of Mn(II) in *P. eryngii* (Martinez 2002).

1.2.3 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are compounds composed of two or more fused benzene rings with or without substituted groups or other linkages. Members of this group of compounds often have toxic and carcinogenic properties (Keith and Telliard 1979). Polycyclic aromatic hydrocarbons are typically found in creosote, oils, tars and sites contaminated with these products. They are also produced via incomplete combustion of carbon materials (Mueller *et al.* 1989). Polycyclic aromatic hydrocarbons are generally very hydrophobic (Table I-2) and they are generally regarded as relatively persistent environmental pollutants. Much of the elimination of PAHs from contaminated sites is a result of microbially mediated remediation, but

other removal mechanisms exist. These include photoxidation, volatilization, adsorption to soil matrixes, and chemical oxidation. Ecologically, PAHs tightly adsorbed to the soil matrix are less available for biodegradation and present a difficult target for remediation (Edwards 1983).

The primary health concern associated with PAHs is their carcinogenicity. Benzo[a]pyrene was one of the first pure chemicals demonstrated to induce tumor formation in laboratory animals (Cook *et al.* 1933). Subsequent work has illustrated that PAHs can be more precisely described as precarcinogens as steps involved in mammalian metabolism of these compounds tend to produce detoxified products as well as proximal and ultimate carcinogens (Miller and Miller 1985).

1.2.3.1 Polycyclic aromatic hydrocabon degradation

1.2.3.1.1 Bioavailability of PAHs

There has been no evidence that crystalline PAHs are available for microbial degradation although colonization of anthracene crystals by bacteria directly has been observed (Tongpim and Pickard 1996). These poorly soluble compounds are only available for microorganisms when they are in the dissolved state. Low water solubility and low dissolution rates means that microbial growth is often substrate-limited even in the presence of excess crystalline PAHs. Thus, the degradation rate often depends on the dissolution rate (Gray *et al.* 1994; Tiehm and Fritzsche 1995), which in turn is influenced by physical and chemical factors such as the mixing rate, particle surface area (i.e. particle size), and the presence of organic solvents and surfactants (Boldrin *et al.* 1993).

Table I-2. Fourteen PAHs on the priority pollutant list established by U. S. EPA (Keith and Telliard 1979) and their water solubility (Mueller *et al.* 1989)

Compound	Aqueous Solubility (mg/l, 25°C)
Naphthalene	31.7
Fluorene	2.0
Phenanthrene	1.3
Anthracene	0.07
Fluoranthene	0.26
Pyrene	0.14
Chrysene	0.002
Benzo[a]anthracene	0.014
Benzo[b]fluoranthene	NA[a]
Benzo[k]fluoranthene	NA
Dibenz0[a,h]anthracene	0.0005
Benzo[a]pyrene	0.003
Indeno[1,2,3-c,d]pyrene	NA
Benzo[g,h,i]perylene	0.0003

NA[a]: Not available

1.2.3.1.2 Microbial degradation of PAHs

Microbes, both prokaryotes and eukaryotes, have the ability to metabolize PAHs and play an important role in PAH degradation in terrestrial and aquatic environments. Fig. I-3 shows pathways of microbial PAH degradation. The rate of PAH degradation is generally inversely proportional to the number of rings in the PAH molecule. Thus, the lower molecular weight PAHs are biodegraded more rapidly than the higher molecular weight compounds (Cerniglia 1992).

1.2.3.1.2.1 Bacteria

Most of the groundwork in the study of PAH metabolism was accomplished by examining bacterial systems. Several gram positive and gram negative bacteria have been isolated with the ability of metabolize PAHs as sole carbon and energy sources (Cerniglia 1993). In most bacteria and some green algae, a principal mechanism for the aerobic metabolism of PAHs involves ring oxidation by dioxygenases to form *cis*- dihydrodiols. These dihydrodiols are transformed further to catechols, which are cleaved by other dioxygenases (Cerniglia 1992). In the bacterial system (Fig. I-3), the initial step in the process is a dioxygenase mediated insertion of molecular oxygen to produce a *cis*-dihydrodiol intermediate. Dehydrogenases and other enzymes then produce intermediates suitable for ring cleavage. Once ring cleavage takes place, the products are typically broken down further to central metabolism intermediates.

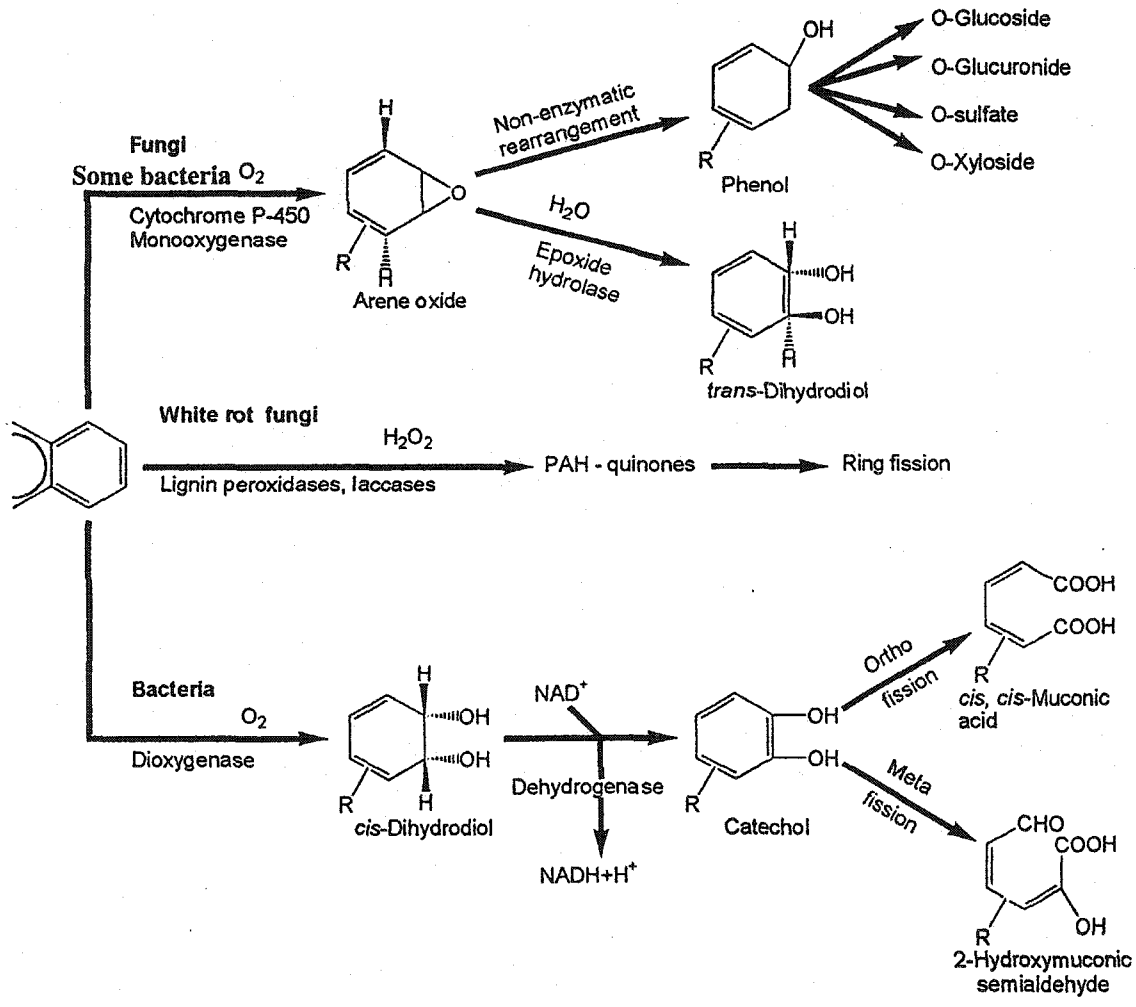

Fig. I-3. Pathways of microbial degradation of PAHs (Cerniglia 1993)

1.2.3.1.2.2 Fungi

In contrast to bacterial systems, fungi tend to transform PAHs by co-metabolism, often to dead-end metabolites (Andersson and Henrysson 1996). Significant detoxificaiton of PAHs takes place because the metabolites formed are often less toxic or mutagenic than their parent compounds (Sutherland 1992). Fig. I-3 shows the preliminary steps involved in fungal metabolism of PAHs. There, cytochrome P_{450} catalyzes the incorporation of one atom of molecular oxygen into the aromatic nucleus and reduces the remaining atom to water. The resulting arene oxide intermediate is carcinogenic and can undergo further metabolism by epoxide hydrolase to form a *trans*-dihydrodiol or undergo non-enzymatic rearrangement to produce a phenol which can form many conjugates as seen in Fig. I-3. Microorganisms that have only these pathways cannot utilize PAHs for carbon source but do achieve detoxification (Sutherland 1992; Cerniglia 1993).

1.2.3.1.2.3 White-rot fungi and their ligninolytic enzymes

Highly condensed PAHs are degraded to a limited extent intracellularly due to low solubility and restricted transport across cell membrane (Eggen and Majcherczyk 1998). White-rot fungi are characterized by their exceptional ability to degrade lignin, the protective polymer in wood, and produce extracellular peroxidases. The nonspecific nature of lignin depolymerization used by white-rot fungi can also be used to degrade a range of persistent environmental pollutants (Barr and Aust 1994).

Some white-rot fungi metabolize PAHs to quinones and other metabolites by mechanisms that do not appear to involve *cis*-dihydrodiols (Fig. I-3).

The possibility of PAH degradation by white-rot fungi was first raised by Bumpus (1989) in a study in which *P. chrysosporium* mineralized benzo[a]pyrene. Later research has shown that this fungus can transform fluorene (George *et al.* 1989), phenanthrene (Barclay 1985; Sutherland *et al.* 1991; Hammel *et al.* 1992), anthracene (Hammel *et al.* 1991), pyrene (Hammel *et al.* 1986), and fluoranthene (Vazquez-Duhalt *et al.* 1994).

Polycyclic aromatic hydrocarbon oxidation by purified LiP and MnP from *P. chrysosporium* has been studied. Lignin peroxidase-mediated PAH metabolism is thought to occur via an 1-electron oxidation to yield quinone products. Substrates with IP of around 7.55 eV, (such as anthracene, benzo[a]anthracene, pyrene, perylene, and benzo[a]pyrene) are thought to be the upper limit of substrates for LiP oxidation. However, white-rot fungal cultures typically transform PAHs with IPs far higher than 7.55 eV (such as phenanthrene). The purified MnP of *P. chrysosporium* has been shown to oxidize twelve 3-6 ring PAHs with IPs up to 8.2 eV in a MnP-lipid peroxidation system (Vazquez-Duhalt *et al.* 1994; Bogan and Lamar 1995; Bogan *et al.* 1996). Bezalel *et al.* (1997) proposed that the mechanism of phenanthrene degradation by *Pleurotus ostreatus* was the involvement of cytochrome P_{450} monooxygenase and epoxide hydrolase in the initial phase I oxidation of phenanthrene to form *trans*-9,10-dihydrodiol. Moen and Hammel (1994) proposed that lipid peroxidation by MnP of *P. chrysosporium* was the basis for phenanthrene oxidation by the intact fungus. Further evidence of MnP-lipid peroxidation of PAH

was observed for *Phanerochaete laevis*, which produced predominantly polar products, with no significant accumulation of quinone (Bogan and Lamar 1996). However, MnP from *N. frowardii* oxidized anthracene and pyrene to quinone products in the same MnP-lipid peroxidation reaction (Guenther *et al.* 1998).

Laccase can catalyze one-electron oxidations of PAHs such as anthracene and benzo[a]pyrene that have IPs less than 7.45 eV (Bezalel *et al.* 1996). The purified laccase from *C. gallica* has been shown to oxidize a range of 3-5 ring PAHs to quinones in the presence of chemical mediators 1-hydroxybenzotriazole and ABTS (Pickard *et al.* 1999b). No correlation was found between oxidation of PAHs and their IPs with the laccase from *T. versicolor* (Majcherczyk *et al.* 1998) or *Coriolopsis gallica* (Pickard *et al.* 1999b).

1.2.3.1.2.4 Bioremediation of PAHs using white-rot fungi

The use of microorganisms, either naturally occurring or introduced, to degrade pollutants is called bioremediation. The aim of bioremediation is to reduce pollutant concentrations to undetectable, non-toxic, or acceptable (i.e. within limits set by regulatory agencies) levels. Often the goal is to completely mineralize organic pollutants to CO_2, or in the case of metals to remove then by sorption or transformation to a less toxic form (Pointing 2001).

In situ PAH bioremediation by white-rot fungi has been studied by various groups and results vary due to different soil conditions and experimental protocols. Andersson and Henrysson (1996) reported that *T. versicolor* mineralized anthracene, benzo[a]anthracene, and dibenzo[a,h]anthracene in soil, whereas *P. chrysosporium*

and *Pleurotus sajor-caju* only partially transformed these PAHs, with significant accumulation of the dead-end metabolite 9,10-anthracenedione. Eggen and Majcherczyk (1998) reported that up to 49% of added benzo[a]pyrene was removed from soil by *P. ostreatus* after 3 months incubation. In soils spiked with a mixture of 3-7 ring PAHs, degradation of 3-4 ringed PAH was not enhanced above levels observed for indigenous (bacterial) microflora upon addition of *Pleurotus* sp. Conversely, the 5-7 ringed PAHs, which were not attacked by indigenous soil bacteria, were removed to 29-42% by *Pleurotus* sp. (Gramss *et al.* 1999). Another study reported significant removal of 3-ring (85-95%) and of 3- and 4-ring PAHs (24-72%) from creosote-contaminated soil by *Phanerochaete sordida* (Davis *et al.* 1993). Eggen (1999) reported that *P. ostreatus* removed 48% of 5-ring PAHs and 87-89% of 3-and 4-ring PAHs from creosote-contaminated soil. A decrease in total petroleum hydrocarbon concentration from 32 g/Kg to 7 g/Kg within 12 months in soil microcosms was observed for *T. versicolor* (Yateem *et al.* 1998).

Bioavailability of PAHs and the growth of the fungus are two important requirements for *in situ* PAH bioremediation. Addition of non-ionic surfactants to soil has been shown to increase bioavailability and PAH degradation by white-rot fungi (Bogan *et al.* 1999). Soil conditions must be favorable for growth of white-rot fungi and factors such as soil moisture, C/N ratio, temperature, and the indigenous microbial populations should be considered (Eggen and Sveum 1999).

The extracellular nature of white-rot fungal ligninolytic enzymes and their low-molecular-mass mediators enhances the bioavailability of pollutants to white-rot fungi. In contrast, bacteria with their cell-associated pollutant catabolism, may not

have access to the sources that contain high molecular weight substrates. One disadvantage of white-rot fungi in bioremediation is that bacteria generally utilize organopollutants as nutritional C and/or N sources, whereas organopollutants in most cases are only oxidized by white-rot fungal enzymes. Therefore, additional C and N sources are required for primary metabolism by white-rot fungi. In addition, white-rot fungi are obligate aerobes, whereas bacteria can transform and mineralize certain pollutants under different dissolved oxygen conditions (Pointing 2001). Combinations of bacteria and fungi in PAH bioremediation systems might be able to overcome the disadvantages of two systems, using white-rot fungi for initial PAH oxidation to produce less toxic and more water-soluble products for bacterial metabolism.

1.2.3.2 Applications of white-rot fungi and their ligninolytic enzymes

1.2.3.2.1 Transformation of environmental pollutants

White-rot fungi and their ligninolytic enzymes have been extensively examined for potential biotechnological applications, especially in the transformation and mineralization of persistent environmental pollutants. In addition to the study of PAH degradation, the use of white-rot fungi and their ligninolytic enzymes in degradation of munition wastes, pesticides, polychlorinated biphenyls, bleach plant effluent, synthetic dyes, synthetic polymers, and wood preservatives have been summarized by Pointing (2001).

Transformations of 2,4,6-trinitrotoluene (TNT) have been studied using the white-rot fungi *P. chrysosporium.* Transformation of TNT often results in formation of dinitrotoluenes (DNTs) as dead-end products by many bacteria and fungi. White-

rot fungi have been shown to be capable of transforming TNT to DNTs with subsequent mineralization to CO_2. Indeed, mineralization of DNTs to CO_2 has been demonstrated using purified MnP (van-Aken *et al.* 1999). Such mineralization has been shown to reduce mutagenicity of aqueous TNT wastes by up to 94%, as measured using the *Salmonella*/microsome bioassay (Donnelly *et al.* 1997).

Biodegradation of pesticides such as 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) has been shown with *P. chrysosporium* (Bumpus and Aust 1987). The white-rot fungi *P. chrysosporium,* plus *Pleurotus ostreatus, Phellinus weirii,* and *Polyporus versicolor* have been shown to be able to mineralize DDT, dicofol, and methoxychlor under ligninolytic growth conditions (Bumpus and Aust 1987).

Numerous studies have shown that white-rot fungi including *Coriolopsis gallica, P. chrysosporium, P. ostreatus,* and *T. versicolor* are capable of removing of polychlorinated biphenyls (PCBs) (Beaudette *et al.* 1998). Mineralization studies show that *Coriolopsis polyzona* (Vyas *et al.* 1994), *P. chrysosporium* (Dietrich *et al.* 1995), and *Trametes versicolor* (Beaudette *et al.* 2000) are capable of mineralizing PCBs.

Oxidation of bleach-plant effluent, with associated decolorization, has been demonstrated for *P. chrysosporium* (Jaspers *et al.* 1994). These studies showed that MnP was responsible for most of the decolorizing activity, with minor involvement of LiP. High decolorization efficiencies by laccase of *T. versicolor* have also been demonstrated (Limura *et al.* 1996).

Degradation of synthetic dyes studies have revealed that white-rot fungi are capable of decolorizing a wide array of azo, triphenylmethane and heterocyclic dyes (Pointing 2000). Most of the work was done by using *P. chrysosporium.* MnPs from *B. adusta* and *P. eryngii* have also been shown to catalyze dye decolorization (Heinfling *et al.* 1998). Two laccase isoenzymes purified from *C. gallica* were able to catalyze decolorization of several synthetic dyes (Rodriguez *et al.* 1999).

Biodegradation of the synthetic polymers polyvinylchloride (PVC) by the white-rot fungi *Pycnoporus cinnabarinus* (Larking *et al.* 1999) and nylon by *P. chrysosporium* and *T. versicolor* under ligninolytic conditions (Deguchi *et al.* 1997) has been reported.

Other compounds that can be efficiently degraded by white-rot fungi include the organic wood preservatives creosote and pentachlorophenol (PCP) (Mileski *et al.* 1988), and BTEX (benzene, toluene, ethylbenzene and xylenes) (Yadav and Reddy 1993).

1.2.3.2.2 Biopulping

Pulp bleaching is achieved by treating pulps with chlorine-based chemicals and the effluents contain toxic chlorinated aliphatic and aromatic compounds. To overcome the environmental problems caused by these polluting effluents, biopulping has been attempted. White-rot fungi with their unique lignin-degrading abilities have been studied. Laccase from *T. versicolor* was demonstrated to be effective in pulp bleaching in the presence of mediators such as ABTS or HBT (Call and Mucke 1994). MnP is capable of oxidizing phenolic lignin substructures via Mn(III)

formation and is also capable of oxidizing non-phenolic lignin substructures via "MnP-mediated lipid peroxidation". The utilization of a MnP-Tween 80 system for pulp bleaching was published by Kondo *et al.* (1994). An increased brightness and decreased pulp kappa number (lignin content) has been achieved in biopulping with white-rot fungi ligninolytic enzymes (Paice *et al.* 1993).

1.2.3.2.3 Polymerization

MnP can catalyze the polymerization of guaiacol, *o*-cresol, 2,6-dimethoxyphenol and other phenolic compounds under defined condition to produce industrially important materials such as polyguaiacol (Iwahara *et al.* 2000).

1.2.3.2.4 Production of natural aromatic flavors

Consumer preference for natural food additives has led to an increasing demand for natural aromatic molecules. White-rot fungi are able to completely degrade lignin, a polymer of substituted *p*-hydroxycinnamyl alcohols, and to metabolize the resulting phenolic monomers (such as *p*-coniferylic and *p*-sinapylic alcohols) into aromatic compounds interest. Compared with other microorganisms, the "volatile spectrum" of white-rot fungi is closest to that of plants and contains many aromatics such as vanillin, benzaldehyde, phenylacetaldehyde, 1-phenylethanone and methylbenzoate (Lonascolo *et al.* 1999).

1.2.4 Enzyme modification

1.2.4.1 Enzyme catalysis in water-organic solvent mixtures

Polycyclic aromatic hydrocarbons are only sparingly soluble in water (Table I-2). Therefore, the use of enzymes in aqueous systems for the oxidation of PAHs would be severely limited by substrate availability. Polycyclic aromatic hydrocarbons are several times more soluble in organic solvents than in water. Organic solvents have been widely used in PAH oxidation systems. However, enzymes are believed to function effectively only in water-rich media and the presence of organic solvents normally decreases enzyme activity.

Enzyme catalysis in organic solvents has received considerable attention and the advantages of using organic solvents include, for example, increased solubility of hydrophobic substrates and favorable shifts of reaction equilibrium (Khmelnitsky and Rich 1999). In the study of PAH oxidation by MnP, on the one hand, water-miscible organic solvents are required to enhance substrate availability. On the other hand, enzyme reactions must take place in aqueous media due to the involvement of H_2O_2 in the reaction system.

Enzymes exhibit their catalytic properties to the full extent only when they have a strictly defined (native) conformation. The conformation of the enzyme molecule in solution is determined by a complicated network of hydrogen bonds and electrostatic and hydrophobic interactions. Water plays an important role in the structure and activity of enzymes by determining the web of electrostatic interactions that influence the final protein conformation (Khmelnitsky *et al.* 1988). When organic solvents are used, if they are more hydrophilic than the surface of the protein, it will

be energetically more favorable for water to enter the solvent. This process will also be driven by the increase in entropy resulting from the release of bound water. If the free energy of this process overcomes the activation energy of the resultant unfolding and reorganization of the protein, denaturation of the enzyme will result. Further, loss of a "shell" of hydration around the enzyme will increase the vulnerability of the enzyme to electrostatic effects from the solvent, leading to further alterations in structure at critical hydrogen bonding and other sites (Russel *et al.* 1992). Beyond effects on conformation, hydration of an enzyme can also play a role in the dynamic nature of enzymatic catalysis and "flexibility". Water molecules can be important in the transient alternations in shape and play an important role in the catalytic cycles of several enzymes (Russel *et al.* 1992).

The addition of increasing concentrations of water-miscible organic solvents produces similar curves in initial enzyme activity when hydrophobic substrates are used, as noted by many researchers (Khmelnitsky *et al.* 1991; van Erp *et al.* 1991; Vazquez-Duhalt *et al.* 1993; Torres *et al.* 1996) (Fig. I-4). When the organic solvent concentration is low, the mass action can limit the biocatalytic activity. When the concentration of organic solvent is increased, the mass transfer limitation is reduced and the biocatalytic activity reaches its maximum value (100% activity). Then, an increase of organic solvent concentration induces a decrease of biocatalytic activity. Threshold concentration (C_{50}) is defined as the specific organic solvent concentration at which half-inactivation occurs.

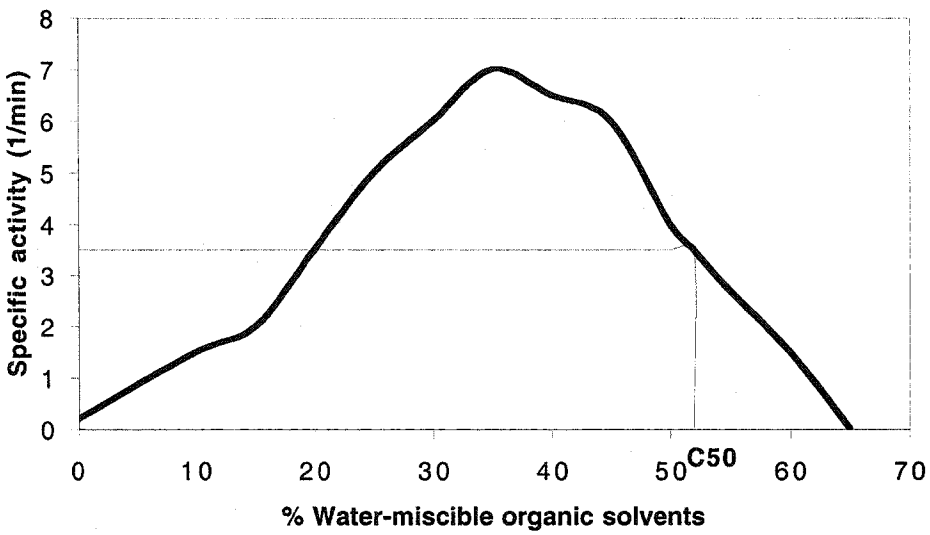

Fig. 1-4. Specific activity curve of enzyme catalysis reactions in different water-miscible organic solvent concentrations (Torres *et al.* 1996).

1.2.4.2 Enzyme modification for improved activity in water-organic solvent mixtures

Researchers have attempted to modify enzymes in various ways to prevent denaturation, mainly due to dehydration of enzyme in the presence of organic solvents. For the same substrate and organic solvent system,C_{50} can be used to evaluate if the modified enzyme has enhanced catalytic activity in the presence of organic solvent. Most of the enzyme modification methods used to improve enzyme activity in non-aqueous organic solvents can be tried in water-organic solvent mixtures. Key among these methods are immobilization, cross-linking, protein engineering, and chemical modification.

1.2.4.2.1 Immobilization

Several researchers have studied the covalent or non-covalent attachment of biocatalysts to solid or soluble supports as a tool for enhancing enzyme in organic solvents. It is hypothesized that binding to these supports aids enzymes in retaining their conformation and activity by locking a portion of their structure in place. Covalent binding of this nature to various supports has been shown to provide stability against fluctuations in ionic concentrations and pH, and to improve activity in organic solvents (Ruiz *et al.* 2000). Laccase has been immobilized on various supports (glass, glass powder, silica gel, and nylon membrane) with improved activity and stability of activity in non-aqueous organic solvents (Ruiz *et al.* 2000).

1.2.4.2.2 Crosslinking

Another attempt to ensure enzymes retain conformation in dehydrating organic solvents is to use crosslinked enzyme crystal technology. Crosslinking results in both stabilization and immobilization of the enzyme without dilution of activity as the protein matrix is both the catalyst and the support (Govardhan 1999). Crosslinked enzyme crystal technology involves two major steps: crystallization of the enzyme and chemical crosslinking of enzyme crystals. Protein crystallization is performed under defined condition using vapor diffusion methods (Govardhan 1999). Glutaraldehyde is a cheap and efficient crosslinker, it binds to and links lysine amino groups on different enzyme moieties (Desantis and Jones 1999). Large scale preparations of crosslinked enzyme crystals from *Pseudomonas cepacia* lipase have been demonstrated to be very successful and many other reports have shown that cross-linking is an inexpensive method to enhance enzyme stability (Collins *et al.* 1998). A first report of catalytically active crossed-linked crystals of chloroperoxidase (Ayala *et al.* 2002) shows this method can be applied to peroxidases.

1.2.4.2.3 Chemical modification with monofunctional polymers

Another approach for increasing enzyme stability in organic solvent involves covalent attachment of polymeric functionalities such as polyethylene glycol (PEG). Attachment of these polymeric groups is often achieved using activation with triazine ring derivatives (e.g. cyanuric chloride). The triazine ring reacts with surface amino groups on the protein under alkaline conditions, covalently linking the polymer

to the enzyme surface. Polyethylene glycol has both hydrophilic and hydrophobic properties. The hydrophilic nature makes it possible to modify enzymes in an aqueous solution; the hydrophobic nature enables modified enzymes to function in a hydrophobic environment. Activated PEG allows the enzyme to be modified under mild conditions (Inada *et al.* 1986). Several reports have shown significant increases in enzyme stability following PEG attachment (Tinoco and Vazquez-Duhalt 1997; Herniaz *et al.* 1999; Koops *et al.* 1999; Wang *et al.* 1999). Another polymer, polyoxyethylene(23)lauryl ether (Brij 35), activated with cyanuric chloride, has been used to increase the stability and activity of a catalase in organic solvents. Typically, the binding of these groups does not significantly change protein structure (Khmelnitsky *et al.* 1988). However, Tinoco and Vazquez-Duhalt (1997) noted an increase in the substrate range of cytochrome c after modification with PEG, indicating that structural changes may take place.

1.2.4.2.4 Chemical modification with small moieties

Another method for increasing enzyme stability is to add small molecular functionalities onto the protein. Ribonuclease A was chemically coupled to D-glucosamine producing mono- and di-glycosylated enzyme which increased specific activity by 80% compared to the unmodified enzyme (DeSantis and Jones 1999). Methylation of carboxylic acid groups is another possible modification method, this method has no specific reaction site and all free carboxylic groups have the potential be modified (Tinoco and Vazquez-Duhalt 1997).

1.2.4.2.5 Protein engineering

Another avenue for increasing activity in organic solvents involves the engineering of amino acid sequences for greater stability. Substantial improvements have been noted with minor sequence alternations and extensive work by Arnold *et al.* (1990) has produced a set of guidelines for engineering protein with enhanced stability in non-aqueous solvents. These design considerations are aimed at increasing conformational stability by maximizing internal crosslinks, van der Waals interactions, hydrogen bonds, and interior electrostatic interactions. In addition, alterations in amino acids at the surface of a protein can be made to increase compatibility with solvent systems.

1.3 Overview of the study

The overall objectives of the work in this thesis were to find a new source of enzyme suitable for PAH degradation. Two uncharacterized MnP-producing *B. adusta* strains (UAMH 7308 and 8258) were chosen as candidates for this purpose because of their ability to degrade PAHs. In addition, these strains produce only MnP in the culture medium, thus simplifying purification. The potential for large-scale enzyme production is high because significant MnP levels are produced. In this study, the MnPs produced by these organisms have been purified and characterized. To prepare this MnP for further PAH oxidation, chemical modification of the enzyme by covalent attachment of polymeric group of PEG was performed. Finally, PAH oxidation by purified native- and chemically-modified MnP were compared and the PAH oxidation products were identified. Polycyclic aromatic hydrocarbon

metabolism and mineralization by living fungal cells was also studied to gain a better understanding the role of MnP in overall PAH metabolism.

2. Materials and Methods

2.1 Microorganisms

Bjerkandera adusta strains UAMH 8258, UAMH 7308 were obtained from the University of Alberta Microfungus Collection and Herbarium (UAMH), Devonian Botanical Gardens, University of Alberta, Edmonton, Alberta, Canada. *B. adusta* ATCC 90940 was obtained from the American Type Culture Collection, Manassas, VA.. The fungi were grown on potato-dextrose agar (Difco, Detroit, MI., U.S.A) at 28 °C for 5-7 days before storage at 4 °C.

2.2 Cultivation conditions

2.2.1 Media

The standard basal medium (SBM) used in this study contained 2.2 mM diammonium tartrate, 56 mM glucose, 1 mg/l thiamine and BIII mineral medium (Tien and Kirk 1988), in 20 mM 2,2-dimethylsuccinate (pH 4.5) buffer. Mn(II) and organic acids were added as inducer to 3-day-old fungal culture in SBM to a final concentration of 1 mM and 5 mM respectively. All the SBM based media were filter sterilized by FP 030/3 filters, with pore size of 0.2 μm (Schleicher and Schuell, Dassel, Germany). Inocula for cereal medium characterization studies were grown in a glucose-malt-yeast extract (GMY) medium, which supported excellent growth of all fungi. It contained (per liter) 10 g glucose, 3.5 g malt extract (Difco), 2.5 g yeast extract (Difco), 2.0 g KH_2PO_4 and 0.5 g $MgSO_4.7H_2O$ (Pickard *et al.* 1999a). The optimized growth medium for *Bjerkandera* sp. BOS55 (ATCC 90940) (Mester and Field 1997) was also used, which combined GMY and SBM (GMY-SBM) by

supplying 40 mM KH_2PO_4, 3 g/l of mycological peptone, 3.5 g/l malt extract and 2 g/l yeast extract to SBM. Mn(II) or Mn(II)-glycolate was also added to final concentrations of 1 mM and 5 mM respectively after 3-day-growth. Other variations on these basic media are described in the Results section.

2.2.2 Fungal inocula

Inocula were prepared by first growing the fungus on potato-dextrose agar plates. Since *B. adusta* does not readily sporulate on agar plates or in submerged culture, transfer is by vegetative mycelium which grows radially from the central inoculum. Then 1 cm^2 of mycelium was taken from the outer edge of the fully-grown plate and transferred into 50 ml liquid medium (GYM) in a Sorvall Omnimixer (Sorvall, Norwalk, CN., U.S.A), homogenized for 5-10 s. After 3days of growth in 500-mL Erlenmeyer flasks at 200 rpm and 28 °C, the cultures were again homogenized. A 5-10% (v/v) inoculum was used for the enzyme production medium. Production medium was 3% rice bran in 60 mM potassium phosphate buffer pH 6.

2.2.3 Scale-up enzyme production

For scale-up enzyme production to 14-L stirred tank reactors, an additional inoculum stage was used from 50ml homogenized fungal pellets to 500 ml GMY medium in a 2-L Erlenmeyer flask which was grown for 3 days, and used without further homogenization. Rice bran (Satin Finish, Wolcott Farms Inc, Willows, CA. U.S.A), 300 g in 3 l 60 mM potassium phosphate buffer, was autoclaved for 30 min at 121°C three times over two days before pumping into 7 l sterile 60 mM potassium

phosphate buffer in a 14-L fermentor (Microferm, New Brunswick, NJ., U.S.A). Growth was at 28° C with an air flow rate of 0.2 v/v/min, stirrer speed 200 rpm. Each day the impeller speed was raised to 500 rpm for 30 s to wash back into the liquid any fungal pellets that had accumulated at the air/ water interface. Samples were taken daily.

2.3 Enzyme purification

When MnP activity reached the maximum, the culture was filtered through cheese cloth and the culture filtrate frozen at -20 °C. When thawed much of the extracellular polysaccharide and some pigment produced by fungus formed a floc and was removed by centrifugation. The supernatant was then concentrated twenty-fold by ultrafiltration (PM-10, Amicon, Lexington, MA.). Polyethyleneimine (PEI) at pH 6.5 was added to the final concentrate to 0.1% and stirred for 15 min at 4 °C. A significant portion of the pigment bound to PEI and was removed by centrifugation. The PEI-treated supernatant was then dialyzed against 10 mM phosphate buffer pH 6 ("buffer") prior to application to a DEAE-cellulose anion-exchange column (Whatman DE-52, Maidstone, England). The column was washed with two volumes of buffer, followed by one volume of 100 mM KCl in buffer, then a shallow, linear gradient from 100 to 300 mM KCl in buffer was used to elute the enzyme. MnP was eluted at about 120 mM KCl. The peak fractions were pooled, the enzyme pool concentrated by ultrafiltration, and the concentrate applied to a size exclusion column (Sephadex G-100; Sigma, St. Louis, Mo., U.S.A) and eluted with 100 mM sodium chloride in buffer. Again, the peak fractions were pooled, concentrated, dialyzed and

applied in 5 mg protein portions to an anion exchange FPLC column (MonoQ HR5/5; Pharmacia). A 100 to 300 mM KCl gradient was used to elute enzyme from the MonoQ column. Additional applications to FPLC were used to confirm the purity of the enzyme. The specific activity and Reinheitzahl (Rz) (A_{407nm}/A_{280nm}) ratio of the pool from each purification step were measured as indicators of purity.

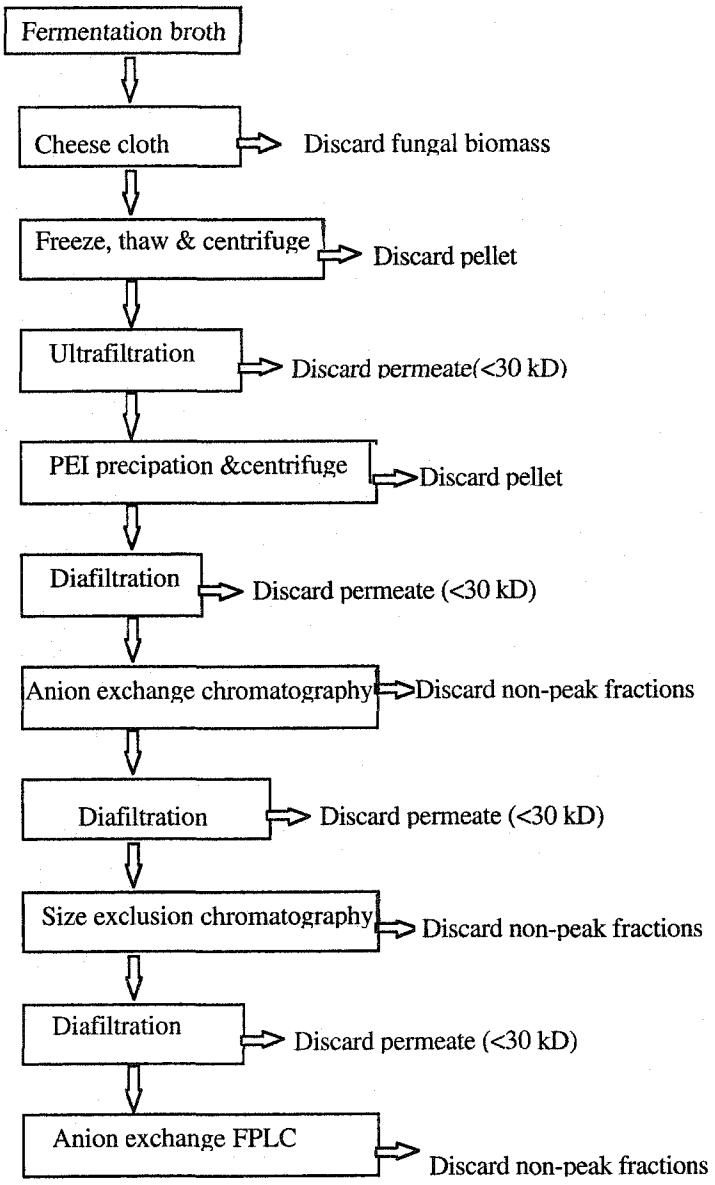

Fig. II-1 Scheme for MnP purification.

2.4 Enzyme assays

Lignin peroxidase was measured by the method of Tien and Kirk (1988) following the H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde at 25 °C. Reaction mixtures contained 4 mM veratryl alcohol in 40 mM succinate buffer, pH 3, and were initialized by the addition of H_2O_2 to a final concentration of 0.4 mM. Absorbance was monitored at 310 nm (ε_{310}=9300 M^{-1} cm^{-1} for veratraldehyde). Manganese peroxidase activity was measured at 30 °C by the H_2O_2-dependent formation of oxidized manganic-malonate complex at 270 nm (ε_{270}=11590 M^{-1} cm^{-1}). Reactions contained 1 mM manganous sulfate in 50 mM malonate buffer (pH 4.5) and 5-50 µl of enzyme sample and were initialized by the addition of H_2O_2 to a final concentration of 0.1 mM (Wariishi *et al.* 1992). Laccase was determined by ABTS oxidation (Wolfenden and Wilson 1982) in a reaction containing 1 mM ABTS in 0.1 M sodium acetate buffer (pH 4.5) with a 5-50 µl enzyme sample. The oxidation was followed at 30 ° C and at 436 nm (ε_{436}=36000 M^{-1} cm^{-1} for ABTS).

2.5 Enzyme characterization

2.5.1 Physical characteristics of purified MnP

The molecular mass of the enzyme was determined by SDS-polyacrylamide gel electrophoresis (15% polyacrylamide gel) using marker proteins (Sigma, St. Louis, MO., U.S.A). The protein mass was also estimated by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, courtesy of

Dr Liang Li, Department of Chemistry, University of Alberta. The analysis for molecular weight was performed as follows: the matrix used in these experiments was α-cyano-4-hydroxycinnamic acid (HCCA), HCCA purchased from Aldrich was first purified by recrystallization from ethanol. A two-layer MALDI deposition method was used. This involves the deposition on the probe tip of a microcrystalline matrix layer via fast evaporation from a 0.7 μl solution of HCCA (12 mg/ml) dissolved in 80% acetone/methanol. One microliter of the sample was mixed with either 1 or 9 μl of a saturated HCCA solution in 40% methanol/0.1% TFA in water. The solution was briefly vortexed and a 0.4-μL portion was deposited on top of the first matrix layer. Once dried, the spot was washed three times with 0.75 μl of room temperature water. The samples were run on a Hewlett Packard LD-TOF system, equipped with delayed extraction, data processing was done with the IGOR Pro software package (Wavemetrics Inc. Lake Oswego, OR., U.S.A).

Isoelectric focussing (IEF) was performed with 5% polyacrylamide gels (pH 3-10) (Bio-Rad) and protein bands were stained with silver nitrate. The IEF standard proteins were purchased from Sigma and fungal isoenzymes of known pI were also used (Pickard and Hashimoto 1988).

The amino acid composition and N-terminal sequence of MnP was done by Alberta Peptide Institute, University of Alberta. The methods were briefly described as follows: the amino acid composition was determined, after hydrolysis of 250 pmol of protein with 6 M HCl at 110°C, using a Biochrom-20 autoanalyser (Pharmacia). The hydrolyzed sample was separated on a C-18 column using 0-70% acetonitrile gradient in 0.1% trifluoroacetic acid (1 ml/min). The N-terminal sequence of MnP

was obtained by automated Edman degradation of 250 pmol of protein in an Applied Biosystems 494 pulsed-liquid protein sequencer.

2.5.2 Enzyme catalytic properties

The oxidation of 2,6-dimethoxyphenol (DMP), ABTS, veratryl alcohol, 4-aminophenol, and hydroquinone was measured at wavelengths of 472, 436, 310, 274 and 253 nm, respectively. The reaction mixture contained final concentrations of 50 mM malonate buffer with pH range from 2-7.5; MnP 0.2 U/ml; substrate 1 mM; H_2O_2 0.1 mM; with 0.5 mM $MnSO_4$ for Mn(II)-dependent peroxidase and no $MnSO_4$ for Mn(II)-independent peroxidase activity.

The Mn(II)-dependent and independent activities of native and chemically modified MnP were measured at their respective optimum pH values of 5 and 3. Kinetic data were obtained from the Lineweaver-Burk plots regression analysis and k_{cat} values were calculated using the molar coefficient of the products: Mn(III)-malonate complex (ε=11590 $M^{-1}cm^{-1}$); oxidized DMP (ε=49600 $M^{-1}cm^{-1}$), ABTS (ε=36000 $M^{-1}cm^{-1}$), veratryl alcohol (ε=9300 $M^{-1}cm^{-1}$) and guaiacol (ε=12100 $M^{-1}cm^{-1}$).

All other assays for enzyme characterization were based on modifying the spectrophotmetric assay for Mn(II)-dependent peroxidase following the formation of the Mn(III)-malonate complex at 270 nm.

2.6 Enzyme modification

Methoxypolyethylene glycol activated with cyanuric chloride (Sigma) was used to chemically modify the enzyme, based on the method described by Vazquez-Duhalt *et al.* (1993) (Fig. II-2). Enzyme and activated PEG in five times molar excess were mixed with 50 mM potassium phosphate buffer, pH 7, and the mixture was gently shaken at 4 °C for 4 days. After dilution in 10 volumes of 20 mM phosphate buffer pH 6, the unreacted activated PEG was removed by ultrafiltration using an Amicon PM-10 membrane, dialysed and concentrated. Trinitrobenzene sulfonate was used to determine the number of amino groups modified (Habeeb 1966).

2.7 *In vivo* metabolism of ^{14}C-labelled PAHs by *B. adusta*

The potential for *B. adusta* to mineralize ^{14}C-labelled PAH was determined in biometer flasks containing 50 ml of rice bran medium: 3% rice bran in 60 mM phosphate buffer (pH 6) or GYM medium (Pickard *et al.* 1999b) inoculated with 5% (v/v) homogenized GMY-grown mycelium. After 3-day-growth at 28 °C, with shaking at 150 rpm, approximately 0.1 μCi of ^{14}C-PAH was added in 1 ml acetonitrile. Potassium hydroxide (1M) was used as the CO_2 trap and the biometer flasks were incubated at 28 °C in the dark with shaking. Triplicate flasks were used for each PAH and autoclaved cells were used as control. At regular intervals, 0.5 ml samples of the KOH trap were removed, mixed with 9 ml of Scintillation Cocktail plus 0.5 ml of 1 M acetic acid. The radioactivity was determined by liquid scintillation counting using a Beckman Model 3800 Liquid Scintillation Counter.

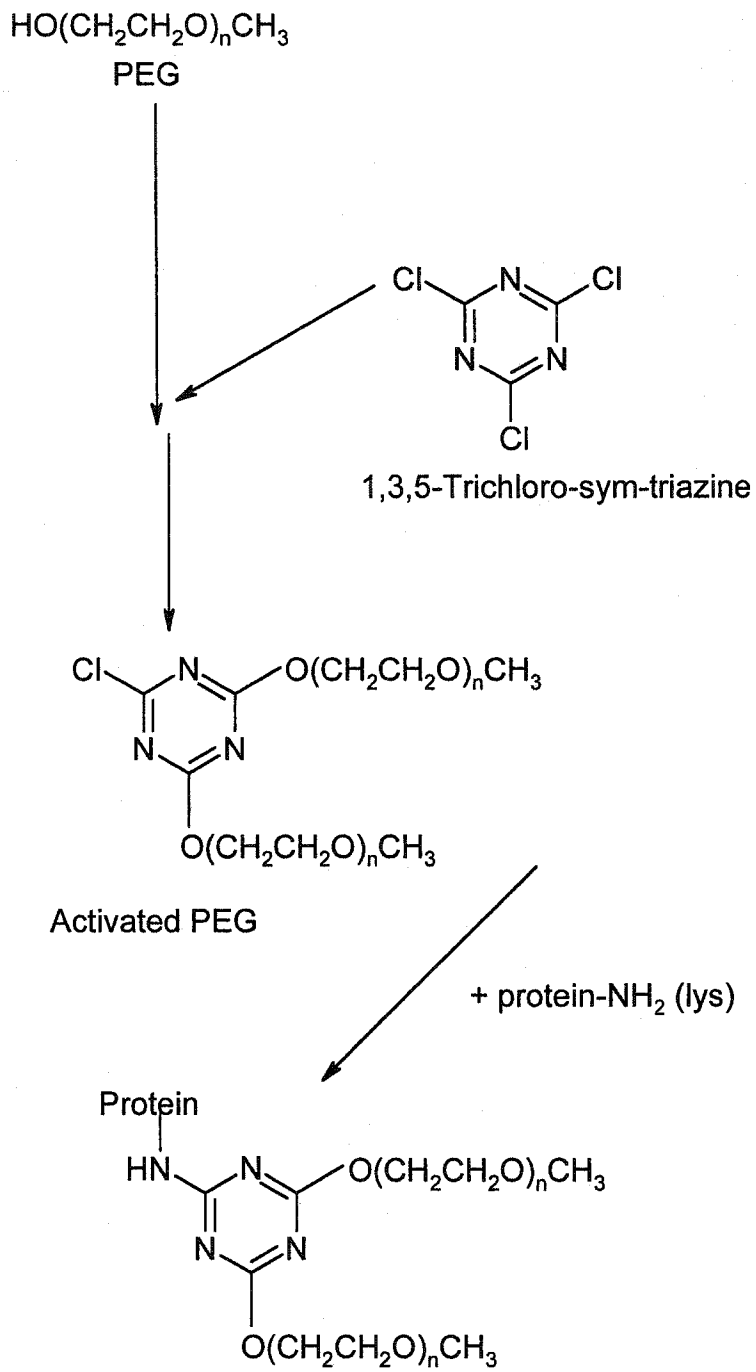

Fig. II-2. Chemical modification of protein with PEG.

For the distribution of the ^{14}C in fungal culture after 40 days incubation, the culture fluid and mycelia were homogenized andextracted three times with 1/3 volumes of dichloromethane. The extracts were combined and 1 ml was counted with liquid scintillation counter, representing the hydrophobic compounds. Then the aqueous homogenate was acidified with HCl to pH 2, and 1/3 volumes of ethylacetate were used to extract the polar organics from the homogenate. The homogenate was washed three times, and 1 ml of this pooled ethylacetate extract used for scintillation counting. The homogenate was resuspended in 100 ml 60 mM phosphate buffer (pH 6) rehomogenized and 1 ml of this homogenate was dried and counted for radio activity, representing biomass and water-soluble metabolites. The mass balance was calculated from the total radioactivity added to each flask.

2.8 *In vivo* fungal PAH metabolism

Inocula were grown in GYM medium at 28°C for 3 d, homogenized and 5% (v/v) used as inoculum for 25 ml of 3% rice bran in 60 mM phosphate buffer (pH 6) or GMY medium in a 125-ml flask. After a further 3-day-growth at 28°C shaker at 150 rpm, 1 ml of PAH stock solution containing 0.125 mg of PAH/ml in acetonitrile was added to give a final concentration of 5 μg PAH/ml. The cultures were extracted with distilled tetrahydrofuran and analyzed by HPLC as described previously (Pickard *et al.* 1999b).

2.9 Enzymatic PAH transformation

Oxidation of PAHs by purified MnP was carried out in glass reaction vials and the reaction monitored by HPLC analysis directly from the vials. For the pH studies, reaction mixtures contained final concentrations of 20 μM of PAH, 20% acetonitrile, 50 mM malonate buffer pH 2-6, 0.5 mM $MnSO_4$ and 1 U/ml enzyme. For the Mn(II)-independent activity, $MnSO_4$ was omitted. Reactions were started by adding H_2O_2 to 0.1 mM and stopped by adding a volume of acetonitrile equal to the reaction volume. All experiments were done in triplicate and the data presented are means of the triplicates.

For the study of effect of water-miscible organic solvents on PAH oxidation, 2-methylanthracene was used as substrate, and different organic solvents were used in Mn(II)-independent oxidation system as described above.

For the study of enzyme stability in acetonitrile, native and chemically modified enzyme were preincubated in 10, 20, and 50% acetonitrile, and 10 ul of sample was taken from each of the mixtures every 10 min and transferred in the Mn(II)-independent reaction mixture as described above.

2.10 Analytical techniques

Analysis of enzymatic reactions by HPLC were carried out on a Spheri-10, RP-18, 10 μm reversed-phase column (100 × 4.6 mm, Brownlee Columns, Perkin Elmer) with isocratic elution using acetonitrile-water (60:40 v/v). The PAH concentration was estimated from the peak area and quantified using a standard curve.

GC/MS samples were prepared by extracting the reaction mixture after acidification to pH 2 with dichloromethane three times. The extracts were pooled, dried over anhydrous sodium sulfate and concentrated under nitrogen. The assay was carried out on a Hewlett-Packard GC (model 6890) coupled to an MS detector (model 5972). The GC/MS was equipped with a type SPB-20 column (30 m by 0.25 mm; Supelco); the temperature program started at 90 °C for 2 min, increasing to 250 °C at 12 °C/min and kept at 250 °C for 18 min.

2.11 Chemical assays

Reducing sugars were determined by the dinitrosalicylic acid method, using D-glucose as a standard (Ghose 1987). Protein content was determined by the method of Bradford (1984). The dye-binding reagent was purchased from BioRad and samples were compared against concurrently prepared serial dilutions of the bovine serum albumin (BSA) as standard.

2.12 Chemicals and radioisotopes

Veratryl alcohol, ABTS and sodium malonate were purchased from Aldrich (Milwaukee, WI., U.S.A.). Glucose, yeast extract and malt extract were purchased from Difco Laboratories (Detroit, MI., U.S.A.). All other chemicals were of reagent grade and were obtained from Sigma (St. Louis, MO., U.S.A.).

1-Methylanthracene, 1-methylphenanthrene, benzo[a]pyrene, benzo[e]pyrene, carbazole, 9-methylanthracene, 2-methylanthracene, anthracene, acenaphathene,

pyrene, fluoranthene, dibenzo thiophene, chrysene, phenanthrene, naphthalene and anthraquinone were obtained from Aldrich (Oakville, Ontario, Canada).

[9,10-^{14}C]Phenanthrene (19.3 mCi/mmol), [4,5,9,10-^{14}C]pyrene (59.5 mCi/mmol), [9,10-^{14}C]anthracene (58 mCi/mmol), [3-^{14}C]fluoranthene (55 mCi/mmol) were purchased from Amersham Life Science (Amersham, UK.).

3. Results

The overall objective of this work was to investigate PAH metabolism by *B. adusta* and to study the involvement of MnP in PAH metabolism. Previous observations of PAH degradation by a variety of white-rot fungi have shown that two uncharacterized *Bjerkandera adusta* strains from UAMH are good candidates for *in vivo* metabolism of PAHs (Pickard *et al.* 1999b). Preliminary results have also shown that cereal bran media supports ligninolytic enzyme production by these two UAMH strains (Pickard *et al.* 1999a).

The results section of this thesis is divided into four parts. Part 1 (section 3.1) deals with the optimization of MnP production conditions and large-scale enzyme production; part 2 (section 3.2) illustrates MnP purification and characterization; part 3 (section 3.3) describes the chemical modification of enzyme for enhanced stability in organic solvent-water system; part 4 (section 3.4 and 3.5) describes PAH oxidation by purified MnP and metabolism by *B. adusta* UAMH 8258 cultures.

3.1 Optimizing MnP production conditions

Culture conditions and medium composition can play a major role in the production of extracellular fungal enzymes. Studies were conducted to optimize MnP production conditions by varying medium composition, growth temperature, initial pH and culture volume to flask volume ratios. Previous studies have shown that *B. adusta* strains UAMH 7308 and 8258 produce high levels of MnP in bran flakes medium (Pickard, *et al.* 1999a). In this work, a well studied *Bjerkandera* sp. BOS55

(ATCC 90940) was used as a reference strain to compare results from this study with published data.

3.1.1 Optimizing MnP production medium

3.1.1.1 Screening of bran sources for enzyme production

It has been shown that a carbon source such as natural lignin from cereal bran (Wang *et al.* 2001; Pickard, *et al.* 1999a), wheat straw (Hofrichter *et al.* 1999), or hardwood Kraft pulp (Miura *et al.* 1998) can effectively support ligninolytic enzyme production in submerged cultures for several fungi. Because different sources of bran have different properties (Table III-1), it is necessary to assay supernatants from fungal cultures grow with these different bran sources for MnP production.

Table III-1. Nutritional information of Kellogg's bran flakes, Quaker oat bran, Satin Finish rice bran and 100% Natural Bran wheat bran.

Bran	Protein (%)	Fat (%)	Carbohydrate (%)	Dietary Fibre (%)
Bran Flakes (BF)	10.3	2	90	15
Oat Bran (OB)	17	9	63	17
Wheat Bran (WB)	16	4	64	42
Rice Bran (RB)	13	20	60	8.5

Three *B. adusta* strains were compared for MnP production in the four different bran sources described above, at 3% (w/v). Samples were taken daily for MnP activity assays. The results of this experiment (Fig. III-1) showed that the best medium for MnP production by three strains in shake flask was

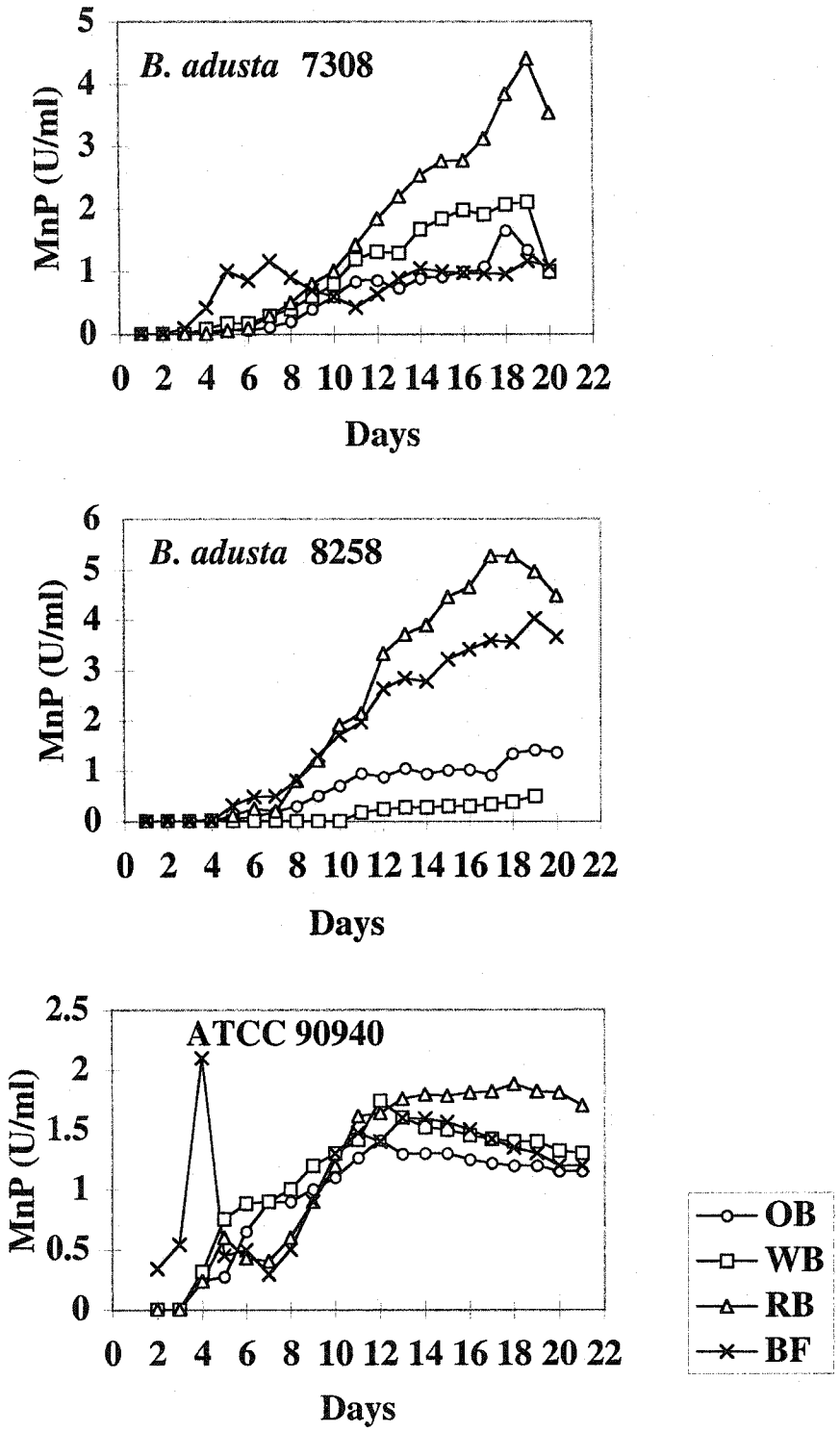

Fig. III-1. Comparison of MnP production in different bran media with 3% bran (w/v), phosphate buffer pH 6, 28 °C, 200 rpm. Triplicate flaks were used. Error bars showing standard deviations were close, as shown in Fig. III-4, and are not shown here.

rice bran (RB). The maximum MnP level produced by *B. adusta* 7308 and *B. adusta* 8258 was about 4.5 U/ml and 5.5 U/ml, respectively, on day 18 in this medium. Strain ATCC 90940 produced up to 1.7 U/ml of MnP in rice bran, and when BF were used, two peaks of MnP activity were consistently observed. The first peak was very sharp, appearing around day 4 and disappearing within 24 h. The second peak reflected that produced by using the other bran media and by the other strains, reaching a maximum after day 12 and remaining at that level for a few days before slowly declining. Triplicate flasks were used. To maximize clarity, error bars were only shown in Fig. III-4.

For each individual fungal strain, enzyme production profiles were different with different bran sources, indicating enzyme production was affected by different bran sources. Rice bran contains the highest percentage of fat and the lowest percentage of dietary fibre, whereas WB bran contains the highest percentage of dietary fibre (Table III-1). In order to find out if increased dietary fibre concentration in RB, and increased soluble starch concentration in RB and WB affect enzyme production, mixtures of RB (1.5%)+WB (1.5%), soluble starch (1%)+RB (2%), and soluble starch (1%)+WB (2%) were used as media to compare enzyme production. Fig. III-2 shows that high levels of MnP production were obtained in RB (2%)+starch (1%) medium by all three strains. MnP peak titres and the incubation times to reach the maximum are shown in Table III-2. Higher enzyme production was obtained in RB medium by UAMH strains, and the addition of soluble starch (1%) in RB (2%) shortened the required incubation time with only a slight reduction in enzyme production.

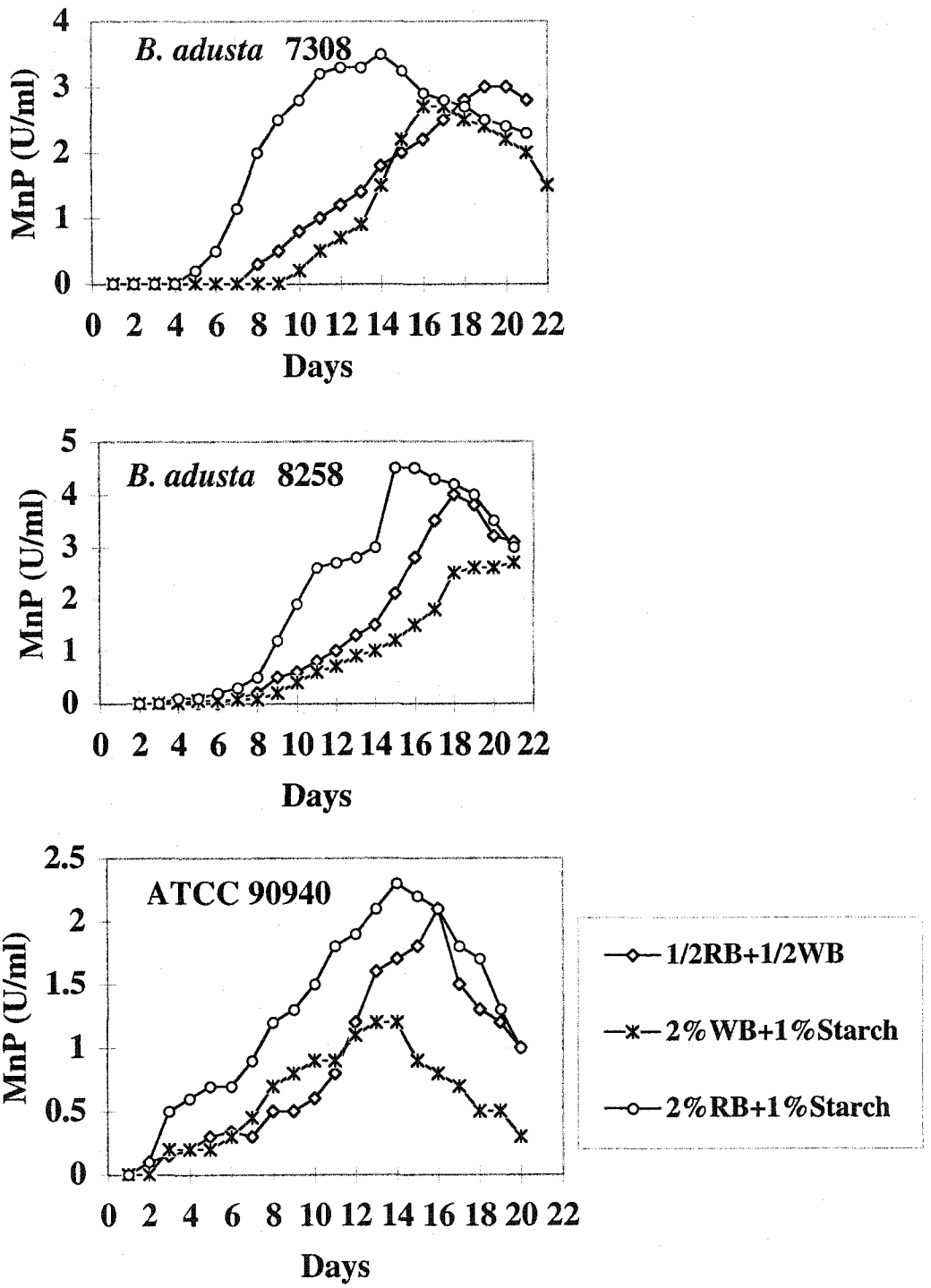

Fig. III-2. Comparison of MnP production in modified bran media (1.5% rice bran (w/v) +1.5% wheat bran (w/v), 1/2RB+1/2WB; 2% wheat bran (w/v)+1% starch (w/v), 2%WB+1%Starch; 2% rice bran (w/v)+1% starch (w/v), 2%RB+1%Starch), phosphate buffer pH 6, 28 °C, 200 rpm.

Table III-2. Comparison of MnP production by *B. adusta* strains: UAMH8258, UAMH 7308 and ATCC 90940.

	UAMH 8258		UAMH 7308		ATCC 90940	
Growth Medium	MnP Activity (±SD)(U/l)	Days to Peak MnP Titre	MnP Activity (±SD)(U/l)	Days to Peak MnP Titre	MnP Activity (±SD)(U/l)	Days to Peak MnP Titre
GMY[a]	0	NA[e]	0	NA	0	NA
SBM[b]	0	NA	0	NA	0	NA
SBM-Mn(II)[c]	23(±11)	10	15(±12)	10	0	NA
SBM-Mn(II)-Organic Acid*[c]	52(±10)	10	43(±10)	10	23(±10)	10
GMY-SBM-Mn(II)[c]	300(±230)	7	200(±120)	7	1300(±200)	7
GMY-SBM-Mn(II)-Glycolate[d]	400(±120)	7	420(±70)	7	1700(±320)	7
3% BF	2100(±780)	12	2200(±250)	16	2100(±350), 1500(±170)	4, 12
3% WB	2500(±160)	20	2500(±470)	18	1700(±470)	15
3% OB	1700(±920)	11	2200(±620)	18	1600(±420)	15
3% RB	5500(±250)	18	4400(±350)	18	1700(±250)	15
1% Starch+2% RB	4500(±370)	15	3500(±440)	14	2200(±450)	14

*Organic acid: gluconic acid, glycolic acid, glyoxylic acid and malonic acid.
[a] GYM: Pickard *et al.* (1999a).
[b] SEM: Tien and Kirk (1988).
[c] GYM-SEM-Mn(II): Mester and Field (1997).
[d] GYM-SEM-Mn(II)-Glycolate: Mester and Field (1997).
[e] NA: Not applicable.

A variety of media used by other researchers to produce manganese peroxidase were also compared using UAMH 7308, 8258 and ATCC 90940 (Table III-2). No laccase or LiP activity was detected in any culture over the 30-day-incubation. No or low MnP was produced by using a rich medium (GYM), N-limited SBM, and addition of Mn(II) or Mn(II) plus organic acids in SBM. GMY-SBM supported MnP production by the three tested strains with the addition of the inducers Mn(II) or Mn(II)-glycolate. All four bran media supported high MnP production by all three strains. In general, *B. adusta* UAMH 7308 produced similar enzyme levels to 8258 in most media, whereas ATCC 90940 produced slightly lower levels than the UAMH strains in most bran media but lower levels in rice bran and higher than the UAMH strains in the GMY-SBM-Mn medium.

An additional experiment following the culture method of Mester and Field (1997) with ATCC 90940 was conducted by adding 5 mL of GMY-SBM in 250 mL serum bottles. The bottles were inoculated with one agar plug, loosely capped for passive aeration and statically incubated under an air atmosphere at 30°C. The MnP titre was 1.6 U/ml before adding Mn(II) and glycolate on day 3, and reached 2.4 U/ml on day 4, confirming the published data with this strain (Mester and Field 1997).

3.1.1.2 Effect of RB concentration on MnP production

To determine the optimal RB concentration for MnP production, 1%, 3% and 5% of RB was used, (Fig. III-3). For *B. adusta* 7308, MnP peaked and leveled off at about the same time in 3% and 5% RB media, although higher MnP production was obtained in 5% RB. For *B. adusta* 8258, MnP production profiles

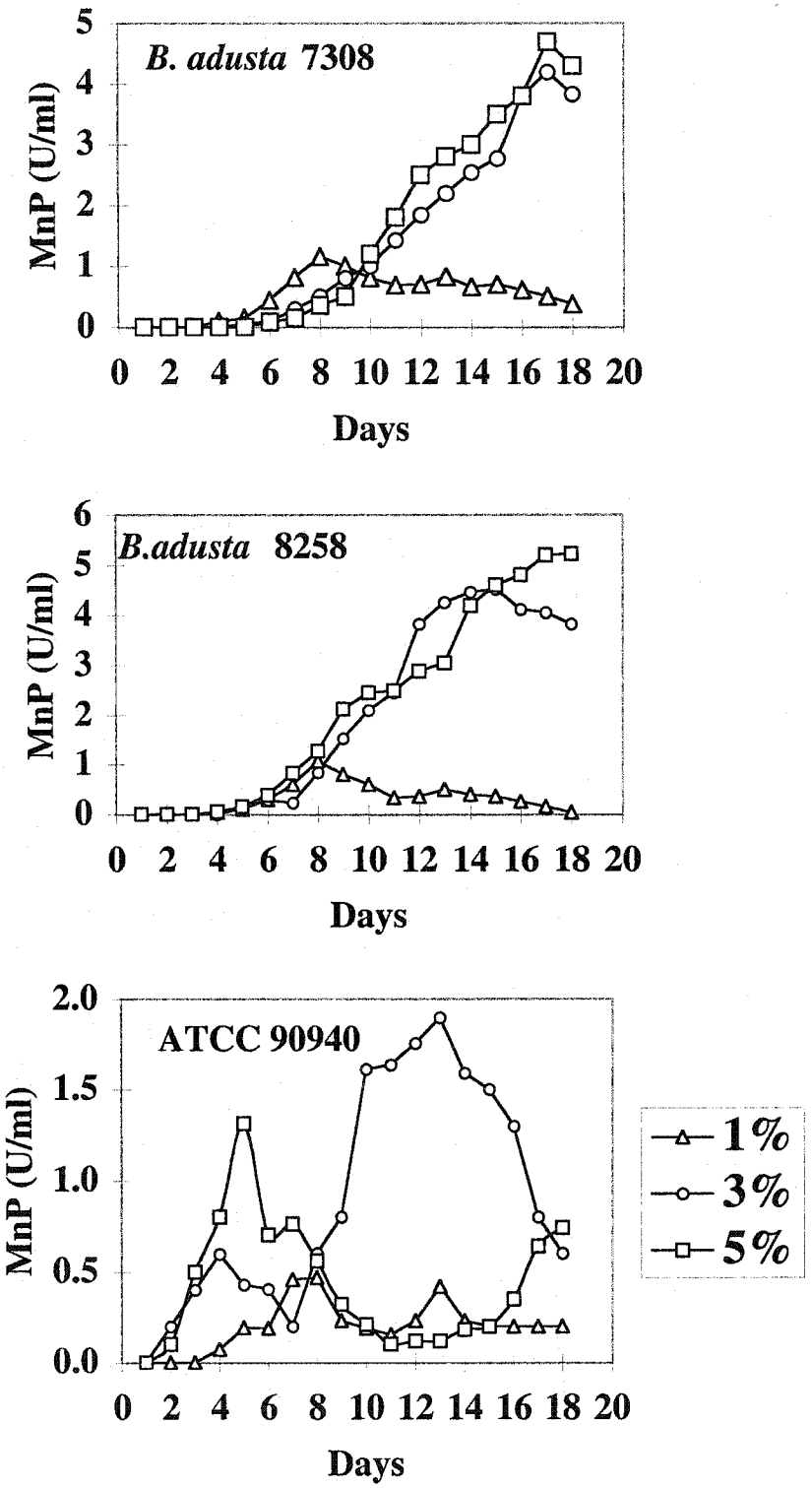

Fig. III-3. Effect of rice bran concentration on MnP production (1% rice bran (w/v), 1%; 3% rice bran (w/v), 3%; 5% rice bran (w/v), 5%); phosphate buffer pH 6, 28 °C, 200 rpm.

in 3% and 5% RB media were the same for the first 15 days, whereas after 15 days, MnP production leveled off in 3% RB but continued to increase in 5% RB. For ATCC 90940, enzyme production profiles are different from those of other two strains in that the highest MnP peak was obtained in 3% rice bran. Although higher enzyme production can be obtained in 5% RB with longer incubation times as using UAMH strains, 3% RB was chosen as the optimum concentration with the increase of solid bran, the viscosity of the medium increased significantly, making it more difficult to sterilize.

3.1.2 Effect of physical conditions on MnP production

3.1.2.1 Effect of medium volume to flask volume ratio on MnP production

To test the effect of rate of aeration level on MnP production, different volumes of medium were added to 500-mL flasks. Fungi were cultivated in 3% RB (pH 6.0, 200 rpm, 27 °C; Fig. III-4) and for all three strains, 200 ml of medium resulted in lower MnP production than with 50, 100, 150 ml medium suggesting that rate of aeration is important for MnP production. For *B. adusta* 7308, high enzyme production was obtained in the cultures with the lowest medium volume to flask volume ratios. For *B. adusta* 8258, mild aeration (100 ml and 150 ml medium in 500-mL flask) was better but the enzyme was produced a few days later than that with higher aeration (50 ml medium in 500 mL-flask). For ATCC 90940, there was little effect of aeration on MnP production and production was lower than for the two UAMH strains.

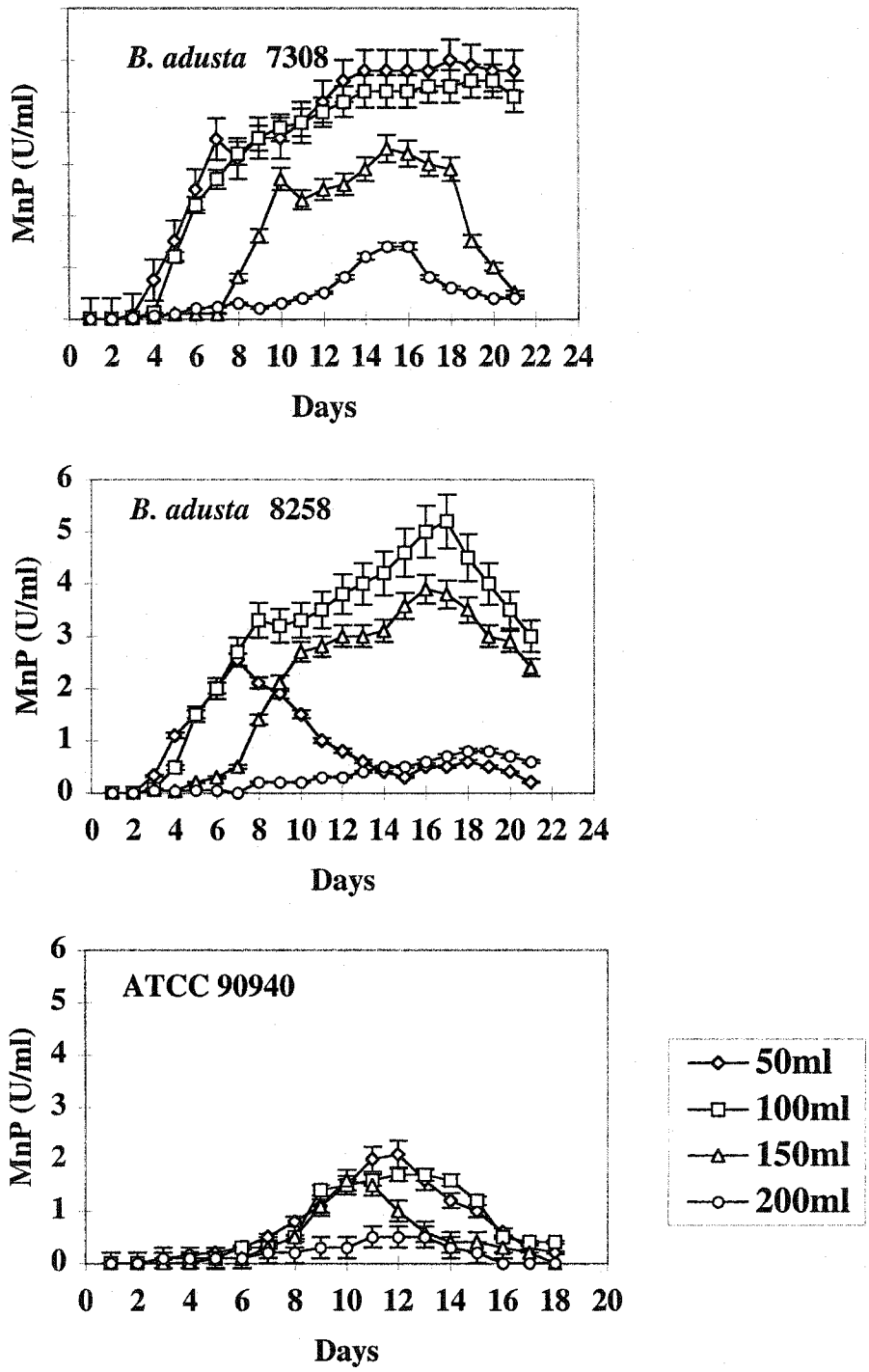

Fig. III-4. Effect of medium volume to flask volume ratios on MnP production (50 ml 3% RB medium in 500-mL flask, 50 ml/500 ml; 100 ml 3% RB medium in 500 mL-flask, 100 ml/500 ml; 150 ml 3% RB medium in 500-mL flask, 150 ml/500 ml; 200 ml 3% RB medium in 500-mL flask, 200 ml/500 ml); phosphate buffer pH 6, 28 °C, 200 rpm.

3.1.2.2 Effect of initial pH on MnP production

Bran medium consisted of insoluble bran suspended in 60 mM phosphate buffer. For all three strains, the optimal starting pH value for MnP production was pH 5.5-6 as shown in Fig. III-5a, 5b, 5c. It is interesting to note that in all cultures with starting pH values from 5.0 to 7.5, enzyme production started at the point that the culture growth had adjusted the pH of the medium to 5. Enzyme production peaked at pH 5.5 and the final pH in all cases was about 6. High starting pH usually produced lower and less stable enzyme levels. The same trends were observed in all three strains.

3.1.2.3 Effect of temperature on MnP production

Of the temperatures tested (Fig. III-6), 27-30°C was the optimal for both MnP production and fungal growth in all three strains. No enzyme production was detected at 37 ^{0}C.

3.1.3 Scale-up MnP production in a 14-L fermentor

Once optimal MnP production conditions had been determined in shake flask cultures, enzyme production was scaled up in order to produce enough enzyme for purification and characterization. The stirred tank reactor (STR) is the most extensively used fermentor in industry. Up to now, there have been no reports on using fermentors to cultivate *B. adusta* strains for MnP production. Based on the shake-flask data, the following culture conditions chosen: 14-L fermentor (working capacity 10 l), 5% inoculum, 3% RB, 60 mM potassium

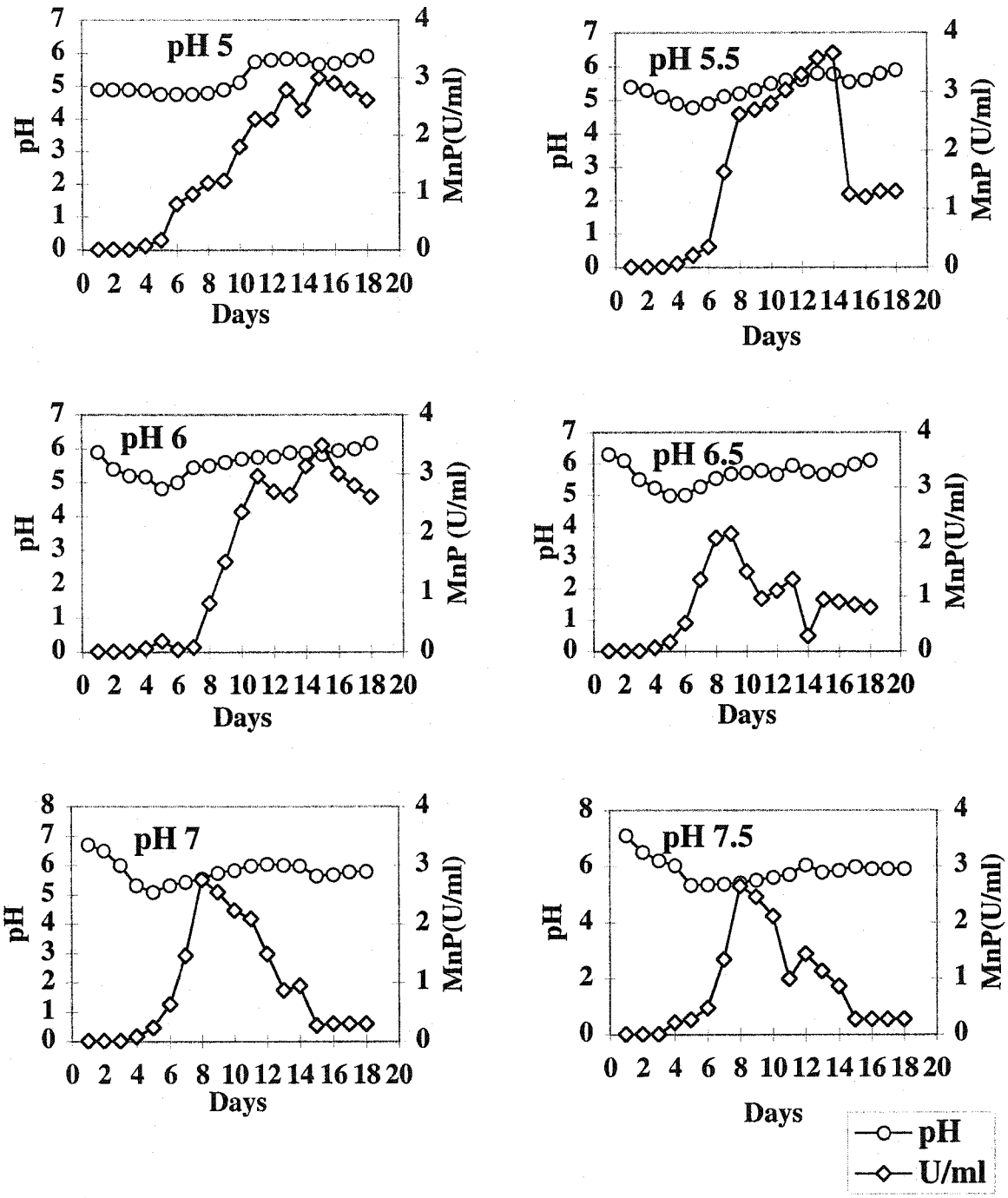

Fig. III-5a. Effect of initial pH on MnP production by *B. adusta* UAMH 7308; rice bran 3%(w/v), 28 °C, 200 rpm.

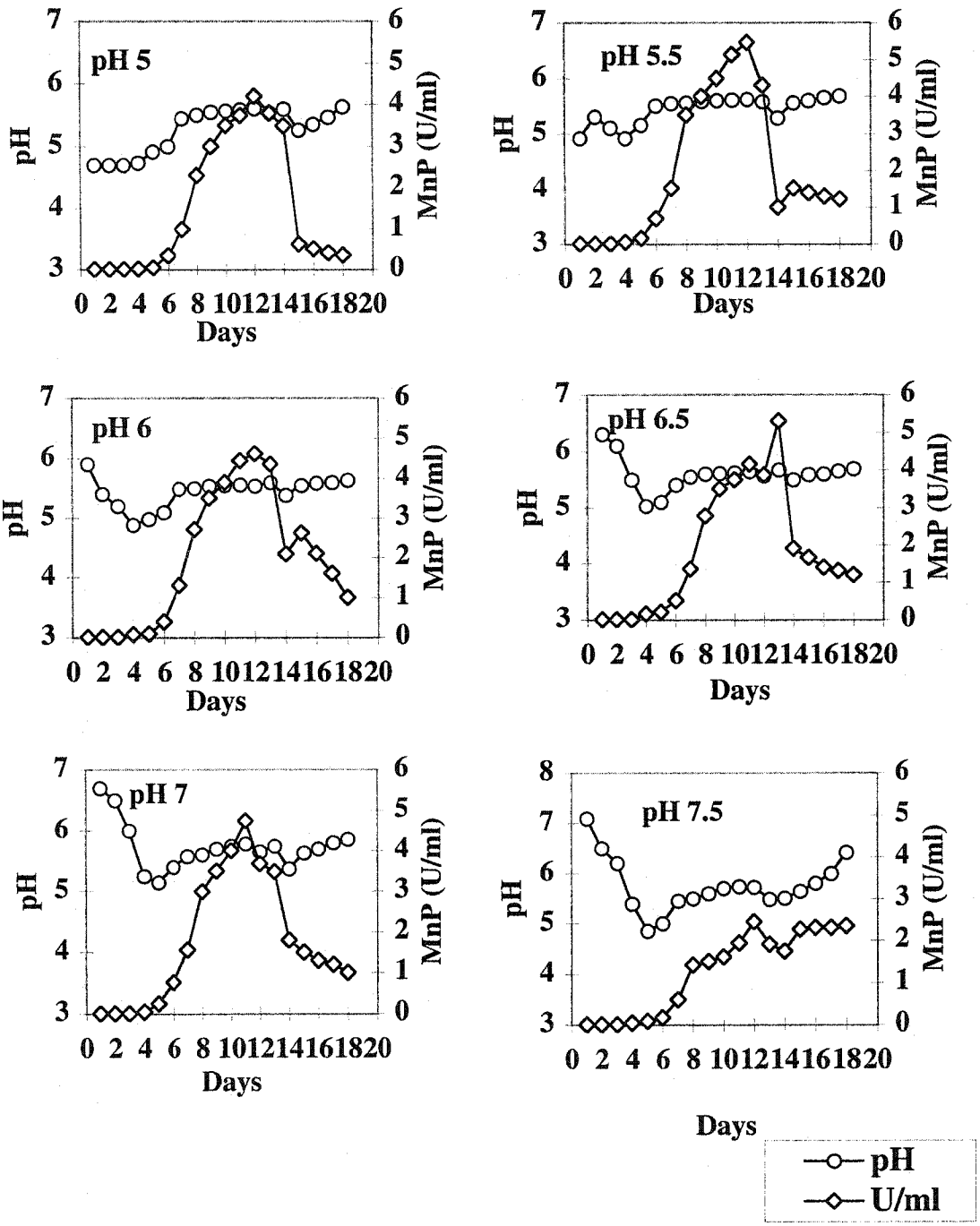

Fig. III-5b. Effect of initial pH on MnP production by *B. adusta* UAMH 8258; rice bran 3% (w/v), 28 °C, 200 rpm.

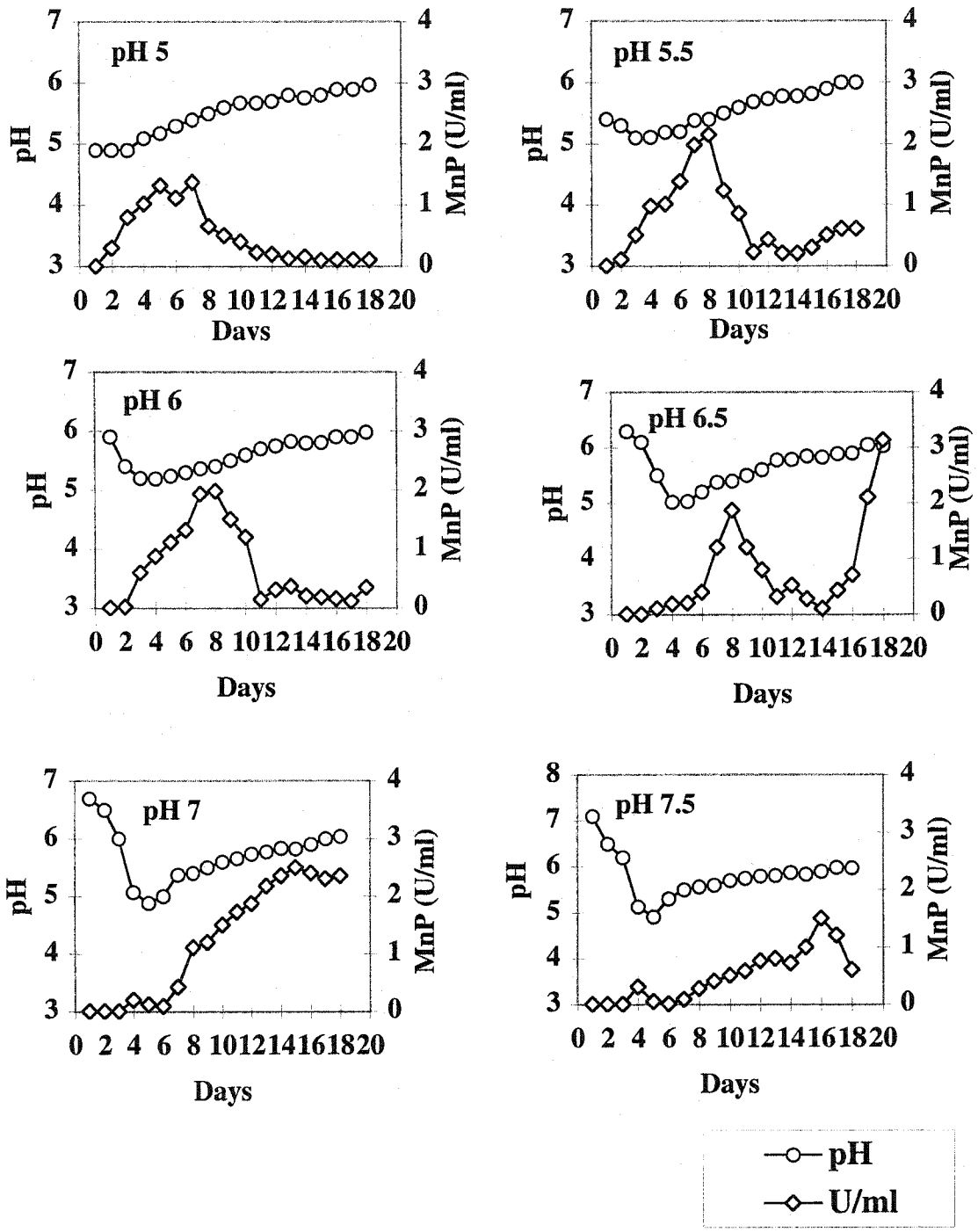

Fig. III-5c. Effect of initial pH on MnP production by ATCC 90940; rice bran 3% (w/v), 28 °C, 200 rpm.

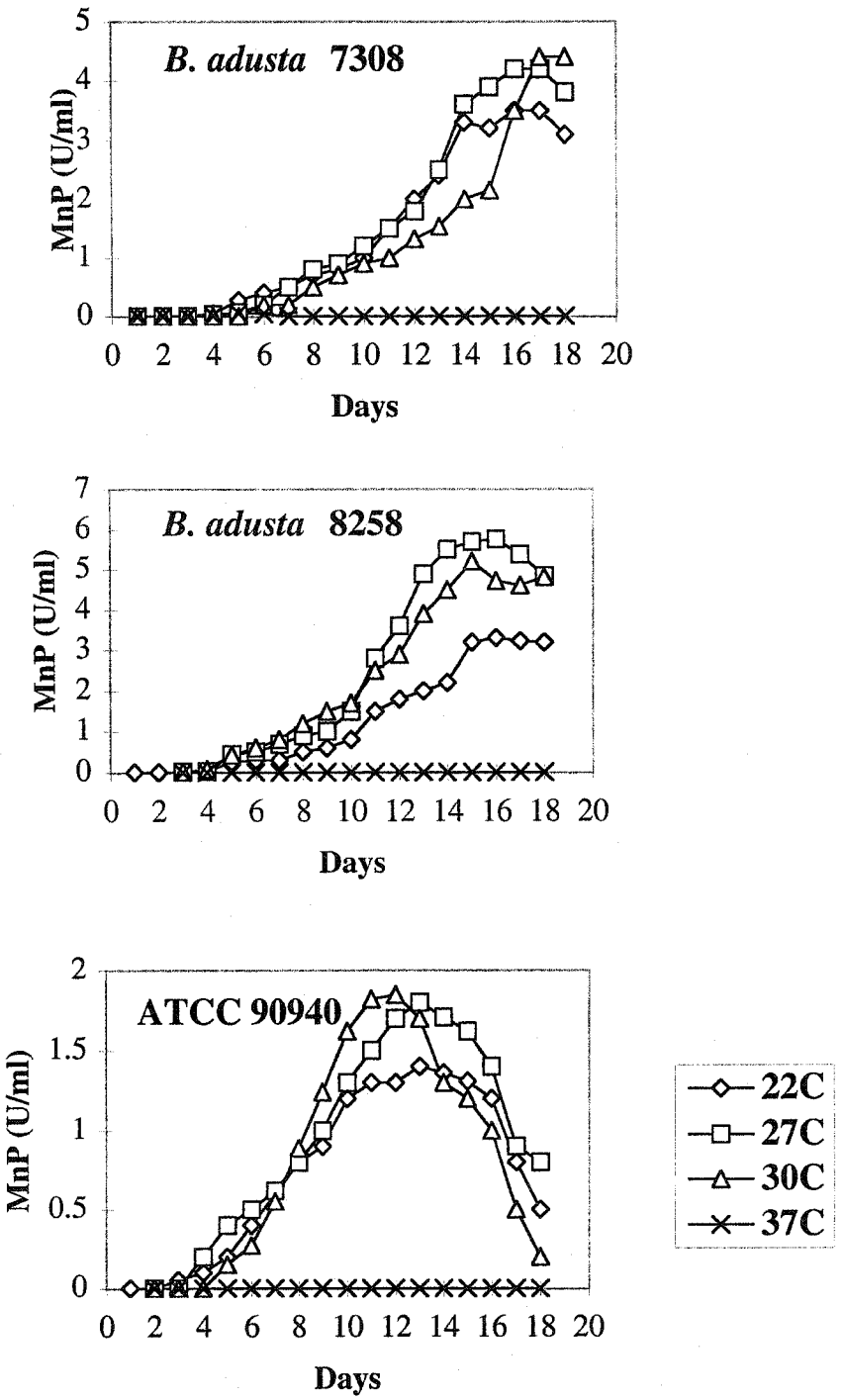

Fig. III-6. Effect of cultivation temperature on MnP production; rice bran 3% (w/v), phosphate buffer pH 6, 200 rpm.

phosphate buffer (pH 6.0), air flow rate 0.2v/vmin, stir speed 200 rpm and a temperature of 27 ^{0}C.

Using these conditions, several MnP production experiments were conducted in a STR using the three *B. adusta* strains. Fig. III-7 shows a photograph of a STR with growing *B. adusta* UAMH 8258, which showed uniform growth of 2-3 mm fungal pellets, and no interface material.

Manganese peroxidase production in shake flasks and in the STR was compared (Fig. III-8). The lower panel shows the production of MnP in a STR. Here maximum MnP production was about 3.5 U/ml on day 9. Thus, production was lower than in 500-mL shake flasks but shorter incubation time was required. Declining reducing sugar concentrations correlated with MnP production and increasing levels of MnP coincided with fluctuations in the level of reducing sugars. However, fluctuations were of a shorter duration than those observed using flasks. Similar methods were used to scale up MnP production by UAMH 7308 and ATCC 90940 (data not shown). For UAMH 7308, it took 14 days to reach a peak MnP production level of 2.5 U/ml in STR. Attempts to produce MnP from strain ATCC 90940 in a STR were less successful as the fungal pellets clustered at the air/ liquid interface and could not be drawn into the liquid. No MnP production by this strain could be detected within 20 days in a STR, but scale up with ATCC 90940 was successful in 2-L Erlenmeyer flasks with 400 ml of medium. These experiments were repeated at least three times. Antifoam was not used in any of the fermentations because of anticipated difficulty of removal during enzyme purification.

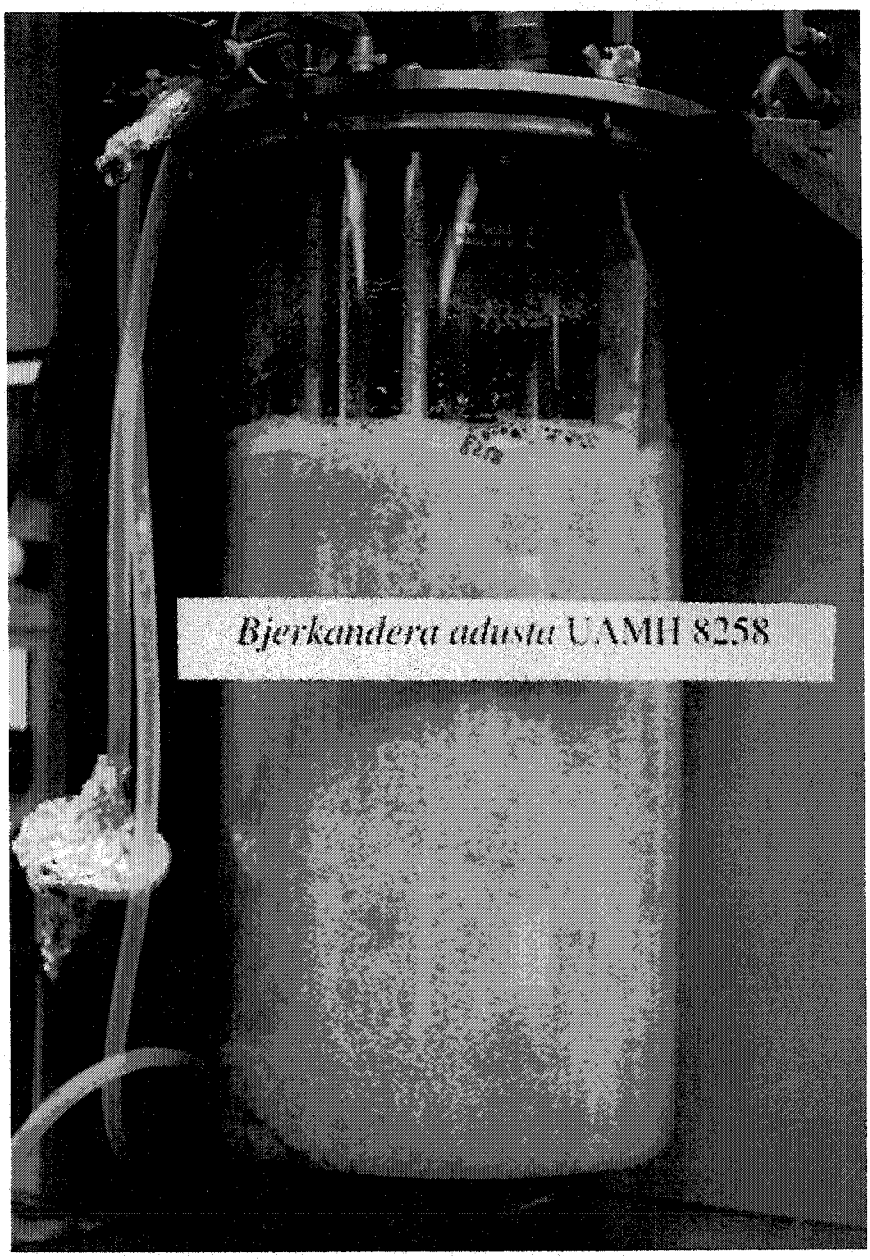

Fig. III-7. Photograph of 14-L stirred tank reactor with growing fungus *B. adusta* UAMH 8258.

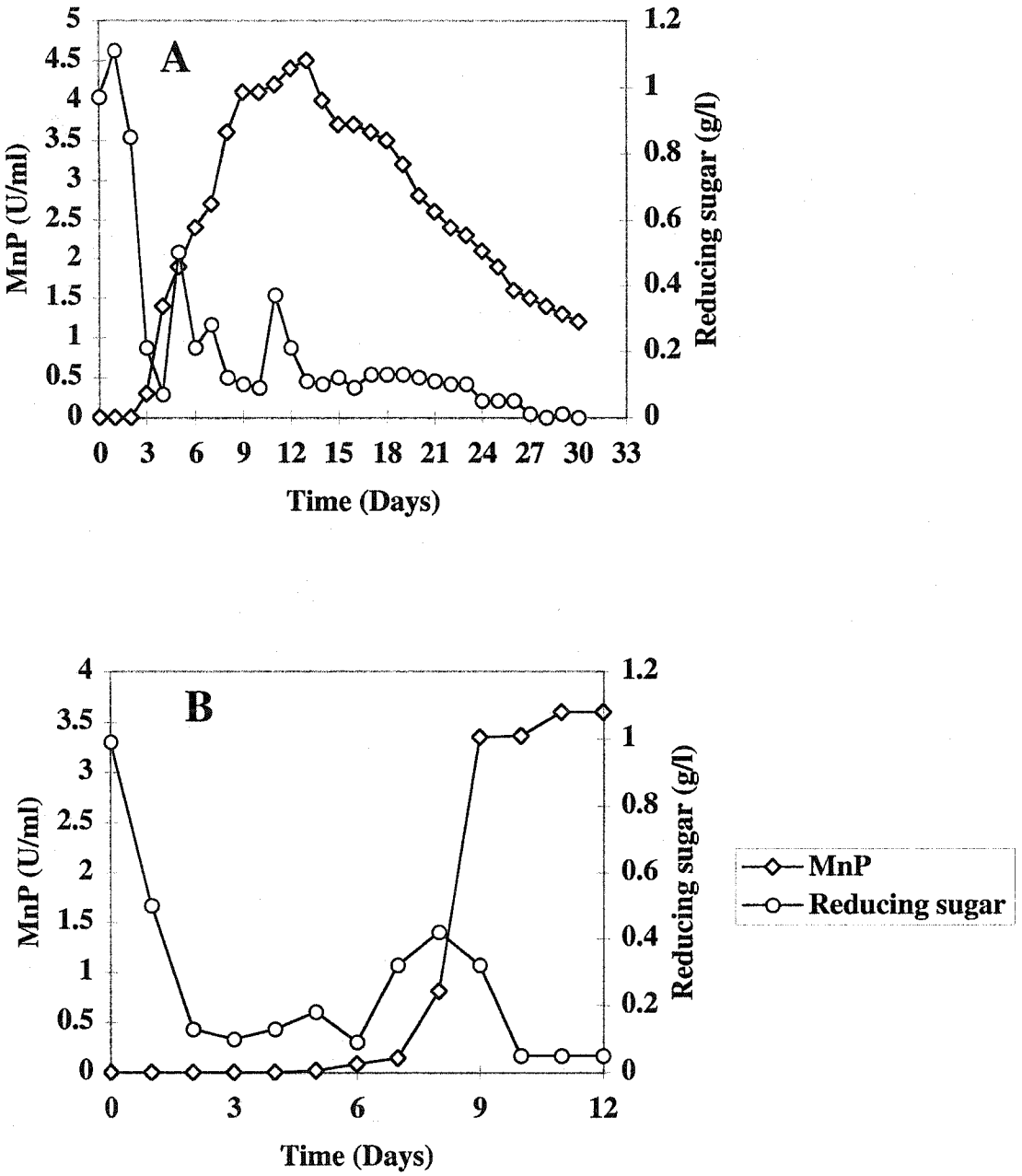

Fig. III-8. Comparison of MnP production by *B. adusta* UAMH 8258 in shake flask (A) and in STR (B).

3.2 Enzyme purification and characterization

3.2.1 Enzyme purification

Manganese peroxidase is an extracellular enzyme, and is therefore secreted into the growth medium which simplifies downstream processing because cell breakage is not required. However, fungi may also secrete a number of other proteins and the medium used in this study also contains proteins that must be separated from the desired protein product. Further, although RB medium induced high levels of enzyme production, a significant amount of interfering pigment is also produced during delignification which imparts a deep brown color to the culture supernatant. The enzyme purification protocol was designed to remove contaminating proteins and pigments.

Once sufficient MnP had been produced, purification was attempted based on the procedure summarized in Fig. II-1. After the sample was applied on a DE-52 column, MnP could be eluted at concentration of 110 mM KCl (Fig. III-9). The peak fractions from DE-52 were concentrated and applied to a size exclusion chromatography column (Sephadex G-100) and eluted with 10 mM potassium phosphate buffer (pH 6) with 100 mM sodium chloride (Fig. III-10). The peak fractions from Sephadex G-100 were concentrated and dialyzed prior to anion exchange FPLC (MonoQ) (Fig. III-11). The peak fractions from FPLC were pooled, specific activity and Reinheitzahl (Rz) (A_{407}/A_{280}) number of the pool were measured. The pool was applied on 15% SDS-PAGE to evaluate purity.

The purification data for MnP from *B. adusta* UAMH 8258 grown on 3% RB are shown in Table III-3. Overall, the enzyme was purified 24-fold and 24% of

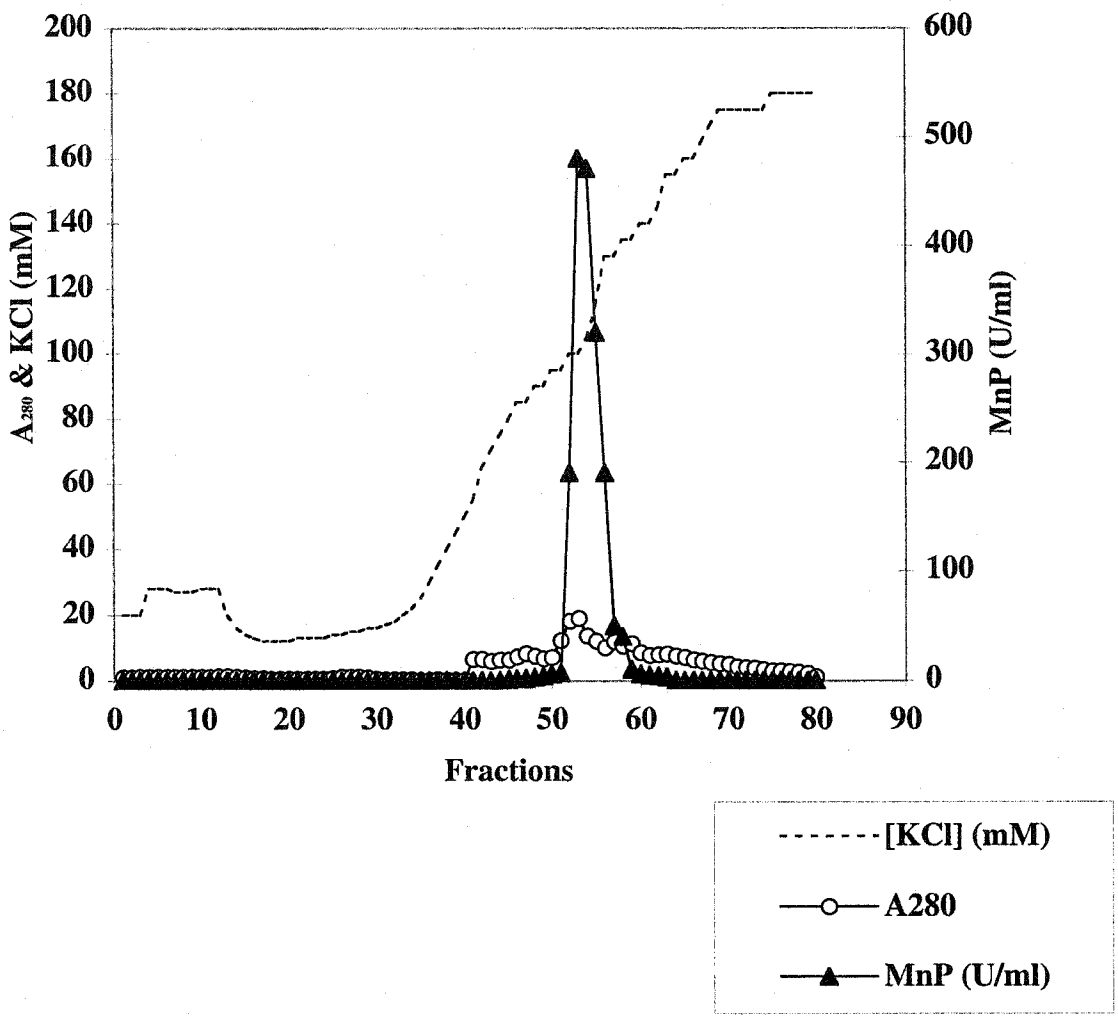

Fig. III-9. Elution profile of MnP from DEAE-cellulose anion-exchange column with linear gradient from 100 mM to 300 mM KCl in 10 mM phosphate buffer pH 6.

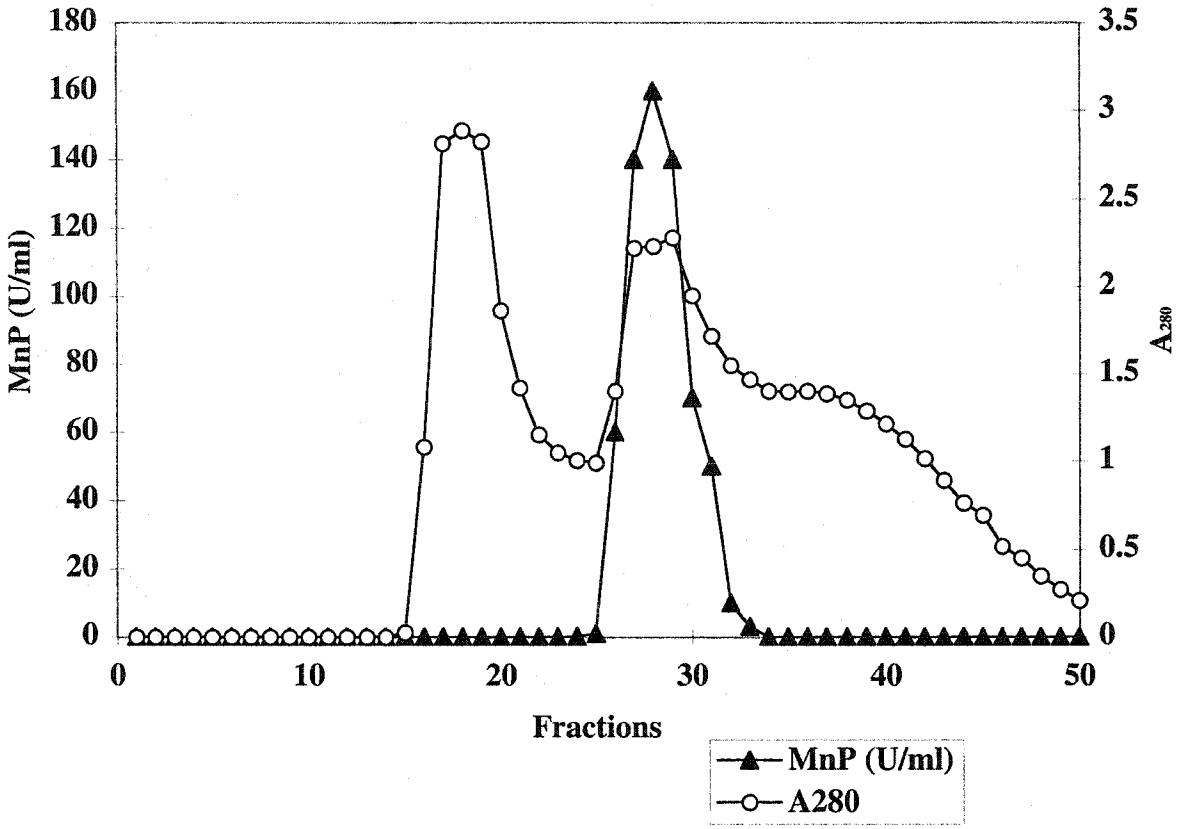

Fig. III-10. Elution profile of MnP from size-exclusion Sephadex G-100 column with 100 mM NaCl buffer.

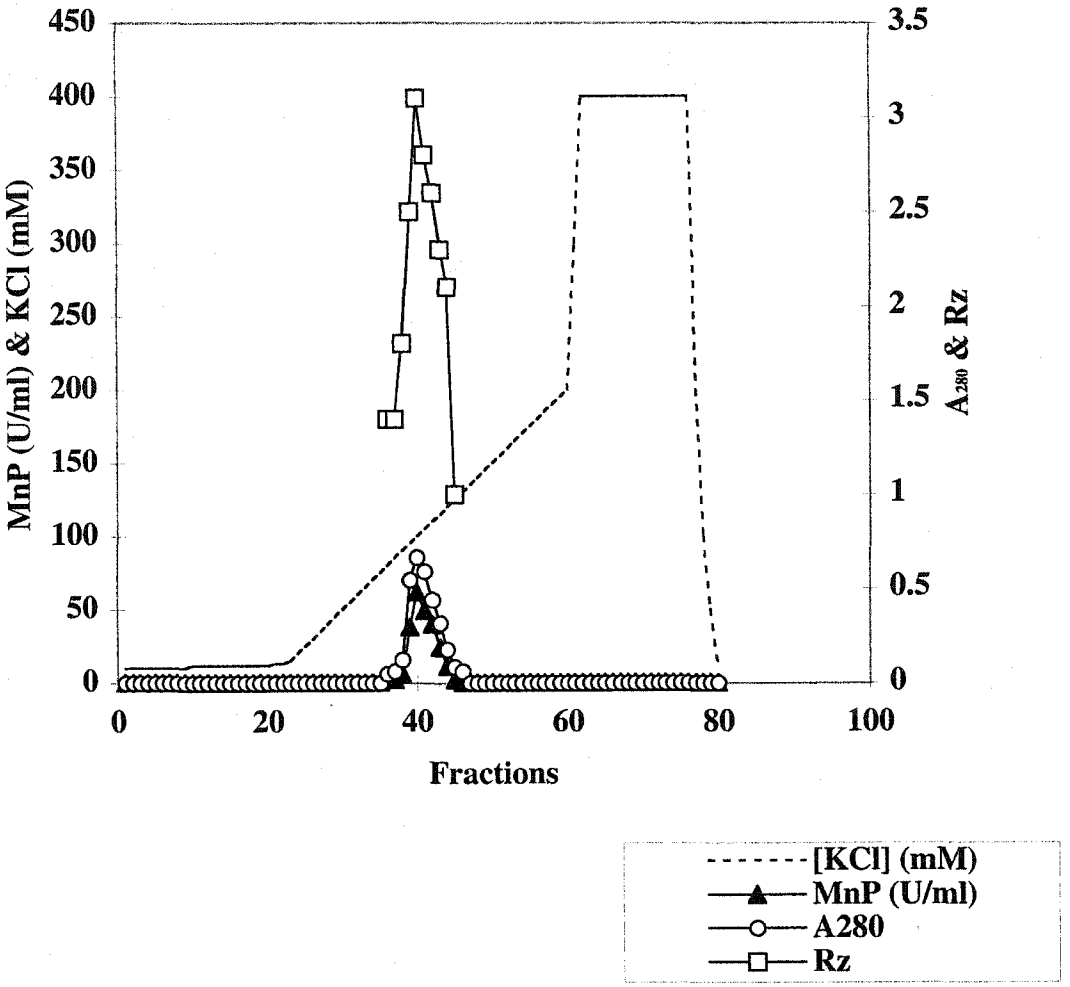

Fig. III-11. Elution profile of MnP from FPLC anion-exchange MonoQ column with linear gradient from 100 mM to 300 mM KCl in 10 mM phosphate buffer pH 6. Then wash with 400 mM KCl in 10 mM phosphate buffer pH 6.

Table III-3. Purification table for MnP from *B. adusta* UAMH 8258.

Purification Step	Volume (ml)	Protein (mg/ml)	Enzyme (U/ml)	Total EU (U)	Specfic Activity (U/mg)	Rz (A_{407}/A_{280})	Yield (%)
Culture Supernatant	6750	0.40	1.8	12150	4.5	0.003	100
Ultrafiltration	300	7.47	39	11700	7.9	0.007	96
PEI Precipitation	270	5.23	40	10800	11.7	0.1	89
DE-52 Pool	50	6.82	156	7800	22.9	0.5	64
Ultrafiltration	5	25.78	1035	7400	40	0.6	60
Sephadex G-100 Pool	50	1.35	88	4400	65	1.5	36
Ultrafiltration	5	11.60	824	4120	71	1.5	34
FPLC Pool[1]	4	7.80	768	3050	98	3	25
FPLC Pool[2]	4	6.70	720	2880	108	4.3	24

FPLC Pool[2]: Repeat FPLC with FPLC Pool[1] peak fractions.

initial activity present in the culture supernatant was recovered as purified protein with an Rz value of 4.3 and specific activity of 108 U/mg. No further increase in Rz or specific activity was observed with additional passages through the same FPLC column or affinity chromatography using a Concanavalin A column (data not presented).

The same purification procedure was used for the MnPs from UAMH 7308 and ATCC 90940. Similar purification levels were achieved except that the FPLC peak fraction of 7308 has a specific activity of 87 U/mg and Rz of 3.5; and the FPLC peak fraction of ATCC 90940 has a specific activity of 98 U/mg and Rz of 4.5.

3.2.2 Enzyme characterization

3.2.2.1 Physical characterization

The purified MnP preparation from *B. adusta* UAMH 8258 was analyzed using gel electrophoresis and mass spectrometry. A single band on 15% SDS-PAGE indicated an apparent molecular weight of 43 kDa, and a single band on isoelectric focusing gel had an isoelectric point of 3.55 (Fig. III-12). MALDI-TOF mass spectrometric analysis produced a single peak indicating a molecular mass of 36.6 kDa. The N-terminal amino acid sequence of this preparation was VAXPDGVNTATNAAXXALFA, where X has a 90% likelihood of being cysteine (Table III-4). The amino acid composition of the pure enzyme was obtained and compared with the MnP from *T. versicolor*, LiP from *P. chrysosporium* and laccase from *T. versicolor* (Table III-5): no tyrosine residues were detected in this MnP preparation.

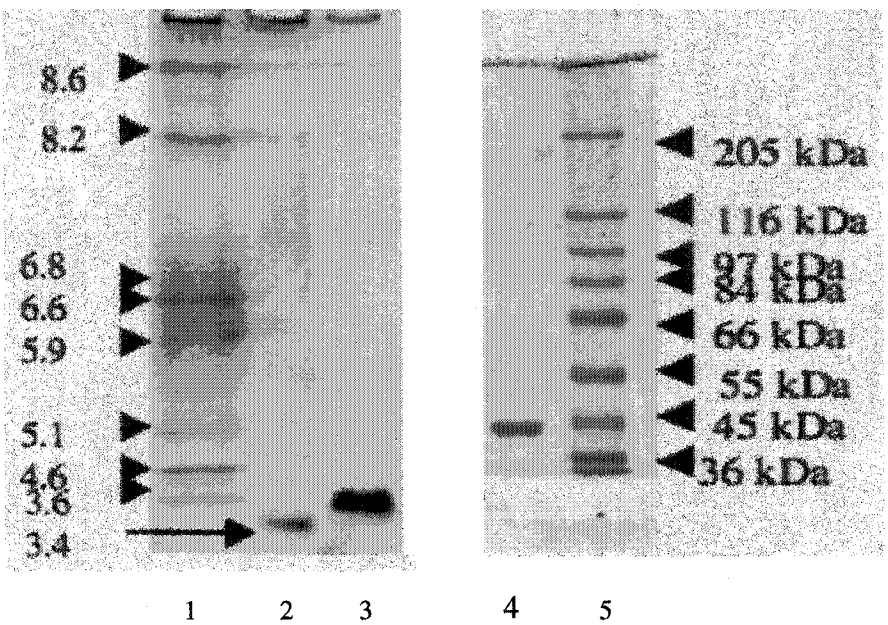

Fig. III-12 Estimation of isoelectric point and molecular weight of manganese peroxidase from *B. adusta* UAMH 8258.

Lanes 1-3 are an IEF gel
Lane 1: IEF standards
Lane 2: Chloroperoxidase from *Caldarimyces fumago* pI=3.4
Lane 3: Manganese peroxidase from UAMH 8258

Lane 4& 5 are 15% SDS-polyacrylamide gel
Lane 4: Manganese peroxidase from UAMH 8258
Lane 5: Molecular weight markers

Table III-4. Comparison of N-terminal sequences of *B. adusta* MnP with related enzymes.

B. adusta 8258 (this study)	**VAXPDGVNTATNAAXXALFA**
B. adusta 7308 (this study)	**VAXPDGVNTATNAAXXALFA**
Bjerkandera sp. BOS55 (this study)	**VAXPDGVNTATNAAXXALFA**
T. versicolor LiP (Limongi *et al.* 1995)	**V*T*CPDGVNTATNAAXX*QLFH***
T. versicolor MnP (Limongi *et al.* 1995)	**VAXPDGVNTATNAAXX*QLFD***
Bjerkandera sp. BOS1 (Palma *et al.* 2000)	**VAXPDGVNTATNAAXX*X*LFA**
Bjerkandera sp. BOS2 (Palma *et al.* 2000)	**VAXPDGVNTATNAAXXALFA**
P. chrysosporium MnP (H4, Palma *et al.* 2000)	***AV*CPDG*TRV*----*SH*AACCA*FP***
P. chrysosporium MnP (H5, Palma *et al.* 2000)	***AV*CPDG*TRV*----TNAACC**
Bjerkandera sp. BOS55 (Mester and Field 1998)	**VACPDGVNTATNAAXXALFA**

Where X has a >90% likelihood of being cysteine (C)

Table III-5. Comparison the amino acid composition of MnPs from *B. adusta* UAMH 8258 and ligninolytic enzymes from other fungi (reactive residues only).

Residue	UAMH 8258 MnP	MnP [a]	LiP [b]	Laccase[c]
Ser	7.4	6.5	6.2	8.8
Lys	1.9	1.1	3.5	0.8
Thr	7.6	7.9	5.9	1
Asp[d]	12.1	9.9	10	14.8
Glu[e]	8.7	11.2	9.7	4.2
Arg	3.1	4.1	2.4	2.9
Cys	ND	2.2	2.2	1.2
Tyr	0	0	0	2.7
His	1.8	2.2	2.7	3.1
Met	1.6	0.5	2.4	1
Trp	ND	0.3	0.8	1.2

[a]MnP: MnP from *T. versicolor* Johansson *et al.* (1993).
[b]LiP: LiP from *P. chrysosporium* Ritch and Gold (1992).
[c]Laccase: Laccase from *T. versicolor* Johansson *et al.* (1995).
Asp[d]=Asp+Asn
Glu[e]=Glu+Gln

The tryptophan content of the prepared MnP was determined using the dye-binding technique with gravimetric analysis using a freeze-dried preparation after extensive dialysis against distilled water (Appendix 7.1). *B. adusta* MnP had a lysine content (1.9%) lower than the 'average' protein (7%) and lower than bovine serum albumin (3%) which is used as a standard in the dye-binding protein assay (Bi0-Rad Protein Assay). Dye binding in this assay relies in part on the lysine content of the protein. Based on a molecular weight of 36.6 kDa by MALDI-TOF, the theoretical Rz for the pure enzyme would be 4.2 assuming the enzyme contains two tryptophan residues, or 8.4 if only one tryptophan is present. Since the purest preparation had an Rz of 4.3, the enzyme probably contains two tryptophan residues. This value has recently been reported for the versatile peroxidase from *Pleurotus eryngii* (Perez-Boada, *et al.* 2002).

3.2.2.2 Catalytic characterization

Five compounds frequently used as substrates for MnP were used to evaluate and compare the catalytic properties of the purified MnP from *B. adusta* UAMH 8258. The pH dependence of oxidation of these five substrates is shown in Fig. III-13. The optimum for Mn(II)-dependent peroxidase activity was pH 4.5 for all five substrates, whereas the optimum pH for Mn(II)-independent peroxidase activity was 3 for 2,6-dimethoxyphenol, ABTS and veratryl alcohol. The maximum reaction rate of the Mn(II)-dependent activity was between higher than that of the Mn(II)-independent activity when 2,6-dimethoxyphenol, veratryl alcohol, 4-aminophenol and hydroquinone were used as substrates (Fig. III-13), but when ABTS

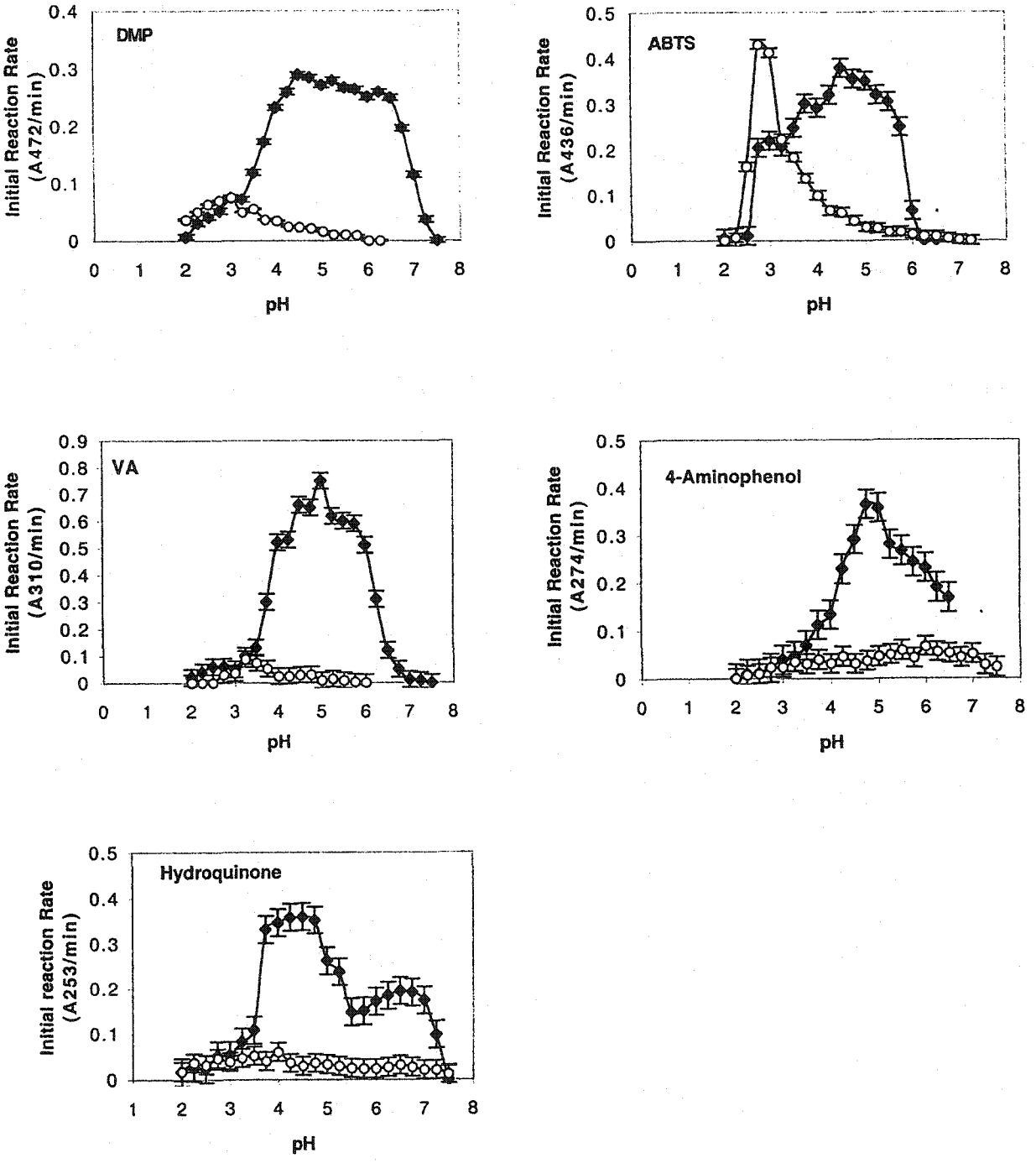

Fig. III-13. Effect of pH on Mn(II)-dependent peroxidase (♦) and Mn(II)-independent peroxidase (o) activity on different substrates. (DMP: 2,6-dimethoxyphenol; VA: veratryl alcohol)

Table III-6. Comparison of steady-state kinetic constants of native and chemically modified manganese peroxidase on Mn(II)-dependent and independent activity.

	Mn(II)-dependent Activity				Mn(II)-independent Activity			
	Native MnP		PEG-MnP		Native MnP		PEG-MnP	
	$K_{M, app}$ (μM)	k_{cat} (min^{-1})	$K_{M, app}$ (μM)	k_{cat} (min^{-1})	$K_{M, app}$ (μM)	k_{cat} (min^{-1})	$K_{M, app}$ (μM)	k_{cat} (min^{-1})
H_2O_2	4.50	13500	7.80	8700				
Mn(II)	17.0	9000	17.7	9700				
DMP	3.66	19200	3.59	13700	145	7200	121	8280
ABTS	15.0	8200	75.0	4680	150	4320	327	3600
VA	432	2400	550	2270	5300	720	5800	690
Guaiacol	222	1900	184	2770	4960	870	4200	1170

was used as substrate, the maximum manganese (II)-dependent and independent activities were about the same (Table III-6).

3.3 Enzyme modification

To enhance enzyme activity in water-miscible organic solvent-water mixtures, covalent modification of the pure MnP was done at pH 7 over 4 days because the enzyme loses activity rapidly at pH 10, the recommended pH for the borate-dependent reaction (Vazquez-Duhalt *et al.* 1992). At pH 7, 45% of the lysine residues of MnP were modified by activated PEG and 95% activity was recovered. To determine the effect of chemical modification on enzyme catalytic properties, the oxidation of Mn(II) and the other substrates were compared (Fig. III-14 and Table III-6). Both the native and modified MnP showed the highest Mn(II) oxidation activity when the H_2O_2 concentration was 0.1 mM (Fig. III-14A). However, the chemically modified MnP showed enhanced stability to increased levels of H_2O_2 compared to the native enzyme. The modification has little effect on Mn(II) and malonate binding (Fig. III-14B, C). Both enzymes reached their highest Mn(II) oxidation activity at 0.5 mM Mn(II) and 50 mM malonate. The optimum pH for both native and modified enzymes was 5 (Fig. III-14D). The highest Mn(II) oxidation rate over 1 minute was obtained when the temperature was around 60 ^{0}C for both enzymes (Fig. III-14E), and the modified form was less susceptible to thermal denaturation than the native enzyme.

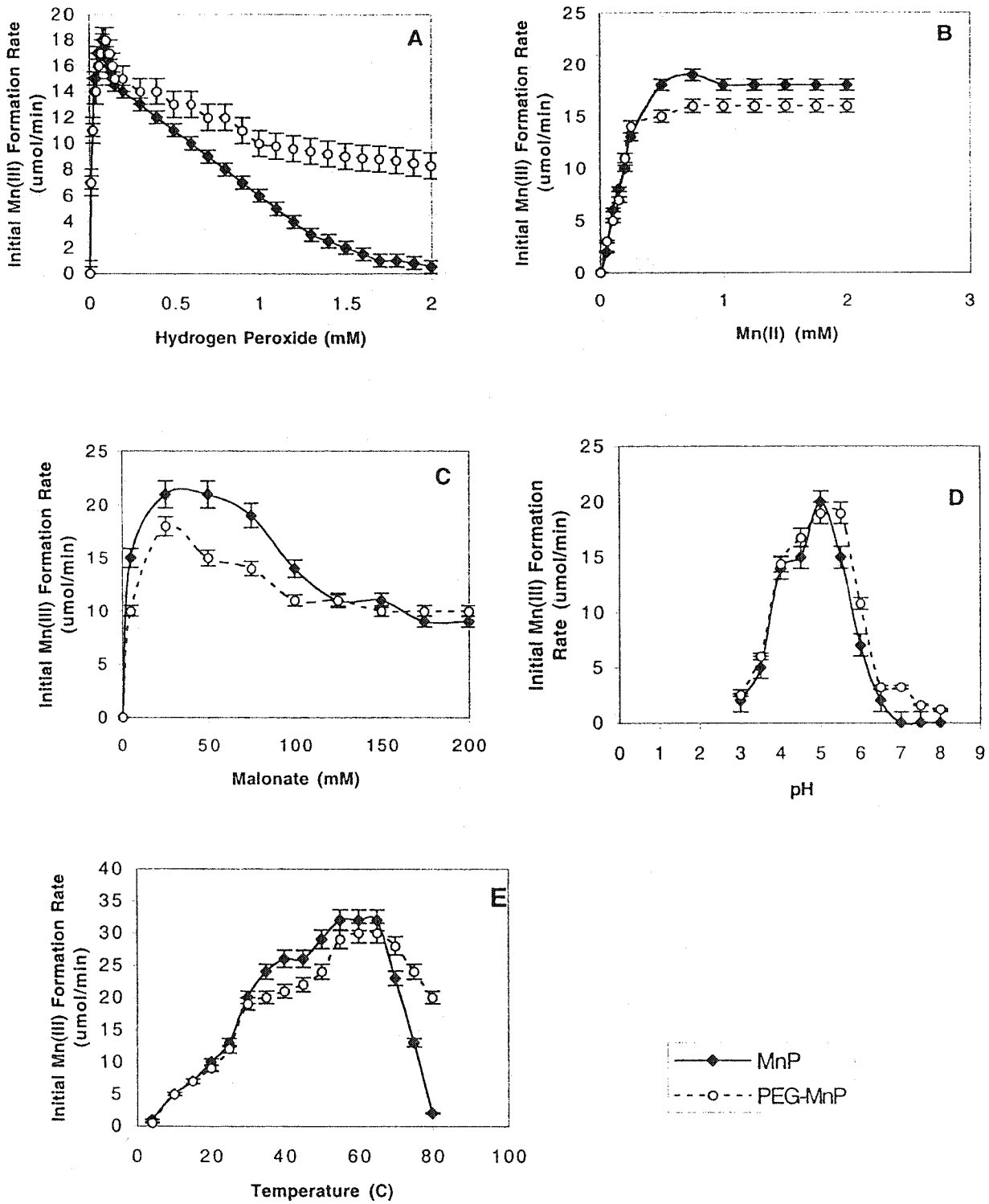

Fig. III-14. Comparison the Mn(II) oxidation activity of native (♦) and PEG- (o) MnP under different conditions.

Chemically modified MnP had an optimum pH 5 for Mn(II)-dependent peroxidase and pH 3 for Mn (II)-independent peroxidase activity (data not shown). The kinetic constants ($K_{M, app}$ and k_{cat}) of Mn(II)-dependent and independent activity at the optimum pH were compared (Table III-6). The kinetic constants showed that the native and modified enzymes had similar affinity for H_2O_2 and Mn(II), and similar $K_{M, app}$ and k_{cat} during the oxidation of other four substrates, but that Mn(II)-dependent activity had lower $K_{M. app}$ and higher k_{cat} than the corresponding Mn(II)-independent activity (Table III-6).

One of the objectives of this study was to find a new source of enzyme suitable for PAH degradation. In PAH oxidation systems, water-miscible organic solvents are usually used to increase the substrate availability. The Mn(II) oxidation ability of native and modified MnP were compared in acetonitrile, N,N-dimethylformamide, tetrahydrofuran, ethanol and methanol (Fig. III-15). In each of these solvents, the chemically modified MnP showed higher manganese (II) oxidation ability than that of the native enzyme. In methanol the effect was the least pronounced, whereas residual activity was seen at high acetonitrile and tetrahydrofuran concentrations for the modified form where the native form was inactive. Athough the previous experiment showed the instantaneous effect of solvent concentration on activity, long term enzyme stability under several conditions was also compared (Fig. III-16). The stability of both enzyme forms was compared under extreme pH, high temperature and in the presence of acetonitrile. The modified MnP showed an enhanced stability at lower pH (pH 2 and 3), in the presence of 30% and 50% of acetonitrile, and at high temperature (65 °C and 90 °C) (Fig. III-16).

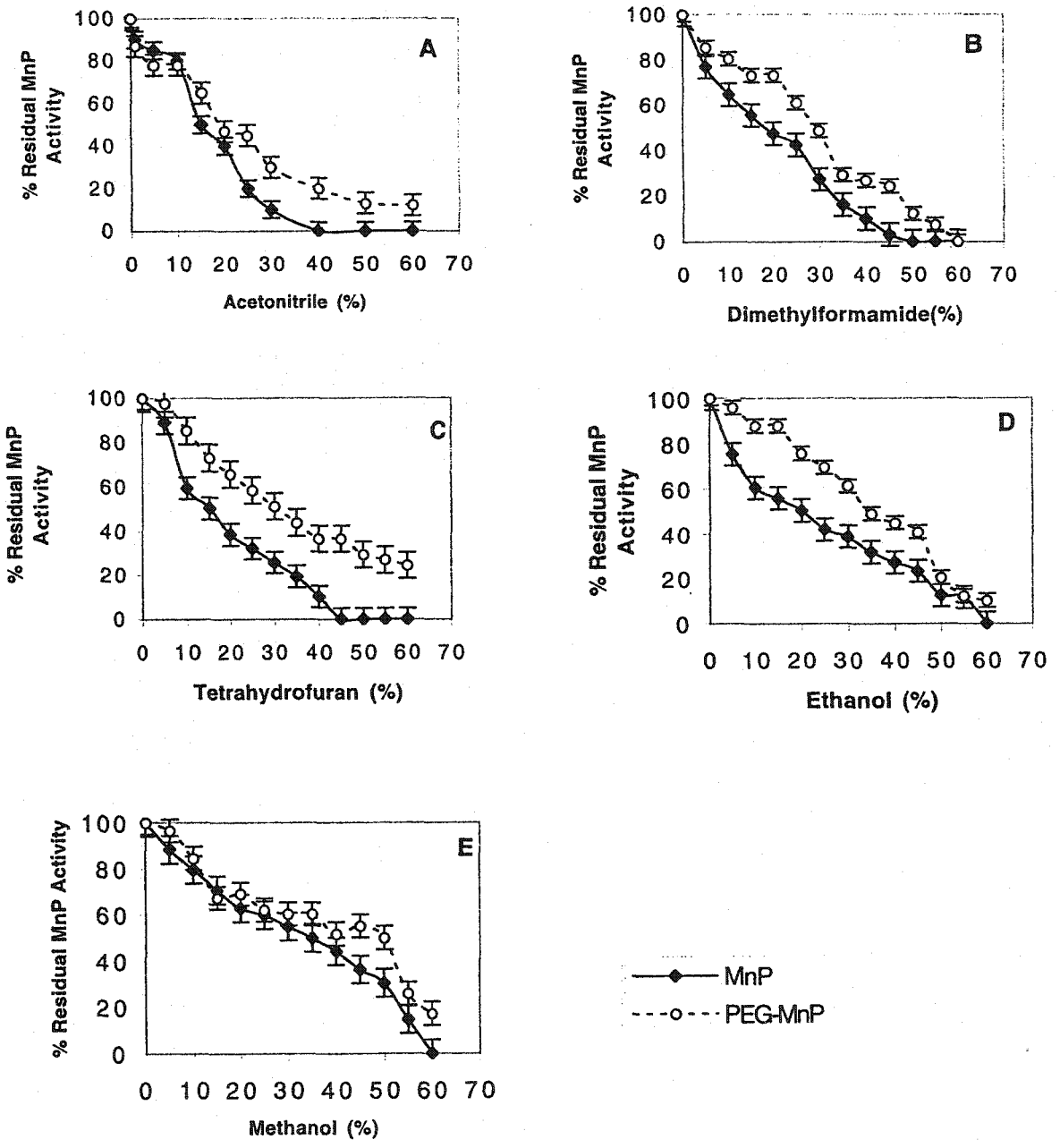

Fig. III-15. Comparison the Mn(II) oxidation activity of native (♦) and PEG- (o) MnP in different organic solvents.

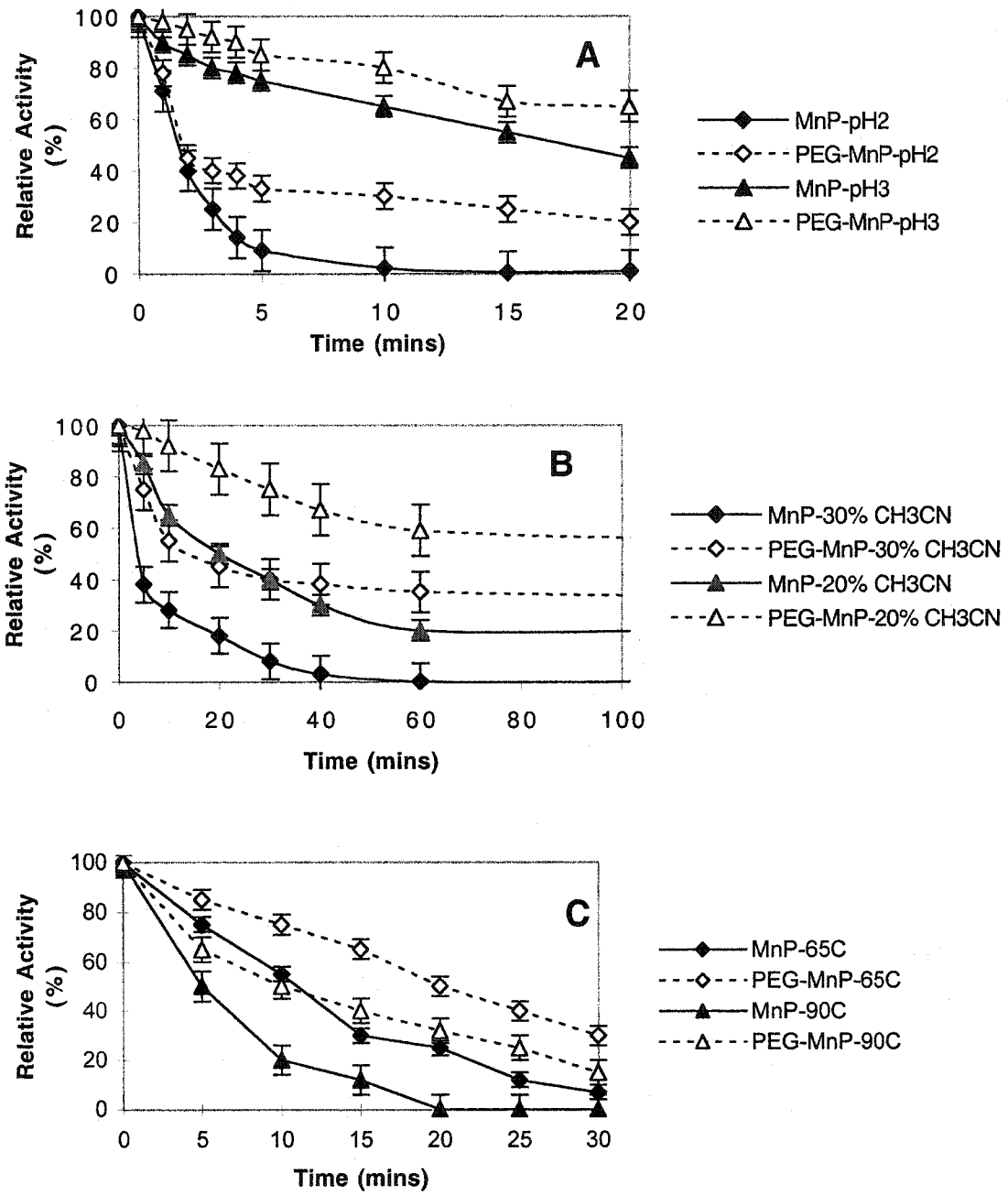

Fig. III-16. Comparison of the stability of native and PEG-MnP for the oxidation of Mn(II) to Mn(III) under acidic pH, presence of acetonitrile and high temperature.

3.4 Polycyclic aromatic hydrocarbon oxidation by MnP from *B. adusta* UAMH 8258

3.4.1 pH optima for PAH oxidation by MnP

The mechanism of MnP oxidation of various aromatic phenols and some phenolic lignin model compounds involves catalytic formation of Mn(III), which, when suitably chelated, acts to oxidize target substrates. It was found that the MnP-Mn(II) system could not be used efficiently to oxidize PAHs. Manganese peroxidase from *B. adusta* UAMH 8258 was purified and assayed for oxidation of the four PAHs in the presence and absence of Mn(II) at different pH values. The pH activity profile for different PAHs is shown in Figure III-17. In all the cases, the reaction medium without Mn(II) showed higher oxidation rates than those containing 0.5 mM $MnSO_4$. In addition, different pH optima were found for PAH oxidation depending on the presence or absence of Mn(II) in the reaction mixture. For anthracene, higher activity without Mn(II) was found at pH 4.0, whereas in the presence of Mn(II) the optimal pH was 3.0. The same behavior was observed with pyrene and 2-methylanthacene. Relatively high oxidation rates were obtained both with and without Mn(II) when benzo[a]pyrene was used as substrate at an optimum pH of 3.5. Conversely, activity in the presence of Mn(II) dropped at pH 4, whereas in the absence of Mn(II) this activity remained high up to pH 4.5.

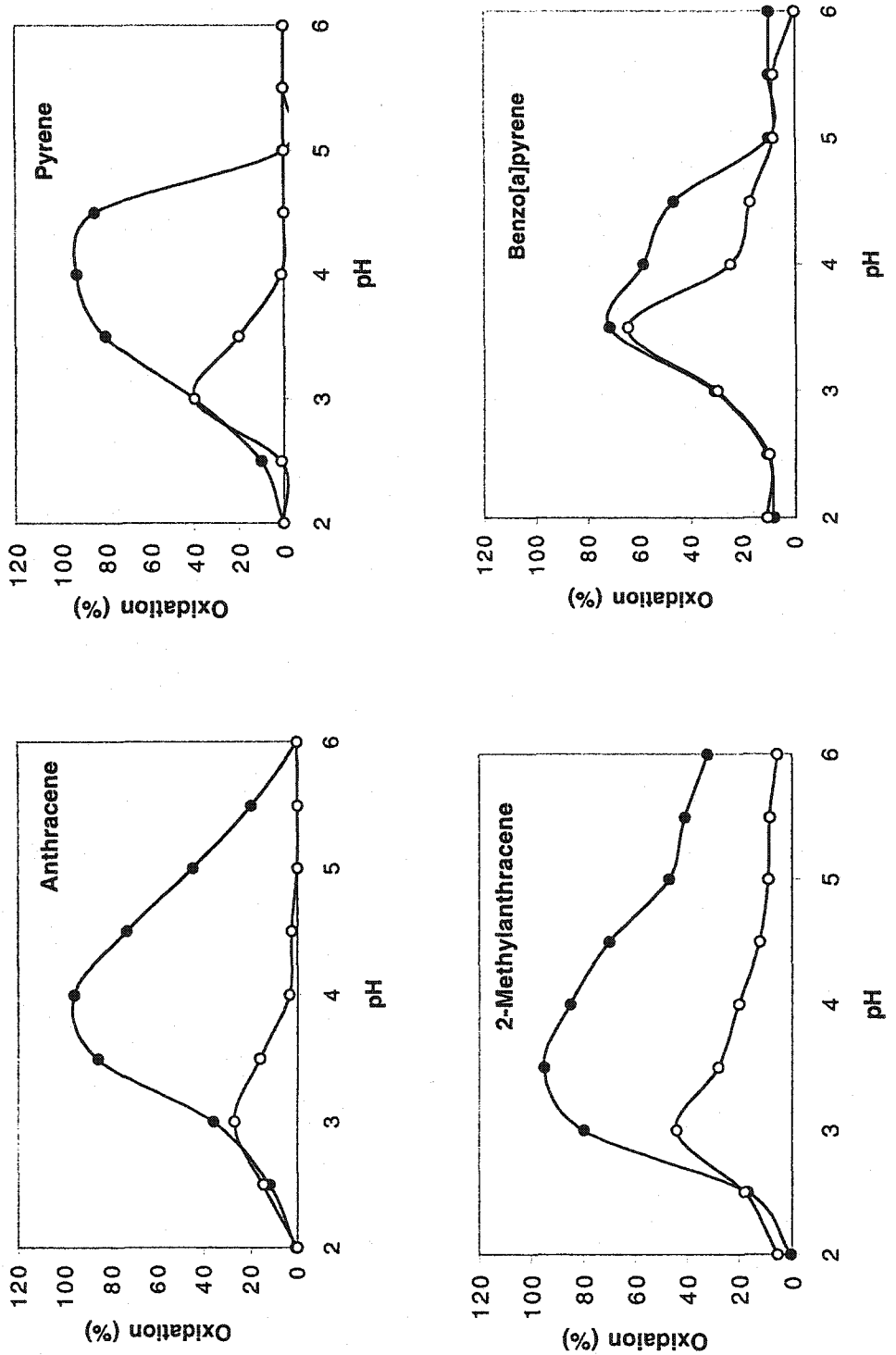

Fig. III-17. Effect of pH on Mn(II)-independent (●) and Mn(II)-dependent (○) MnP activity in PAH oxidation.

Table III-7. Oxidation of aromatic compounds by MnP and PEG-MnP at pH 4 in the absence of Mn(II).

		MnP	PEG-MnP
PAH	IP (eV)[a]	Specific activity (min^{-1})	Specific activity (min^{-1})
9-Methylanthracene	7.25	52.0 (±2.7)[b]	57.7 (±2.5)
1-Methylanthracene	NA[c]	22.4(±1.7)	27.2(±1.6)
2-Methylanthracene	7.37	12.4 (±1.1)	14.4 (±1.4)
Anthracene	7.41	2.5 (±0.01)	2.9 (±0.01)
Benzo[a]pyrene	7.41	0.3 (±0.04)	0.4 (±0.04)
Pyrene	7.42	0.01 (±0.01)	0.01 (±0.005)
Benzo[e]pyrene	7.43	NR[d]	NR[d]
Chrysene	7.60	NR	NR
1-Methylphenanthrene	7.70	NR	NR
Acenaphthene	7.76	NR	NR
Phenanthrene	7.91	NR	NR
Dibenzothiophene	7.93	NR	NR
Fluoranthene	7.95	NR	NR
Naphthalene	8.15	NR	NR

[a]Photoelectron spectroscopy values (http://webbook.nist.gov)
[b]Values in parentheses are standard deviations.
[c]NA, not available
[d]NR, no recation

3.4.1.2 Substrates of MnP

A collection of polyaromatic compounds were tested for oxidation with MnP and PEG-MnP at pH 4 in a Mn(II)-independent reaction (Table III-7). For PAHs, a clear correlation between ionization potential and oxidation rate was found. All of the substrates that were oxidized had an ionization potential of 7.55 eV or lower. Aromatic heterocycles, such as carbazole, did not follow this trend. The chemically modified enzyme followed the same rule although a slightly higher specific activities were obtained. The kinetic constants for the Mn-independent oxidation of 2-methylanthracene at pH 4 were determined and the values obtained were a k_{cat} of 145 min^{-1}, $K_{M,app}$ for the aromatic substrate of 23.8 μM and $K_{M,app}$ for hydrogen peroxide of 0.2 mM.

3.4.1.3 Effect of Mn(II) on PAH oxidation

The effect of Mn(II) in PAH oxidation was assayed with 2-methylanthracene as substrate. Increasing the Mn(II) concentration at pH 4 caused a decrease of the PAH oxidation rate. The Lineweaver-Burk plot shows noncompetitive inhibition (Fig. III-18), suggesting the presence of different binding sites for Mn(II) and 2-methylanthracene. The analysis of the Mn(II) inhibition data shows an inhibition constant K_I of 20 μM.

3.4.1.4 Polycyclic aromatic hydrocarbon oxidation products

The products of PAH oxidation by purified MnP from *B. adusta* UAMH 8258 were extracted and analyzed by GC/MS. The mass spectral data is shown in

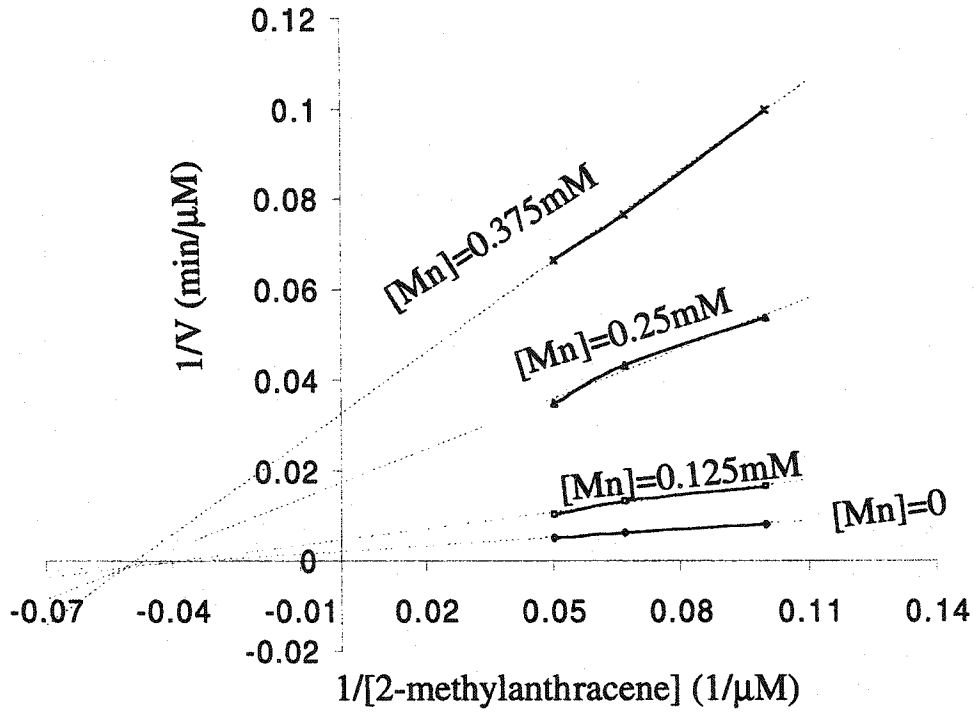

Fig. III-18. Lineweaver Burk plot for the oxidation of 2-methylanthracene by *B. adusta* MnP enzyme at different manganese concentration.
A plot of the slope of these rates against the manganese concentration produced a curve, indicating noncompetitive inhibition.

Table III-8. Mass spectral data of products from PAH compounds oxidized by manganese peroxidase from *B. adusta* UAMH 8258 without addition of Mn(II).

Substrate	Products	Mass spectral ions (*m/z*)
Anthracene	Anthraquinone	209 (15), 208 (100) **[M$^+$]**, 207 (16), 181 (14), 180 (80) **[M$^+$-28]**, 179 (12), 153 (10), 152 (78) **[M$^+$-56]**, 151 (35), 150 (20), 126 (10), 77 (10), 76 (44), 63 (15)
1-Methylanthracene	1-Methylanthraquinone	223 (16), 222 (100) **[M$^+$]**, 221 (30), 194 (15) **[M$^+$-28]**, 166 (10) **[M$^+$-56]**, 165 (55)
2-Methylanthracene	2-Methylanthraquinone	223 (16), 222 (100) **[M$^+$]**, 221 (11), 208 (10), 207 (15), 194 (35) **[M$^+$-28]**, 166 (20) **[M$^+$-56]**, 165 (72), 164 (10), 163 (10)
9-Methylanthracene	Anthraquinone	209 (15), 208 (100) **[M$^+$]**, 207 (16), 181 (14), 180 (80) **[M$^+$-28]**, 165 (10), 152 (70) **[M$^+$-56]**, 151 (20), 150 (15), 126 (10), 76 (44), 63 (15)
Pyrene	1,8-Pyrenedione	234 (19), 233 (18), 232 (88) **[M$^+$]**, 205 (13), 204 (33) **[M$^+$-28]**, 176 (17) **[M$^+$-56]**, 174 (15), 150 (14), 88 (35), 87 (15), 75 (12), 57 (11)
Benzo[a]pyrene	Benzo[a]pyrenedione	283 (22), 282 (100) **[M$^+$]**, 255 (11), 254 (56) **[M$^+$-28]**, 227 (15), 226 (76) **[M$^+$-56]**, 225 (27), 224 (44), 113 (37), 112 (24), 100 (9)

Table III-8. The products from all of the PAHs tested showed the loss of two CO groups and abundant [M^+]-28 or [M^+]-(2)(28) ions, suggesting that products are aromatic diones. The oxidation products from anthracene, 1- and 2-methylanthracenes were anthraquinone, 1- and 2-methylanthraquinone, respectively. The product from 9-methylanthracene oxidation with MnP was identified as anthraquinone, demonstrating a demethylation reaction. Pyrene yielded pyrenedione as shown by the [M^+] 232, 204 and 176 *m/z* ions. After spectrophotometric analysis, the purified pyrenedione has been identified as 1,8-pyrenedione analysis (Fatiadi *et al.* 1965). The benzo[a]pyrene product also showed the loss of two CO groups, sugesting the product was benzo(a)pyrenedione.

3.4.1.5 Effect of different water-miscible organic solvents on PAH oxidation by MnP from *B. adusta* UAMH 8258

2-Methylanthracene, which has relatively low water solubility (7.9 ug/l, Eastcott *et al.* 1988) was chosen as a model compound to study the effect of water-miscible organic solvents on PAH oxidation. With an increase in the water-miscible organic solvent concentration, PAH water solubility increased. PAH solubility was higher in the solution containing dimethylsulfoxide and tetrahydrofuran than the solutions containing ethanol and acetonitrile (Appendix 7.2). Since different water-miscible organic solvents on one hand enhance the substrate availability for enzyme oxidation, on the other hand deactivate the enzyme, the overall effect of different concentrations of water-miscible organic solvents on PAH oxidation was studied and

a curve showing the theoretical catalytic ability of an enzyme with increasing concentrations of a water-miscible organic (Fig. I-4) was obtained. Fig. III-19 shows that the addition of different water-miscible organic solvents at different concentrations enhanced 2-methylanthracene oxidation to different extents. The maximum initial oxidation rate (8 nmol/min) was obtained in the system containing 20% acetonitrile, while 5% dimethylsulfoxide or tetrahydrofuran increased the initial 2-methylanthracene oxidation rate to 5 nmole/min. Ethanol had very little effect on the oxidation rate with slight increases being observed up to 35%.

3.4.1.6 Comparison 2-methylanthracene oxidation by native and chemically modified MnP in the presence of acetonitrile

The presence of organic solvents can enhance 2-methylanthracene oxidation by MnP (Fig. III-19). It showed that the native enzyme can be deactivated in acetonitrile and that a chemically modified enzyme has enhanced stability in acetonitrile (Fig. III-16). The results confirmed that the modified enzyme also has enhanced stability in acetonitrile during 2-methylanthracene oxidation. Fig. III-20 shows that when incubated with 10% acetonitrile, the native and chemically modified MnP had same residual activity, whereas in 20% acetonitrile, the chemically modified enzyme showed higher residual activity than the native enzyme. This enhancement was also observed in 30% and 50% acetonitrile. Given that 20% acetonitrile was the optimum concentration for 2-methylanthracene oxidation, it is interesting to note that after 1 h the native enzyme retains 45% activity whereas the modified enzyme retains 70%.

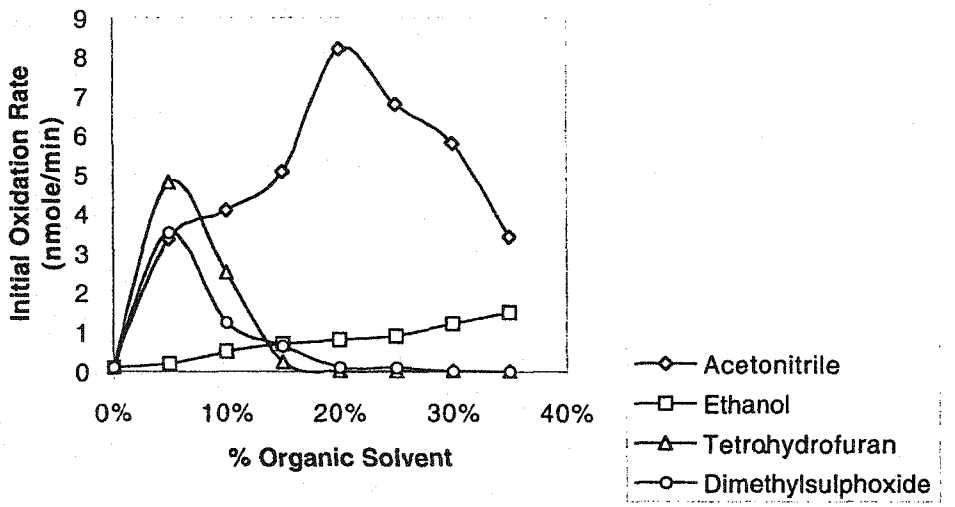

Fig. III-19 Effect of different organic solvents on 2-methylanthracene initial oxidation rate by MnP from *B. adusta* UAMH 8258.

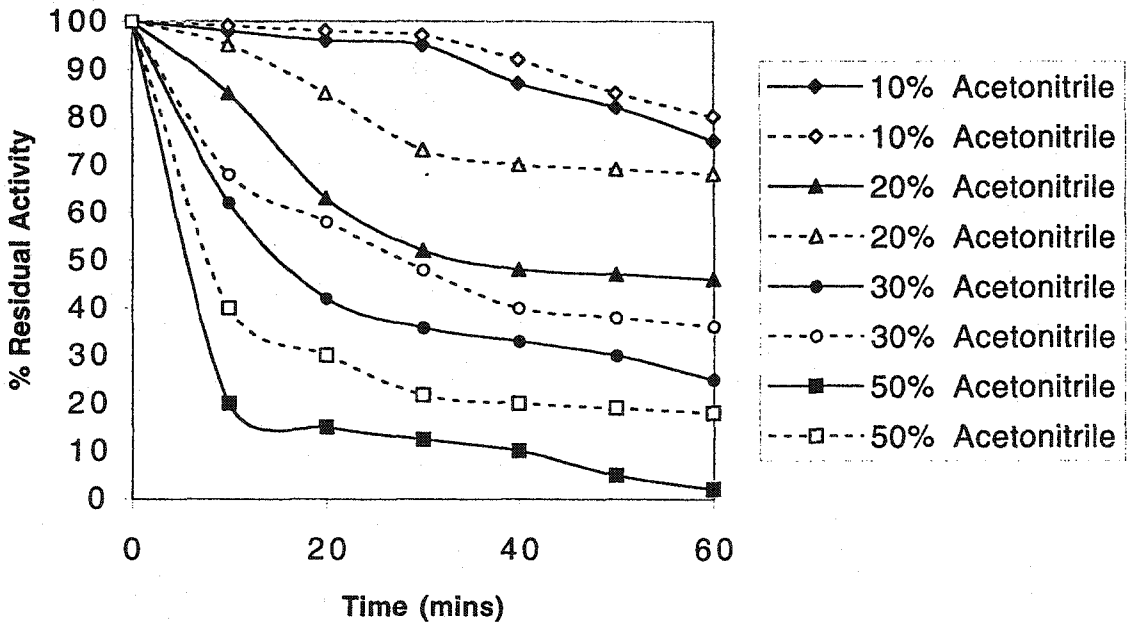

Fig. III-20. Effect of different acetonitrile concentration on the stability of native (dark symbol and PEG-MnP (open symbols) from *B. adusta* UAMH 8258 for 2-methylanthracene oxidation.

3.5 Polycyclic aromatic hydrocarbon metabolism by *B. adusta* UAMH 8258 cultures

3.5.1 Metabolism of ^{14}C-labelled PAHs by *B. adusta* UAMH 8258

In the previous studie of Pickard *et al.* (1999b), *B. adusta* UAMH 8258 and 7308 were shown to have higher PAH oxidation ability than that of the well studied *P. chrysosporium* under ligninolytic condition (BF medium). These two UAMH strains produce only MnP and in order to understand the roles of MnP in PAH metabolism, ^{14}C-labelled PAH metabolism by *B. adusta* UAMH 8258 cultures under ligninolytic and non-ligninolytic conditions were studied. From previous studies, we know that these two *B. adusta* strains produce high levels of MnP in media that contain natural lignin sources, such as RB and BF (ligninolytic conditions), and no MnP production in GYM medium (non-ligninolytic condition) (Pickard *et al.* 1999a; Wang *et al.* 2001).

After a 40-day-incubation, the percentage distribution of radioactivity from ^{14}C-labelled PAHs in the various fractions was determined (Table III-9). From this it is clear that little, if any, PAH mineralization occurred during this extended period, and these data indicated that cometabolism occurred during growth in the two media. The dichloromethane extractable material represents mainly non-metabolized PAHs and ranged from a low of 10% for fluoranthene to a high of 37% for phenanthrene. The acid-extractable ethylacetate fraction comprised the polar organics, and ranged from a low of 7% for anthracene to a high of 25% for phenanthrene. Except for phenanthrene, most of the radioactive material was found in the aqueous fraction. For

Table III-9. Percentage distribution of ^{14}C from ^{14}C-labelled PAHs in *B. adusta* UAMH 8258 cultures Incubate for 40 d.

	Anthracene		Phenanthrene		Pyrene		Fluoranthene	
Fraction	GYM	RB	GYM	RB	GYM	RB	GYM	RB
CO_2	0	2	0	2	3	4	0	2
Hydrophobics[a]	32	17	30	37	25	27	10	12
Polar organics[b]	7	9	25	24	11	9	12	8
Water Soluble + Biomass	49	66	14	5	47	43	55	64
Total	88	94	69	68	86	83	77	85

[a]Extracted with methylene chloride
[b]Extracted with ethylacetate, pH 2

phenanthrene most of the radioactivity was found as unreacted material (methylene chloride extract) or as slightly oxidized products (ethylacetate extract). There was no clear difference in radioactivity distribution under ligninolytic or non-ligninolytic growth conditions. Overall recovery of radioactivity ranged from 68% for phenanthrene to 94% for anthracene. Low recovery of radioactivity was observed with phenanthrene under both ligninolytic (68%) and non-ligninolytic conditions (69%). The reasons for this low recovery are not clear, but one of the possibilities is the condensation or polymerization of the products, yielding a polymer that cannot be efficiently extracted.

3.5.2 Polycyclic aromatic hydrocarbon transformation by living fungal cultures

A variety of other PAHs and carbazole were used to study cometabolism reactions of *B. adusta* UAMH 8258 under ligninolytic (RB medium) and non-ligninolytic (GYM medium) conditions. By plotting the amount of residual PAH and mycelium dry weight over time, the PAH disappearance rate could be calculated from the slope of the linear portion of the curve (Table III-10). PAH disappearance rates under ligninolytic and non-ligninolytic conditions were not different for the MnP substrates: carbazole, 9-methylanthracene, 2-methylanthracene and anthracene. For acenaphthene, fluoranthene, phenanthrene, which are not substrates of MnP, the rate of disappearance in GYM medium was higher than that in RB medium. Although pyrene can be oxidized by MnP, the oxidation rate was very low compared with the other substrates and once again, the rate of disappearance in GYM was higher that that in RB. For chrysene, which is not a substrate of MnP, no oxidation

Table III-10. Disappearance rate of PAHs and carbazole in *B. adusta* UAMH 8258 cultures

PAH	Rate of disappearance (nmol h^{-1} mg dw^{-1})	
	GYM	RB
Anthraquinone	0.29	0.53
Carbazole	0.75	0.75
9-Methylanthracene	1.09	1.09
2-Methylanthracene	0.76	0.87
Anthracene	1.87	1.79
Acenaphthene	0.97	0.89
Pyrene	1.33	1.00
Fluoranthene	1.00	0.42
Chrysene	0.00	0.18
Phenanthrene	1.69	0.38

in GYM and very low degradation in RB. Because chrysene has an extremely low water solubility, and the final acetonitrile concentration used to increase water solubility of PAHs was 4% for all the tests, the undetectable or very low degradation rate might due to limited substrate availability in the culture. This result indicates that under ligninolytic conditions, MnP plays important role in PAH oxidation, whereas under non-ligninolytic conditions, other enzyme systems, such as the cytochrome P_{450} system are most likely important.

4. Discussion

As in the results section of this thesis, discussion of the results is divided into four parts: MnP production, purification and characterization, chemical modification and polycyclic aromatic hydrocarbon oxidation by purified MnP and metabolism by *B. adusta* UAMH cultures.

4.1 Optimizing enzyme production conditions

4.1.1 Optimizing MnP production medium in shake flasks

Early studies showed that production of ligninolytic enzymes by *P. chrysosporium,* the most-investigated white-rot fungus, and related lignin-degrading fungi require strict N-limited growth conditions, usually achieved by using Kirk's medium (SBM) or modification thereof (Kirk *et al.* 1986; Hatakka 1994; Buswell *et al.* 1995; Moilanen *et al.* 1996). In contrast, results (Table III-2) and several other observations, such as in the studies of Mester *et al.* (1996), Hofrichter and Fritsche (1997), and Scheel *et al.* (2000) showed that ligninolytic activities were partly repressed by using SBM (N-limited medium) and MnP production in cultures of *Bjerkandera, Clitocybula dusenii* b11 and *Nematoloma frowardii* b19 were stimulated by N-sufficient medium. However, our results showed no MnP production in GYM medium for three *Bjerkandera* strains UAMH 8258, UAMH 7308 and ATCC 90940, where glucose and N were present in excess. The observation of heavy growth in this medium suggesting that in addition to N-sufficient medium, other physiological factors must be involved in triggering MnP production. Mester *et al* (1996) found that the addition of Mn(II) and small organic acid such as gluconic acid, glycolic acid,

glyoxylic acid and malonic acid could induce MnP production in *B. adusta.* Scheel *et al* (2000) provided molecular genetic evidence to show the inducing effect of Mn(II) on the expression of MnP. Results (Table III-2) also show that the addition of Mn(II) or Mn(II) and glycolate induce MnP production in N-sufficient medium (GYM-SBM), but not in N-limited medium (SBM). This Mn(II) induction mechanism is different from the Mn(II) induction effect observed in *P. chrysosporium* (Brwon *et al.* 1991), which only occurs under N-limited conditions.

All bran preparations examined in this study supported MnP production to different extents (Table III-2), where MnP titres in four different kinds of bran were higher than in the other tested media except for strain ATCC 90940. There was no increase in MnP production when Mn(II) and glycolate were added as inducers in bran media (data not shown), indicating the natural lignin sources (bran) contain unknown inducers that can trigger MnP production. The same conclusion was drawn by Ha *et al.* (2002) because high levels of enzyme production were obtained in glucose-peptone medium supplied with WB.

Rice bran supported MnP production by all three strains, particularly for UAMH strains 7308 and 8258. Comparison of the nutrient information of RB with the other brans revealed that RB has the highest percentage of fat and the lowest percentage of dietary fibre (Table III-1). Enhancing dietary fibre (by adding WB) or soluble starch concentration in RB did not trigger higher MnP production (Fig. III-2). Therefore it might be the high fatty acid concentration rather than high dietary fibre or carbohydrate concentration results in higher MnP production in RB. Although the induction mechanism is not clear, one possibility is that metabolic products from fatty

acids, such as organic acids, may play a role in MnP induction. The other possibility is that fatty acid are more readily used carbon source. Certainly fatty acids are not inhibitory to fungal growth, and their presence may have the added benefit of acting as foam depressants during growth in the STR.

4.1.2 Optimizing MnP production conditions

Strains UAMH 8258 and 7308 are able to produce high levels of MnP in submerged culture without the need for chemical inducers or pure oxygen. Thus, as an initial step for further applications at a larger scale, different MnP production conditions were evaluated in batch experiments in shake flasks. Published methods for MnP production in submerged culture, used the following conditions: 1/5-2/5 medium volume to flask volume ratio, 150 -275 rpm, 30 °C, flush with pure oxygen, initial pH 4.5-6.5 (Moreira *et al.* 2000; Scheel *et al.* 2000, Hofrichter and and Fritsche 1997). The optimized conditions are similar to the conditions described above except without flushing the culture with pure oxygen. It is interesting to notice that when different initial pH levels were used, enzyme production started when the cultures adjusted the pH to 5 and that enzyme production peaked when the pH reached 5.5 (Fig. III-5a, 5b, 5c). Moreira *et al* (2000) found that a drastic change of pH and redox potential coincided with the maximum MnP activity. These parameters can be considered as indicative of the initiation of the enzyme production stage and an on-line sensor might be developed to monitor the production of MnP in the future.

4.1.3 Scale-up MnP production

Excellent MnP production in STR was achieved, following optimization in shake flasks (Fig. III-8). With RB as growth medium, large amounts of biomass were produced. Thus, at the enzyme production stage, aeration is poor but increasing the agitation rate is undesirable because shearing of fungal mycelia and cell breakage would release non-specific proteases. Increased air flow rate also results in the formation of bigger air bubbles with increased shear stress on fungal mycelia.

To reduce shear stress, immobilized cultures could be used. Many attempts to scale-up ligninolytic enzyme production have been made using immobilized white-rot fungi on different materials, such as silicon tubes (Willershausen *et al.* 1987), nylon (Rodriguez-Couto *et al.* 1999), polyurethane (D'Annibale *et al.* 1998; Rodriguez-Couto *et al.* 1999), and polypropylene (Ziegenhagen and Hofrichter 2000). In these experiments, fungi absorbed on these carriers spontaneously. It was noticed that the media that used in all these fungal immobilization studies was Kirk's basal medium or modified basal medium, which could not support growth of large amounts of biomass. The addition of carrier material should allow for a larger area for attachment and growth. However, under these condition, the addition of carrier materials might further decrease oxygen and nutrient diffusion efficiency.

4.2 Enzyme purification and characterization

In choosing to study the application of fungal enzymes, the choice of producing fungus is important, not only for the volumetric enzyme content but also for other factors such as the length of the growth period, the ease of scale-up, and

purification. As a result of the screening study here and earlier work, *B. adusta* UAMH 8258 was chosen to produce MnP. The purification procedure involved mainly anion-exchange chromatography and size-exclusion for the removal of contaminating pigment because only one enzyme form was shown to be present in the culture broth of *B. adusta* and no other lignin-degrading enzymes were detected under the conditions used (Pickard *et al.* 1999b).

4.2.1 Physical characteristics of MnP

The observed molecular weight value for purified MnP from *B. adusta* UAMH 8258 by SDS-PAGE and MALDI-TOF mass spectrometry were different, 43 kDa and 36.6 kDa respectively. Because MnP is a glycoprotein and the SDS-PAGE molecular weight estimate method is affected by the carbohydrate content of a glycoprotein, mass spectrometry is a more accurate reflection of the true molecular weight. Ruiz-Duenas *et al.* (1999) also reported that MnP from *Pleurotus eryngii* had a molecular weight of 43 kDa by SDS-PAGE and 37 kDa by MALDI-TOF.

The N-terminal sequence of MnP from *B. adusta* UAMH 8258 is identical to MnP from *B. adusta* UAMH 7308, the published sequence of BOS55 (ATCC 90940) (Mester and Field 1998) and the MnP isoenzyme (BOS2) from BOS55 (Palma *et al.* 2000). The N-terminal sequence of MnP from *B. adusta* was found to have high homology with MnP (only one amino acid different) and LiP (2 amino acids different) from *T. versicolor,* but low homology with MnPs from *P. chrysosporium* (less than 50% identity) (Table III-4). When the amino acid composition of MnP from *B. adusta* UAMH 8258 is compared with MnP from *T. versicolor* (Johansson 1993),

LiP from *P. chrysosporium* (Ritch *et al.* 1992), and laccase from *T. versicolor* (Joensson *et al.* 1995) (Table III-5), it is striking to note that no tyrosine is present. In fact, no tyrosine appears in any of the 59 MnPs described (www.ncbi.nlm.nih.gov/entrez/protein.html) or in LiP from *P. chrysosporium* (Ritch and Gold 1992). These MnP forms contained either one or two tryptophan residues per molecule.

Enzyme purity is based on a number of independent factors, and must be supported by constant specific activity after purification and the presence of a single protein species by physical techniques. For hemoproteins, the ratio of heme to protein is an added criterion of purity and the Reinheitzahl or Rz ratio, the ratio of absorbances due to heme and the aromatic residues in the protein (A_{407}/A_{280}) is often quoted. Contaminating proteins and pigments can lower the Rz number by increasing absorbance at 280 nm without affecting absorbance at 407 nm. The published Rz values of MnPs from different sources are variable (Table IV-1), ranging from 1.85 to 450. All MnPs cited in Table IV-1 are purified enzymes, and all showed a single band on SDS-PAGE. It is possible that purified MnPs from different sources have different Rz numbers due to the different absorbances of MnPs at 280 nm (the molar extinction coefficient for a purified enzyme at $\varepsilon(280_{nm})(mM^{-1}cm^{-1})$=(no. of tryptophan)(5500)+(no. of tyrosine)(1490)+(no. of cystine)(125) (Pace *et al.* 1995)). As we know there is no tyrosine in the current studied MnPs, and since the absorbance at 280 nm is caused most by tryptophan, the different Rz values for the purified MnPs are likely due to their different tryptophan contents. An observed Rz of 4.3 for this enzyme is based on two tryptophan residues per molecule (Appendix 7.1).

Table VI-1. Molecular weight, Rz and pI of isoenzymes of MnP from different strains.

	MW (Da) SDS-PAGE (MALDI-TOF)	Rz	pI
P. chrysosporium SC-26 (Sasaki *et al.* 2001)	NA[a]	1.85	NA[a]
P. chrysosporium IZU-154 (Matsubara *et al.* 1999)	43,000	5.7	5.1,4.9,4.5,3.7
P. eryngii (Ruiz-Duenas *et al.* 1999)	43,000	450	NA[a]
P. chrysosporium (Paszczynski *et al.* 1988)	NA[a]	3.6	NA[a]
P. chrysosporium (Wariishi *et al.* 1992)	NA[a]	6	NA[a]
N. frowardii b19 (Schneegaβ *et al.* 1997)	44,000	6	3.2
C. subvermispora (Lobos *et al.* 1994)	NA[a]	NA[a]	4.58,4.52,4.49,4.43, 4.31,4.22,4.13
Bjerkandera sp. BOS1 (Palma *et al.* 2000)	45,000	NA[a]	3.45
Bjerkandera sp. BOS2 (Palma *et al.* 2000)	44,000	NA[a]	3.40
Bjerkandera sp. BOS55 (Mester and Field 1998)	43,000	NA[a]	3.88
B. adusta (Yoshida *et al.* 1996)	46,500	NA[a]	3.9
B. adusta UAMH 8258	43,000 (36,600)	4.3	3.55
P. eryngii (Martinez 2002)	43,000 (36,000)	4.2	NA[a]
B. adusta UAMH 7308 (this study)	43,000 (36,600)	3.5	3.55
Bjerkandera sp. BOS55 (this study)	43,000 (36,600)	4.5	3.55

4.2.2 Catalytic characteristics of MnP

Like the novel type of peroxidase from the *Pleurotus* species (Martinez *et al.* 1996), MnP from *B. adusta* UAMH 8258 also has both Mn(II)-dependent and independent activities. The kinetic constants of MnP from *B. adusta* UAMH 8258 (Table III-6) are slightly different from the MnP isoenzyme from *Bjerkandera* sp. BOS55 (Mester and Field 1998) and MnP isoenzyme from *P. eryngii* (Table IV-1).

Table IV-2. Comparison of steady-state kinetic constants of MnP isoenzymes on Mn(II)-independent activity.

	$K_{M, app}$ (μM)		
	Bjerkandera sp. BOS55[a]	*P.* eryngii[b]	*B. adusta* UAMH 8258
H_2O_2	31	6-9	4.5
Mn(II)	51	<20	17
DMP	41	300	144.5
VA	116	3000	5300

[a]: Mester and Field (1998)
[b]: Camarero *et al.*(1999)

The kinetic constant $K_{M, app}$ of MnP from UAMH 8258 for H_2O_2 and Mn(II) are lower than those of BOS55 and $K_{M, app}$ for DMP and VA are higher. These differences in data for *B. adusta* strains may be due to laboratory methodology and of only minor significance. However, the $K_{M, app}$ values of MnP from *B. adusta* UAMH 8258 described above are similar to the isoenzyme from *P. eryngii* (Martinez *et al.* 1996).

4.3 Comparison of native and modified enzyme

The polyethylene-glycolation (PEG-) of MnP has no significant effect on Mn(II) and malonate binding, and MnP-dependent activity on some phenolic substrates (Fig. III-14 and Table III-6). Presumably because the Mn(II)-binding site has three acidic amino acid residues (E35, E39, D179) and PEG-modification occurs mainly on lysine amino groups, the Mn(II)-binding site will remain unaffected. However, one observation about the modified enzyme was the enhanced stability in high concentrations of H_2O_2. It is well known that native heme-enzymes deactivate in excess H_2O_2 due to the reaction of H_2O_2 with (in this case, MnP-) compound (II) to form MnP compound (III), the deactivated enzyme form (Wariishi *et al.* 1992). Since the peroxide binding site on MnP compound II has not been studied, the mechanism of this enhanced resistance by PEG-modification is not clear.

Chemical modification with monofunctional polymers has been utilized by several researchers to modulate enzyme activity in organic solvents and at high temperature, for enzymes such as lipase, chymotrypsin, subtilisin and catalase. Oxidative enzymes have also been examined, with HRP, cytochrome c and LiP, all exhibiting enhanced stability in the presence of water-miscible organic solvents (Khmelitsky *et al.* 1988; van Erp *et al.* 1991; Vazquez-Duhalt *et al.* 1993). As anticipated, PEG-modified MnP showed higher catalytic activity than native MnP when organic solvents were present and also had enhanced thermal and organic solvent stability (Fig. III-16). The results also showed that PEG-modified enzyme had a significant enhancement of enzyme stability in acidic environment (Fig. III-16), which may be important because the optimum for Mn(II)-independent oxidation is

pH 3. It was found that the modification protects the enzyme better in low pH (2-5) than in high pH (pH 8-10) (data not shown), perhaps because the PEG modification is a covalent addition to the lysine amino groups, thus preventing lysine protonation and subsequent conformational change at low pH.

4.4 Polycyclic aromatic hydrocarbon oxidation by purified MnP from *B. adusta* UAMH 8258

Bjerkandera adusta UAMH 8258 produces a MnP, different from *P. chrysosporium* MnPs, that can efficiently oxidize Mn(II) to Mn(III), and can also carry out Mn(II)-independent activity on aromatic substrates (Heinfling *et al.* 1998a; Mester and Field 1998; Wang *et al.* 2001, 2002). Similar MnP-LiP hybrid peroxidases have been reported in *P. eryngii* ((Martinez *et al.* 1996; Ruiz-Dueñas *et al.* 1999a and b; Heinfling *et al* 1998a, 1998b), *Pleurotus pulmonarius* (Camarero *et al.* 1996), and *Pleurotus ostreatus* (Sarkar *et al.* 1997). PAH oxidation with this MnP from *B. adusta* UAMH 8258 showed different pH optima according to the presence or the absence of Mn (Fig. III-17): the Mn-dependent oxidation of PAHs showed lower optimal pH and specific activity than the Mn-independent oxidation. Veratryl alcohol oxidation, which is considered to be of the LiP type, has a pH optimum of 3.0, whereas the optimum pH for the oxidation of Mn(II) to Mn(III) is 4.5 (Heinfling *et al.* 1998).

A clear correlation was found between the specific activity of MnP from *B. adusta* UAMH 8258 and the IP of the PAH substrate. Aromatic substrates with an IP lower than 7.43 eV were oxidized by the purified enzyme, and the lower the IP the

faster the oxidation rate (Table III-7). The biocatalytic mechanism of peroxidases involves the production of free radicals, so it is not surprising that the energy involved in the removal of one electron from the substrate is correlated to the peroxidase oxidation rate. A threshold value of the IP for compounds to be substrates has been found for many peroxidases. Lignin peroxidase oxidizes PAHs with IP ≤ 7.55 eV (Hammel *et al.* 1986; Vazquez-Duhalt *et al.* 1994), whereas MnP oxidizes PAHs with IP up to 8.2 eV (Bogan *et al.* 1995, 1996). The highest IP for hemoglobin-mediated oxidation of PAHs was 8.0 eV (Torres and Vazquez-Duhalt, 2000) and chloroperoxidase was able to modify PAHs with IP lower than 8.2 eV (Vazquez-Duhalt *et al.* 2001). On the other hand, no correlation was found between oxidation of PAHs and their IP with laccase from *T. versicolor* (Majcherczyk *et al.* 1988) or from *C. gallica* (Pickard *et al.* 1999b).

In this study, the MnP oxidation products were the corresponding quinones (Table III-8). A carbon-carbon bond cleavage was detected in the Mn(II) independent-oxidation of 9-methylanthracene, which presumably demethylated to form anthraquinone. Ligninolysis also involves release of methyl groups as methanol. Quinones are the common oxidation products of peroxidases on PAHs (Vazquez-Duhalt 1998). The relative rates of oxidation of anthracene and the methylanthracenes reflected the electron-withdrawing properties of the methyl groups, making the adjacent carbons electron-deficient and thus more susceptible to oxidation: 9-methyl > 1-methyl > 2-methyl > anthracene.

The fundamental difference between the Mn-dependent and the Mn-independent oxidations is of course the lack of the mediating oxidant, Mn(III). The

partial noncompetitive inhibition of Mn(II) on the oxidation of PAHs (Fig. III-18) suggests that the Mn(II) and the 2-methylanthracene bind reversibly, randomly, and independently at different sites. The three-dimensional model of the *P. eryngii* hybrid enzyme showed a Mn(II) binding site near the internal heme propionate, a long range electron transfer mechanism for aromatic substrates has been proposed (Camarero *et al.* 1999). Despite the possibility of direct electron transfer from the aromatic substrate to the heme environment, long range electron transfer has been also detected in LiP from *P. chrysosporium* (Shoemaker *et al.* 1994). Thus the PAH is oxidized by the enzyme directly, and shares with Mn(II) for the oxidizing power of the enzyme. Long range electron transfer may be responsible for H_2O_2-dependent peroxidase inactivation, and protein engineering has been proposed as a solution (Valderrama *et al.* 2002).

Here, the chemically modified enzyme cannot only enhance enzyme stability in the presence of acetonitrile for Mn(II)-dependent activity but also has the same effect on enzyme for Mn(II)-independent activity in direct oxidation of PAHs (Fig. III-16, Fig. III-20). The modification did not change the substrate specificity of during both Mn(II)-dependent and independent activity (Table III-6, Table III-7), indicating the enzyme structure is not changed by modification. A hypothesis explaining the increase in oxidation of PAHs after modification is that given the amphipathic nature of the polymers used to modify MnP in this study, increases in the surface hydrophobicity or relaxed enzyme conformation could have taken place (Vazquez-Duhalt *et al.* 1992). Thus in addition to trapping water at the enzyme surface, the modification could have played a role in increasing the rate of reaction by

partitioning hydrophobic PAHs from solution to the enzyme surface. A combination of these effects may explain the enhanced catalytic ability of chemically modified enzyme in PAH oxidation.

4.5 Polycyclic aromatic hydrocarbon metabolism by *B. adusta* UAMH 8258 culture

To determine if *B. adusta* was able to mineralize ^{14}C-PAH or to what extent the fungus was able to metabolize these polycyclic compounds. White rot fungi are well known for their ability to metabolize a large range of xenobiotics (Aust 1990; Field *et al.* 1993). It was found that active lignin-degrading strains of *B. adusta* are able to cometabolize several PAHs, but only a very limited amount of mineralization occurred (Table III-9). Anthracene, pyrene and fluoranthene were converted mainly to water soluble products or adsorbed to biomass, whereas phenanthrene was mainly unchanged with the major fraction extracted into dichloromethane. No clear differences were found between the ligninolytic and non-ligninolytic growth media, and previous experiments showed that after a 40-day-incubation an end-point had been reached. In addition, the data in Table III-10 show that the initial rate of PAH metabolism was similar under the two growth conditions, even if the growth rates of the fungus differed. As in the case of other white rot fungi, it seems that the metabolic degradation of PAHs by *B. adusta* requires both intracellular enzymatic systems, such as cytochrome P_{450} (Masaphy *et al.* 1995; Bezalel *et al.* 1996) and extracellular oxidative enzymes (Kirk *et al.* 1990; Bogan and Lamar 1996; Pickard *et al.* 1999b).

It is generally accepted that the ligninolytic enzymes, LiP, MnP and laccase are capable, under optimized conditions, of oxidizing PAHs with an IPs less than 7.6 eV. For PAHs with a higher IPs, a cytochrome P_{450} monoxygenase activity is likely the enzyme responsible. Since growing *B. adusta* cultures were able to metabolize a wide range of PAHs in this study, it can be concluded similarly that a cytochrome P_{450} monoxygenase activity is likely responsible for the oxidation of PAHs with an IP above 7.7 eV. However, the Mn(II)-independent activity exhibited by *B. adusta* MnP is clearly capable of oxidation of anthracene and its monomethyl isomers in the absence of competing substrates such as Mn(II). This is the first demonstration of PAH oxidation by MnP from *B. adusta* with Mn(II)-independent activity.

4.6 Concluding remarks

The goals of this project were to investigate the metabolism of PAHs by *B. adusta.* In order to do this, large amounts of MnP were produced and enzyme characteristics were determined, including PAH oxidizing ability.

In choosing to study aspects of PAH metabolism by enzymes from white-rot fungi, the choice of producing fungus is important, not only for the volumetric enzyme content but also for other factors such as the ease and reliability of growth, the speed of enzyme production, and the ease of enzyme purification. Sometimes reproducing published enzyme production data from fungal strains obtained from culture collections is difficult and comparison with literature values depends to an extent on laboratory methodology. For these reasons, two relatively uncharacterized culture collection strains were chosen to compare with a well studied ATCC strain.

In this study, *B. adusta* strains UAMH 7308 and 8258 produce levels of MnP equivalent to or exceeding those produced by ATCC 90940 (BOS55) under similar growth conditions. These enzyme yields, expressed as Mn-malonate units, after about 15-day-incubation in shake flasks, were to 4.4 U/ml by UAMH 7308, 5.5 U/ml by UAMH 8258 and 1.7 U/ml by BOS55. Scale-up as submerged cultures in 14-L stirred tank reactors produced 3.5 U/ml by UAMH 8258 and 2.5 U/ml by 7308 in a shorter time period, among the highest MnP production levels described. These results indicate that the two strains of *B. adusta*, UAMH 7308 and 8258, are good candidates for large scale enzyme production and that the RB medium used supports higher levels of enzyme production than most previously described media.

The purification of these enzymes was typical of other ligninolytic enzymes from white-rot fungi and produced preparations of high purity, no isoenzymes were isolated. Characterization of the enzyme indicated that MnP produced by *B. adusta* UAMH 8258, has both Mn(II)-dependent and independent activities. The PEG-modified MnP exhibited little difference in catalytic properties but has greater resistance to loss of activity in the presence of H_2O_2 and organic solvents. The modified enzyme also showed enhanced stability at high temperature, low pH and in the presence of organic solvents such as acetonitrile.

Polycyclic aromatic hydrocarbon oxidation studies using purified MnP from *B. adusta* UAMH 8258 have shown that PAHs with IPs of 7.43 eV or lower can be oxidized by MnP without addition of Mn(II), and the presence of Mn(II) noncompetitively inhibited PAH oxidation. The PAH metabolites of the Mn(II)-independent reaction were identified as the corresponding quinones. This is the first

report of PAH oxidation by MnP from white-rot fungi. The chemically modified MnP displayed the same catalytic characteristics as the native MnP in PAH oxidation and greater PAH oxidation rate due to the enhanced stability in the presence of organic solvent.

In the metabolic degradation of PAHs by *B. adusta* living fungal systems involved both intracellular enzymatic systems (such as cytochrome P_{450}) and extracellular oxidative enzymes. The role of MnP in PAH metabolism is to oxidize PAH to more soluble quinones. This sets up the possibility for further metabolism by other enzymes or microorganisms.

5. Future study

5.1 Enzyme production

The production of ligninolytic enzymes using solid-substrate fermentation has been used successfully demonstrated by the Vazquez-Duhalt group (personal communication). Economically, this type of process presents many advantages such as superior volumetric productivity, reduced energy requirements, simple handling and improved product recovery. One of the great advantages of solid-state processes in ligninolytic enzyme production could be the possible reduction of the production costs by using agriculture wastes.

Continuous production of fungal enzymes from immobilized fungal mycelium is one of the most promising ways of enhancing ligninolytic enzyme production. Many studies have shown that the immobilized fungal mycelium can be reused and maintained in its idiophase, MnP production starts immediately after the addition of fresh medium.

Recombinant production of MnP is another way of achieving high ligninolytic enzyme production. Several *P. chrysosporium* MnPs have been cloned and hyperproducing strains could be generated. Isolating and cloning the gene for MnP from *B. adusta* may be accomplished by probing genomic cDNA libraries with an oligonucleotide based on the N-terminal amino acid sequence of the enzyme.

Promising results have been obtained in the production of MnP in transgenic plants. Expression of *P. chrysosporium* MnP has been achieved in alfalfa and tobacco plants and active MnP has been expressed in cell suspension cultures of tobacco.

5.2 Enzyme modification

The chemical modification utilized in this study were only a small sample of the chemical modifications possible. There are numerous methods and chemical groups for modification of proteins. As the oxidation site for aromatic compounds on MnP has been studied, site-directed mutagenesis could be used to protect the oxidation site in the presence of organic solvents.

5.3 Polycyclic aromatic hydrocarbon metabolism by white-rot fungi

It is clear that aromatic compounds with IPs lower than 7.43 eV can be oxidized by purified MnP from *B. adusta*. White-rot fungi can oxidize PAHs with much higher IPs, intracellular enzyme cytochrome P_{450} has been considered as one of the most important enzymes involved in oxidation of PAHs with higher IP. This enzyme catalyzes the incorporation of only one atom of oxygen into the aromatic ring to eventually yield the corresponding dihydrodiol and resulting in accumulation of that transformed product. In fungal PAH mineralization, fungal cultures can transform PAHs to many polar productions indicating that cytochrome P_{450} is not the only enzyme involved in initial oxidation or further transformation of PAHs. More studies are needed to find out the other enzymes involved in PAH oxidation.

5.4 Using mixed bacterial and fungi system to enhance PAH degradation

Two-step degradation of PAH by white-rot fungi and soil microorganisms are promising in PAH bioremediation. Using white-rot fungi as a first step to oxidize

PAH and to produce less toxic and more water-soluble products may allow for further metabolism by other fungi and bacteria to obtain higher PAH mineralization rates.

6. Literature Cited

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.

Aifa, M. S., Sayadi, S. and Gargouri, A. (1999) Heterologous expression of lignin peroxidase of *Phanerochaete chrysosporium* in *Aspergillus niger. Biotechnol. Lett.* **21,** 849-853.

Ander, P. (1994) The cellobiose-oxidizing enzymes CBQ and CBO as related to lignin and cellulose degradation. *FEMS Microbiol. Rev.* **13,** 297-312.

Andersson, B. E. and Henrysson, T. (1996) Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white-rot fungi. *Appl. Microbiol. Biotechnol.* **46,** 647-652.

Archibald, F. S., Bourbonnais, R., Jurasek, L., Paice, M. G. and Reid, I. D. (1997) Kraft pulp bleaching and delignification by *Trametes versicolor. J. Biotechnol.* **53,** 215-236.

Arnold, F. (1990) Engineering enzymes for non-aqueous solvents. *Trends Biotechnol.* **8,** 244-248.

Aust, S. D. (1990) Degradation of environmental pollutants by *Phanerochaete chrysosporium. Microb. Ecol.* **20,** 197-209.

Ayala, M., Baratto, M. C., Basosi, R., Vazquez-Duhalt, R. and Pogni, R. (2001) Spectroscopic caracterization of a manganese-lignin peroxidase hybrid isoenzyme produced by *Bjerkandera adusta* in the absence of manganese: evidence of a protein centred radical by hydrogen peroxide. *J. Mol. Catalysis B: Enzymatic* **16,** 159-167.

Ayala, M., Horjales, E., Pickard, M. A. and Vazquez-Duhalt, R. (2002) Cross-linked crystals of chloroperoxidase. *Biochem. Biophys. Res. Commun.*, (accepted).

Bao, W., Fukushima, Y., Jensen, K. A., Moen, M. A. and Hammel, K. E. (1994) Oxidative degradation of non-phenolic lignin during lipid peroxidation by fungal manganese peroxidase. *FEBS Lett.* **354,** 297-300.

Barclay, C. D., Farquhar, G. F. and Legge, R. L. (1995) Biodegradation and sorption

of polyaromatic hydrocarbons by *Phanerochaete chrysosporium*. *Appl. Microbiol. Biotechnol.* **42,** 958-963.

Beaudette, L. A., Davies, S., Fedorak, P. M., Ward, O. P. and Pickard, M. A. (1998) Comparison of biodegradation and mineralization as methods for measuring loss of selected polycholorinated biphenyl congeners in cultures of four white rot fungi. *Appl. Environ. Microbiol.* **64,** 2020-2025.

Beaudette, L. A., Ward, O. P., Pickard, M. A. and Fedorak, P. M. (2000) Low surfactant concentration increases fungal mineralization of a polychlorinated biphenyl congener but has no effect on overall metabolism. *Lett. Appl. Microbiol.* **30,** 155-160.

Bezalel, L., Hadar, Y. and Cerniglia, C. E. (1996) Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* **62,** 292-295.

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

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.

Blodig, W., Doyle, W. A., Smith, A. T., Winterhalter, K. H., Choinowski, T. H. and Piontek, K. (1998) Autocatalytic formation of hydroxy group at C_β of Trp171 in lignin peroxidase. *Biochem.* **37,** 8832-8838.

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., Schoenike, B., Lamar, R. T. and Cullen, D. (1996) Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **62,** 2381-2386.

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

Bogan, B. W., Lamar, R. T., Burgos, W. D. and Tien, M. (1999) Extent of humification of anthracene, flouranthene and benzo[a]pyrene by *Pleurotus ostreatus* during growth in PAH-contaminated soils. *Lett. Appl. Microbiol.* **28,** 250-254.

Boldrin, B., Tiehm, A. and Fritzsche, C. (1993) Degradation of phenanthrene, fluorene,
fluoranthene and pyrene by a *Mycobacterium* sp. *Appl. Environ. Microbiol.* **59,** 1927-1930.

Bourbonnais, R. and Paice, M. G. (1992) Demethylation and delignification of Kraft pulp by *Trametes versicolor* laccase in the presence of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate). *Appl. Microbiol. Biotechnol.* **36,** 823-827.

Bourbonnais, R., Paice, M., Reid, I., Lanthier, P. and Yaguchi, M. (1995) Lignin oxidation by laccase isoenzymes from *Trametes versicolor* and role of the mediator 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) in Kraft lignin depolymerization. *Appl. Environ. Microbiol.* **61,** 1876-1880.

Bradford, M. (1984) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248-254.

Brock, J. B., Rieble, S. and Gold, M. H. (1995) Purification and characterization of a 1,4-benzoquinone reductase from the basidiomycete *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **61,** 3076-3081.

Brown, J. A., Alic, M. and Gold, M. H. (1991) Manganese peroxidase gene transcription in *Phanerochaete chrysosporium*: activation by manganese. *J. Bacteriol.* **173,** 4101-4106.

Brown, J. A., Li, D., Alic, M. and Gold, M. H. (1993) Heat shock induction of manganese peroxidase gene transcription in *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **59,** 4295-4299.

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.

Bumpus, J. A. and Aust, S. D. (1987) Biodegradation of DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane] by the white-rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **53,** 2001-2008.

Buswell, J. A. and Odier, E. (1987) Lignin degradation. *Crit. Rev. Biotechnol.* **6,** 1-60.

Buswell, J. A., Cai, Y. J. and Chang, Y. J. (1995) Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production, by *Lentinula edodes. FEMS Microbiol. Lett.* **128,** 81-88.

Call, H. and Mucke, I. (1997) History, overview and applications of mediated

ligninolytic systems, especially laccase-mediator-systems (Lignozyme-process). *J. Biotechnol.* **53,** 163-202.

Camarero, S., Bockle, B., Martinez, M. J. and Martinez, A. T. (1996) Manganese-mediated lignin degradation by *Pleurotus pulmonarius. Appl. Environ. Microbiol.* **62,** 1070-1072.

Camarero, S., Sarkar, S., Ruiz-Duenas, F. J., Martinez, M. J. and Martinez, A. T. (1999) Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase activities. *J. Biol. Chem.* **274,** 10324-10330.

Cameron, M. D., Post, Z. D., Stahl, J. D., Haselbach, J. and Aust, S. D. (2000) Cellubiose dehydrogenase-dependent biodegradation of polyacrylate polymers by *Phanerochaete chrysosporium. Environ. Sci. Pollut. Res.* **7,** 130-134.

Caramelo, L., Martinez, M. J. and Martinez, A. T. (1999) A search for ligninolytic peroxidases in the fungus *Pleurotus eryngii* involving alpha-keto-gamma-thiomethybutyric acid and lignin model dimers. *Appl. Environ. Microbiol.* **65,** 916-922.

Cerniglia, C. E. and Heitkamp, M. A. (1989) Microbial degradation of polycyclic aromatic hydrocarbon in the aquatic environment. In *Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment.*, (ed. U. Varanasi), pp. 41-68, CRC Press, Inc., Fl.

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

Chen, L. L., Chang, H. M. and Kirk, T. K. (1982) Aromatic acids produced during degradation of lignin in spruce wood by *Phanerochaete chrysosporium. Holzforschung.* **36,** 3-9.

Chen, J. C. and Chang, H. M. (1985) Chemistry of lignin biodegradation. In *Biosynthesis and biodegradation of wood components.*, (ed. T. Higuchi), pp. 535-556, Academic Press, Orland, Fl.

Collins, A. M., Maslin, C. and Davis, R. J. (1998) Scale-up of a chiral resolution using cross-linked enzyme crystals. *Org. Process Res. Dev.* **2,** 400-406.

Conesa, A., van den Hondel, C. A. M. J. J. and Punt, P. J. (2000) Studies on the production of fungal peroxidase in *Aspergillus niger. Appl. Environ. Microbiol.* **66,** 3016-3023.

Cook, J., Hewett, C. and Hieger, I. (1933) Isolation of a cancer-producing hydrocarbon from coal tar. II. Isolation of 1,2- and 4,5-benzopyrenes,

perylene and 1,2-benzanthracene. *J. Chem. Soc.*, 396-398.

Crawford, R. L. (1981) Lignin biodegradation and transformation. Wiley-Interscience, New York.

D' Anibale, A., Crestini, C., Di Mattia, E. and Giovannozzi-Sermanni, G. (1996) Veratryl alcohol oxidation by manganese-dependent peroxidase from *Lentinus edodes*. *J. Biotechnol.* **48,** 231-239.

Davis, M. W., Glaser, J. A., Evans, J. W. and Lamar, R. T. (1993) Field evaluation of the lignin-degrading fungus *Phanerochaete sordida* to treat creosote contaminated soil. *Environ. Sci. Technol.* **27,** 2572-2576.

Deguchi, T., Kakezawa, M. and Nishida, T. (1997) Nylon biodegradation by lignin-degrading fungi. *Appl. Environ. Microbiol.* **63,** 329-331.

DeSantis, G., Berglund, P., Stabile, M. R., Gold, M. H. and Jones, J. B. (1998) Site-directed mutagenesis combined with chemical modification as a strategy for altering the specificity of the S1 and S1' pockets of subtilisin *Bacillus lentus*. *Biochemistry* **37,** 5968-5973.

DeSantis, G. and Jones, J. B. (1999) Chemical modification of enzymes from enhanced functionality. *Curr. Opin. Biotechnol.* **10,** 324-330.

Dietrich, D., Hickey, W. J. and Lamar, R. T. (1995) Degradation of 4,4'-dichlorobiphenyl, 3,3',4,4'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl by the white-rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **61**, 3904-3909.

Donnelly, K. C., Chen, J. C., Huebner, H. J., Brown, K. W., Autenrieth, R. L. and Bonner, J. S. (1997) Utility of four strains of white-rot fungi for the detoxification of 2,4,6-trinitrotoluene in liquid culture. *Environ. Toxicol. Chem.* **16,** 1105-1110.

Du, P., Collins, J. R. and Loew, G. H. (1992) Homology modeling of a heme protein, lignin peroxidase, from the crystal structure of cytochrome c peroxidase. *Protein Eng.* **5**, 679-691.

Dunford, H. B. (1999) Heme peroxidases, John Wiley and Sons, Inc., New York.

Eastcott, L., Shiu, W. Y. and Mackay, D. (1988) Environmentally relevant physical-chemical properties of hydrocarbons: a review of data and development of simple correlations. *Oil & Chem. Pol.* **4,** 191-216.

Eaton, D. C. (1985) Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*: a ligninolytic fungus. *Enzyme Microb. Technol.* **7,** 194-196.

Edwards, N. T. (1983) Polycyclic aromatic hydrocarbons (PAHs) in the terrestrial environment: a review. *J. Environ. Quality.* **12,** 427-441.

Eggen, T. (1999) Application of fungal substrate from commercial mushroom production-*Pleurotus ostreatus*-for bioremediation of creosote contaminated soil. *Int. J. Biodet. Biodegrad.* **44,** 117-126.

Eggen, T. and Sveum, P. (1999) Decontamination of aged creosote polluted soil: the influence of temperature, white-rot fungus *Pleurotus ostreatus. Int. J. Biodet. Biodegrad.* **41,** 111-117.

Eriksson, K.-E. L. (1990) Biotechnology in the pulp and paper industry. *Wood Sci. Technol.* **29,** 79-101.

Eriksson, K.-E. L., Pettersson, G., Volc, J. and Musilek, V. (1986) Formation and partial characterization of glucose-2-oxidase, a H_2O_2 producing enzyme in *Phanerochaete chrysosporium. Appl. Microbiol. Biotechnol.* **23,** 257-262.

Farrell, R. L., Murtagh, K. E., Tien, M., Mozuch, M. D. and Kirk, T. K. (1989) Physical and enzymatic properties of lignin peroxidase isoenzymes from *Phanerochaete chrysosporium. Enzyme Microb. Technol.* **11,** 322-328.

Field, J. A., Boelsma, F., Baten, H. and Rulkens, W. H. (1995) Oxidation of anthracene in water/solvent mixtures by the white-rot fungus, *Bjerkandera* sp. strain BOS55. *Appl. Microbiol. Biotechnol.* **44,** 234-240.

Field, J. A., de Jong, E., Feijoo-Costa, G. and de Bont, J. A. M. (1993) Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. *Trends Biotechnol.* **11,** 42-49.

Forney, L. J., Reddy, C. A., Tien, M. and Aust, S. D. (1982) The involvement of hydroxyl radical derived from hydrogen peroxide in lignin degradation by the white-rot fungus *Phanerochaete chrysosporium. J. Biol. Chem.* **257,** 11455-11462.

George, E. J. and Neufeld, R. D. (1989) Degradation of fluorene in soil by fungus *Phanerochaete chrysosporium. Biotechol. Bioeng.* **33,** 1306-1310.

Ghose, T. K. (1987) Measurement of cellulase activities. *Pure Appl. Chem.* **59,** 257-268.

Gianfreda, L., Xu, F. and Bollag, J. (1999) Laccases: a useful group of oxidoreductive enzymes. *Bioremed. J.* **3,** 1-25.

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. Commun.* **114,** 1077-1083.

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., Akileswaran, L. and Gold, M. H. (1986) Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **251,** 688-696.

Gold, M. H., Wariishi, H. and Valli, K. (1989) Extracellular peroxidases involved in lignin degradation by the white-rot basidiomycete *Phanerochaete chrysosporium. ACS Symp. Ser.* **389,** 127-140.

Gold, M. H. and Alic, M. (1993) Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Microbiol. Rev.* **67,** 605-622.

Govardhan, C. (1999) Crosslinking of enzymes from improved stability and performance. *Curr. Opin. Biotechnol.* **10,** 331-335.

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

Gray, M. R., Banerjee, D. K., Fedorak, P. M., Hashimoto, A., Mashiyah, J. H. and Pickard, M. A. (1994) Biological remediation of anthracene-contaminated soil in rotating bioreactors. *Appl. Microbiol. Biotechnol.* **40,** 933-940.

Guenther, T., Sack, U., Hofrichter, M. and Laetz, M. (1998) Oxidation of PAH and PAH-derivatives by fungal and plant oxidoreductaes. *J. Basic. Microbiol.* **38,** 113-122.

Ha, H. C., Honda, Y., Watanabe, T. and Kuwahara, M. (2001) Production of manganese peroxidase by pellet culture of the lignin-degrading basidiomycete *Pleurotus ostreatus. Appl. Microbiol. Biotechnol.* **23,** 437-442.

Habeeb, A. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.* **14,** 328-336.

Hall, P. L., Glasser, W. G. and Drew, S. W. (1980) Enzymatic transformations of lignin. In *Lignin biodegradation: microbiology, chemistry, and potential applications.*, (eds. T. K. Kirk, T. Higuchi and H. M. Chang), pp. 33-49, CRC Press, Boca Raton, FL.

Hammel, K. E., Tien, M., Kalyanaraman, B. and Kirk, T. K. (1985) Mechanism of oxidative C_{α}-C_{β} cleavage of a lignin model dimer by *Phanerochaete chrysosporium* ligninase. Stoichiometry and involvement of free radicals. *J. Biol. Chem.* **260**, 8348-8353.

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

Hammel, K. E., Green, B. and Gai, W. Z. (1991) Ring fission of anthracene by a eukaryote. *Proc. Natl. Acad. Sci. USA.* **88,** 10605-10608.

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.

Harris, R. Z., Wariishi, H., Gold, M. H. and Ortiz de Montellano, P. R. (1991) the catalytic site of manganese peroxidase. Regiospecific addition of sodium azide and alkylhydrazines to the heme group. *J. Biol. Chem.* **266,** 8751-8758.

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

Heinfling, A., Martinez, M. J., Martinez, A. T., Bergbauer, M. and Szewzyk, U. (1998a) Purification and characterization of peroxidases from the dye-decolorizing fungus *Bjerkandera adusta. FEMS Microbiol. Lett.* **165,** 43-50.

Heinfling, A., Ruiz-Duenas, F. J., Martinez, M. J., Bergbauer, M., Szewzyk, U. and Martinez, A. T. (1998b) A study on reducing substrates of manganese-oxidizing peroxidase from *Pleurotus eryngii* and *Bjerkandera adusta. FEBS Lett.* **428,** 141-146.

Hernaliz, M. J., Sanchez-Montero, J. M. and Sinisterra, J. V. (1999) Modification of purified lipases from *Candida rugosa* with polyethylene glycol: a systematic study. *Enzyme Microb. Technol.* **24,** 181-190.

Herpoel, I., Asther, M. and Sigoillot, J. C. (1999) Design and scale up of a process for manganese peroxidase production using the hypersecretory strain *Phanerochaete chrysosporium* I-1512. *Biotechnol. Bioeng.* **65,** 468-473.

Higuchi, T. (1990) Lignin biochemistry: biosynthesis and biodegradation. *Wood Sci. Technol.* **24,** 23-63.

Hilden, L., Johansson, G., Pettersson, G., Li, J., Ljungquist, P. and Henriksson, G.

(2000) Do the extracellular enzymes cellobiose dehydrogenase and manganese peroxidase form a pathway in lignin biodegradation? *FEBS Lett.* **477,** 79-83.

Hofrichter, M. and Fritsche, W. (1997) 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., Vares, T., Kalsi, M., Galkin, S., Scheibner, K., Fritsche, W. and Hatakka, A. (1999a) 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, K., Scheibner, K., Galkin, S., Sipila, J. and Hatakka, A. (1999b) Mineralization and solubilization of synthetic lignin by manganese peroxidases from *Nematoloma frowardii* and *Phlebia radiata. J. Biotechnol.* **67,** 217-228.

in der Wiesche, C., Martens, F. and Zadrazil, F. (1996) Two-step degradation of pyrene by white-rot fungi and soil microorganisms. *Appl. Microbiol. Biotechnol.* **46,** 635-659.

Inada, Y., Takahashi, K., Yoshimoto, T., Ajima, A., Matsushima, A. and Saito, Y. (1986) Application of polyethylene glycol-modified enzymes in biotechnological processes: organic solvent-soluble enzymes. *Trends Biotechnol.* **7,** 190-194.

Iwahara, K., Honda, Y., Watanabe, T. and Kuawahara, M. (2000) Polymerization of guaiacol by lignin-degrading manganese peroxidase from *Bjerkandera adusta* in aqueous organic solvents. *Appl. Microbiol. Biotechnol.* **54,** 104-111.

Jaspers, C. J., Jiminez, G. and Penninck, M. J. (1994) Evidence for a role of manganese peroxidase in the decolorization of Kraft pulp bleach plant effluent by *Phanerochaete chrysosporium*: effects of initial culture conditions on enzyme production. *J. Biotechnol.* **37,** 229-234.

Jensen, K. A., Bao, W., Kawai, S., Srebotnik, E. and Hammel, K. E. (1996) Manganese-dependent cleavage of nonphenolic lignin structures by *Ceriporiopsis subvermispora* in the absence of lignin peroxidase. *Appl. Environ. Microbiol.* **62,** 3679-3686.

Joensson, L., Sjoestroem, K., Haeggstroem, I. and Nyman, P. O. (1995) Characterization of a laccase gene from the white-rot fungus *Trametes versicolor* and structural features of basidiomycete laccases. *Biochim.*

Biophys. Acta. **1251,** 210-215.

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.

Johansson, T., Welinder, K. G. and Nyman, P. O. (1993) Isoenzymes of lignin peroxidase and manganese (II) peroxidase from the white-rot basidiomycete *Trametes versicolor*. II. Partial sequences, peptide maps, amino acid and carbohydrate compositions. *Arch. Biochem. Biophys.* **300,** 57-62.

Johnson, T. M. and Li, J. K. (1991) Heterologous expression and characterization of an active lignin peroxidase from *Phanerochaete chrysosporium* using recombinant baculovirus. *Arch. Biochem. Biophys.* **291,** 371-378.

Johnson, F., Loew, G. H. and Du, P. (1994) Homology models of two isoenzymes of manganese peroxidase: prediction of a Mn(II) binding site. *Protein Struct. Funct. Genet.* **20,** 312-319.

Joshi, D. K. and Gold, M. H. (1993) Degradation of 2,4,5-trichlorophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **59,** 1779-1785.

Kapich, A. N., Jensen, K. A. and Hammel, K. E. (1999) Peroxyl radicals are potential agents of lignin biodegradation. *FEBS Lett.* **461,** 115-119.

Keith, L. and Telliard, W. (1979) Priority pollutants: A perspective view. *Environ. Sci. Technol.* **13,** 416-423.

Kelley, H. C. and Reddy, C. A. (1986) Purification and characterization of glucose oxidase from ligninolytic cultures of *Phanerochaete chrysosporium. J. Bacteriol.* **166,** 269-274.

Kennes, C. and Lema, J. (1994) Degradation of major compounds of creosotes (PAH and phenols) by *Phanerochaete chrysosporium. Biotechnol. Lett.* **16,** 759-764.

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.

Khmelnitsky, Y. L., Levashow, A. V., Klyachko, N. L. and Martinek, K. (1988) Engineering biocatalytic systems in organic media with low water content. *Enzyme Microb. Technol.* **10,** 711-724.

Khmelnitsky, Y. L., Belova, A., Levashov, A. and Mozhaev, V. (1991) Relationship between surface hydrophilicity of a protein and its stability against

denaturation by organic solvents. *FEBS Lett.* **284,** 267-269.

Khmelnitsky, Y. L. and Rich, J. O. (1999) Biocatalysis in nonaqueous solvents. *Curr. Opin. Chem. Biol.* **3,** 47-53.

Kirk, T. K., Harkin, J. M. and Cowling, E. B. (1968) Oxidation of guaiacyl- and veratryl-glycerol-β-gualacyl ether by *Polyporus versicolor* and *Stereum frustulatum. Biochim. Biophys. Acta.* **165,** 134-144.

Kirk, T. K. and Chang, H. M. (1974) Decomposition of lignin by white-rot fungi. I. Isolation of heavily degraded lignins from decayed spruce. *Holzforschung.* **28,** 217-222.

Kirk, T. K. and Chang, H. M. (1975) Decomposition of lignin by white-rot fungi. II. Characterization of heavily degraded lignins from decayed spruce. *Holzforschung.* **29,** 56-64.

Kirk, T. K., Croan, S., Tien, M., Murtagh, E. and Farrell, R. L. (1986) Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. *Enzyme Microbiol. Technol.* **8,** 27-32.

Kirk, T. K. and Cullen, D. (1998) Environmentally friendly technologies for the pulp and paper industry. In *Enzymology and molecular genetics of wood degradation by white rot fungi.*, (eds. R. A. Young and M. Akhtar), pp. 273-308, John Wiley & Sons, New York.

Kirk, T. K. and Farrell, R. L. (1987) Enzymatic 'combustion': the microbial degradation of lignin. *Annu. Rev. Microbiol.* **41,** 465-505.

Kirk, T. K., Tien, M., Kersten, P. J., Kalyanaraman, B., Hammel, K. E. and Farrell, R. L. (1990) Lignin peroxidase from fungi: *Phanerochaete chrysosporium. Methods Enzymol.* **188,** 159-170.

Klibanov, A. M. (2001) Improving enzymes by using them in organic solvents. *Nature* **409,** 241-246.

Kondo, R., Harazono, K. and Sakai, K. (1994) Bleaching of hardwood Kraft pulp with manganese peroxidase secreted from *Phanerochaete sordida* YK-624. *Appl. Environ. Microbiol.* **60,** 4359-4363.

Kotterman, M. J. 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.

Kuan, I. C. and Tien, M. (1993) Stimulation of Mn peroxidase activity: a possible role of oxalate in lignin biodegradation. *Proc. Natl. Acad. Sci.* **90,** 1242-1246.

Kutsuki, H. and Gold, M. H. (1982) Generation of hydroxyl radical and its involvement in lignin degradation by *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* **109,** 320-327.

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

Larking, D. M., Crawford, R. L., Christie, G. B. Y. and Lonergan, G. T. (1999) Enhanced degradation of polyvinyl alcohol by *Pycnoporus cinnabarinus* after pretreatment with Fenton's reagent. *Appl. Environ. Microbiol.* **65,** 1798-1800.

Lewtas, J. (1993) Complex mixtures of pollutants: characterizing the cancer risk of polycyclic organic matter. *Environ. Health Perspect* **100,** 211-218.

Li, D., Alic, M., Brown, J. A. and Gold, M. H. (1995) Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress, and molecular oxygen. *Appl. Environ. Microbiol.* **61,** 341-345.

Limura, Y., Hartikainen, P. and Tatsumi, K. (1996) Dechlorination of tetrachloroguaiacol by laccase of white-rot basidiomycete *Coriolus versicolor. Appl. Microbiol. Biotechnol.* **45,** 434-439.

Lin, S. Y. and Dence, C. W. (1992) Methods in lignin chemistry, Springer-Verlag, Berlin.

Lobos, S., Larrain, J., Salas, L., Cullen, D. and Vicuna, R. (1994) Isoenzymes of manganese-dependent peroxidase and laccase produced by the lignin-degrading basidiomycete *Ceriporiopsis subvermispora. Microbiology* **140,** 2691-2698.

Lobos, S., Larrondo, L., Salas, L., Karahanian, E. and Vicuna, R. (1998) Cloning and molecular analysis of a cDNA and the Cs-mnp1 gene encoding a manganese peroxidase isoenzyme from the lignin-degrading basidiomycete *Ceriporiopsis subvermispora. Gene* **206,** 185-193.

Lomascolo, A., Stentelaire, C., Asther, M. and Lesage-Meessen, L. (1999) Basidiomycetes as new biotechnological tools to generate natural aromatic flavours for the food industry. *Trends Biotechnol.* **17,** 282-289.

Lundell, T. and Hatakka, A. (1994) Participation of Mn(II) in the catalysis of laccase, manganese peroxidase and lignin peroxidase from *Phlebia radiata. FEBS*

Lett. **348,** 291-296.

Majcherczyk, A., Johannes, C. and Huttermann, A. (1998) Oxidation of polycyclic aromatic hydrocarbons (PAHs) by laccase of *Trametes versicolor. Enzyme Microbiol. Technol.* **22,** 335-341.

Martinez, M. J., Ruiz-Duenas, F. J., Guille, F. and Martinez, A. T. (1996) Purification and catalytic properties of two manganese-peroxidase isoenzymes from *Pleurotus eryngii. Eur. J. Biochem.* **237,** 15412-15417.

Martinez, M. J., Bockle, B., Camarero, S., Guillen, F. and Martinez, A. T. (1996a) MnP isoenzymes produced by two *Pleurotus* species in liquid culture and during wheat straw solid-state fermentation. In *Enzymes for pulp and paper processing*, (ed. T. W. Jeffries, and Viikari, L.), pp. 183-196, ACS Press, WA.

Martinez, M. J. and Martinez, A. T. (1996b) Characterization of MnP isoenzymes of *Pleurotus eryngii* exhibiting Mn-independent activities on 2,6-dimethoxyphenol and veratryl alcohol. In *Biotechnology in the pulp and paper industry: recent advances in applied and fundamental research.*, (eds. K. Messner and E. Srebotnik), pp. 417-420, Facultas-Universitatsverlag, Vienna.

Martinez, A. T. (2002) Molecular biology and structure-function of lignin-degrading heme peroxidases. *Enzyme Microbiol. Technol.* **30,** 425-444.

Masaphy, S., Levanon, D., Henis, Y., Venkateswarlu, K. and Kelly, S. L. (1995) Microsomal and cytosolic cytochrome P_{450} mediated benzo(a)pyrene hydroxylation in *Pleurotus pulmonarius. Biotechnol. Lett.* **17,** 969-974.

Matsubara, M., Suzuki, J., Deguchi, T., Miura, M. and Kitaoka, Y. (1996) Characterization of manganese peoxidases from the hyperlignolytic fungus IZU-154. *Appl. Environ. Microbiol.* **62,** 4066-4072.

Mester, T. and Field, J. A. (1997) Optimization of manganese peroxidase production by the white rot fungus *Bjerkandera* sp. strain BOS55. *FEMS Microbiol. Lett.* **155,** 161-168.

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., Pena, M. and Field, J. A. (1996) Nutrient regulation of extracellular peroxidases in the white-rot fungus *Bjerkandera* sp. BOS55. *Appl. Microbiol. Biotechnol.* **44,** 778-784.

Mester, T. and Tien, M. (2001) Engineering of a manganese-binding site in lignin peroxdase isozyme H8 from *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* **284,** 723-728.

Meulenberg, R., Rijnaarts, H. H. M., Doddema, H. J. and Field, J. A. (1997) Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. *FEMS Microbiol. Lett.* **152,** 45-49.

Mileski, G. J., Bumpus, J. A., Jurek, M. A. and Aust, S. D. (1988) Biodegradation of pentachlorophenol by the white-rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **54,** 2885-2889.

Miller, M. A., Hazzard, J. T., Mauro, J. M., Edwards, S. L., Simons, P. C., Tollin, G. and Kraut, J. (1988) Site-directed mutagenesis of yeast cytochrome c peroxidase shows histidine 181 is not required for oxidation of ferrocytochrome c. *Biochem.* **27,** 9081-9088.

Miller, E. and Miller, J. (1985) Some historical perspectives on the metabolism of xenobiotic chemicals to reactive electrophiles. In *Bioactivation of Foreign Compounds*, (ed. M. Anders), pp. 3-28, Academic Press, Orlando.

Miura, M., Yoshihisa, K., Masaaki, K. and Tomoaki, N. (1998) Biobleaching of hardwood Kraft pulp with cellulase-deficient mutant from hyperligninolytic fungus IZU-154. *Appl. Biochem. Biotechnol.* **73,** 113-126.

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.

Moreira, M. T., Feijoo, G., Sierra-Alvarez, R., Lema, J. and Field, J. A. (1997) Manganese is not required for biobleaching of oxygen-delignified Kraft pulp by the white rot fungus *Bjerkandera* sp. strain BOS55. *Appl. Environ. Microbiol.* **63,** 1749-1755.

Moreira, M. T., Feijoo, G. and Lema, J. M. (2000a) Manganese peroxidase production by *Bjerkandera* sp. BOS55. *Bioprocess Eng.* **23,** 657-661.

Moreira, M. T., Torrado, A., Feijoo, G. and Lema, J. (2000b) Manganese peroxidase production by *Bjerkandera* sp. BOS55. *Bioprocess Eng.* **23,** 663-667.

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.

Mueller, J. G., Chapman, P. J. and Pritchard, P. H. (1989) Creosote-contaminated sites: their potential for bioremediation. *Environ. Sci. Technol.* **23,** 1197-1201.

Nishida, A. and Eriksson, K.-E. L. (1987) Formation, purification and partial characterization of methanol oxidase, a H_2O_2-producing enzyme in *Phanerochaete chrysosporium. Biotechnol. Appl. Biochem.* **9**, 325-338.

Pace, C. N., Vajdos, F., Fee, L., Grimsky, G. and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* **4,** 2411-2423.

Paice, M. G., Reid, I., Bourbonnais, R., Archibald, F. S. and Jurasek, L. (1993) Manganese peroxidase, produced by *Trametes versicolor* during pulp bleaching, demethylates and deligninifies Kraft pulp. *Appl. Environ. Microbiol.* **59,** 260-265.

Palma, C., Martinez, A. T., Lema, J. M. and Martinez, M. J. (2000) Different fungal manganese-oxidizing peroxidases: a comparison between *Bjerkandera* sp. and *Phanerochaete chrysosporium. J. Biotechnol.* **77,** 235-245.

Paszczynski, A., Huynh, V. B., and Crawford, R. L. (1986) Comparison of ligninase-1 and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **244,** 750-765.

Paszczynski, A., Crawford, R. L. and Huynh, V. (1988) Manganese peroxidase of *Phanerochaete chrysosporium*: purification. *Methods Enzymol.* **161,** 264-270.

Pease, E. A., Andrawis, A. and Tien, M. (1989) Manganese-dependent peroxidase from *Phanerochaete chrysosporium.* Primary structure deduced from cDNA sequence. *J. Biol. Chem.* **264,** 13531-13535.

Pease, E. A., Aust, S. D. and Tien, M. (1991) Heterologous expression of active manganese peroxidase from *Phanerochaete chrysosporium* using the baculovirus expression system. *Biochem. Biophys. Res. Commun.* **179,** 897-903.

Perez-Boada, M., Doyle, W. A., Ruiz-Duenas, F. J., Martinez, M. J., Martinez, A. T. and Smith, A. T. (2002) Expression of *Pleurotus eryngii* versatile peroxidase in *Escherichia coli* and optimization of *in vitro* folding. *Enzyme Microbiol. Technol.* **30,** 518-524.

Pickard, M. A. and Hashimoto, A. (1988) Stability and carbohydrate composition of chloroperoxidase from *Caldariomyces fumago* grown in fructose-salts medium. *Can. J. Microbiol.* **34,** 998-1002.

Pickard, M. A., Vandertol, H., Roman, R. and Vazquez-Duhalt, R. (1999a) High

production of ligninolytic enzymes from white rot fungi in cereal bran medium. *Can. J. Microbiol.* **48,** 1351-1358.

Pickard, M. A., Roman, R., Tinoco, R. and Vazquez-Duhalt, R. (1999b) 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., Bucher, V. V. C. and Vrijmoed, L. L. P. (2000) Dye decolorization by sub-tropical basidiomycetous fungi and the effect of metals on decolorizing ability. *World J. Microbiol. Biotechnol.* **16,** 199-205.

Pointing, S. B. (2001) Feasibility of bioremediation by white-rot fungi. *Biochem. Bioeng.* **3,** 25-33.

Poulos, T. L., Edwards, S. L., Wariishi, H. and Gold, M. H. (1993) Crystallographic refinement of lignin peroxidase at 2A. *J. Biol. Chem.* **268,** 4429-4440.

Ramachandra, M., Crawford, D. L. and Hertel, G. (1988) Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete *Streptomyces viridosporus. Appl. Environ. Microbiol.* **54,** 3057-3063.

Reinhammar, B. (1984) Laccase. In *Copper proteins and copper enzymes, Vol. 3,* (ed. R. Lontie), pp. 1-36, CRC Press, Boca Raton, FL.

Reyes, P., Pickard, M. A. and Vazquez-Duhalt, R. (1999) Hydroxybenzotriazole increases the range of textile dyes decolorized by immobilized laccase. *Biotechnol. Lett.* **21,** 875-880.

Ritch, T. G. J. and Gold, M. H. (1992) Characterization of a highly expressed lignin peroxidase-encoding gene from the basidiomycete *Phanerochaete chrysosporium. Gene* **118,** 73-80.

Rivela, I., Rodriguez-Couto, S. and Sanroman, A. (2000) Extracellular ligninolytic enzyme production by *Phanerochaete chrysosporium* in a new solid-state bioreactor. *Biotechnol. Lett.* **22,** 1443-1447.

Rodriguez, E., Pickard, M. A. and Vazquez-Duhalt, R. (1999) Industrial dye decolorization by laccase from ligninolytic fungi. *Curr. Microbiol.* **38,** 27-32.

Rodriguez-Couto, S., Longo, M. A., Cameselle, C. and Sanroman, A. (1999) Production of manganese peroxidase and laccase in laboratory-scale bioreactors by *Phanerochaete chrysosporium. Bioprocess Eng.* **20,** 531-535.

Ruiz, A., Malave, A., Felby, C. and Griebenow, K. (2000) Improved activity and

stability of an immobilized recombinant laccase in organic solvents. *Biotechnol. Lett.* **22,** 229-233.

Ruiz-Duenas, F. J., Martinez, M. J. and Martinez, A. T. (1999a) Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii. Mol. Microbiol.* **31,** 223-235.

Ruiz-Duenas, F. J., Martinez, M. J. and Martinez, A. T. (1999b) Heterologous expression of *Pleurotus eryngii* peroxidase confirms its ability to oxidize Mn(II) and different aromatic substrates. *Appl. Environ. Microbiol.* **65,** 4705-4707.

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.

Russel, A., Chatterjee, S., Rapanovich, I. and Goodwin, J. (1992) Mechanistic enzymology in organic solvents. In *Biomolecules in organic solvents.*, (ed. A. Gomez-Puyou), CRC Press Inc., Boca Raton, FL.

Sarkar, S., Martinez, A. T. and Martinez, M. J. (1997) Biochemical and molecular characterization of a manganese peroxidase isoenzyme from *Pleurotus ostreatus. Biochem. Biophys. Acta.* **1339,** 23-30.

Sasaki, T., Kajino, T., Li, B., Sugiyama, H. and Takahashi, K. (2001) New pulp biobleaching system involving manganese peroxidase immobilized in a silica support with controlled pore sizes. *Appl. Environ. Microbiol.* **67,** 2208-2212.

Scheel, T., Hofer, M., Ludwig, S. and Holker, U. (2000) Differential expression of manganese peroxidase and laccase in white-rot fungi in the presence of manganese or aromatic compounds. *Appl. Microbiol. Biotechnol.* **54,** 686-691.

Schneegaβ, 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.

Sethuraman, A., Akin, D. E. and Eriksson, K.-E. L. (1999) Production of ligninolytic enzymes and synthetic lignin mineralization by bird's nest fungus *Cyathus stercoreus. Appl. Microbiol. Biotechnol.* **52,** 689-697.

Shimada, M. and Higuchi, T. (1983) Recent advances in lignin biodegradation research. In *Recent advances in lignin biodegradation research.*, (eds. T. Higuchi, H. M. Chang and T. K. Kirk), pp. 195-208, Uni Publishers., Tokyo.

Shoda, M. and Kim, S. J. (1999) Purification and characterization of a novel peroxidase from *Geotrichum candidum* Dec 1 involved in decolorization of

dyes. *Appl. Environ. Microbiol.* **65,** 1029-1035.

Shoemaker, H. E., Lundell, T. K., Floris, R., Glumoff, T., Winterhalter, K. H. and Piontek, K. (1994) Do carbohydrates play a role in the lignin peroxidase cycle? Redox catalysis in the endergenic region of the driving force. *Bioorg. Med. Chem.* **2,** 509-519.

Smith, A. T., Santama, N., Dacey, S., Edwards, M., Bray, R. C. and Thorneley, R. N. F. (1990) Expression of a synthetic gene for horseradish peroxidase C in *Escherichia coli* and folding and activation of the recombinant enzyme with Ca(II) and heme. *J. Biol. Chem.* **265,** 13335-13343.

Smith, G. E., Summers, M. D. and Fraser, M. J. (1983) Production of human *B*-interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* **3,** 2156-2165

Stewart, P., Whitwam, R. E., Kersten, P. J., Cullen, D. and Tien, M. (1996) Efficient expression of a *Phanerochaete chrysosporium* manganese peroxidase gene in *Aspergillus oryzae. Appl. Environ. Microbiol.* **62,** 860-864.

Sundaramoorthy, M., Kishi, K., Gold, M. H. and Poulos, T. L. (1994) The crystal structure of manganese peroxidase from *Phanerochaete chrysosporim* at 2.06-A resolution. *J. Biol. Chem.* **269,** 32759-32767.

Sundaramoorthy, M., Kishi, K., Gold, M. H. and Poulos, T. L. (1997) Crystal structures of substrate binding site mutants of manganese peroxidase. *J. Biol. Chem.* **272,** 17574-17580.

Sutherland, J. B. (1992) Detoxification of polycyclic aromatic hydrocarbons by fungi. *J. Ind. Microbiol.* **9,** 53-62.

Sutherland, J. B. and Aust, S. D. (1996) The effects of calcium on the thermal stability and activity of manganese peroxdase. *Arch. Biochem. Biophys.* **332,** 128-134.

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

ten Have, R. and Teunissen, P. J. M. (2001) Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chem. Rev.* **101,** 3397-3413.

Thurston, C. (1994) The structure and function of fungal laccases. *Microbiol.* **140,** 19-26.

Tiehm, A. and Fritsche, C. (1995) Utilization of solubilized and crystalline mixtures

of polycyclic aromatic hydrocarbons by a *Mycobacterium* sp. *Appl. Microbiol. Biotechnol.* **42,** 964-968.

Tien, M. and Kirk, T. K. (1983) Lignin-degrading enzyme from *Phanerochaete chrysosporium. Science* **221,** 661-663.

Tien, M. and Kirk, T. K. (1988) Lignin peroxidase of *Phanerochaete chrysosporium. Methods Enzymol.* **161B,** 238-248.

Tien, M. and Tu, C.-P. D. (1987) Cloning and sequencing of a cDNA for a ligninase from *Phanerochaete chrysosporium. Nature* **326,** 520-523.

Tinoco, R. and Vazquez-Duhalt, R. (1997) Chemical modification of cytochrome C improves their catalytic properties in oxidation of polycyclic aromatic hydrocarbons. *Enzyme Microbiol. Technol.* **21,** 1-5.

Tongpim, S. and Pickard, M. A. (1996) Growth of *Rhodococcus* S1 on anthracene. *Can. J. Microbiol.* **42,** 289-294.

Torres, E., Tinoco, R. and Vazquez-Duhalt, R. (1996) Solvent hydrophobicity predicts biocatalytic behavior of lignin peroxidase and cytochrome *c* in aqueous solution of water-miscible organic solvents. *J. Biotechnol.* **49,** 59-67.

Torres, E. and Vazquez-Duhalt, R. (2000) Chemical modification of hemoglobin improves biocatalytic oxidation of PAHs. *Biochem. Biophys. Res. Commun.* **273,** 820-823.

Tuor, U., Wariishi, H., Schoemaker, H. E. and Gold, M. H. (1992) Oxidation of phenolic arylglycerol β-aryl ether lignin model compounds by manganese peroxidase from *Phanerochaete chrysosporium*: oxidative cleavage of an α-carbonyl model compound. *Biochem.* **31,** 4986-4995.

Urzua, U., Kersten, P. J. and Vicunna, R. (1998) Manganese peroxidase-dependent oxidation of glyoxylic and oxalic acids synthesized by *Ceriporiopsis subvermispora* produces extracellular hydrogen peroxide. *Appl. Environ. Microbiol.* **64,** 68-73.

Valderrama, B., Ayala, M. and Vazquez-Duhalt, R. (2002) Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes. *Chem. Biol.* **9,** 1-20.

Valli, K. and Gold, M. H. (1991) Degradation of 2,4-dichlorophenol by the lignin-degrading fungus *Phanerochaete chrysosporium. J. Bacteriol.* **173,** 345-352.

van Erp, S., Kamenskaya, E. and Khmelnitsky, Y. L. (1991) The effect of water content and nature of organic solvent on enzyme activity in low-water media.

Eur. J. Biochem. **202,** 379-384.

van-Aken, B., Godefroid, L. M., Peres, C. M., Naveau, H. and Agathos, S. N. (1999a) Manganese-dependent peroxidase of the white-rot basidiomycete *Phlebia radiata. J. Biotechnol.* **68,** 159-169.

van-Aken, B., Godefroid, L. M., Peres, C. M., Naveau, H. and Agathos, S. N. (1999b) Mineralization of ^{14}C-U-ring labelled 4-hydroxylamino-2,6-dinitroroluene by manganese-dependant peroxidase of the white-rot fungus *Phlebia radiata. J. Biotechnol.* **68,** 159-169.

Vazquez-Duhalt, R. (1998) Hemoproteins as biocatalyst for the oxidation of polycyclic aromatic hydrocarbons. In *Advances in bioprocess engineering II,* (eds. E. Galindo and O. T. Ramirez), pp. 183-207, Kluwer Academic Publishers.

Vazquez-Duhalt, R., Ayala, M. and Marquez-Rocha, F. (2001) Biocatalytic chlorination of aromatic hydrocarbons by chloroperoxidase of *Caldariomyces fumago. Phytochem.* **58,** 929-933.

Vazquez-Duhalt, R., Fedorak, P. M. and Westlake, D. W. S.(1992) Role of enzyme hydrophobicity in biocatalysis in organic solvents. *Enzyme Microbiol. Technol.* **14,** 837-841.

Vazquez-Duhalt, R., Semple, K., Westlake, D. W. S. and Fedorak, P. M. (1993) Effect of water miscible organic solvents on the catalytic activity of cytochrome c. *Enzyme Microbiol. Technol.* **15,** 936-943.

Vazquez-Duhalt, R., Westlake, D. W. S. and Fedorak, P. M. (1994) Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. *Appl. Environ. Microbiol.* **60,** 459-466.

Vazquez-Duhalt, R., Westlake, D. W. S. and Fedorak, P. M. (1995) Kinetics of chemically modified lignin peroxidase and enzymatic oxidation of aromatic nitrogen-containing compounds. *Appl. Microbiol. Biotechnol.* **42,** 675-681.

Vicuna, R. (2000) Ligninolysis: a very peculiar microbial process. *Mol. Biotechnol.* **14,** 173-176.

Vyas, B. R. M., Bakowski, S., Sasek, V. and Matucha, M. (1994) Degradation of anthracene by selected white-rot fungi. *FEMS Microbiol. Ecol.* **14,** 65-70.

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.

Wang, P., Woodward, C. and Kaufman, E. (1999) Poly(ethylene glycol)-modified ligninase enhances pentachlorophenol biodegradation in water-solvent mixtures. *Biochem. Bioeng.* **64,** 290-297.

Wang, Y., Vazquez-Duhalt, R. and Pickard, M. A. (2001) Effect of growth conditions on the production of manganese peroxidase by three strains of *Bjerkandera adusta*. *Can. J. Microbiol.* **47,** 277-282.

Wang, Y., Vazquez-Duhalt, R. and Pickard, M. A. (2002) Purification, characterization and chemical modification of manganese peroxidase from *Bjerkandera adusta* UAMH 8258. *Curr. Microbiol.* **43**, 77-87.

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. *Biochemistry.* **27,** 5365-5370.

Wariishi, H., Dunford, H. B., MacDonald, I. D. and Gold, M. H. (1989) Manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *J. Biol. Chem.* **264,** 3335-3340.

Wariishi, H., Morohoshi, N. B., Dunford, H. B. and Gold, M. H. (1987) Degradation of lignin by the extracellular enzymes of *Coriolus versicolor* VII. Effective degradation of syringyl type *B*-aryl ether lignin model compound by laccase III. *Mokuzai Gakkaishi,* 892-898.

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*. *J. Biol. Chem.* **267,** 23688-23695.

Watanabe, T., Katayama, S., Enoki, M., Honda, Y. and Kuwahara, M. (2000) Formation of acyl radical in lipid peroxidation of linoleic acid by manganese-dependent peroxidase from *Ceriporiopsis subvermispora* and *Bjerkandera adusta*. *Eur. J. Biochem.* **267,** 4222-4231.

Westermark, U. and Eriksson, K.-E. L. (1974) Carbohydrate-dependent enzyme quinone reduction during lignin degradation. *Acta. Chem. Scand.* **B28,** 204-208.

Willershausen, H., Jager, A. and Graf, H. (1987) Ligninase production of *Phanerochaete chrysosporium* by immobilization in bioreactors. *J. Biotechnol.* **6,** 231-235.

Winkelmann, G. (1992) Microbial degradation of natural products., VCH, New York.

Wolfenden, B. S. and Wilson, R. L. (1982) Radical cations as reference chromogens in studies of one-electron transfer reactions: pulse radiolysis studies of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate). *J. Chem. Soc. Perkin Trans.* **2,** 805-812.

Yadav, J. S. and Reddy, C. A. (1993) Degradation of benzene, toluene, ethylbenzene and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* **59,** 756-762.

Yateem, A., Balba, M. T., Al-Awadhi, N. and El-Nawawy, A. S. (1998) White-rot fungi and their role in remediating oil-contaminated soil. *Environ. Int.* **24,** 181-187.

Yoshida, S., Yonehara, S. and Minami, S. (1996) Production and characterization of ligninolytic enzymes from *Bjerkandera adusta* grown on wood meal/wheat bran culture and production of these enzymes using a rotary-solid fermenter. *Mycosci.* **37,** 417-425.

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

Ziegenhagen, D. and Hofrichter, M. (2000) A simple and rapid method to gain high amounts of manganese peroxidase with immobilized mycelium of the agaric white-rot fungus *Clitocybula dusenii. Appl. Microbiol. Biotechnol.* **53,** 553-557.

7. Appendix

7.1. Estimation of Rz (A_{407}/A_{280}) ratio

For hemoproteins, the Reinheitzahl (Rz) ratio is one of the important criterion of enzyme purity. As the Rz ratio from different sources are variable (ranging from 1.85 to 450). It is useful if a theoretical Rz ratio of a hemoprotein could be estimated using some known physical characteristics of enzyme such as molecular weight and amino acid composition. Here is a example of estimation of Rz ratio for purified MnP from *B. adusta* UAMh 8258.

In this example, two different methods were used to get protein concentration: 1) freeze dry extensively dialyzed purified MnP preparation, then use gravimetric method, 15 μg protein in 10 μl sample, protein concentration=1.5 g/l.

2) Bio-Rad dye-binding method (BSA as standard), 4.77 μg protein in 10 μl sample, protein concentration=0.477 g/l.

From SDS-PAGE, most quoted molecular weight of MnP is 43,000 Da, whereas from MALDI-TOF study, the molecular weight is 36,600 Da. The protein concentration of enzyme can be converted to molar concentration:

(1.5 g/l)/43000 g/mol=3.5×10^{-5} (M) (gravimetric method and MW=4.3 kDa)

(1.5 g/l)/36600 g/mol=4.1×10^{-5} (M) (gravimetric method and MW=36.6 kDa)

(0.477 g/l)/43,000 g/mol=1.1×10^{-5}(M) (Bio-Rad and MW=43 kDa)

(0.477 g/l)/36,600 g/mol=1.3×10^{-5}(M) (Bio-Rad and MW=36.6 kDa)

One of the MnP samples has A_{407}=1.919 and A_{280}=0.64. We assume that A_{407} is contributed only by hemoprotein, A_{280} is contributed by hemoprotein and other contaminated proteins and pigment. The theoretical molar coefficient of hemoprotein can be calculated using A_{407}=1.919.

$\Sigma(407)=1.919/3.5\times10^{-5}=5.48\times10^{4}$ ($M^{-1}cm^{-1}$)=54.8 $mM^{-1}cm^{-1}$ (gravimetric and MW=4.3 kDa)

$\Sigma(407)=1.919/4.1\times10^{-5}=4.6\times10^{4}$ ($M^{-1}cm^{-1}$)=46 $mM^{-1}cm^{-1}$ (gravimetric and MW=36.6 kDa)

$\Sigma(407)=1.919/1.1\times10^{-5}=1.74\times10^{5}$ ($M^{-1}cm^{-1}$)=174 $mM^{-1}cm^{-1}$ (Bio-Rad and MW=43 kDa)

$\Sigma(407)=1.919/1.3\times10^{-5}=1.48\times10^{5}$ ($M^{-1}cm^{-1}$)=148 $mM^{-1}cm^{-1}$ (Bio-Rad and MW=36.6kDa)

$\Sigma(280)$=(no. tryptophan)(5500)+(no. tyrosine)(1490) to predict theoretical A_{280}.

$\Sigma(280)=5500(M^{-1}cm^{-1})$ (1 tryptophan) or

$\Sigma(280)=11000(M^{-1}cm^{-1})$ (2 tryptophan)

Rz ratio can be estimated as:

$Rz=\Sigma(407)/\Sigma(280)=54.8/5.5=9.96$ (1 tryptophan) or 54.8/11=4.98 (2 tryptophan) with gravimetric method (based on MW=43 kDa)

*$Rz=\Sigma(407)/\Sigma(280)=46/5.5=8.4$ (1 tryptophan) or 46/11=4.2 (2 tryptophan) with gravimetric method (based on MW=36.6 kDa)

$Rz=\Sigma(407)/\Sigma(280)=174/5.5=31.6$ (1 tryptophan) or 174/11=15.8 (2 tryptophan) with Bio-Rad protein assay method (based on MW=43 kDa)

$Rz=\Sigma(407)/\Sigma(280)=148/5.5=27$ (1 tryptophan) or 148/11=13.5 (2 tryptophan) with

Bio-Rad protein assay method (based on MW=36.6 kDa).

* the most accurate value

7.2. Effect of different organic solvents on water solubility of 2-methylanthracene

2-Methylanthracene was added in glass bottle with different concentrations of ethanol, acetonitrile and dimethylsulfoxide and tetrahydrofuran, the bottle was sealed with teflon cap. After stirred for 24 h, the solution was filtrated with glass wool, and the filtrate was assayed by HPLC, the 2-methylantracene concentration was obtained using concentration vs. HPLC peak area standard curve.

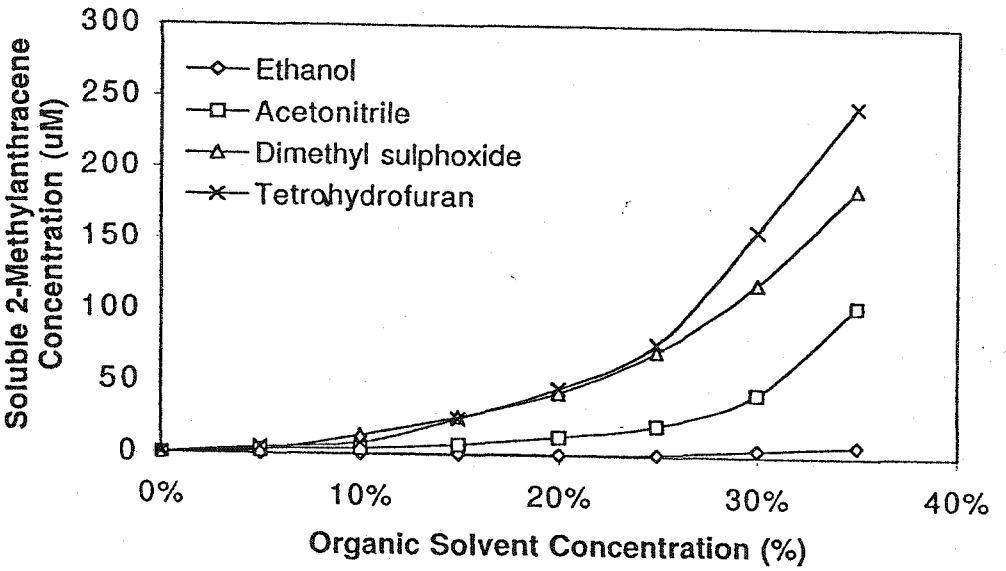

Fig. VI-1. Effect of different water-miscible organic solvents on water solubility of 2-methylanthracene.

The concentration of 2-methylanthracene increased with the increase of organic solvent concentration. Ten percent tetrahydrofuran, acetonitrile and dimethylsulfoxide is needed to get accurate detection of 2-methylanthracene using our HPLC method. Thirty percent ethanol is needed to dissolve 2-methylanthracene to the water at the detectable level.