ENZYMATIC DEGRADATION OF ANTHRACENE, DIBENZOTHIOPHENE AND PYRENE BY MANGANESE PEROXIDASE IN MEDIA CONTAINING ACETONE

Gemma Eibes[1], Tomas Cajthaml[2], Maria Teresa Moreira[1], Gumersindo Feijoo[1]* & Juan M. Lema[1]

[1]Department of Chemical Engineering. School of Engineering. University of Santiago de Compostela. E-15782 Santiago de Compostela. SPAIN

[2] Institute of Microbiology. Academy of Sciences of Czech Republic. Prague. CZECH REPUBLIC.

[*]Corresponding author:
e-mail: eqfeijoo@lugo.usc.es
Phone: 34-981563100 Ext. 16776
Fax: 34-981528050

ABSTRACT

The high hydrophobicity of polycyclic aromatic hydrocarbons (PAHs) greatly hamper their degradation in liquid media. The use of an organic solvent can assist the degradative action of ligninolytic enzymes from white rot fungi. The enzymatic action of the enzyme manganese peroxidase (MnP) in media containing a miscible organic solvent, acetone (36% v/v), was evaluated as a feasible system for the *in vitro* degradation of three PAHs: anthracene, dibenzothiophene and pyrene. These compounds were degraded to a large extent after a short period of time (7, 24 and 24 h, respectively), at conditions maximizing the MnP-oxidative system. The initial amount of enzyme present in the reaction medium was determinant for the kinetics of the process. The order of degradability, in terms of degradation rates was as follows: anthracene > dibenzothiophene > pyrene. The intermediate compounds were determined using gas chromatography-mass spectrometry and the degradation mechanisms were proposed. Anthracene was degraded to phthalic acid. A ring cleavage product of the oxidation of dibenzothiophene, 4-methoxybenzoic acid, was also observed.

Keywords: enzymatic degradation; manganese peroxidase (MnP); anthracene; pyrene; dibenzothiophene; miscible solvent

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are pollutants produced via natural and anthropogenic sources, generated during the incomplete combustion of solid and liquid fuels or derived from industrial activities. The environmental impact associated may cause a potential health risk due their mutagenic and carcinogenic potential. Important drawbacks greatly hamper the naturally biological degradation of PAHs (Cerniglia, 1992; Shuttleworth and Cerniglia, 1995). Due to their hydrophobicity, this type of compounds tends to accumulate on the soil organic matter and thus, their desorption from soil limits their availability for the microorganisms to biodegrade these pollutants.

The extracellular enzymes produced by white rot basidiomycetes is believed to underlie the ability of these fungi to degrade PAHs and other organopollutants (Bumpus, 1989; Field et al., 1993; Bezalel et al., 1996; Novotny et al., 2004). The catalytic action of these peroxidases generates more polar and water-soluble metabolites, such as quinones, which are more susceptible to further degradation by indigenous bacteria present in soils and sediments (Brodkorb and Legge, 1992; Meulenberg et al., 1997). However, the degradative action of the extracellular enzymes is limited by the Ionization Potential (IP) of the xenobiotic compound, which is related to the ease of removal of a π-electron from an aromatic molecule. The oxidative activity of manganese peroxidase (MnP) is mediated through the production of manganese ions, acting as freely diffusible oxidants. In a way to reproduce the degradative action of MnP, manganic acetate was found to be incapable of oxidising PAHs with IPs equal or greater than that of chrysene (approximately 7.8 eV), which gives an idea about the threshold value for the PAHs degradation by the catalytic action of MnP (Cavalieri and Rogan, 1985).

Little work has been developed about *in vitro* oxidation of PAHs by MnP and it has been focused on the determination of the limit IP mentioned above (Bogan and Lamar, 1995, 1996; Bogan et al., 1996; Sack et al., 1997a; Wang et al., 2003). Günther et al., (1998) have reported the degradation of 30% anthracene and 12% pyrene by MnP from *Nematoloma frowardii* after 24 h of reaction (initial concentration: 10 mg L^{-1}). The addition of mediating agents, such as reduced glutathione, increased the oxidative strength of MnP so that anthracene was completely reduced and 60% of pyrene was degraded after 24 h.

Considering another approach to assist the catalytic action of the enzyme, the possibility to enhance the solubility of PAHs by the use of an organic solvent is proposed (Kotterman et al., 1994; Field et al., 1995, 1996). This system could be implemented in the management of wastes containing PAHs previous to the enzymatic treatment. However, the degradative action can be hindered by the presence of the solvent and its effect on the enzymatic activity. In a previous work (Eibes et al., 2005), different water-miscible organic solvents (acetone, methyl-ethyl-ketone, methanol and ethanol) were evaluated to increase the solubility of anthracene. Bearing in mind the maximal solubilisation of anthracene and the minimum loss of MnP activity, acetone was selected as the optimal cosolvent, allowing to enhance 143-fold the anthracene solubility for an acetone concentration of 36% (v/v).

In this research work, the degradation of three PAHs: anthracene, pyrene and dibenzothiophene by a MnP crude preparation of *Bjerkandera* sp. BOS55 in media containing acetone was investigated. The selected PAHs present a recalcitrant structure with 3 or 4 aromatic rings, low solubility in water and IP values over and below the limit established for the enzyme. The degradation rates were evaluated for different initial MnP concentrations, in order to establish a direct relation for both of them.

Besides, the mechanisms of the enzymatic degradation of anthracene, dibenzothiophene and pyrene were proposed.

MATERIALS AND METHODS

Chemicals

Anthracene, dibenzothiophene and pyrene were obtained from Janssen Chimica (98% purity). Acetone was obtained from Panreac (chemical purity).

All PAHs present a complex structure, low water solubility: 0.07, 0.14 and 1.47 mg L^{-1} for anthracene, pyrene and dibenzothiophe, respectively (Mackay and Shiu, 1997; Hassett et al., 1980) and high IPs: 7.4, 7.5 and 8.1 eV for anthracene, pyrene and dibenzothiophene, respectively (Vázquez-Duhalt et al., 1994). To attain a PAH concentration in liquid phase of 5 mg L^{-1}, acetone was added in different proportions to ensure total solubilisation of the added PAH (25-28 μM): 36% for dibenzothiophene and anthracene, and 45% for pyrene.

MnP production

MnP was obtained from *Bjerkandera* sp. BOS55 (ATCC 90940). The fungus was grown in a 10-L fermenter, BIOSTAT®E B. Braun-Biotech International, (Melsungen, Germany) on skimmed cheese whey medium (Feijoo et al., 1999; Moreira et al., 2001). Once the peak production of MnP was detected, the fermentation was stopped by decreasing temperature and the extracellular liquid was vacuum filtered. High molecular weight polysaccharides were precipitated by freezing-thawing and removed by filtration. Crude enzyme was concentrated by ultrafiltration using a 10-kDa cut-off type YM-10 membrane (Amicon, USA), and then it was centrifuged for 10 min at 20,000 × *g*. Neither lignin peroxidase (LiP) nor laccase activities were detected in any of the separated fractions during the procedure.

MnP activity assays

The MnP activity was determined spectrophotometrically (Cecil CE 7200, UK) at 30 ºC and 468 nm, as described by Field et al. (1992). The method is based on the oxidation of 2,6-dimethoxyphenol (2,6-DMP) by the MnP system to form a quinone dimer. The molar extinction coefficient at this wavelength is 49,600 $M^{-1}cm^{-1}$ (Wariishi et al., 1992).

The MnP assays were carried out with 200 μL sodium malonate (stock solution: 250 mM, pH 4.5), 50 μL 2,6-DMP (stock solution: 20 mM), 50 μL $MnSO_4$ (stock solution: 20 mM), 50 μL of the sample whose MnP activity is to be determined and 550 μL water. The reaction was started by the addition of 100 μL H_2O_2 (stock solution: 4 mM), ending up with a volume of 1 mL. One unit (U) was defined as the amount of enzyme that releases 1 μmol of the dimeric product of 2,6-DMP oxidation per min measured at 468 nm.

PAH degradation assays

Oxidation of PAHs was carried out in 100-mL Erlenmeyer flasks, sealed with teflon plugs, with magnetic stirring at room temperature, i.e. 23 ºC. The reaction mixture (50 mL) consisted of acetone 36% (dibenzothiophene and anthracene) or 45% (pyrene), 5 mg L^{-1} PAH (25-28 μM), 20 μM Mn^{2+}, 20 mM malonic acid (pH 4.5), continuous addition of 5 μM min^{-1} H_2O_2 (defined for the volume of the reactor) and MnP activities specified for each case. Samples were withdrawn periodically and disappearance of each PAH was determined by HPLC. The evolution of MnP concentration was spectrophotometrically determined. To verify that degradation took place only due to an enzymatic oxidation, controls were run in parallel in the absence of MnP.

Analytical determinations

A HP 1090 HPLC, equipped with a diode array detector, a 4.6×200 mm Spherisorb ODS2 reverse phase column (5 μm; Waters) and a HP ChemStation data processor were used for determining the PAH concentration. The injection volume was set at 10 μL and the isocratic eluent was pumped at a rate of 1 mL min^{-1}. The PAH concentrations were determined monitoring the absorbance at 254 nm for anthracene, 240 nm for pyrene and 235 nm for dibenzothiophene. The isocratic eluent consisted on 80% acetonitrile:20% water (v/v).

Degradation products were analyzed by gas chromatography coupled with mass spectrometry (GC-MS, GCQ, Finnigan, USA). For structure elucidation, electron impact and chemical ionization mass spectrometry as well as MS-MS technique were used. The GC instrument was equipped with split/splitless injector and a DB-5MS column was used for separation (30 m, 0.25 mm id, 0.25 μm film thickness). The temperature program started at 60 °C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 150 °C at a rate of 25 °C min^{-1}. The second temperature ramp was up to 260 °C at a rate of 10 °C min^{-1}, this temperature being maintained for 20 min. The solvent delay time was set to 4 min. The transfer line temperature was set to 280 °C. Mass spectra were recorded at 1 scan s^{-1} under electron impact at 70 eV, mass range 50–450 amu. The excitation potential for the MS/MS product ion mode applied was 0.5 V, and 0.9 V in the case of more stable ions. Methane was used as a medium for chemical ionisation. The extracts were directly injected without any derivatisation. Moreover, the samples were trimethylsililated with aliquot volume of N,O-bis(trimethylsilyl)trifluoroacetamide (60 min, 60 °C) (Cajthaml et al., 2002).

RESULTS

Enzymatic degradation of PAHs

Degradation of anthracene was studied in the MnP-oxidative system. A previous work consisted on the optimisation of several environmental conditions such as malonic acid concentration and H_2O_2 loading rate, concluding that the higher degradation rate was obtained with 20 mM malonic acid (pH 4.5), 20 μM Mn^{2+}, 5 μM min^{-1} and at room temperature (23 ºC) (Eibes et al., 2005). In the present work, the effect of the initial MnP concentration was evaluated in order to improve the kinetics of anthracene degradation (Table 1). The higher enzymatic concentration (550 U L^{-1}) led to the higher anthracene degradation rate (3.22 μM h^{-1}) and consequently, to the higher kinetics constant (first order kinetics, 0.488 h^{-1}). In these conditions, 23 μM of anthracene were degraded in 7 h of reaction (Figure 1a). Anthraquinone, the main reaction product, was measured during the experiment, and the final concentration was 12 μM, which represented 52% of the degraded anthracene (data not shown). A control experiment was performed in absence of MnP; it was observed a slight decrease on anthracene concentration (9%), but there were no traces of anthraquinone. The continuous addition of hydrogen peroxide reduced the acetone concentration from 36% to 28% which caused a slight diminution of soluble anthracene in this control.

The experiments at lower MnP concentrations (60, 140 and 210 U L^{-1}) were stopped when MnP activity decreased below 10 U L^{-1} (4, 5 and 6 h, respectively), which corresponded with a distinct change in the slope of anthracene degradation. Therefore, the minimum enzyme requirements for degradation of anthracene was beyond 10 U L^{-1}.

The experiments with pyrene were carried out under the same optimised environmental conditions considered in the assays with anthracene and at various initial enzymatic concentrations: 210, 540, 1180 and 1310 U L^{-1} (Table 1). In comparison with

anthracene, the amounts of MnP assayed were higher, not only because of lower degradation percentages (11, 19, 53 and 61%, respectively) but also higher inactivation rates of MnP (data not shown).

Figure 1b shows the degradation of 17 μM pyrene after 24 h, at an initial MnP concentration of 1180 U L^{-1}. The control in absence of enzyme, showed no change in pyrene concentration in the course of the experiment, verifying the degradative action of MnP for the *in vitro* system.

Three initial MnP concentrations were assayed for experiments of dibenzothiophene degradation: 170, 570 and 1340 U L^{-1} (Table 1). The last experiment led to a nearly complete degradation after 24 h (95%) (Figure 1c). The slight diminution of the dibenzothiophene concentration in the control experiment (15%) was probably due to the reduction in solvent concentration during the experiment (from 36 to 20%) as a result of the hydrogen peroxide addition.

Kinetics of enzymatic degradation

The initial enzymatic concentration greatly affected the degradation kinetics for the studied PAHs. Therefore, in order to determine the relationship between these two variables (E_0 and k), linear regressions were considered for the experiments with anthracene, pyrene and dibenzothiophene (Figure 2). The regression coefficients obtained indicate that the data fitted well to the linear equation. Notice that the intercept value is not zero for the three equations, and it is more significant for pyrene and dibenzothiophene, which implies a residual degradation not dependent on MnP concentration. The slope value of the equations gives an idea of how recalcitrant each PAH is. Anthracene is the less recalcitrant one, whereas dibenzothiophene is 12-fold

more recalcitrant and pyrene 34-fold. This order of degradability is not consistent with the order relating to its IP values: anthracene < pyrene < dibenzothiophene.

Mechanisms of degradation

Table 2 lists retention data and mass spectral characteristics of the detected degradation products. Possible degradation sequences are given in Figure 3. In all cases, except for anthraquinone, only traces of intermediate compounds were detected (0.5-1% of the stoichiometric concentration expected for total degradation), indicating that no significant accumulation of these compounds took place and immediate degradation occurred after formation.

Most of the intermediates were confirmed by comparison with chemical standards (Table 2). Phthalic acid was identified as dehydrated form and trimethylsilyl (TMS) derivative. A structure of dihydroxyanthrone was suggested using electron impact fragmentation. The fragmentation pathways of MS-MS generated product ions showed a loss of water molecules from the molecular ion indicating possible ortho position of two hydroxyl groups ($M\text{-}H_2O$= m/z 210$^{+\bullet}$). Other fragmentations suggested a loss of one hydroxyl (m/z 209) and further a loss of carbonyl group (m/z 181). Ion m/z 152 (m/z 181-COH) appeared to be stable under our MS-MS conditions. Another fragmentation could be explained by a loss of oxygen from m/z 209 producing ion m/z 193 and further formation of m/z 165 after a loss of carbonyl.

DISCUSSION

Little work has been performed on the *in vitro* degradation of PAHs by MnP and the results obtained showed that the degradation was minimal and not optimized (Gunther et al., 1998; Wang et al., 2003). Moreover, several authors stated that the initial degradations of anthracene, dibenzothiophene or pyrene in living cultures of ligninolytic fungi do not depend on the ligninolytic activity, and suggested that cytochrome P-450 monooxygenase could be the responsible for this initial step (Bezalel et al., 1996; Gramss et al., 1999; Verdin et al., 2004).

The development of an efficient degradation system for polyaromatics based on the use of peroxidases *in vitro* requires their increased solubility by using cosolvents. MnP was demonstrated to be resistant to acetone, being scarcely deactivated even at high solvent concentrations (Eibes et al., 2005). An effective system must be defined for the enzymatic action of MnP by improving the action of the enzyme and the cofactors involved. The potential of the system: MnP-H_2O_2-Mn^{2+} consisted on a continuous treatment combining the action of the enzyme, Mn^{2+}, an organic chelator and hydrogen peroxide in the presence of acetone to increase the PAHs solubilities.

Theoretically, the enzymatic system should be efficient provided that the oxidation potential of a particular compound was lower than that supplied by the enzymatic cycle. The *in vitro* system was proven to oxidize anthracene and pyrene with crude MnP with an IP lower than 7.8 eV (Cavalieri and Rogan, 1985). However, this limitation was overcome when the system was capable to oxidize dibenzothiophene efficiently (IP: 8.1 eV), yielding an almost total degradation after 24 h. In the case of LiP, Vázquez-Duhalt et al. (1994) demonstrated that its IP threshold value, 7.6 eV (Hammel et al., 1992), was slightly higher for alkylaromatic and heteroaromatic polycyclic compounds (8.0 eV). Therefore, bearing in mind the degradation of

dibenzothiophene attained in this work, it could be stated that MnP is capable to oxidise heterocyclic compounds with an IP equal or below 8.1 eV.

A major factor considered for the increased efficiency of the process was the level of MnP concentration necessary. The capacity of the system was enhanced at a higher MnP concentration. This phenomenon was attributed to the kinetics of the process. The degradation rate of anthracene was higher than those of dibenzothiophene and pyrene, as the k values for each PAH support. A short period of 7 h is required to the complete removal of anthracene, while a longer period of 24 h-reactions is needed for dibenzothiophene. Pyrene led to the slowest kinetics and it was not achieved the complete oxidation under the studied conditions. The experiments with different amounts of enzyme showed that a lower MnP concentration could not complete the PAH degradation for a prolonged period, while a higher MnP concentration increased the rate of degradation for anthracene, dibenzothiophene and pyrene.

During the degradation of anthracene by MnP, the formation of anthrone was detected, which was an expected intermediate, and it was followed by the appearance of 9,10-anthraquinone (Cerniglia, 1992). This compound was produced at high molar yields, around 50%. Anthraquinone has been earlier described as the common oxidation product in *in vitro* reactions of peroxidases (Hammel, 1995). Further oxidation resulted in the formation of phthalic acid as it was observed in ligninolytic cultures of *Phanerochaete chrysosporium* (Hammel et al., 1991). To our knowledge this is the first report when ring cleavage of a PAH representative appeared by MnP without any present redox mediator like glutathione or unsaturated lipids (Moen and Hammel, 1994; Sack et al., 1997b). It was detected also a structure that was assigned as dihydroxyanthrone with ortho hydroxyl radicals. This compound, together with production of 1-hydroxypyrene from pyrene, indicates a direct hydroxylation by $^{\bullet}OH$

radicals during oxidative process. On the other hand, it was not detected any formation of pyrenediones (Kästner, 2000). Dibenzothiophene was transformed to dibenzothiophene sulfone (Bezalel et al., 1996; Ichinose et al., 2002) and, a ring cleavage product 4-methoxybenzoic acid, was detected.

We can conclude that the addition of acetone increased the solubility of PAHs and it did not hamper significantly the MnP activity. Crude MnP was sufficient to initiate and promote the degradation of anthracene and pyrene (IPs: 7.4 and 7.5, respectively). Evenmore, the hererocyclic compound dibenzothiophene with an IP of 8.1 could be degraded by MnP, which suggests the application of this enzyme in the degradation of other heterocyclic compounds with IPs higher than the threshold value for the MnP. From the intermediate compounds detected in the degradation of anthracene and pyrene, we concluded that •OH radicals were involved during oxidative process.

Acknowledgements

This work was funded by the Spanish Commission of Science and Technology (CICYT-Project VEM2003-20089-C02-00), by grant KJB6020308 of the Grant Agency of the Academy of Science of the Czech Republic and by Institutional Research Concept No. AV0Z50200510. Gemma Eibes would like to express her gratitude to the Spanish Ministry of Science and Technology for her financial support (BES-2002-2809).

REFERENCES

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

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

Bogan, B.W., Schoenike, B., Lamar, R.T., Cullen, D., 1996. Expression of *lip* genes during growth in soil and oxidation of anthracene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 62, 3697-3703.

Brodkorb, T.S., Legge, R.L., 1992. Enhanced biodegradation of phenanthrene in oil tar-contaminated soils supplemented with *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 58, 3117-3121.

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

Cajthaml, T., Moder, M., Kacer, P., Sasek, V., Popp, P., 2002. Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. J. Chromatogr. A 974, 213-222.

Cavalieri, E.L., Rogan, E.G., 1985. Role of radical cations in aromatic hydrocarbon carcinogenesis. Environ. Health Perspect. 64, 69-84.

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

Eibes, G., Lu Chau, T., Feijoo, G., Moreira, M.T., Lema, J.M., 2005. Complete degradation of anthracene by Manganese Peroxidase in organic solvent mixtures. Enzyme Microb. Technol. 37, 365-372.

Feijoo, G., Moreira, M.T., Roca, E., Lema, J.M., 1999. Use of cheese whey as a substrate to produce manganese peroxidase by *Bjerkandera* sp. BOS55. J. Ind. Microbiol. Biotechnol. 23, 86-90.

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

Field, J.A., de Jong, E., Feijoo, G., de Bont, J.A.M., 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol. 11, 44-49.

Field, J.A., Boelsma, F., Baten, H., 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., Vledder, R.H., van Zeist, J.G., Rulkens, W.H., 1996. The tolerance of lignin peroxidase and manganese-dependent peroxidase to miscible solvents and the in vitro oxidation of anthracene in solvent:water mixtures. Enzyme Microb. Technol. 18, 300-308.

Gramss, G., Kirsche, B., Voight, K.D., Günther, T., Fritsche, W., 1999. Conversion rates of five polycyclic aromatic hydrocarbons in liquid cultures of fifty-eight

fungi and the concomitant production of oxidative enzymes. Mycol. Res. 103, 1009-1018.

Günther, T., Sack, U., Hofrichter, M., Latz, M., 1998. Oxidation of PAH and PAH-derivatives by fungal and plant oxidoreductases. J. Basic Microbiol. 38, 113-122.

Hammel, K.E., 1995. Mechanisms for polycyclic aromatic hydrocarbon by ligninolytic fungi. Environ. Health Perspect. 103, 41-43.

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

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

Hassett, J.J., Means, J.C., Banwart, W.L., Wood, S.G., Ali, S., Khan, A., 1980. Sorption of Dibenzothiophene by soils and sediments. J. Environ. Qual. 9, 184-186.

Ichinose, H., Nakamizo, M., Wariishi, H., Tanaka, H., 2002. Metabolic response against sulfur-containing heterocyclic compounds by the lignin-degrading basidiomycete *Coriolus versicolor*. Appl. Microbiol. Biotechnol. 58, 517-526.

Kästner, M., 2000. Degradation of Aromatic and Polyaromatic Compounds. Wiley VCH, Weinheim.

Kotterman, M.J.J., Heessels, E., de Jong, E., Field, J.A., 1994. The physiology of anthracene biodegradation by the white-rot fungus *Bjerkandera* sp. strain BOS55. Appl. Microbiol. Biotechnol. 42, 179-186.

Mackay, D., Shiu, W.Y., 1977. Aqueous solubility of polynuclear aromatic hydrocarbons. J. Chem. Eng. Data 22, 399-402.

Meulenberg, R., Rijnaarts, H.H.M., Doddema, H.J., Field, J.A., 1997. Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. FEMS Microbiol. Lett. 154, 45-49.

Moen, M.A., 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.

Moreira, M.T., Palma, C., Mielgo, I., Feijoo, G., Lema, J.M., 2001. *In vitro* degradation of a polymeric dye (Poly R-478) by manganese peroxidase. Biotechnol. Bioeng. 75, 362-368.

Novotny, C., Svobodova, K., Erbanova, P., Cajthaml, T., Kasinath, A., Lang, E., Sasek, V., 2004. Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. Soil Biol. Biochem. 36, 1545-1551.

Sack, U., Hofrichter, M., Fritsche, W., 1997a. Degradation of polycyclic aromatic hydrocarbons by manganese peroxidase of *Nematoloma frowardii*. FEMS Letters 152, 227-234.

Sack, U., Hofrichter, M., Fritsche, W., 1997b. Degradation of phenanthrene and pyrene by *Nematoloma frowardii*. J. Basic Microbiol. 37, 287-293.

Shuttleworth, K.L., Cerniglia, C.E., 1995. Environmental aspects of PAH biodegradation. Appl. Biochem. Biotechnol. 54, 291-302.

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

Verdin, A., Sahraoui, A.L.H., Durand, R., 2004. Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. Int. Biodeterior. Biodegrad. 53, 65-70.

Wang, Y., Vazquez-Duhalt, R., Pickard, M.A., 2003. Manganese-lignin peroxidase hybrid from *Bjerkandera adusta* oxidizes polycyclic aromatic hydrocarbons more actively in the absence of manganese. Can. J. Microbiol. 49, 675-682.

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

CAPTIONS TO FIGURES

Figure 1. Time course of PAH disappearance (■), MnP enzymatic activity (○) during *in vitro* treatment. A control assay without MnP was run in parallel (□). (a): anthracene; (b): pyrene and (c): dibenzothiphene.

Figure 2. Kinetics constants of anthracene (●), pyrene (▲) and dibenzothiophene (■) as a lineal function of initial enzymatic activity. $k_{ANT} = 8.7 \cdot 10^{-4} \cdot E_0 + 0.0014$ ($r^2 = 0.97$); $k_{PYR} = 2.4 \cdot 10^{-5} \cdot E_0 + 0.0084$ ($r^2 = 0.97$); $k_{DBT} = 7.0 \cdot 10^{-5} \cdot E_0 + 0.0139$ ($r^2 = 1.00$);

Figure 3. Intermediate compounds from the degradation of anthracene (a), pyrene (b) and dibenzothiophene (c) in experiments with MnP from *Bjerkandera* sp. BOS55.

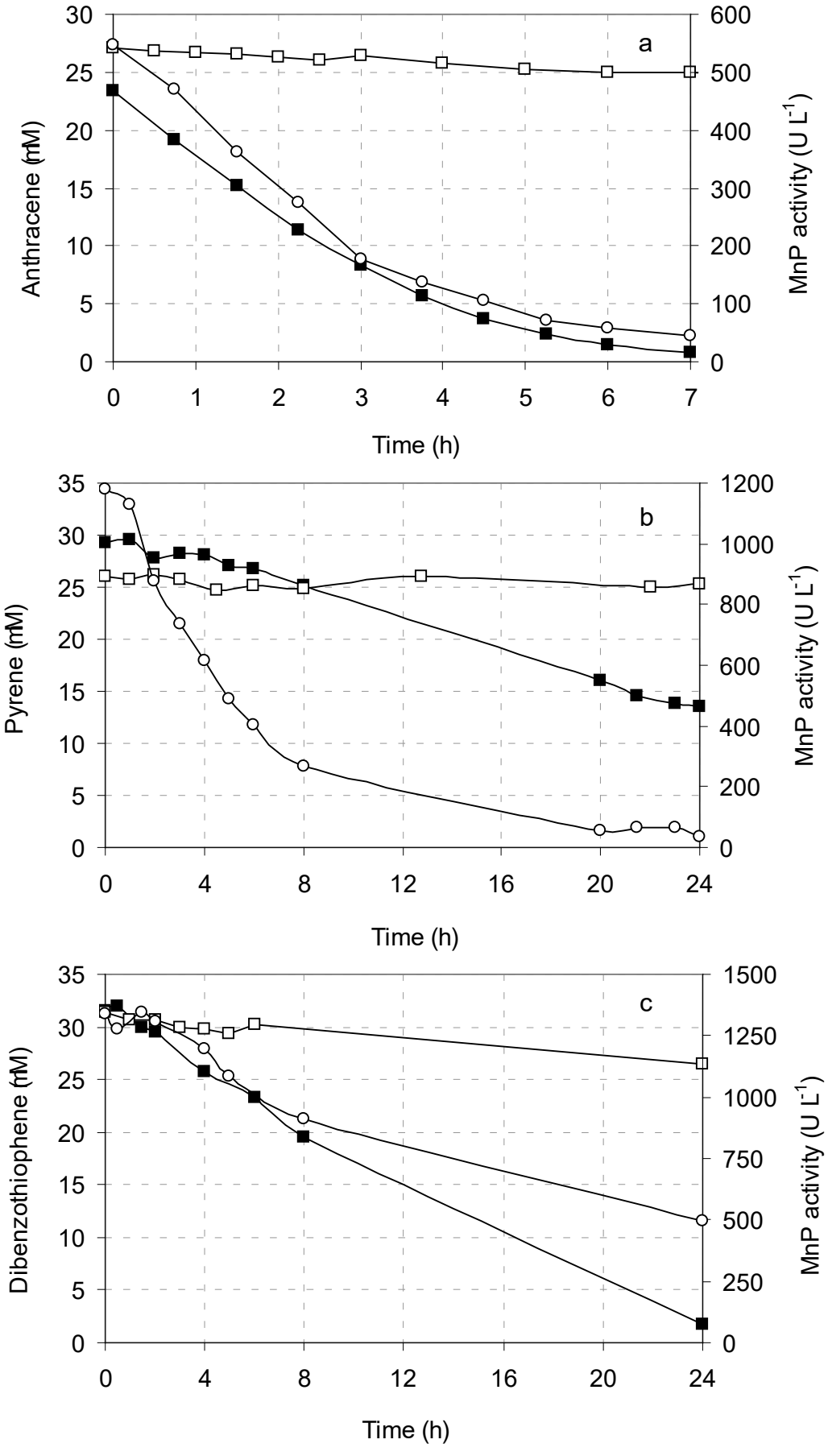
a
Anthracene (mM)
MnP activity (U L-1)
Time (h)
b
Pyrene (mM)
MnP activity (U L-1)
Time (h)
c
Dibenzothiophene (mM)
MnP activity (U L-1)
Time (h)

Figure 1.

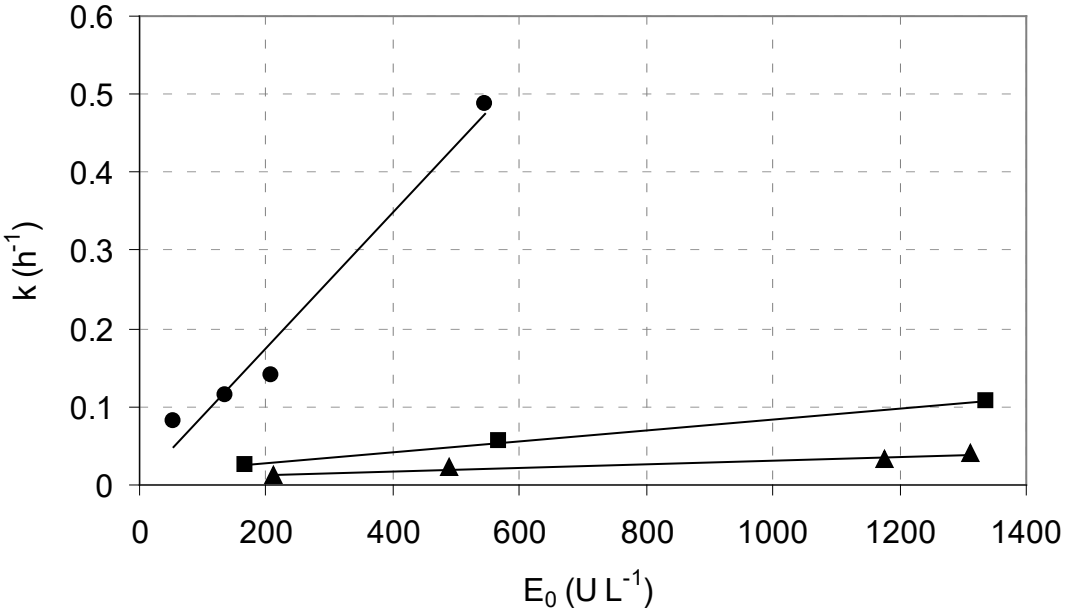

Figure 2.

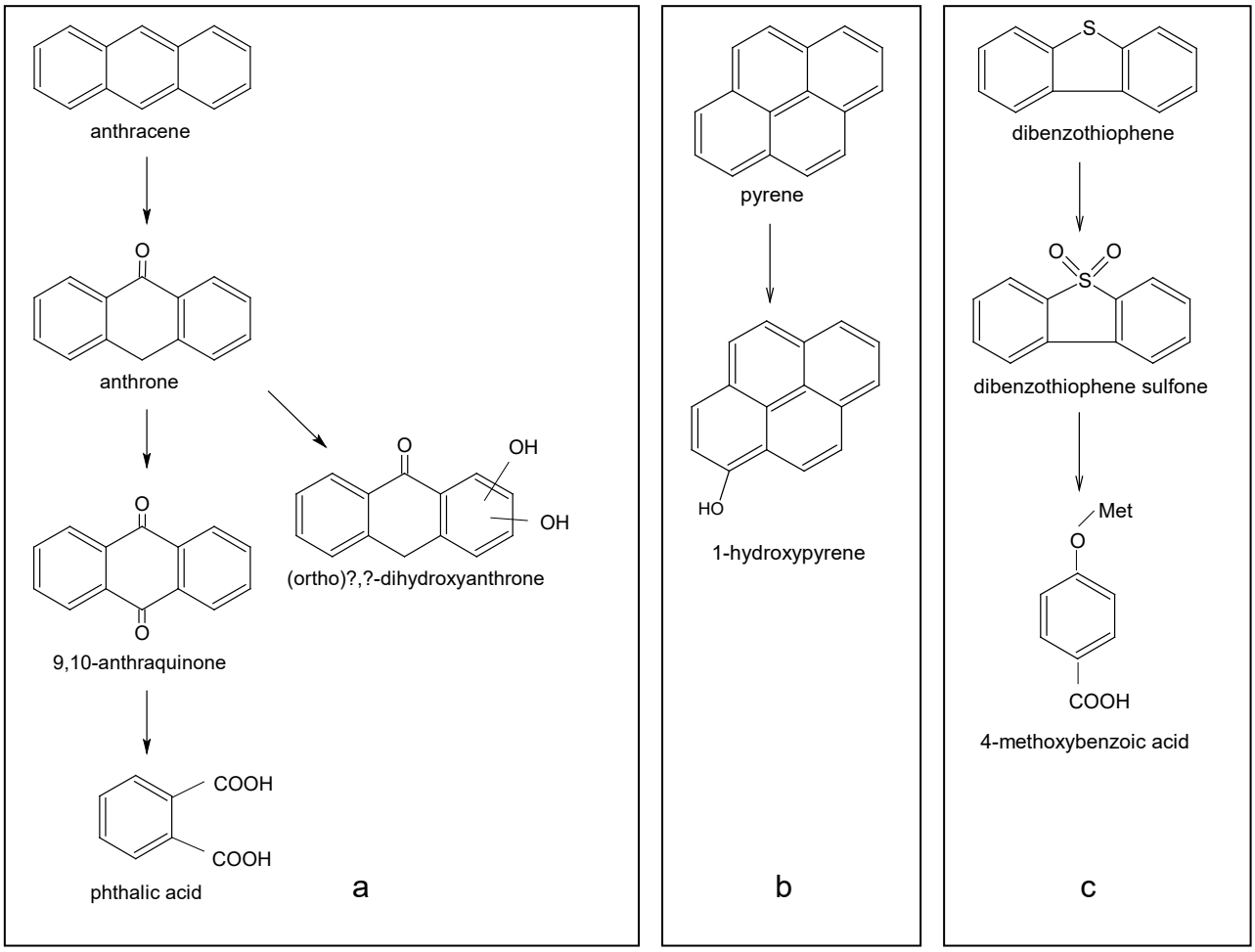
anthracene
O
anthrone
O
O
9,10-anthraquinone
COOH
COOH
phthalic acid
O
OH
OH
(ortho)?,?-dihydroxyanthrone
a
pyrene
HO
1-hydroxypyrene
b
S
dibenzothiophene
O
O
S
dibenzothiophene sulfone
Met
O
COOH
4-methoxybenzoic acid
c

Table 1. Biodegradation of ANT, PYR and DBT with MnP at different initial enzymatic concentration

PAH	Initial enzyme E_0 (U L^{-1})	Reaction duration (h)	Average PAH degradation rate (μmol L^{-1} h^{-1})	First order kinetics	
				k (h^{-1})	r^2
ANT	60	4	1.78	0.081	0.983
	140	5	2.04	0.114	0.991
	210	6	2.15	0.140	0.979
	550	7	3.22	0.488	0.983
PYR	210	6	0.28	0.012	0.937
	540	9	0.55	0.023	0.980
	1180	24	0.65	0.034	0.985
	1310	24	0.54	0.040	0.980
DBT	170	6	1.063	0.023	0.985
	570	24	0.934	0.055	0.998
	1340	24	1.241	0.121	0.961

Table 2. Retention data and electron impact mass spectral characteristics degradation products

t_R (min)	MW (CI)	Parent compound	m/z of fragment ions (relative intensity)	Compound suggestion
6.72	148	ANT	148 (2.3), 104 (100), 76 (41.2), 50 (20.4)	phthalic anhydride*§
10.65	310	ANT	310 (3.7), 295 (57.6), 265 (6.4), 221 (27.5), 193 (3.8), 147 (100), 73 (53.1)	phthalic acid di-TMS*
13.00	194	ANT	194 (100), 165 (98.4), 139 (49.6), 81 (37.1)	Anthrone*
13.51	208	ANT	208 (100), 180 (64.2), 152 (58.8), 126 (4.4), 76 (5.9)	9,10-anthracenedione*
14.83	226	ANT	226 (100), 210 (41.5), 209 (44.7), 208 (36.8), 194 (21.1), 193 (23.7), 165 (34.2), 152 (52.6)	(ortho) ?,?-dihydroxyanthrone
19.23	218	PYR	218 (100),189 (40.3), 95 (13.9)	1-hydroxypyrene*
7.7	152	DBT	152 (74.6), 135 (100), 107 (14.5), 92 (10), 77 (20.5)	4-methoxybenzoic acid*
15.03	216	DBT	216 (100), 187(27.6), 168 (17), 160 (21.3), 139 (18.4), 136 (20.2)	dibenzothiophene sulfone*

* structures were later identified by comparison with standards

§ dehydrated form of the metabolite